Carbonaceous Resource Recovery Through Flexible Engineered Biological Systems and Platforms - Process Engineering and Systems Biology

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ABSTRACT

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This dissertation research represents efforts towards development of organic waste fueled bio-refineries, capable of achieving circular economy and resource recovery through conversion of the embedded chemical energy and nutrients present in the organic waste, into biochemical products of commercial value. The specific objectives were:

1. *Evaluation of anaerobic fermentation derived volatile fatty acid as a recovery platform:* Anaerobic fermentation of organic wastes is a cost and energy efficient way of recovering the inherent chemical energy potential associated with such waste streams. Additionally, since the carbon is recovered in the form of short chain volatile fatty acids (VFA), the high solubility in water results in fairly easy recovery and handling than the gas phase endpoints (methane, hydrogen). It also opens up the possibility to biologically or chemically redirect those VFA to high-value endpoints. Anaerobic fermentation of such streams to recover VFA also results in reducing the organic strength of the waste streams, thereby achieving waste treatment and sanitation in a far more sustainable manner than the existing practices of the organic waste management and wastewater treatment, which were inherently designed with the goal of removal of contaminants and

are focused upon sequential removal of those undesirable 'contaminants' including carbon (energy), nitrogen and phosphorus.

The pipeline consists of two major steps, namely (i) Production and recovery of carbon in the form of volatile fatty acids through anaerobic fermentation and, (ii) Biological or chemical conversion of VFA to other endpoints including but not limited to biofuels, bio-plastics, butanol, organic acids and solvents and dihydrogen.

2. *Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids:*
   Lipid accumulation in the oleaginous yeast *Cryptococcus albidus* was evaluated using mixtures of volatile fatty acids (VFA) as substrates. In general, batch growth under nitrogen limitation led to higher lipid accumulation using synthetic VFA. During batch growth, an initial COD:N ratio of 25:1 mg COD:mg-N led to maximum intracellular lipid accumulation (28.33±0.74% g/g dry cell weight), which is the maximum reported for *C. albidus* using VFA as the carbon source, without compromising growth kinetics. At this feed COD:N ratio, chemostat cultures fed with synthetic VFA yielded statistically similar intracellular lipid content as batch cultures (29.88±1.92%, g/g). However, batch cultures fed with VFA produced from the fermentation of food waste, yielded a lower lipid content (14.99±0.06%, g/g). The lipid composition obtained with synthetic and food-waste-derived VFA was similar to commercial biodiesel feedstock. We therefore demonstrate the feasibility of linking biochemical waste treatment and biofuel production using VFA as key intermediates.

3. *Genome sequencing of oleaginous yeast Cryptococcus albidus and evaluation of its genetic and biotechnological potential:*

We reported the complete draft genome sequence of *Cryptococcus albidus var. albidus*, an oleaginous yeast, which can utilize various organic carbon sources for lipid synthesis. The basidiomycetous oleaginous yeast has been gaining popularity as a non-conventional yeast with the ability to metabolize and transform diverse organic substrates. The 24.8 Mb genome of *C. albidus* was sequenced and the metabolic reconstruction revealed that *C. albidus* contains several essential pathways for metabolism of various carbon sources (including glucose, sucrose, glycerol, acetic, propionic and butyric acids), accumulation of carbon compounds (tri-acyl glycerol (TAGs) and glycogen) and for assimilation of various nitrogen (ammonia, nitrate, nitrite, and urea) and sulfur sources (sulfate, sulfite, thiosulfate). It is also capable of secreting enzymes of industrial significance.

Here, we presented a comprehensive overview of the biology and biotechnology of *C. albidus*, specifically focusing on its microbial physiology, metabolic pathways and its potential for production of commercially and industrially important chemicals.

4. *Evaluation of the global transcriptomic and proteomic responses of the Cryptococcus albidus to nitrogen limitation:*

‘Non-ideal’ carbon sources could be an ideal substrate for economically feasible lipid production by oleaginous yeast *Cryptococcus albidus*; however, there have been no studies thus far, on biochemical pathways governing its oleaginity and metabolism. Here, we report for the first time, a comprehensive account of the transcriptome and proteome level changes in continuous cultures of *Cryptococcus albidus* in response to nitrogen limitation. Proteome and differential gene expression data revealed a tight co-regulation

of nitrogen and carbon metabolism, wherein nitrogen limitation resulted in a complete redistribution of carbon flux throughout the cellular processes, including nitrogenous compound recycling, autophagy and cessation of nucleic acid and ribosome biosynthesis. Lipid accumulation by *C. albidus* does not seem to involve transcriptional regulation but is a passive consequence of carbon flux redistribution during nitrogen limitation. This study therefore, provides a valuable resource to understand oleaginity and metabolism of alternate carbon sources by *C. albidus* and provides opportunities for metabolic re-engineering of its lipid production pathways.

5. *Organic waste fueled biorefineries: future perspectives on production of chemicals of industrial significance from volatile fatty acids:*

   The focus of this review was to propose alternate bio-based pipelines for recovery and conversion of organic waste streams into high value commercial products, using VFA as central precursors for further aerobic/anaerobic carbon cycling. Herein, we present various pathways, microorganisms, culture conditions and current status of bio-based production of certain building-block chemicals such as adipic acid, butanol, organic acids such as citric, malic and succinic acids. These chemicals have the highest potential to be economically produced from VFA since the pathways for their bioconversion either exist natively or have been metabolically engineered. Nevertheless further research would be needed to reduce the costs and enhance productivity.

In conclusion, this dissertation represents the first attempt at a systematic evaluation of a VFA based resource recovery platform. During the first phase, microbial conversion of volatile

fatty acids into lipids by the yeast *C. albidus* was evaluated and nitrogen limitation was identified as the inducer of lipogenesis in *C. albidus* through operation of batch and chemostat cultures. Next, the genome of *C. albidus* was sequenced and transcriptome and proteome level changes were evaluated in conjunction to understand the metabolic basis of nitrogen-mediated oleaginity in *C. albidus.* This genomic and the comparative transcriptome and proteome data is expected to help further elucidate factors driving lipid accumulation in *C. albidus* and contribute toward bioprocess development and optimization for engineered lipid production from 'waste' streams. Finally, the feasibility of microbial conversion of VFA into several other bio-based chemicals of commercial value was also evaluated.

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CHAPTER 1

INTRODUCTION

The increase in waste generation due to rapid global urbanization, coupled with stringent waste disposal regulations and increasing public awareness relating to environmental impact of improper waste handling has resulted in the need to design and operate improved waste management systems (Zhao and Deng 2014). Existing waste management practices tend to be prohibitively expensive due to their extremely large energy footprint. According to US EPA, about 3–4% of total energy use in the US is devoted to water treatment and 45 million tons of greenhouse gases are emitted into the atmosphere every year from waste treatment alone(Marshall, LaBelle et al. 2013).

Lack of waste management also poses serious health risks in most developing countries where, without a waste management facility in place, the untreated waste ends up directly in the local environment resulting in its pervasive contamination with pathogens. The reluctance toward developing adequate or appropriate waste management systems stems mainly from the economic burden it places on its end users or local governments(Strande June 2014).

If we recognize that ‘waste’ streams are truly speaking ‘enriched’ streams, then we can at the very least recover energy, chemicals, and nutrients and connect the end products to other sectors and industries. Moreover, monetization of these waste-derived chemicals could be an economic incentive towards building and operating these engineered recovery systems and processes, which could provide access to sanitation for many underserved or unserved communities.

Recovery and reuse of resources present in various feedstocks, including organic waste streams can be a viable pathway towards realization of a circular economy. Widespread adoption and implementation of resource recovery depends on several factors including engineering feasibility, technology maturity and reliability, capital and operating costs, recovered product resale value and environmental impacts. As such, several specific waste streams may be readily used to obtain value added compounds, as is the case of biodiesel production from spent cooking oils. From an elemental composition perspective, organic waste is a tremendous resource comprised of nutrient rich organic material, microorganisms, undigested organics such as paper, plant residues, oils, or faecal material, inorganic materials and moisture. The two components in the organic waste streams that are technically and economically feasible to recycle are nutrients (primarily nitrogen and phosphorus) and energy (carbon)(Tyagi and Lo 2013).

Of these, in general, organic carbon is present in most waste streams at an average oxidation state of zero, C(0). During traditional waste treatment processes, a fraction of this organic carbon is converted to biomass while the remainder is oxidized to carbon dioxide (CO_2, +4) either aerobically, anoxically or anaerobically. In terms of its amenability for conversion into commercially attractive products, CO_2 is perhaps the least attractive substrate among various possible waste treatment endpoints for carbon (methane, organic acids and alcohols etc. Alternatively, the influent organic carbon and biomass can be reduced under anaerobic conditions to generate methane (CH_4, -4), which is a source of heat and power. Indeed, production of CH_4 has emerged as a principal endpoint for readily usable energy around the globe. Most current efforts towards biological recovery of carbon from organic waste streams have largely focused upon the process of anaerobic digestion, during which complex biopolymers are sequentially converted by microorganisms in the absence of oxygen to

ultimately form methane (CH_4), inorganic nutrients and carbon dioxide (CO_2). However, biogas produced through anaerobic digestion is a mixture of several gases (CO_2, H_2, H_2S, NH_3) and moisture with a maximum methane content of about 60% (Weiland 2010). Therefore, for any further practical use, all gas contaminants as well as moisture must be removed, and the upgraded gas must have a methane content of more than 95% in order to fulfill the quality requirements of the different gas appliances. In order to ensure that the scrubbed biogas does not contain bacteria and molds that could create unacceptable risks for human health and equipment, the application of HEPA filters is also necessary. These secondary and tertiary treatments make the recovery of methane from biogas rather costly (Weiland 2010). Therefore, in most cases, biogas is either released or flared after addition of pure methane gas. Yet, methane is only one of many possible endpoints for carbon recovery through anaerobic microbial platforms and pathways.

To understand the complement of anaerobic carbon recovery endpoints, it would be beneficial to first summarize the anaerobic reactions involved in the commonly employed process of anaerobic digestion (AD). AD is comprised of a series of biological processes, which involve biodegradation of complex biopolymers by microorganisms in the absence of oxygen to ultimately form methane (CH_4), inorganic nutrients and carbon dioxide (CO_2). The process occurs in four stages: a) hydrolysis, b) acidogenesis, C) acetogenesis and d) methanogenesis (Grady, Daigger et al. 1999).

During hydrolysis, the complex biopolymers present in the organic feedstock are converted extracellularly into smaller monomers to render them amenable for further conversion. During fermentation, acidogenic bacteria transform the products from hydrolysis into short chain volatile fatty acids (VFA) such as butyric, propionic and acetic acid. Fermentation also results in the

production of ketones, alcohols, hydrogen and CO_2. These products of acidogenesis are further transformed by bacteria into hydrogen, carbon dioxide and acetic acid in a process known as acetogenesis. Finally, methanogenic archaea produce methane either by reduction of carbon dioxide with hydrogen or by cleavage of acetic acid molecules.

Conventionally, AD has been regarded as an effective technology to combine treatment and microbiological stabilization of many diverse waste streams including sewage, food waste, fecal sludge and wastewater with resource recovery by the conversion of organic carbon to methane-containing biogas (CH_4-biogas). CH_4-biogas can be used directly in heating or electricity generation or post-upgradation, it can be injected into the natural gas grid. However, currently, the production of CH_4-biogas is in clear disadvantage compared with natural gas extraction, which has become far more cost-competitive (Kleerebezem, Joosse et al. 2015). On the other hand, the use of VFA produced during fermentation might be regarded as an alternative and advantageous channel to facilitate resource recovery from waste streams. In this sense, the fermenter output could also be manipulated to produce the most favorable VFA composition for the downstream process.

VFA are produced as intermediates during the acidogenesis step through the process of anaerobic fermentation. These VFA are present in liquid form and can therefore be recovered fairly easily without any additional high cost pretreatment steps(Chang, Kim et al. 2010) and are highly amenable to further biological or chemical conversion into upcycled products of greater commercial value. Chemically, VFA (table 1) are short chain (C1:0 - C5:0) carboxylic acids comprised mainly of formate, acetate, propionate, butyrate, and valerate, and are especially attractive, since they can be produced from highly diverse organic waste streams including waste activated sludge(Zhang, Chen et al. 2009), food waste(Lim, Kim et al. 2008), dairy

waste(Demirel and Yenigun 2004) and municipal solid waste(Sans, Mata-Alvarez et al. 1995) among others. Such waste-derived VFA can be chemically or biologically transformed for diverse applications including lipid production (and subsequent conversion to biodiesel) by oleaginous (oil-producing or accumulating) microorganisms. Therefore, VFA can be the ideal intermediate for highly versatile applications including sanitation and commercial production of valuable chemicals while addressing the problem of the organic waste disposal in a technologically sustainable manner.

Table 1 Properties of major VFA produced during anaerobic fermentation

VFA	Formula		Molecular weight (g/mol)	Density at 25°C (g/mL)	pKa
	Molecular	Structural			
Formic acid C1:0	CH_2O_2	HCOOH	46.03	1.220	3.77
Acetic acid C2:0	$C_2H_4O_2$	CH_3COOH	60.05	1.049	4.76
Propionic acid C3:0	$C_3H_6O_2$	CH_3CH_2COOH	74.08	0.988	4.87
Butyric acid C4:0	$C_4H_8O_2$	$CH_3(CH_2)_2COOH$	88.11	0.953	4.82
Valeric acid C5:0	$C_5H_{10}O_2$	$CH_3(CH_2)_3COOH$	102.13	0.930	4.82

Volatile fatty acids are produced during the acidogenesis step of the anaerobic digestion process (section 1.1, figure 1) and are further converted into biogas during the subsequent methanogenesis phase. The process of anaerobic digestion in its prevalent form is a rather

inefficient way of recovering the carbon and the chemical energy associated with the organic waste streams since only a fraction of recovered carbon is in the form of methane gas, and this fraction is usually unusable without costly pretreatment steps such as scrubbing. Herein, we propose microbial conversion of such VFA into lipids, which are an alternate and far more commercially and energetically viable endpoint. The work presented in this dissertation demonstrates the feasibility of biological conversion of organic waste derived VFA into long chain fatty acids through oil producing (oleaginous) yeast *Cryptococcus albidus* (Vajpeyi and Chandran 2015). We have further sequenced and studied the genome, transcriptome and proteome of *C. albidus* under various growth conditions to ascertain the most optimal growth conditions to maximize the lipid yield from VFA. Lipogenesis is usually induced upon exhaustion of a nutrient, typically nitrogen or phosphorus. Under the growth limitation conditions, the consumed chemical oxygen demand (COD) is channeled toward intracellular carbonaceous storage compounds such as lipids, rather than to more cellular biomass.

It is also of keen interest to understand the metabolic bias in carbon uptake exhibited by oleaginous microorganisms under growth limiting conditions. Relative carbon to nitrogen ratio (COD:N) has long been implicated to play a role in oleaginity and based primarily on empirical evidence, it is assumed the nitrogen can act as a metabolic 'switch' to divert carbon away from catabolic tricarboxylic acid cycle (TCA) towards anabolic fatty acid biosynthesis under nitrogen limited growth conditions. In our previous study, the intracellular lipid content increased by 52% w/w (from 19% to 29%) in *C. albidus* cultures subjected to nitrogen limitation

The overall objective of this research was to develop an integrated VFA based platform technology, which is capable of capturing the carbon present in various organic waste

streams in the form of VFA and then biologically or chemically transform those VFA into various products of commercial value.

1.1 Anaerobic digestion

Anaerobic digestion comprises of a series of biological processes, which involve biodegradation of complex biopolymers by microorganisms in the absence of oxygen to form methane, inorganic nutrients and carbon dioxide (Figure 1). The process occurs in three basic stages: Bacterial Hydrolysis, Acid formation and Methanogenesis as the result of the activity of the consortium of microbes present in the waste.

1.1.1 Hydrolysis

In the first stage, the complex biopolymers present in the waste are hydrolyzed into smaller monomers to access the energy potential of the material. Hydrolytic and fermentative bacteria excrete enzymes like cellulose, protease and lipase to catalyze the reaction to produce amino acids, monosaccharides, Purines and pyrimidines, glycerol and long chain fatty acids.

$$C_6H_{10}O_4 + 2\ H_2O \rightarrow C_6H_{12}O_6 + 2\ H_2 \quad (1)$$

1.1.2 Acidogenesis

In the second stage, acidogenic bacteria transform the products of the first reaction into short chain volatile acids, ketones, alcohols, hydrogen and carbon dioxide. The principal acidogenesis stage products are propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), acetic acid (CH_3COOH), formic acid ($HCOOH$), lactic acid ($C_3H_6O_3$), ethanol (C_2H_5OH) and methanol (CH_3OH). The microbial consortia are responsible for the various products of the acidogenesis,

e.g., *syntrophobacter wolinii*, a propionate decomposer and *sytrophomonos wolfei*, a butyrate decomposer. Other acid formers are *clostridium sp.*, *peptococcus anerobus*, *lactobacillus*, and *actinomyces.*

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (4)$$

1.1.3 Acetogenesis

In the third stage, known as acetogenesis, the rest of the acidogenesis products, i.e. the propionic acid, butyric acid and alcohols are transformed by acetogenic bacteria into hydrogen, carbon dioxide and acetic acid. Hydrogen plays an important intermediary role in this process, as the reaction will only occur if the hydrogen partial pressure is low enough to thermodynamically allow the conversion of all the acids. Such lowering of the partial pressure is carried out by hydrogen scavenging bacteria, thus the hydrogen concentration of a digester is an indicator of its health.

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad (5)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (6)$$

$$CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + 2H_2 + H^+ \quad (7)$$

1.1.4 Methanogenesis

Methane is produced in the final stage of anaerobic digestion by bacteria called methanogens eg. *methanobacterium, methanobacillus, methanococcus* and *methanosarcina*. Methane can be produced either by reduction of carbon dioxide or by cleavage of acetic acid molecules. Methane production is higher from the reduction of carbon dioxide but low concentration of hydrogen results in cleavage of acetic acid as the major methane forming mechanism in the anaerobic digester. Following reactions occur during methanogenesis:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (8)$$

$$2C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COOH \quad (9)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (10)$$

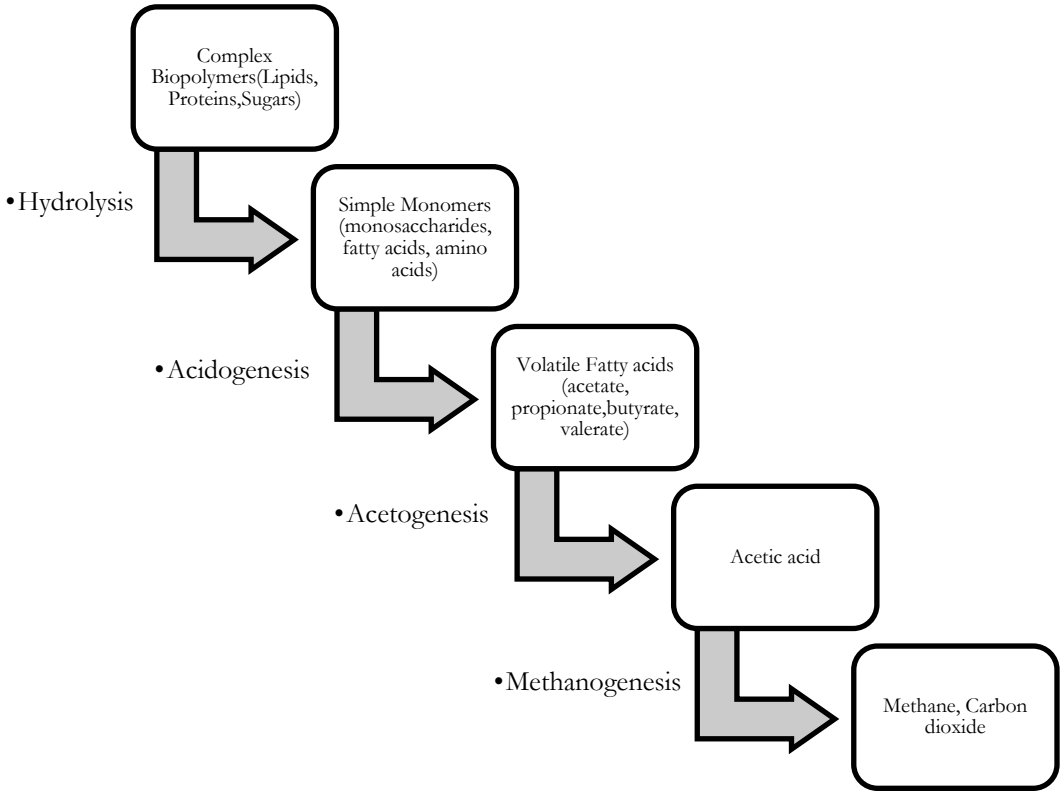

Figure 1 **Outline of various steps involved in anaerobic digestion process. VFA are formed in the second step through acidogenesis of hydrolyzed biomass.**

1.2 Operational parameters affecting the shortcut anaerobic fermentation for VFA production

VFA can be produced from anaerobic fermentation of a variety of organic feedstocks such as food waste (Lim, Kim et al. 2008), wastewater activated sludge (Zhang, Chen et al. 2009, Morgan-Sagastume, Valentino et al. 2014), dairy waste (Demirel and Yenigun 2004) and the organic fraction of municipal solid waste (OFMSW) (Sans, Mata-Alvarez et al. 1995). Concentration of VFA and yields using selected feedstocks are shown in Table 2.

Table 2 Distribution of volatile fatty acids from anaerobic fermentation of a variety of substrates

Reference	(Morgan-Sagastume, Valentino et al. 2014)	(Demirel and Yenigun 2004)	(Sans, Mata-Alvarez et al. 1995)	(Vajpeyi and Chandran 2015)	(Jung, Moon et al. 2013)
Substrate	Sludge	Dairy waste	OFMSW	Food waste	Food waste + sludge
Operation	Batch	Continuous	Continuous	Continuous	Batch
Fermentation time (d)	6	NA	NA	NA	NA
Temp (°C)	42	35	37	37	35
pH	5.5-6.5	8.6	6.1	6.5	6.0
HRT (d)*	NA	0.5	6	2	NA
VFA (g COD/L)	7.0	1.6	23.1	10.1	34.0
VFA yield (gCOD/gCODini)	0.20	NA	NA	0.38	NA
Acetic (%)	38	13.1	35.4	51.5	11.6
Butyric (%)	18	20.0	24.8	34.6	62.7
Propionic (%)	14	39.7	15.8	13.8	7.9
Valeric (%)	NA	27.2	19.1	NA	NA
Iso-valeric (%)	11	NA	2.7	NA	NA
Iso-butyric (%)	5	NA	1.9	NA	NA
Caproic (%)	1	NA	NA	NA	NA
Lactic (%)	10ppm	NA	NA	NA	0.8
Ethanol (%)	NA	NA	NA	NA	17.1

*Hydraulic retention time (HRT). ^Solids retention time (SRT).

To date not all the mechanisms determining the product distribution in anaerobic fermentation have been identified (Kleerebezem, Joosse et al. 2015). However, the clear influence of some operation parameters such as pH, retention time, temperature and loading rate on the yield and distribution of VFA achieved by fermentation has been observed.

1.2.1 pH

pH plays an important role in maximizing the volatile fatty acid content in the fermenter. Maximization of VFA accumulation is achieved through reduction in methanogenic activity in the anaerobic fermentation system. Methanogens are extremely sensitive to the pH, preferring a neutral pH and their growth is inhibited under both acidic and basic conditions(Chen, Cheng et al. 2008). Under basic conditions (pH>8), ammonia toxicity caused by the increase in free ammonia concentration results in a decreased methanogen activity and consequently, increased VFA accumulation. Methanogenesis has also been reported to be inhibited under acidic conditions (pH<6.5), however, the specific mechanism of inhibition remains unclear. It has been postulated that a lower pH results in protonation of acetate and leads to a lower substrate availability for acetoclastic methanogens (Ye, Jin et al. 2012). Acetogenesis can also lead to accumulation of high concentrations of VFA and could lower the pH of the system, which can inhibit the growth of methanogens and thus the cleavage of the acetic acid to form methane. The pH of the system is a function of total VFA concentration, alkalinity, CO_2 fraction and the buffering capacity of the system. Nevertheless, in most cases pH is controlled at values around 6.0, where methanogens are inhibited and the production of VFA is maximized (Wang, Yin et al. 2014). It has been reported that for fermentations at pH between 5.0 and 6.5 the main produced

VFA are butyric and acetic acids (Ren, Chua et al. 2007, Wang, Yin et al. 2014, Vajpeyi and Chandran 2015). A recent literature review suggested that in terms of the fermentation pH, acidic neutral and alkaline conditions favored the VFA production from sewage, food waste and sludge fermentation respectively (Lee, Chua et al. 2014). However, a dynamic acidification of fermentations starting at alkaline pH has shown to benefit the formation of VFA when utilizing a variety of substrates including sugars and cheese whey (Davila-Vazquez, Alatriste-Mondragón et al. 2008). In this sense, alkaline pH shocks at startup of uncontrolled-pH sewage fermentation improved the production of VFA, compared to steady alkaline pH conditions, due to effective inhibition of methanogens and extra protein-COD available after degradation at eventually attained acidic pH (Khiewwijit, Temmink et al. 2015). Likewise, alkaline pH imposed at the beginning of food waste fermentation led to greater VFA production by means of enhancing proteins and fats solubilization and hydrolysis, in contrast to fermentation initiated at acidic or neutral pH (Dahiya, Sarkar et al. 2015).

1.2.2 Coupled impact of retention time and temperature

Retention time refers to the time that the waste stream spends in the fermenter. The hydraulic retention time (HRT) of a system is calculated by the equation:

$$HRT = V/Q \quad (11)$$

where, V = reactor volume (L)

Q= Volumetric flow rate (L/day)

Biologically, only those microorganisms whose doubling time is shorter than the retention time can be kept inside the fermenter. Therefore, retention time is one of the best parameters to control or manipulate the microbial populations inside the reactor(Ljupka Arsova 2010). Based on the microbial growth kinetics, shorter residence times (HRT~ 4 days) can cause the washout of methanogens and proliferation of acidogens in the system, resulting in higher VFA concentrations.

However, the VFA yield from fermentation is highly determined by the fraction of biodegradable organic carbon in feedstock available to be hydrolyzed and the rate of hydrolysis. Therefore, the influence of SRT on VFA yield and speciation depends also on the nature of waste being fermented. Commonly, higher SRT values between 8 to 12 d yield greater VFA concentrations when fermenting wastewater sludge at temperatures between 20-25 ºC (Miron, Zeeman et al. 2000, Feng, Wang et al. 2009). However, increasing the temperature during sludge fermentation to 50ºC might accelerate hydrolysis and acidogenesis to maximize VFA productivity at SRT values as low as 5 d (Xiong, Chen et al. 2012). Similarly, shorter SRT ranging 0.25 to 4 d are sufficient when fermenting readily biodegradable substrates such as food waste and carbohydrates, at temperatures between 30-37 ºC (Arooj, Han et al. 2008, Davila-Vazquez, Cota-Navarro et al. 2009, Jiang, Zhang et al. 2013, Wang, Yin et al. 2014). Although operation at higher SRT could lead to increased VFA production, an increased risk of methanogenesis, feedback inhibition by the fermentation products (including hydrogen) must be considered as part of overall process optimization.

1.2.3 Loading rate

Organic loading rate determines the biological conversion capacity of the system. Higher loading rates can result in accumulation of volatile fatty acids in the fermenter slurry and result in lowering of the system pH, which can inhibit the growth of the methanogenic bacteria. It also results in proliferation of acidogenic bacteria, which can further increase the VFA concentration on the system. Thus, keeping the loading rate high in the fermenter operation leads to better yield of VFA. In studies focused on the production of hydrogen, yielding also VFA, the H_2 production rates were proportionally linked to increasing OLR, when fermenting substrates such as glucose, lactose and cheese whey (Van Ginkel and Logan 2005, Davila-Vazquez, Cota-Navarro et al. 2009). However, a recent survey (Lee, Chua et al. 2014), suggested a lack of a systematic impact of organic loading rate on VFA yield and speciation likely because of interacting effects of other factors such as pH, HRT and type of waste being fermented.

1.2.4 Effect of ammonia concentration

Ammonia is produced by the biological degradation of nitrogenous matter in the feedstock, present mostly in the form of proteins and urea. Above a certain threshold, free-ammonia exerts toxic impacts on overall fermentation and digestion processes (Grady, Daigger et al. 2011). Several mechanisms for ammonia inhibition have been proposed, such as a decrease in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme reaction. Ammonium ion (NH_4^+) and free ammonia (NH_3) are the two principal forms of inorganic ammonia nitrogen in aqueous solution. Free ammonia has been suggested to be the main cause of inhibition since it is freely membrane-permeable. The hydrophobic ammonia molecule may diffuse passively into the cell and cause proton imbalance, thus disrupting enzyme activity (Chen, Cheng et al. 2008). Among the microorganisms involved in anaerobic digestion,

methanogens are extremely sensitive to the ammonia concentration with several methanogens being inhibited at an ammonia concentration of 4-6 g/L (Jarrell Kf Fau - Saulnier, Saulnier M Fau - Ley et al.). As ammonia concentrations were increased in the range of 4-5.7 g NH_3–N L^{-1}, acidogenic populations in the granular sludge were hardly affected while the methanogenic population lost 56.5% of its activity(Koster and Lettinga 1988). Thus, when methanogenesis is inhibited by ammonia, the concentration of VFA in the system increases and this leads to a decrease of pH. The decrease of pH partly counteracts the effect of ammonia due to a decrease in the free ammonia concentration(Ljupka Arsova 2010). This phenomenon is called "inhibited steady state".

1.3 VFA to products

Recovery of VFA generated during anaerobic fermentation can be used to valorize and beneficiate the waste feedstocks. The main products obtained from waste-derived VFA, so far, include bioplastics, lipids, biofuels, solvents, electricity and few speciality chemicals. Nonetheless, there is a pressing need for further efforts towards product recovery and utilization to fully realize the potential for organic carbon recovery(Kleerebezem, Joosse et al. 2015).

1.3.1 Bioplastics

Biological production of polyhydroxyalkanoates (PHA) production is one of the several research directions followed for organic carbon recovery from waste streams. An increased awareness on the environmental insults from the use of artificial plastics and sourcing them directly from fossil carbon has encouraged the use of natural biopolymers such as PHA. PHA are biodegradable polyesters accumulated inside microbial cells as storage compound under nutrient (carbon,

nitrogen, phosphorous) limitation. At room temperature PHA are solid, which facilitates separation from the liquid production broth. Similar to petroleum-based plastics, PHA have suitable characteristics for production of thermostable biodegradable plastics or as precursors for chemical synthesis (Chen 2009).

There is a wide variety of PHAs (around 80) that can be produced biologically, with the most common form being polybetahydroxybutyric acid (PHB) and poly-betahydroxyvaleric acid (PHV). The market price of these industrially relevant PHA ranges from approximately US$ 13 to 15 per kg PHA (Moita and Lemos 2012). Recent market analyses have projected that the global demand for PHAs will increase from approximately 10,000 metric tons per year in 2013 to 34,000 metric tons per year in 2018 (Salehizadeh and Van Loosdrecht 2004) translating to an overall market value ranging from US$130 to 510 million per year.

Presently, the high price of biodegradable plastics compared to petroleum-based plastics, urges the need to reduce production costs for PHA. There is considerable effort focused on mixed microbial fermentation on various feedstocks included waste streams as a cost-effective production strategy (Serafim, Lemos et al. 2008, Castilho, Mitchell et al. 2009, Morgan-Sagastume, Hjort et al. 2015). Given that feedstock price could contribute up to 30% of the overall cost of PHA production, the use of low value waste streams as feedstocks for mixed microbial fermentation may be rather favorable (Choi and Lee).

PHA production scheme from wastewater was proposed following a three-stage system composed by 1) production of a VFA-rich stream through wastewater anaerobic fermentation and acidogenesis, 2) selection and enrichment of PHA-storing microorganisms and 3) accumulation of PHA utilizing the VFA-rich stream generated in the first step as substrate.

Finally, produced PHA during accumulation phase is then extracted and purified (Serafim, Lemos et al. 2008).

Extensive reviews of PHA production using mixed microbial culture fermentation have been presented (Dobroth, Hu et al. 2011, Moita and Lemos 2012). PHA yield from mixed culture fermentation can range from 0.08 to 0.4 g PHA/g substrate using controlled substrates like glucose and glycerol. Recent studies on lab-scale fermentation of pure substrates such as acetate or lactate have reported maximum PHA yield of 0.9 g PHA/g VSS (Johnson, Jiang et al. 2009, Jiang, Marang et al. 2012). A direct comparison of PHA yield from some of these studies is precluded by different operating strategies followed therein.

Using real complex substrates maximum PHA accumulation in the range of 0.5 g PHA/ g biomass has been observed recently (Serafim, Lemos et al. 2008). PHA accumulation of 0.75 PHA/ g VSS (Albuquerque, Torres et al. 2010) and 0.77 g PHA/ g dry-biomass have been reported for fermented molasses and fermented paper mill effluents, respectively (Jiang, Marang et al. 2012). Higher PHA yields were correlated to an effective PHA-storing microbial enrichment, with a specific incidence of bacteria related to *Plasticicumulans acidovorans*. Pilot scale production of PHA shown comparable PHA contents of 0.70 g PHA/g VSS (Tamis, Lužkov et al. 2014) and 0.3-0.4 g PHA/ g VSS (Morgan-Sagastume, Valentino et al. 2014, Morgan-Sagastume, Hjort et al. 2015).

In terms of operation parameters, it was found that feeding strategy from pulse to continuous, increased the accumulation of intracellular PHA and promoted the diversification of the obtained PHA, when utilizing a mixture of synthetic VFA (Albuquerque, Martino et al. 2011). Recently it was found that in cultures dominated by *Plasticicumulans acidovorans* butyrate used as sole substrate led to an increase of PHA yield, compared with the cases when acetate alone and

acetate/butyrate combination were used, explained by the cellular lowered need of energy to transform butyrate to PHA (Marang, Jiang et al. 2013).

1.3.2 Hydrogen

Many studies have focused on the production of not only VFA but also dihydrogen (H_2), concurrently during fermentation. In general, fermentation of wastes rich in carbohydrates such as glucose and sucrose result in ,higher hydrogen and VFA yields (Kim and Kim 2012, Wu, Chu et al. 2013). However, hydrogen production has been also been observed during fermentation of waste streams including dairy waste (Davila-Vazquez, Cota-Navarro et al. 2009), food waste (Dahiya, Sarkar et al. 2015) and food waste combined with activated sludge (Jung, Moon et al. 2013). The concurrent preponderance of acetate and butyrate over other VFA has been regarded as an indicator of suitable conditions for hydrogen production during anaerobic fermentation of sugar-rich substrates. In contrast, the presence of other VFA such as propionate and short chain alcohols such as ethanol suggests consumption of a fraction of the produced hydrogen towards the formation of more reduced end products (Hawkes, Dinsdale et al. 2002). Recently, a theoretical analysis taking into account current prices of technology, labor and availability of feedstock, showed the feasibility of producing hydrogen from food waste at industrial scale. Expectedly, it was found that the hydrogen price was the main parameter affecting the projected profit, which could be guaranteed sustained by increasing H_2 prices (Han, Fang et al. 2016).

Hydrogen can also be produced from electrohydrolysis of VFA by means of applying a low electrical potential (< 0.2 V) though electrodes immersed in a VFA solution in microbial electrolysis cells (MECs). By providing an appropriate voltage, electrons from a metal electrode (anode) are transported to reduce water or VFA protons at the cathode to yield hydrogen.

Following a similar mechanism it is feasible also to recover electricity from VFA rich streams, by using microbial fuel cells (MFCs) where a biofilm formed at the anode transforms organic compounds to protons, electrons and CO_2, electrons are transported through a circuit, while protons diffuse through a membrane to an aerobic chamber where oxygen, electrons and protons are combined at the cathode producing water and driving an electrical current. The main challenges for an extensive application of MECs and MFCs are the expensive electrodes and the low current or hydrogen production achieved from waste-generated VFA. The need for optimizing the distribution of VFA to a predominant acetate solution has been identified as an important strategy to enhance the performance of both MECs and MFCs (Lee, Chua et al. 2014).

1.3.3 Lipids

The global production of biofuels such as biodiesel has tripled in the last decade leading to a closer evaluation of the resource footprint. Lipids are among the primary substrates for biodiesel production. The biggest cost of biodiesel production is usually the lipid feedstock, which can account for 40-80% of the total cost (Mondala, Liang et al. 2009, Leung, Wu et al. 2010). Biodiesel production entails converting the lipid content of a feedstock into fatty acids alkyl (methanol) esters (FAMEs). Production of FAMEs from biosolids or wastewater organics can be approached either by the direct conversion of the lipid fraction or select free fatty acids to FAMEs via catalyzed transesterification or esterification (Chongkhong, Tongurai et al. 2009, Mondala, Liang et al. 2009). A far more viable strategy is to actually convert the non-lipid fraction in these feedstocks through VFA as intermediates using the oleaginous organisms described below.

The biological conversion of VFA into long chain fatty acids or lipids is accomplished by oleaginous microorganisms, which can biologically convert a wide array of carbon sources into lipids and can accumulate lipids. Lipogenesis in these microorganisms is induced upon limitation or exhaustion of a co-nutrient, typically nitrogen or phosphorus. Under such conditions, the consumed COD is channeled toward intracellular carbonaceous storage compounds, rather than to more cellular biomass.

Among oleaginous microorganisms yeast *Cryptococcus curvatus* has the ability to accumulate storage lipids up to 25%, on a dry weight basis, when grown with glycerol as carbon source under limited nitrogen conditions (Meesters, Huijberts et al.). Although *Cryptococcus curvatus* showed the capability of storing up to 65% of lipids when fed with pure acetate at pH 7.0 and 30 °C, the yield with a VFA-containing fermentation effluent was only 13%, attributed to the high nitrogen content culture broth (Chi, Zheng et al. 2011). Another yeast strain, *Cryptococcus albidus*, was used recently to achieve a 28% of intracellular lipid accumulation using a mixture of synthetic VFA as the carbon source, at a COD/N ratio of 25:1, while close to 15% accumulation was attained when grown on VFA from food waste fermentation (Vajpeyi and Chandran 2015). Recently it was suggested that lipid content of oleaginous microorganisms could be further increased through metabolic engineering techniques (Liang and Jiang 2013)

1.3.4 Microorganisms available for lipid production

Although bacteria, microalgae, fungi, and yeast can all produce and store lipids (Table 3), not all of them are suitable for enhanced lipid production. Yeast and fungi are considered as the most favorable oleaginous microorganisms since bacteria on average accumulate lower amount of lipids than microalgae and yeasts (Table 3).. Furthermore, the relative fatty acid composition of

the lipids accumulated by oleaginous yeast fungi, and microalgae (Table 3), is generally more suitable for downstream use in biodiesel production (Meng, Yang et al. 2009). The fatty acid composition of lipids varies widely with microorganisms, culture conditions and carbon source (Table 4). Therefore, it is essential that the microorganism chosen for lipid production is able to metabolize the carbon source for growth and lipid storage and the lipids stored should have relatively low levels of saturated and poly unsaturated fats and high levels of monounsaturated fats(Hoekman, Broch et al. 2012). The oleaginous yeast *Cryptococcus albidus* can accumulate storage lipids up to 65% on a dry weight basis when it grows under limited nitrogen conditions (Liang and Jiang). *Cryptococcus albidus* was chosen for our experiments since it can efficiently consume VFA for growth (specific VFA uptake rate sVUR= 88.7±3.8 mg-COD/g-cell/h). The maximum intracellular lipid accumulation was (28.33±0.74% w/w) with a maximum specific growth rate (μ_m = 0.04±0.00 h^{-1}) at a COD:N ratio of 25:1. Additionally, the microbial lipids obtained from the VFA contained about 70% unsaturated fats with a linoleic acid (C18:2) content at about 15%, which is very similar to the commercial feedstock used for biodiesel production (~75% unsaturated fats in jatropha oil (Berchmans and Hirata 2008)).

Table 3 Intracellular lipid content of some oleaginous microorganisms

Microorganism	Culture conditions/ Carbon source	Lipid content (% dry weight)	Reference
MICROALGAE			
Chlorella protothecoides	Heterotrophic	49	(Gao, Zhai et al. 2010)
Chlorella sp.	Phototrophic	32.6–66.1	(Hsieh and Wu 2009)
Chlorella vulgaris	Phototrophic	20–42	(Feng, Li et al. 2011)
Dunaliella sp.	Phototrophic	12.0–30.12	(Araujo, Matos et al. 2011)
Haematococcus pluvialis	Phototrophic	15.61–34.85	(Damiani, Popovich et al. 2010)
Neochloris oleoabundans	Phototrophic	7–40	(Li, Horsman et al. 2008)
*Pseudochlorococcum*sp.	Phototrophic	24.6–52.1	(Li, Han et al. 2011)
YEAST			
Cryptococcus albidus	VFA	28.8	(Vajpeyi and Chandran 2015)
Lipomyces starkeyi	Glucose and xylose	61	(Zhao, Kong et al. 2008)
*Rhodosporidium toruloides*Y4	Glucose (Batch culture)	48	(Li, Zhao et al. 2007)
Rhodotorula glutinis	Monosodium glutamate wastewater	20	(Xue, Miao et al. 2008)
Trichosporon fermentans	Glucose	62.4	(Zhu, Zong et al. 2008)
Yarrowia lipolytica	Industrial glycerol	43	(Papanikolaou and Aggelis 2002)

MOLD

Cunninghamella echinulata	Xylose	57.5	(Fakas, Papanikolaou et al. 2009)
Mortierella isabellina	Xylose	65.5	(Fakas, Papanikolaou et al. 2009)
Mortierella isabellina	High glucose	50–55	(Papanikolaou, Komaitis et al. 2004)
Mucorales fungi	Sunflower oil	42.7–65.8	(Čertík, Baltészová et al. 1997)

BACTERIA

Acinetobacter baylyi	Sodium gluconate and glycerol	12.4	(Santala, Efimova et al. 2011)
Alcanivorax borkumensis	Pyruvate	23	(Kalscheuer, Stöveken et al. 2007)
Mycobacterium tuberculosis	Limiting nutrient	11.9	(Bacon, Dover et al. 2007)
Nocardia globerula	Pristine and acetate	49.7	(Alvarez, Souto et al. 2001)
Rhodococcus opacus	High glucose	38	(Kurosawa, Boccazzi et al. 2010)

Table 4: Relative fatty acid composition of different oleaginous microorganisms classes

Microorganisms	Relative fatty acid (% of total lipid)					
	Palmitic acid [C16:0]	**Palmitoleic acid [C16:1]**	**Stearic acid [C18:0]**	**Oleic acid [C18:1]**	**Linoleic acid [C18:2]**	**Linolenic acid [C18:3]**
Microalgae	12–21	55–57	1–2	58–60	4–20	14–30
Yeast	11–37	1–6	1–10	28–66	3–24	1–3
Fungi	7–23	1–6	2–6	19–81	8–40	4–42
Bacterium	8–10	10–11	11–12	25–28	14–17	–

1.3.5 Biochemistry of lipid production

De novo synthesis of fatty acid (FA) takes place in the cytosol and is followed by elongation and desaturation in the endoplasmic reticulum(Klug and Daum). The initial step of FA synthesis is catalyzed by acetyl-CoA-carboxylase (EC 6.4.1.2). The elongation of carbon chain of fatty acids is mainly dependent on the reaction of two enzyme systems, acetyl-CoA carboxylic enzyme (ACC) and fatty acid synthase (FAS) (EC 2.3.1.85) in most microorganisms.

Acetyl-CoA is the precursor for FA synthesis in the cells. In case of different VFA, acetate can be directly converted to acetyl-CoA and butyrate undergoes β-oxidation to yield two acetyl-CoA molecules. Propionate, being an odd chain acid cannot directly undergo β-oxidation and it must

be converted to propionyl-CoA and enter the TCA cycle via methlymalonyl-CoA inter-conversion to succinyl-CoA (figure 2)(Fradinho, Oehmen et al. 2014).

The first step is of FA synthesis is the irreversible formation of malonyl-CoA from acetyl-CoA catalyzed by ACC. Once malonyl-CoA is synthesized, it is transferred by malonyl-CoA:ACP transacetylase (MAT), one of the fatty acid synthase (FAS) multi-enzymatic complex subunits, to form malonyl-acyl-carrier protein (malonyl-ACP). The FAS transfers the malonyl moiety to acyl-carrier protein (ACP) to use it as a carbon source for the synthesis of long chain fatty acids, mainly C16 and C18, like palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), oleic acid (C18:1) and linoleic acid (C18:2). Each cycle of C2 addition is initiated by a reaction catalyzed by a β -ketoacyl-ACP synthase (KAS) and involves the condensation of a malonyl-ACP with an acyl acceptor. At last, acyl-ACP-thioesterase (FAT) cleaves the acyl chain and liberates the fatty acid(Liang and Jiang). The overall reaction for the synthesis of palmitate from acetyl-CoA can be considered in two parts. First, the formation of sever malonyl-CoA molecules:

$$7\ \text{Acetyl-CoA} + 7CO_2 + 7\text{ATP} \rightarrow 7\ \text{Malonyl-CoA} + 7\text{ADP} + 7P_i$$

followed by seven cycles of condensation and reduction:

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \rightarrow$$

$$\text{palmitate} + 7CO_2 + 8\text{CoA} + 14\ \text{NADP}^+ + 6H_2O$$

1.3.6 Metabolic regulation of microbial lipids in oleaginous microorganisms

It is of keen interest to determine the metabolic bias in carbon uptake exhibited by oleaginous microorganisms. The response of *C. albidus* to nutrient limitation can be explained by certain key metabolic reactions involved in carbon and nitrogen assimilation. Following nitrogen exhaustion, the enzyme adenosine monophosphate (AMP) deaminase (EC 3.5.4.6), which catalyzes the conversion of AMP to inosine monophosphate (IMP) and ammonium (Figure 2), is activated to scavenge intracellular nitrogen(Ratledge 2002).

$$AMP \rightarrow IMP + NH_4^+$$

This depletion of AMP causes allosteric inhibition of the activity of the enzyme isocitrate dehydrogenase (IDH, EC 1.1.1.41), which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate during the tricarboxylic acid (TCA) cycle (Ratledge 2004). α -ketoglutarate is a nitrogen transporter and forms the backbone of the glutamate family of amino acids and is essential for nitrogen assimilation in the cells. At limiting nitrogen concentrations, the flux through the TCA cycle slows down, resulting in buildup of citrate inside the mitochondrion(Liu, Zhao X Fau - Wang et al. 2011). Consequently, citrate is transported outside the mitochondria and is cleaved by the enzyme ATP: Citrate lyase (ACL, EC 2.3.3.8) to form acetyl co-A and the excess acetyl co-A is directed towards fatty acid synthesis for storage (Ratledge 2004).

$$Citrate + ATP + CoA \rightarrow Acetyl\text{-}CoA + Oxaloacetate + ADP + P_i$$

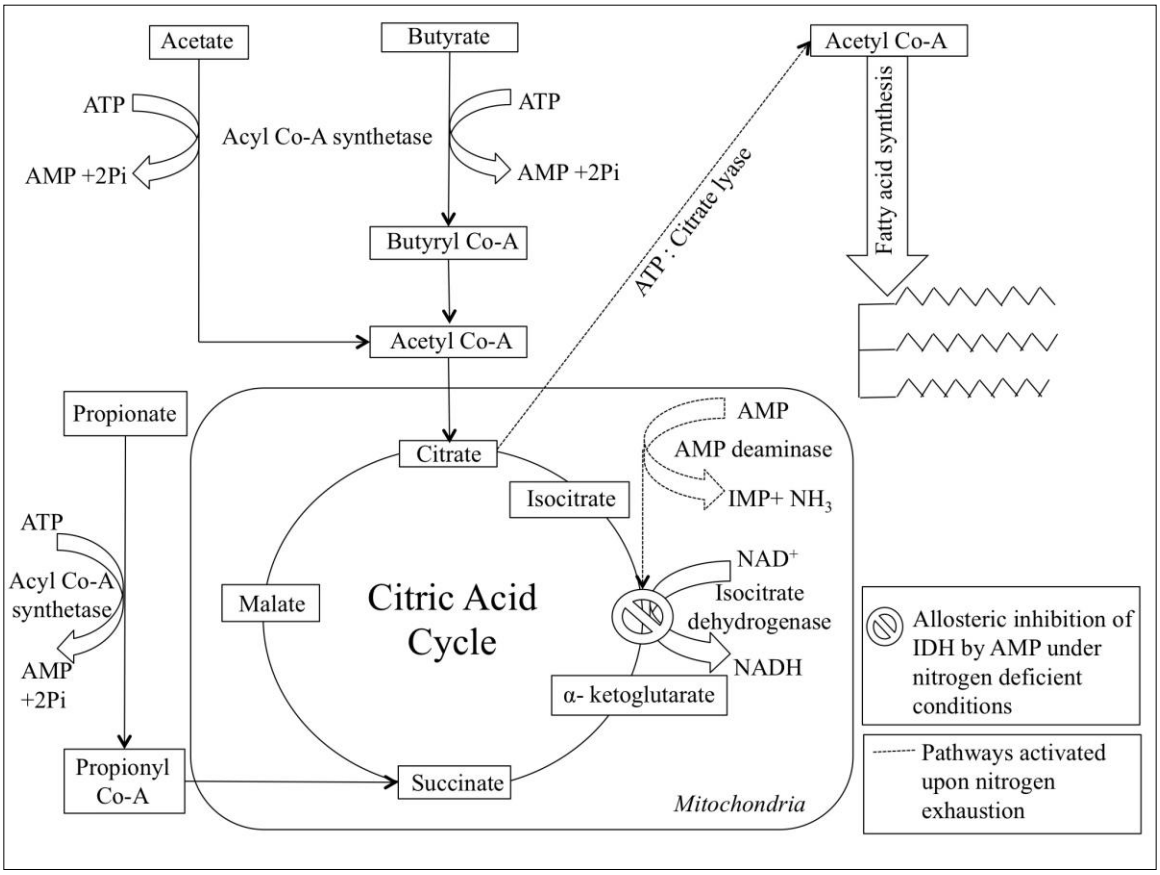

Figure 2 Biochemical conversions of various volatile fatty acids under nitrogen sufficient and deficient conditions. Adapted from (Zheng, Chi et al. 2012) with some modifications.

1.4 Factors affecting lipid accumulation by *Cryptococcus albidus*

1.4.1 Initial VFA concentration

In order to achieve high cell densities and lipid storage, it is essential to use high initial VFA concentration. However very high initial VFA concentration (>5 g/L) may have an inhibitory effect on the cell growth and the lipid accumulation(Fei, Chang et al. 2011). In our experiments,

when the initial VFA concentration was increased to 10,000 mg COD/L, no cell growth or lipid accumulation was observed presumably due to substrate inhibition on account of toxicity of acetic acid at higher concentrations(Vajpeyi and Chandran 2015). Inhibition of organic acids on cell growth occurs due to chemical interference with the membrane transport of the phosphates. This results in greater expenditure of ATP and, consequently lipid accumulation would also be inhibited by very high initial VFA concentration(Georgina Rodrigues 2000).

1.4.2 Relative VFA composition

Lipid content of the cells can also be sensitive to the relative VFA composition in the medium. Lipid yield coefficient of *C. albidus* has been found to increase significantly for the VFA mixtures containing higher percentage of acetic acid(Fei, Chang et al. 2011), presumably due to preference for acetic acid over other VFA(Lim, Kim et al. 2008). In our experiments, we observed that acetate was completely exhausted by the end of the exponential growth phase and propionic and butyric acid were utilized only after the depletion of acetate. Slower consumption rates of longer chain acids compared to acetate may be attributed to their different metabolic fate after intake. Propionate, an odd chain carboxylic acid, cannot be cleaved directly to acetyl-CoA and it must be converted to propionyl-CoA and enter the TCA cycle via methlymalonyl-CoA inter-conversion to succinyl-CoA. The rate of propionate metabolism is therefore likely controlled by the rate of its decarboxylation of acetyl-CoA. In case of butyrate, the higher number of biochemical transformations that it must undergo including β-oxidation to acetoacetyl-CoA and then further cleavage to acetyl-CoA may be the cause of its slower uptake (Vajpeyi and Chandran 2015).

1.4.3 COD/N ratio

The relative initial carbon to nitrogen ratio (COD:N) has been reported to affect the extent of intracellular lipid accumulation(Immelman, du Preez et al. 1997). Optimal nitrogen concentration varies with the oleaginous species but lipid accumulation is always high when nitrogen is present as the growth limiting substrate. Upon nitrogen exhaustion, the cell proliferation stops and the lipids are stored in the existing cells, thus yielding higher lipid accumulations in cells.

1.5 Microbial lipids as a feedstock for biodiesel production

1.5.1 Fatty acid composition of the lipids for biodiesel production

The composition of fatty acids plays a crucial role in the quality of biodiesel that can be produced. Unsaturated fats are generally preferred for biodiesel production since they have a lower melting point and consequently the biodiesel will have lower gel point and would have better cold flow properties i.e. the tendency of fuel to solidify at lower temperatures. However, excessive unsaturated fats are more prone to oxidation and rancidification and the biodiesel made from such fats would usually require an oxidative stabilizer to be used safely as fuel (Atadashi, Aroua et al. 2010). To achieve the best compromise between the cold flow properties and the oxidative stability i.e. the susceptibility of unsaturated fats to self-oxidation, the optimum

feedstock should have relatively low levels of saturated and poly unsaturated fats and high levels of monounsaturated fats(Hoekman, Broch et al. 2012).

1.6 Research hypothesis and objectives

The overall objective of this research was to develop a VFA based resource recovery platform for biological transformation of organic waste into lipids and to utilize –omics based studies to understand the biochemical basis of lipid production to maximize the growth kinetics and lipid productivity. Accordingly, the following hypotheses were proposed:

1. Nitrogen limitation can result in enhanced lipid accumulation in *C. albidus:* Nutrient limitation induced stress has been known to trigger carbon storage in different forms including glycogen, polyhydroxyalkanoates and lipids by various microorganisms. We propose that nitrogen limitation in cultures *C. albidus* can trigger enhanced lipid accumulation.
2. Nitrogen limitation may also impact the quality and chemical composition of lipids: Biochemical reduction of palmitic acid for production of unsaturated fatty acids is endergonic and requires reduced equivalents such as NADPH. Therefore, we propose that the chemical composition of microbial lipids may also be affected by the nitrogen availability due to its impact on cellular energy metabolism.
3. VFA generated from fermentation might contain inhibitory compounds which can negatively impact growth: While VFA are the dominant form of carbon during anaerobic fermentation, the fermentate may also contain additional fermentation products such as short chain alcohols or other dicarboxylic acid which may have an adverse inhibitory effect on the growth and lipid accumulation by *C. albidus.* Furthermore, biological

hydrolysis of nitrogenous compounds may release ammonium into fermentate, thereby altering the intended COD:N ratio and partially relieving the nitrogen limitation.

4. Lipid accumulation by *C. albidus* under nitrogen limitation is a consequence of a broader system-wide stress response, which causes overexpression of FAS: Nitrogen limitation in *C. albidus* results in carbon flux redistribution from oxidative to storage pathways, causing an overexpression of the fatty acid synthase enzyme system and resulting in excessive lipid production.
5. Reduced carbon flux through TCA leads to buildup of carbon inside the mitochondria, which is then channeled to fatty acid biosynthesis: Based on glucose based study on lipid accumulation, the slowdown of tricaboxylic acid (TCA) cycle under nitrogen limitation results in a buildup of pyruvate inside the mitochondria, which is transported to cytosol as citrate and cleaved back to acetyl-CoA to be channeled towards fatty acid biosynthesis.

Accordingly, the following objectives were used to test the hypotheses:

1. Test the impact of nitrogen limitation on lipid accumulation by *C. albidus.*
2. Test the lipid speciation under different COD:N ratios.
3. Compare the growth and lipid accumulation under synthetic and food waste derived VFA.
4. Compare the transcriptome and proteome of *C. albidus* under nitrogen limited and nitrogen sufficient growth conditions.
5. Propose a carbon cycling model using the –omics data to assess the carbon inventory of the cell under nitrogen limitation.

This dissertation consists of five chapters.

Chapter 1 presents an introduction to the concept and the necessity of resource recovery and then presents an overview of biological methods available to attain resource recovery from organic waste. It also discusses the current efforts into biological upcycling of those VFA into chemicals and products of greater commercial value such as lipids, bioplastics and hydrogen gas.

Chapter 2 presents the feasibility of lipid production from VFA using the oleaginous yeast *C. albidus*. It further characterizes the optimum growth conditions needed to maximize the lipid accumulation (quantity) and attain the most ideal lipid composition (quality).

Chapter 3 presents the complete genome sequence of *C. albidus* and discusses the potential biotechnological application of *C. albidus* through and evaluation of its genome and its genetic capabilities.

Chapter 4 presents the transcriptomic and proteomic responses of *C. albidus* to understand the biochemical basis of its oleaginity. It represents the first attempt to evaluate the proteomic and transcriptomic changes in conjunction to allow for a much deeper understanding of the pathways and factors governing the lipid production in *C. albidus*.

Chapter 5 discusses the broader societal need for this research and potential challenges to its scale up. It also presents future perspectives and potential application of VFA for production of several other industrially important chemicals through a thorough evaluation of microorganisms and biological pathways available for such conversions.

CHAPTER 2

MICROBIAL CONVERSION OF SYNTHETIC AND FOOD WASTE DERIVED VOLATILE FATTY ACIDS TO LIPIDS

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2.1 Introduction

During the past decade, global biofuel production has tripled. As a result, biofuels have come under particular scrutiny owing to the significant resource stress they exert in terms of substrates, including agricultural products and water. Biofuels such as bio-ethanol and biodiesel have contributed to an increase in the food prices either directly through use of food crops towards bio-ethanol production or indirectly through use of agricultural land mass for cultivation of oil crops for biodiesel production (Bouriazos, Ikonomakou et al. 2014). Accordingly, alternate substrates for improving the longer term sustainability of biofuel production need to be explored. In this study, we evaluated the use of volatile fatty acids (VFA), for lipid production (and subsequent conversion to biodiesel) by oleaginous (oil-producing or accumulating) microorganisms. Oleaginous microorganisms can biologically convert a wide array of carbon sources into lipids and can accumulate lipids in excess of 65% of their dry cell mass.

Oleaginous microorganisms have been employed for lipid production with a wide variety of commercial carbon sources including glucose (Hansson and Dostálek 1986), starch, sucrose, xylose (Li, Liu et al. 2010) ethanol and lactose (Papanikolaou, Diamantopoulou et al. 2010). However, none of these strategies have so far proven viable for commercial biodiesel production either due to the high cost of the substrate, glucose, which accounts for about 80% of the total production cost (Fei, Chang et al. 2011). Among alternate less expensive carbon sources for lipid production are volatile fatty acids (VFA). VFA are especially attractive, since they can be produced by anaerobic fermentation of different nonedible feed stocks such as organic waste streams including waste activated sludge (Zhang, Chen et al. 2009), food waste (Lim, Kim et al. 2008), dairy waste (Demirel and Yenigun 2004), municipal solid waste (Sans, Mata-Alvarez et al. 1995) among others. Traditionally, anaerobic treatment of organic waste has been focused on

biogas production through methanogenesis. Alternately, VFA produced at wastewater treatment plants have mainly been considered for use in biological nutrient removal operations. Herein, we explored the possibility of re-directing the VFA to lipids by the oleaginous yeast *Cryptococcus albidus,* for downstream use in biodiesel production.

The principal objectives of this study were to determine lipid and cell yields and specific growth rates of *C. albidus* batch cultures subjected to various degrees of nitrogen limitation and different carbon sources and to characterize the speciation of the accumulated lipids and evaluate the potential for use as a commercial biodiesel feedstock.

2.2 Materials and Methods

2.2.1 Batch cultivation of C. albidus cultures

Cryptococcus albidus (ATCC 10672) was obtained from American Type Culture Collection (ATCC, Manassas, VA). *C. albidus* cultures were propagated through a monthly subculture on liquid yeast mold (YM) broth (BD Difco, Sparks, MD). The nutrient medium for all the experiments contained, per liter: 3g KH_2PO_4, 1g $MgSO_4 \cdot 7H_2O$, 15 mg $FeCl_3 \cdot 6H_2O$, and 7.5 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $CuSO_4.5H_2O$ (ACS grade chemicals, Fisher Scientific, Pittsburgh, PA). Ammonium chloride (NH_4Cl) was used as the nitrogen source and VFA were used as the sole carbon and energy source. All the equipment and solutions for microbial cultivation were steam sterilized at 121^{o}C and 15 psi for 15 min. VFA were sterilized using a 0.2 μm syringe filter (VWR scientific, Bridgeport, NJ) and added to the autoclaved medium upon cooling to prevent losses by volatilization. 24-hour-old *C. albidus* cultures in the late log phase grown on YM broth were used as the inoculum (10% v/v). Batch cultivation was performed in aerobic 6.0 L glass reactors (Bellco glass, Vineland, NJ) at a temperature of 23°C to stationary phase (96 hours, data not shown). Reactor pH was measured using an autoclavable pH electrode (Cole Parmer, Vernon

hills, IL) and automatically controlled (Jenco 3676, San Diego, CA) at 6.0 ± 0.1 through automated addition of 2.0 mol/L HCl or NaOH solutions. The above temperature and the pH were chosen based upon a previous study by (Fei, Chang et al. 2011). Sterile lab air (filtered through 0.2 um filter, Millipore MTGR 05010 Bedford, MA) was supplied at 360 L/h to keep the dissolved oxygen concentration near saturation.

2.2.2 Effect of nitrogen concentration during batch growth

The initial VFA concentration was kept constant at 6500 mg COD/L and five different initial nitrogen concentrations of 1300, 260, 130, 52 and 26 mg NH_3-N/L were tested which correspond to an initial COD:N ratio of 5:1, 25:1, 50:1, 125:1 and 250:1 respectively.

2.2.3 Effect of different carbon sources and VFA concentrations on batch growth

VFA produced from anaerobic fermentation of food waste and a synthetic 5:1:4 VFA mixture of acetic: propionic: butyric acid (expressed as COD, which was reflective of fermenter output) were used as carbon and energy sources in distinct experiments. The impact of nitrogen limitation on batch cultures was tested using three different initial synthetic VFA concentrations of 2600, 6500 and 10,000 mg COD/L, while keeping the initial ammonia-nitrogen concentration at 260 mg-N/L.

2.2.4 Chemostat cultivation

Duplicate chemostats (V = 6L) were operated at a hydraulic retention time (HRT) of 3 days. The influent VFA and nitrogen concentrations were 6500 mg COD/L and 260 mg-N/L corresponding to the COD:N ratio of 25:1 obtained from batch cultivation described above. The remaining growth medium composition and all other operation conditions were same as for the batch cultures.

2.2.5 Cell growth and reactor performance measurement

20 mL samples were periodically withdrawn from the batch and chemostat reactors using a sterile sampling port assembly. Cell concentrations were measured as absorbance of the culture broth at 600 nm ($OD_{600,}$ Genesys-20 spectrophotometer, Thermoscientific, Waltham, MA). Cell samples were centrifuged (10,000 rpm, 9133 x g, 10 min) and filtered using a 0.2 μ_m syringe filter (VWR scientific, Bridgeport, NJ) for VFA and nitrogen measurements. VFA concentrations were determined using colorimetry (Hach volatile acids reagent set (2244700). Ammonia concentrations were measured using an ion-selective electrode (Thermofisher scientific, Waltham, MA).

For the batch cultures, maximum specific cell growth rate (μ_m), the specific VFA and nitrogen uptake rates (sVUR and sNUR) and the relative nutrient uptake ratio (ΔCOD:ΔN) were calculated using following equations:

$$\ln\left(\frac{ODexp}{OD_o}\right) = \mu_m * t_{exp} \qquad \text{Equation (1)}$$

$$\text{sVUR} = \frac{1}{\text{X}_\text{f}} * \frac{\text{COD}_\text{o} - \text{COD}_\text{f}}{\text{t}_f} \qquad \text{Equation(2)}$$

$$\text{sNUR} = \frac{1}{\text{X}_\text{f}} * \frac{\text{N}_\text{o} - \text{N}_\text{f}}{\text{t}_f} \qquad \text{Equation (3)}$$

$$\frac{\Delta\text{COD}}{\Delta\text{N}} = \frac{\text{COD}_\text{o} - \text{COD}_\text{f}}{\text{N}_\text{o} - \text{N}_\text{f}} \qquad \text{Equation(4)}$$

where OD, COD, N, X and t represent the optical density at 600nm, VFA concentration in mg COD/L, ammonia concentration (mg-N/L), cell concentration (mg/L) and time of incubation, respectively. The subscripts 'o', 'exp' and 'f' represent the concentrations at the initiation, end of exponential phase and end of batch incubation, respectively. The end of exponential growth phase was inferred visually from the time series profiles of OD during batch growth. The end of

the incubation was defined as the time point past which VFA, NH_3-N and cell densities (as OD) were nearly invariant.

VFA composition was determined using gas chromatography with flame ionization detection (GC-FID) with MXT-1 column, 60m, 0.53mm ID (Restek , Bellfonte, PA) . The flame ionization detector was operated at 240^{o}C with an initial temperature of 150^{o}C for 5 min subsequently ramping up 10^{o}C/min to 240^{o}C.

2.2.6 Anaerobic fermentation of food waste

Anaerobic fermentation was performed in a 6.0 L-jacketed vessel (Bellco glass, Vineland, NJ) maintained at 37°C by recirculating water from a hot water bath. The pH in the fermenter was adjusted at 6.5 by automated addition of 1:1 1M $NaHCO_3$ and 1M NaOH solution. The anaerobic fermenter was operated at a HRT of 2 days to limit methanogenesis and enhance production of VFA. To prepare the feed, food waste was obtained from the University cafeteria. The total solid (TS) and total volatile solid (TVS) content in the raw undiluted food waste was determined gravimetrically by drying 200 mg food waste in a furnace (Thermoscientific, Asheville, NC) at 105^{o}C and 550^{o}C respectively until no further decrease in the weight was observed. The chemical parameters including the total and soluble COD (tCOD and sCOD), total nitrogen (TN) and VFA were determined using colorimetry (Hach kits 2125915, 2672245 & 2244700, Loveland, CO). The COD loading rate of the fermenter were 13000 mg COD/L/day. The food waste was blended in a mixer and suitably diluted using de-ionized (DI) water to achieve a final concentration of 26000 mg COD/L. The fermentate was centrifuged at 10,000 rpm for 20 min (Beckman Coulter, Avanti J 26, Pasadena, CA) and the supernatant was collected and sterilized using a 0.2 μ_m syringe filter (VWR scientific, Bridgeport, NJ) to be used as the carbon source for *C. albidus* cultivation.

2.2.7 Lipid extraction and determination of fatty acid composition

Lipids were extracted according to the Folch wash method(Folch, Lees et al. 1957) with some modifications. Briefly, cell pellet was lysed by ultrasonication at 70 Hz for 10 min. followed by mechanical disruption using 0.4-0.5 mm glass beads for 30 min on a vortex mixer. Lipids were extracted using a 2:1 chloroform:methanol mixture, the purified chloroform layer was evaporated to dryness under a N_2 stream. The lipids were immediately weighed and transesterified using 12% boron trifluoride (BF_3) and the fatty acid methyl esters (FAME) components were analyzed using SRI instruments 8610C GC-FID (Torrance, CA) with MXT-WAX column, 30m, 0.53mmID (Restek , Bellfonte, PA). The injector temperature was 40^{o}C, ramped at 6^{o}C to 200^{o}C and then to 250^{o}C at 15^{o}C/min.

2.3 Results and discussion

2.3.1 Effect of nitrogen concentration on the lipid and biomass yield

The relative initial carbon to nitrogen ratio (COD:N) has been reported to affect the extent of intracellular lipid accumulation(Immelman, du Preez et al. 1997). In our experiments, nitrogen was considered growth limiting if the supplied nitrogen was exhausted before the VFA. Herein, nitrogen limiting conditions (NH_3-N $\leq$ 260 mg/L, COD:N $\geq$ 25:1) resulted in statistically higher intracellular lipid content (p=0.03). The maximum intracellular lipid content (28.81±2.06%) was achieved at an excessively high COD:N ratio of 250:1, but this occurred at the expense of a lower kinetics (μ_m = 0.02±0.00 h^{-1}) (Table 5). In contrast, when excess nitrogen (COD:N = 5:1) was fed, the growth kinetics (0.04±0.00 h^{-1}) were higher (p=0.02) but the intracellular lipid content was significantly lower (19.59±0.16%, p=0.03). Of the different COD:N ratios tested, a COD:N ratio of 25:1 was the most favorable for maximizing both intracellular lipid accumulation (28.33±0.74% w/w) and growth kinetics (0.04±0.00 h^{-1}). To date, this is the

highest reported degree of lipid accumulation in *C. albidus* using VFA as the carbon source. Previous studies have reported similar optimal COD:N ratios in different oleaginous species under diverse growth conditions. COD:N ratios of 24:1, 26:1 and 27:1 were reported for optimal lipid production by the oleaginous yeast *Rhodosporidium toruloides* on distillery wastewater (Ling, Nip et al. 2014), oleaginous fungus *Cunninghamella echinulata* on tomato hydrolysate waste (Fakas, Papanikolaou S Fau - Galiotou-Panayotou et al. 2008) and oleaginous fungi *Mucor circinelloides* on acetate (Immelman, du Preez et al. 1997) respectively. These results suggest that the metabolic pathways for lipid accumulation across different oleaginous species might be triggered under similar degree of nutrient limitation, despite differences in their stoichiometric demands.

The different COD:N ratios also impacted the relative COD and nitrogen uptake ratio, ΔCOD/ΔN (w/w, Equation 4). At non-limiting feed NH_3 concentrations (COD:N =5:1), the relative uptake ratio (ΔCOD/ΔN) was 33.10±7.24 g COD:gN. Based on the empirical formula of $CH_{1.65}O_{0.54}N_{0.14}$ (von Stockar and Liu 1999), the stoichiometric COD:N composition of yeast cells is 16.34 g COD:g N). Therefore, under non-limiting nitrogen supply, the fraction of carbon supplied, which is assimilated is roughly 51% (COD:COD), well in keeping with the calculated theoretical yield of microbial growth on acetate (50% COD/COD for a electron capture efficiency of 50%) (Bruce E. Rittmann 2001).

However, at progressively nitrogen limiting conditions (increasing initial COD:N ratios), the cells exhibited an increase in the relative ΔCOD/ΔN uptake ratio. At an initial COD:N ratio of 25:1, the ΔCOD/ΔN increased by 141% to 79.84±8.76 w/w (p=0.03) and even further to 186.69±28.31 w/w for an initial COD:N ratio of 250:1. These results show that *C. albidus* cultures responded to nitrogen limitation by taking up disproportionately higher levels of organic

carbon, potentially for intracellular storage. This finding was supported further by the COD and nitrogen based biomass yield coefficients ($Y_{X/COD}$ and $Y_{X/N}$) obtained from the batch experiments. Essentially, under different degrees of nitrogen limitation, the extent of COD consumption (5639.29 ± 483.09 mg/L) and observed yield of biomass per gram of COD consumed ($Y_{X/COD}$ = 0.19 ±0.01 g/g, which includes biomass carbon and storage carbon) was found to be statistically invariant (Figure 3). In contrast, the yield coefficient $Y_{X/N}$ was 5.5 fold higher for a feed COD:N ratio of 250:1, compared to 5:1 (Figure 3). Similar observations were reported previously in nitrogen starved and nitrogen sufficient cultures of *Chlorella vulgaris* using a range of nitrate concentrations as the nitrogen source. It was observed that progressive nitrogen limitation did not cause a proportional decrease in the biomass concentration but the yield coefficient, $Y_{X/N}$, increased from 1.6 to 10 when initial assimilative N-source (nitrate, in that case) was decreased from 2000 mg-N/L to 40 mg-N/L(Griffiths, van Hille Rp Fau - Harrison et al. 2014). These trends in $Y_{X/N}$ suggest that under nitrogen limitation and exhaustion, the same unit carbon was consumed. However, the consumed carbon was channeled towards intracellular carbonaceous storage compounds, rather than to more cellular biomass. Therefore, *C. albidus* responds to nitrogen limitation by altering the relative substrate uptake efficiency ($\Delta COD/\Delta N$) and increasing intracellular carbon storage including lipid storage.

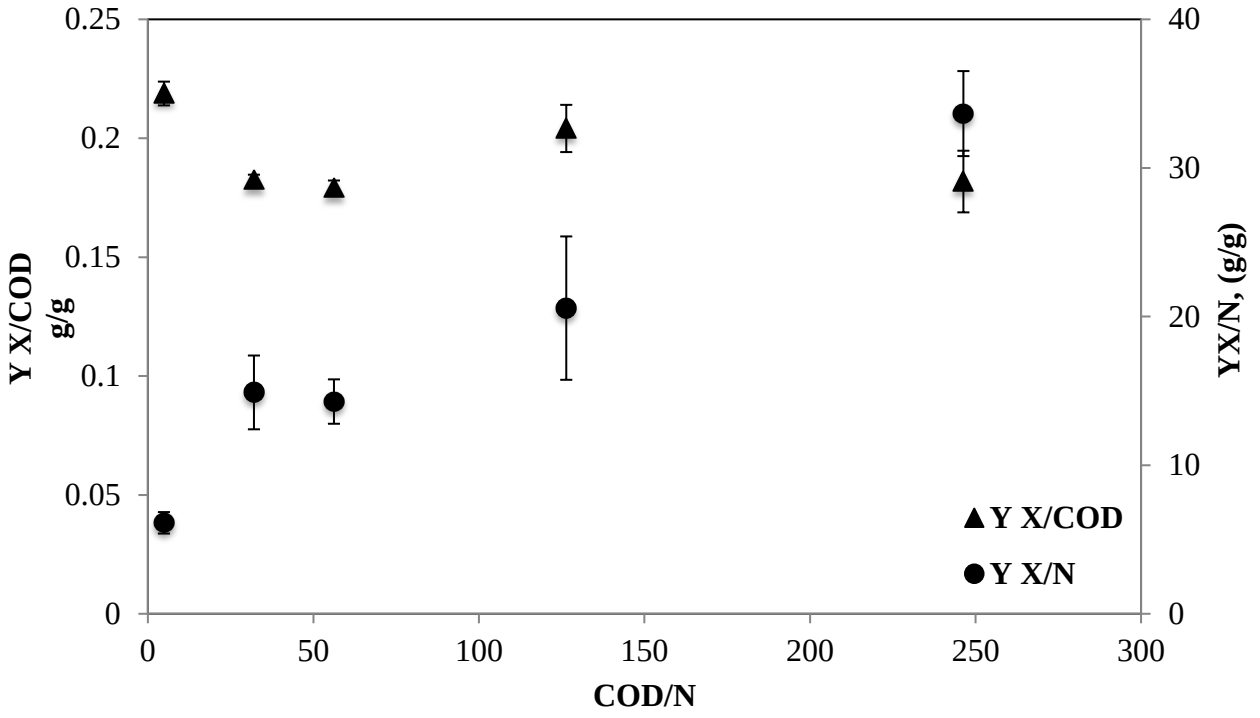

Figure 3 Effect of initial nitrogen concentration on the COD based biomass yield coefficient and nitrogen based biomass yield coefficient. The initial VFA concentration was 6500 mg COD/L in all experiments. Yield coefficients expressed in weight of biomass produced per gram substrate consumed.

The response of *C. albidus* to nitrogen limitation can be explained by certain key metabolic reactions involved in carbon and nitrogen assimilation. Following nitrogen exhaustion, the enzyme adenosine monophosphate (AMP) deaminase (EC 3.5.4.6), which catalyzes the conversion of AMP to inosine monophosphate (IMP) and ammonium, is activated to scavenge intracellular nitrogen(Ratledge 2002). This depletion of AMP causes allosteric inhibition of the activity of the enzyme isocitrate dehydrogenase (IDH, EC 1.1.1.41), which catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate during the tricarboxylic acid (TCA)

cycle (Ratledge 2004). alpha-ketoglutarate is a nitrogen transporter and forms the backbone of the glutamate family of amino acids and is essential for nitrogen assimilation in the cells. At limiting nitrogen concentrations, the flux through the TCA cycle slows down, resulting in buildup of citrate inside the mitochondrion(Liu, Zhao X Fau - Wang et al. 2011). Consequently, citrate is transported outside the mitochondria and is cleaved by the enzyme ATP: Citrate lyase (ACL, EC 2.3.3.8) to form acetyl co-A and the excess acetyl co-A is directed towards fatty acid synthesis for storage (Ratledge 2004). Indeed, in nitrogen limited cultures of oleaginous yeast *Lipomyces starkeyi* intracellular citrate concentrations increased by ~80% and the specific activity of IDH enzyme dropped from 0.023 to 0.005 U/g once the nitrogen source was exhausted (Tang, Zhang et al. 2009). In another study, the N-content of the *Chlorella vulgaris* decreased from 9% to 0.5% of the dry cell weight upon nitrogen exhaustion (Griffiths, van Hille Rp Fau - Harrison et al. 2014). A down-regulation of IDH enzyme and a concomitant increase in the levels of ACL enzyme and intracellular lipids under nitrogen starved conditions were also observed in *Chlamydomonas reinhardtii* cultures (Wase, Black et al. 2014). Therefore, these are several consistent lines of evidence that nitrogen limitation is an effective lipogenesis inducer in the oleaginous microorganisms, as experimentally observed during our study

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Table 4 The effect of different initial COD:N ratio on the biomass growth, specific growth rate and lipid production by *C. albidus*. The initial VFA concentration was 6500 mg COD/L in all experiments and the initial ammonia-N concentrations were varied.

Initial COD:N ratio	**5:1**	**25:1**	**50:1**	**125:1**	**250:1**
Biomass concentration (g/L)	1.3±0.0	1.1±0.1	1.0±0.0	1.1±0.0	0.97±0.0
Lipid concentration (g/L)	0.26±0.0	0.32±0.0	0.21±0.0	0.27±0.0	0.27±0.0
% Intracellular lipid accumulation (g/g)	19.6±0.2	28.3±0.7	21.3±0.1	24.1±0.2	28.8±2.1
$Y_{L/\Delta COD}$ (mg/g)	44.1±0.7	52.4±0.9	32.1±1.1	40.8±0.0	41.2±2.0
Maximum specific growth rate μ_m (h^{-1})	0.041±0.0	0.040±0.0	0.040±0.0	0.038±0.0	0.023±0.0
sVUR (mg-COD/g-cell/h)	64.2±1.1	88.7±3.8	91.4±15.9	91.4±17.2	86.5±20.3
sNUR (mg-N/g-cell/h)	1.8±0.3	1.3±0.3	1.2±0.1	0.96±0.0	0.4±0.1
ΔCOD/ΔN (g/g)	33.1±7.2	79.8±8.8	80.1±7.1	100.7±19.3	186.7±28.3

2.3.2 The effect of initial VFA concentrations on the lipid and biomass yield

As described above, at an initial VFA concentration of 2600 mg COD/L (COD:N= 10:1), the cell growth was limited by the carbon source (and not nitrogen). The VFA mixture was completely exhausted in 48h, following which the cells entered stationary phase (data not shown). The relative nutrient uptake ratio, $\Delta COD/\Delta N$ (62.23±1.00 w/w) was statistically similar (p=0.11) to that of the batch with excess nitrogen (COD:N = 5:1). The specific VFA and nitrogen utilization rates (sVUR = 65.25±0.15 h^{-1} and sNUR = 1.06±0.00 h^{-1}) were also similar (p=0.07 and 0.46) to the batch cultures at the feed COD:N ratios of 5:1 and 10:1. In contrast, when the initial VFA concentration was increased to 10,000 mg COD/L, no cell growth or lipid accumulation was observed presumably due to substrate inhibition on account of toxicity of acetic acid at higher concentrations. High concentrations of acetate interfere with the membrane transport of the phosphates (Georgina Rodrigues 2000). Acetate was shown to inhibit the growth of the oleaginous yeast *Brettanomyces bruxellensis* at concentrations higher than 2100 mg COD/L and growth was completely inhibited at acetate concentrations higher than 4200 mg COD/L (Yahara, Javier et al. 2007). However, we found that the *C. albidus* was not inhibited by even higher initial acetate concentrations as high as 3250 mg COD/L (calculated as 50% of total initial COD of 6500mg/L). This further suggests that *C. albidus* might also be a good candidate for high throughput lipid production as it can efficiently convert high strength organic waste streams to lipids, without the need for substrate dilution.

2.3.3 Differential VFA utilization rates

Acetate was consumed more rapidly with a maximum specific substrate utilization rate of 47.91±4.24 mg-acetate/g biomass/h compared to 3.97±1.54 mg-propionate/g biomass/h for propionate and 7.42±3.01 mg-butyrate/g biomass/h for butyrate. Acetate was completely

exhausted by the end of the exponential growth phase and propionic and butyric acid were utilized only after the depletion of acetate (Figure 4). The trends herein are in good agreement with those from previous studies, in which higher specific uptake rates have been reported for acetate (1.50±0.14 d^{-1}) compared to propionate (0.07±0.00 d^{-1}) and butyrate (0.09±0.06d^{-1}) in the mixed bacterial cultures engaged in polyhydroxyalkanoate production (Fradinho, Oehmen et al. 2014). Slower consumption rates of longer chain acids compared to acetate may be attributed to their different metabolic fate after intake. Propionate, an odd chain carboxylic acid, cannot be cleaved directly to acetyl-CoA and it must be converted to propionyl-CoA and enter the TCA cycle via methlymalonyl-CoA inter-conversion to succinyl-CoA. The rate of propionate metabolism is therefore likely controlled by the rate of its decarboxylation of acetyl-CoA. In case of butyrate, the higher number of biochemical transformations that it must undergo including β-oxidation to acetoacetyl-CoA and then further cleavage to acetyl-CoA may be the cause of its slower uptake(Fradinho, Oehmen et al. 2014). Therefore, in mixed substrate systems, acetate will likely be preferably used for growth over other VFA (Albuquerque, Carvalho et al. 2013). The factors influencing the VFA output from fermentation are fairly well understood and a predominantly acetate product is possible through process manipulations which might lead to improved lipid yield downstream.

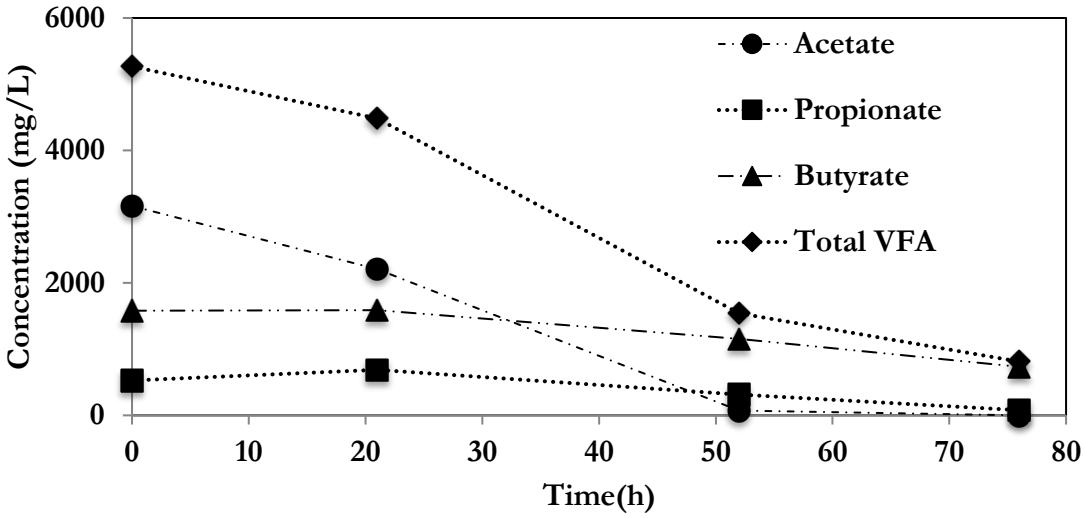

Figure 4 Rate of consumption of different VFA in batch cultures of C. albidus. The initial VFA and nitrogen concentrations were 6500 mg-COD/L and 260 mg-N/L, respectively. Error bars represent standard deviation of three replicate measurements.

2.3.4 Fatty acid composition of the lipids accumulated by C. albidus in batch cultures

In all cases with synthetic VFA, the fatty acids accumulated were predominantly palmitic (C16:0), oleic (C18:1), and linoleic acid (C18:2), corresponding to those of soybean and jatropha oil, which are used as feedstock for biodiesel production in the States and the European Union(Seo, Han et al. 2014) . The fatty acid composition in the nitrogen limited batches (COD:N≥ 25:1) was statistically similar (p=0.43) to palmitic (C16:0), oleic (C18:1), linoleic (C18:2) acid at 27±1.58%, 50±1.32% and 15±1.29% respectively (Table 5). In general, similar fatty acid compositions were reported for yeast *Cryptococcus curvatus* (Chi, Zheng et al. 2011), yeast *Rhodotorula mucilaginosa* (Li, Liu et al. 2010) and for algae *Laminaria japonica* (Xu, Kim et al.) under different growth medium and conditions. This suggests that the metabolic pathways of lipid accumulation could be conserved across the oleaginous species across different

feedstocks and growth conditions, which makes *Cryptococcus albidus* a good candidate for biodiesel production from highly diverse organic waste streams.

The composition of fatty acids also plays a crucial role in the quality of biodiesel that can be produced. To achieve the best compromise between the cold flow properties *i.e.* the tendency of fuel to solidify at lower temperatures and the oxidative stability *i.e.* the susceptibility of unsaturated fats to self-oxidation, the optimum feedstock should have relatively low levels of saturated and poly unsaturated fats and high levels of monounsaturated fats (Hoekman, Broch et al. 2012). We found that the microbial lipids obtained from the VFA contained about 70% unsaturated fats with a linoleic acid (C18:2) content at about 15%, which is very similar to the commercial feedstock used for biodiesel production (~75% unsaturated fats in jatropha oil). Therefore, in addition to the high degree of lipid accumulation of *C. albidus* when grown on VFA with nitrogen limitation, the appropriate composition of the accumulated lipids further renders this process highly suitable for biodiesel production.

Table 5 Percentage composition of lipids obtained under different cultivation conditions

Lipids	Batch with different initial COD:N					VFA from food waste fermentation	Chemostat
	5:1	25:1	50:1	125:1	250:1		
C 14:0	0.28±0.0	0.21±0.0	1.5±1.7	1.5±1.7	0.19±0.1	10±0.4	0.52±0.2
C 16:0	20.8±1.9	28.7±1.4	26.4±1.3	27.3±0.1	29.9±0.7	31.4±1.7	36.8±0.9
C 16:1	0.24±0.1	0.57±0.2	0.69±0.2	0.61±0.1	0.89±0.0	2.0±0.0	0.96±0.4
C 18:0	2.2±0.1	2.4±0.1	2.2±0.3	1.6±0.6	3.1±0.1	4.0±0.0	2.7±2.0
C 18:1	66.3±2.4	50.4±1.2	50.5±0.7	52.8±2.6	49.8±0.1	36.2±2.3	37.4±3.2
C 18:2	9.1±0.4	15.3±0.7	16.7±0.2	14.0±3.5	13.9±0.7	3.5±0.4	19.1±0.3
C 18:3	0.9±0.1	1.9±0.2	1.7±0.4	1.9±0.1	1.7±0.1	0.9±0.0	2.3±0.3
C 20:0	ND	ND	ND	ND	ND	11.5±0.8	ND
C 20:1	0.15±0.0	0.35±0.1	0.33±0.0	0.33±0.0	0.42±0.0	0.52±0.0	0.29±0.1

2.3.5 Effect of different carbon sources on the lipid and biomass yield

Raw food waste and fermentate composition. The raw undiluted food waste used to prepare the fermenter feed had a total solid and total volatile solid content of (TS = 20.10±4.63% w/w) and (TVS = 16.22±0.15% w/w) respectively. The chemical parameters, including the tCOD, sCOD, TN and VFA were 312±30.88 g/Kg, 79.6±18 g/Kg, 15.6±0.7 g/Kg and 6.2±3.9 g/Kg respectively. The food waste fermentation process was concluded after 24 d of continuous operation at a 2d HRT. At this point, the average fermentate COD concentration was 27065±7048 mg/L, which consisted on an average of 52.6±10.9% soluble COD (sCOD) while the sCOD consisted of 71.3±22.7% VFA on average. The conversion of the influent COD load to VFA was 38.7±12.9% and the VFA concentration in the fermentate was 10068±3367 mg COD/L. The most common VFA present in the fermentate were acetate (51.5±0.6%), propionate (13.8±1.4%) and butyrate (34.6±0.1%), all expressed as mass.

Batch growth on both synthetic VFA and the sterilized VFA stream from the anaerobic food-waste fermenter resulted in similar biomass concentration (p=0.11, Table IV). However, the intracellular lipid concentration (14.9±0.1%) and the maximum specific growth rate (0.021±0.00 h^{-1}) were statistically lower for fermentate VFA (p=0.02, Table IV). Similarly, in a previous study, the oleaginous yeast *Cryptococcus curvatus* also showed poor cell growth and a decrease in the intracellular lipid content (13.5%) when using food waste fermentation effluent as the growth medium (Chi, Zheng et al. 2011), pointing to the presence of as yet unknown inhibitors in the fermentate. Another possible cause of lower lipid yield could be the high protein content of the food waste, which can effectively lower the COD:N ratio below the favorable range (COD:N≥25). Based on our previous results, food waste fermentate TKN concentrations were in the range 1410.8±516.2 mg-N/L(Ljupka Arsova 2010) resulting in an effective feed COD:N ratio

in our *C. albidus* batch cultures of 2.87:1. Although not attempted in this study, the observed differences in the kinetics (μ_m) and extent of lipid accumulation between synthetic VFA and VFA present in fermentate point to the necessity for independent optimization of lipid production from different waste streams, which is not entirely unexpected.

In general, the relative content of unsaturated lipids (proportion of lipids with 1,2 or 3 carbon-carbon multiple bonds), using synthetic VFA at all initial COD:N ratios of 5:1 (76.7±0.5),25:1 (68.5±0.4) , 50:1(69.9±0.7), 125:1 (69.7±1.0) and 250:1 (66.7±0.7) was higher than on fermentate (43.1±2.1), as shown in Table 6. Previously, (Hansson and Dostálek 1986) reported that the degree of unsaturation of fatty acids in chemostat *C. albidus* cultures is directly proportional to the specific growth rate. While this argument certainly holds for this study, given the lower maximum specific growth rate (Table 7) and corresponding lower degree of lipid unsaturation (Table 6) with fermentate, more metabolic level studies are needed to elucidate specific mechanisms of differential lipid speciation.

Table 6 Effect of different COD sources on cell growth and lipid accumulation by C. albidus.

Carbon source	Synthetic VFA mixture (initial COD:N=25:1)	VFA from anaerobic fermenter
Biomass Concentration (g/L)	1.1±0.1	0.96±0.0
Maximum specific growth rate μ_m (h^{-1})	0.040±0.0	0.021±0.0
% Intracellular lipid accumulation (g/g)	28.3±0.7	14.9±0.1
$Y_{L/\Delta COD}$ (mg/g)	52.4±0.9	30.8±0.6

2.3.6 Biomass and lipid production during chemostat cultivation

At an operational HRT of 3 days (D = 0.33 d^{-1}), the steady state biomass concentration was 1.02±0.07 g/L and the intracellular lipid content was 29.88 ± 1.92%. The biomass yield coefficients normalized to influent COD and nitrogen concentrations ($Y_{X/COD}$ = 0.18±0.01 and $Y_{X/N}$= 9.05±0.29) were statistically similar to those in the batch culture (*p*=0.18 and 0.52, respectively). The composition of the lipids produced was also largely similar to those from the batch cultures (Table 5). Chemostat cultures could be advantageous over the batch cultures since any transient changes in the available COD:N ratio can be eliminated under the steady state operation and the cells can be maintained under continuous and sustained nitrogen limitation. Furthermore, chemostat operation can also allow the cell growth rate to be manipulated through the dilution rate, which can be used to optimize kinetics and yields of lipid production. Although not optimized herein, an increase in intracellular lipid content with decreasing dilution rates (increasing HRT values) has been shown previously using glucose and complex organics, but not with VFA (Hansson and Dostálek 1986) .

2.3.7 Techno-economic assessment of lipid production:

The production of lipids by first converting non-lipid carbon to VFA as key intermediates by anaerobic fermentation is more advantageous from than just focusing on the inherent lipid content of organic waste streams such as food waste, sewage sludge or fecal sludge. In our experiments, we were able to achieve an overall conversion of 20.29 mg lipid/ g-COD from food waste influent with a corresponding lipid yield from VFA of 52.4 mg lipid/gVFA-COD.

The choice of the feedstock for lipid production can account for up to 80% of the total biodiesel production cost (Fei, Chang et al. 2011). Further, the cost of lipid production itself can vary

significantly between microorganisms and feedstocks. For lipid production using yeast based processes, (Seo, Han et al. 2014) reported a final lipid cost of $2.19/kg lipids produced using *Cryptococcus curvatus* fed with algal residue, while (Fei, Chang et al. 2011) reported $0.49/kg and $3.15/kg from *C. albidus* growing on VFA and glucose respectively. In comparison, the cost of current commercial feed stock like Jatropha and soybean oil is $0.76/kg and $0.77/kg (Seo, Han et al. 2014). Using the lipid yield coefficient ($Y_{L/S}$ = 40.96 kg lipid/ ton VFA) for VFA produced from food waste as the carbon source and a VFA production cost of $30-$100/ ton (Fei, Chang et al. 2011), the overall lipid production cost from food waste was US$ 0.81-2.53/kg.

Correspondingly, for our process, the overall cost of biodiesel production for a 10 million gallon per year system as modeled after (Haas, McAloon et al. 2006) equaled $0.71-$2.23 /L (as summarized along with added assumptions in Table 7). In contrast, for algal biodiesel, (Ratledge and Cohen 2008) reported a very high cost of $21/L for algal biodiesel, while (Davis, Aden et al. 2011) reported the unit cost to be $2.25/L. It must be noted that this analysis does not even take into account the positive impact on the environment considering the reduced discharge of food waste (or other organic wastes) without treatment. In sum, the production of lipids from waste derived VFA using oleaginous yeast such as *C. albidus* offers an economically viable prospect for sustainably linking waste management and biofuel production, while minimizing reliance on food crop for energy use.

Table 7 Summary of unit costs for 10 million gallons/year biodiesel production using C. albidus.

All costs are in US$ unless otherwise mentioned

Costs for biodiesel production	Cost of VFA production	
Carbon source cost	$30/ton	$100/ton
Lipid yield from *C. albidus* (kg lipid/ton VFA)	40.96	40.96
Lipid cost ($/lb)	0.33	1.11
Raw material cost[a] ($/L biodiesel)	0.68	2.20
Utilities cost [b] ($/L biodiesel)	0.012	0.012
Labor cost [c] ($/L biodiesel)	0.013	0.013
Supplies cost [d] ($/L biodiesel)	0.004	0.004
General works cost [e] ($/L biodiesel)	0.003	0.003
Depreciation@10% capital cost/year ($/L biodiesel)	0.03	0.03
Glycerol value [f] ($/L biodiesel)	-0.034	-0.034
Gross cost [g] ($/L biodiesel)	0.71	2.23
Gross cost [h] ($/Kg biodiesel)	0.81	2.53

[a] Raw material includes cost of lipid, methanol, sodium methoxide, hydrochloric acid, sodium hydroxide and water.

[b] Utilities including gas and electricity (0.06$/kWh), taken from US energy Information Administration(USEIA).

[c] Labor includes operating, maintenance, supervisory and fringe benefit costs.

[d] Supplies include operating and maintenance supplies.

[e] General works include administrative costs, property insurance and taxes.

[f] Glycerol co-product when sold as 80% crude solution at a market value of 0.15$/lb

[g] Sum of all the costs minus the co-product value.

[h] Calculated from density of biodiesel 0.88 kg/L reported by (Alptekin and Canakci 2008)

2.4 Conclusions

Batch cultivation of *C. albidus* was optimized to maximize intracellular lipid accumulation, by imposing nitrogen limitation at initial VFA concentrations as high as 6500 mg COD/L. In general, the produced microbial lipids were of similar composition to soybean and jatropha oil, both of which are used for commercial biodiesel production. In sum, we demonstrate the potential for a VFA based flexible microbial platform for the bioconversion of organic 'waste' streams to lipids. This proposed link to 'waste' derived lipids could also lead to more sustainable pathways for biofuel production than through agricultural resources alone.

2.5 Acknowledgements

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CHAPTER 3

GENOME SEQUENCE AND BIOTECHNOLOGICAL APPLICATIONS OF THE VERSATILE OLEAGINOUS YEAST *Cryptococcus albidus*

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3.1 Introduction

Recovery and reuse of resources present in various feedstocks, including organic waste streams can be a viable pathway towards realization of a circular economy. Organic waste is a tremendous resource comprised of energy and nutrient rich organic material and is highly amenable to biological transformation into chemicals of commercial value. As such, several specific waste streams may be readily used to obtain value added compounds, as is the case of biodiesel production from spent cooking oils. From a chemical perspective, the major constituents of nearly all organic waste streams can be classified under carbon, nitrogen and phosphorous compounds. Of these various components, carbon is perhaps the most versatile and most lucrative for further biological transformation. Traditionally, biological approaches to carbon recovery from organic waste have largely focused upon methane production through the process of anaerobic digestion. However, methane is considered a fairly low value product and therefore, efforts are now being directed towards biotransformation of organic waste associated carbon into products of much higher commercial value such as biofuels, bioplastics, organic acids and other building block chemicals for various industries. These research efforts towards realization of a circular, bio-based economy have brought to prominence certain 'non-conventional' yeasts, named as such due to the limited attention these yeasts have gotten compared to the so called conventional yeasts such as *Saccharomyces cerevisiae.* The basidiomycetes oleaginous yeast, *Cryptococcus albidus* is one such non-conventional yeast which has recently gained importance mostly due to its ability to efficiently convert certain alternate carbon sources into lipids (Vajpeyi and Chandran 2015).

Cryptococcus albidus var. albidus was first isolated from air in Japan by Saito in 1922 and it was initially described as *Torula albida*. Later, it was transferred it to Torulopsis by Lodder, while

Skinner brought it into the genus Cryptococcus in 1947 after starch formation was observed. *C. albidus* colonies are cream-color to pale pink, with the majority of colonies being smooth with a mucoid appearance. This species is morphologically and genetically very similar to *Cryptococcus neoformans*, but can be differentiated because it is phenol oxidase-negative, and, when grown on niger or birdseed agar, *C. neoformans* produces melanin, a known virulence factor, causing the cells to take on a brown color while the *C. albidus* cells stay cream-color. It also differs from *Cryptococcus neoformans* because of its ability to assimilate lactose, but not galactose. This species is also considered unique because its strains have a maximum temperature range from between 25°C and 32°C. *C. albidus* has been isolated from diverse natural environments including in soil in symbiotic relationship in plant roots(Oro, Ciani et al. 2016), environments contaminated with oil spills (Yalçın, Çorbacı et al. 2014), arsenic, heavy metals (Vadkertiová and Sláviková 2006) and paper and pulp mill effluents (Singhal, Choudhary et al. 2009). *C. albidus* is halotolerant (tolerance to high salinity) and has the highest reported potential among the *Cryptococcus* spp. to grow at low water potentials and also demonstrates a remarkable ability to grow and survive in arid and low nutrient environments (Vishniac 1995), which confers a significant potential competitive advantage over other species.

Although, *Cryptococcus sp.* has traditionally been associated with pathogenicity in humans due to cryptococcosis caused by *C. neoformans* and *C. gattii*, the species *Cryptococcus albidus* has no known pathogenicity and is generally recognized as safe (GRAS) by the US Food and drug administration (FDA). The complete genome of *Cryptococcus albidus var. albidus* was recently sequenced and published (Vajpeyi and Chandran), which allowed a deeper metabolic understanding of the biochemical pathways present in this yeast. The key advantage this yeast seems to offer is the high versatility of the substrates that it can metabolize. It was demonstrated

that this yeast can consume non-ideal carbon sources such as volatile fatty acids (acetate, propionate, butyrate) (Fei, Chang et al. 2011, Vajpeyi and Chandran 2015), glycerol (Souza, Schwan Rf Fau - Dias et al.) and, xylose (Biely P Fau - Vrsanska, Vrsanska M Fau - Kratky et al.) in addition to glucose. The ability of *C. albidus* yeast to perform assimilatory reduction of nitrate(Fei, Chang et al. 2011) is quite unique and is generally not found in other basidiomycetous yeast or even other oleagionous yeast such as *Yarrowia lipolytica* (Siverio 2002). However, the molecular mechanisms and biochemical pathways utilized by *C. albidus* are not known. The availability of its genome can potentially allow manipulation and regulation of biosynthesis in *C. albidus* and metabolic engineering for value-added chemical production.

Herein, using the newly sequenced genome of *Cryptococcus albidus var. albidus*, we discuss the genetic and biotechnological potential of the oleaginous yeast *Cryptococcus albidus*, focusing first on the biochemical pathways encoded to metabolize various substrates, followed by their potential biotechnological applications.

3.2 Material and methods

Cryptococcus albidus (ATCC 10672) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultivated on YM (yeast and mold) medium in a 100 mL shake flask culture at room temperature (T=25^{o}C). Cells were harvested from 100 μL culture broth (~2x10^{7} cells) by centrifuging at 16,000xg for 10 minutes at 4^{o}C followed by automated extraction of genomic DNA from the resulting pellet using Qiacube^{TM} (Qiagen, Valencia, CA).

A 400 bp genome library of *C. albidus* was prepared and sequenced using Ion-torrentPGM^{TM} (Thermofisher scientific, Waltham, MA). The raw sequence data contained 3,163,623 reads with median length of 362 bp, resulting in 44.8-fold coverage. The raw sequence reads were

assembled using SPADES assembler (Bankevich, Nurk et al. 2012) (v3.1.0) with a k-mer size of 21. This led to a genome assembly containing 834 contigs (each with sequence length>500bp). The total consensus genome length was 24,807,186 with a median contig length, N50 of 114,383bp and a G+C% content of 52.7%.

The genome was further analyzed to assign open reading frames (ORFs) with evidence based annotation pipeline AUGUSTUS(Stanke and Waack). A phylogenetic tree was constructed for *C. albidus* using the internal transcribed regions (ITR 1 and ITR2) (figure 6), and *Cryptococcus neoformans gattii* was identified as the closest related fully sequenced genome available. AUGUSTUS was trained with *C. neoformans gattii* annotation data and 8637 putative ORFs were identified in the assembled *C. albidus* genome. The putative ORFs were translated into corresponding protein sequences and 6110 known proteins were annotated using NCBI BLASTP(Altschul, Gish W Fau - Miller et al.) (protein vs. protein alignment)(Conesa, Gotz S Fau - Garcia-Gomez et al.). Additional functional annotation of predicted genes was performed using using BlastKOALA (figure 5, 7) (KEGG tools, http://www.genome.jp/blastkoala)(Kanehisa, Sato et al.)

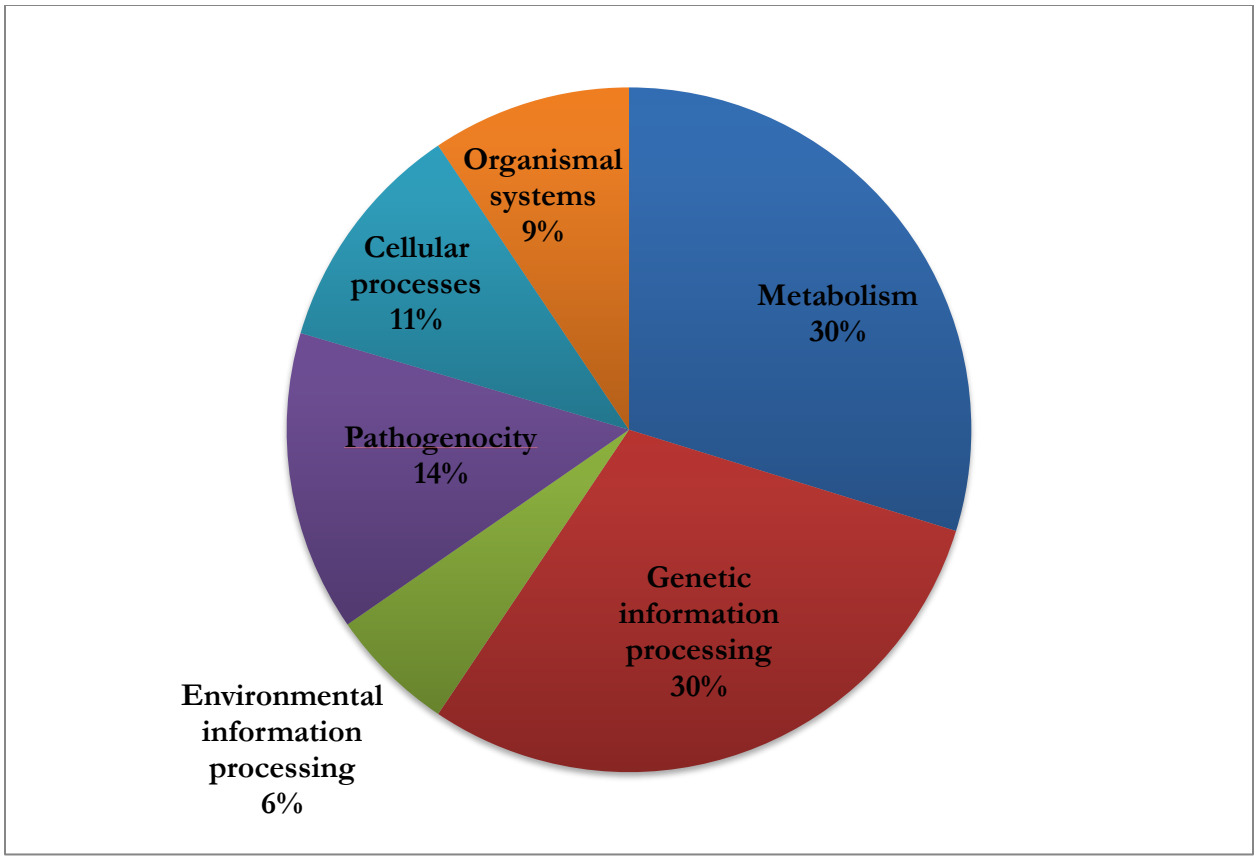

Figure 5 Functional classification of proteins identified in *C. albidus*

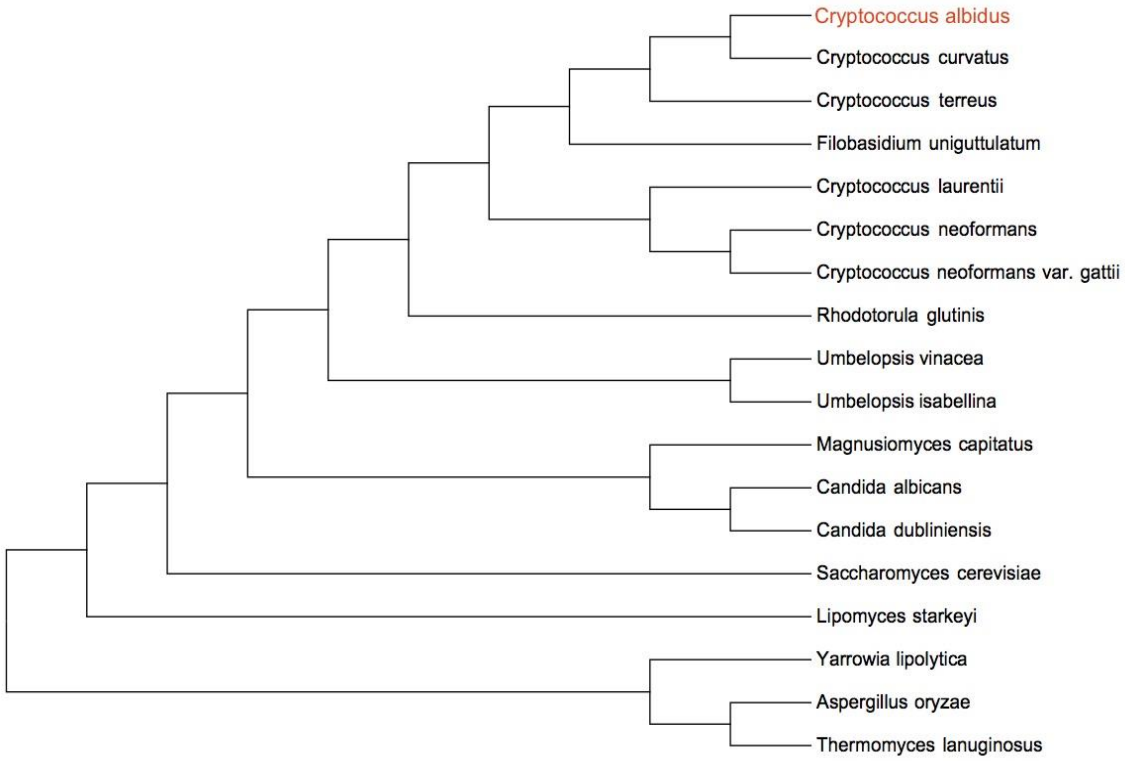

Figure 6 Phylogenetic tree for *C. albidus* constructed using the internal transcribed regions (ITR 1 and ITR2).

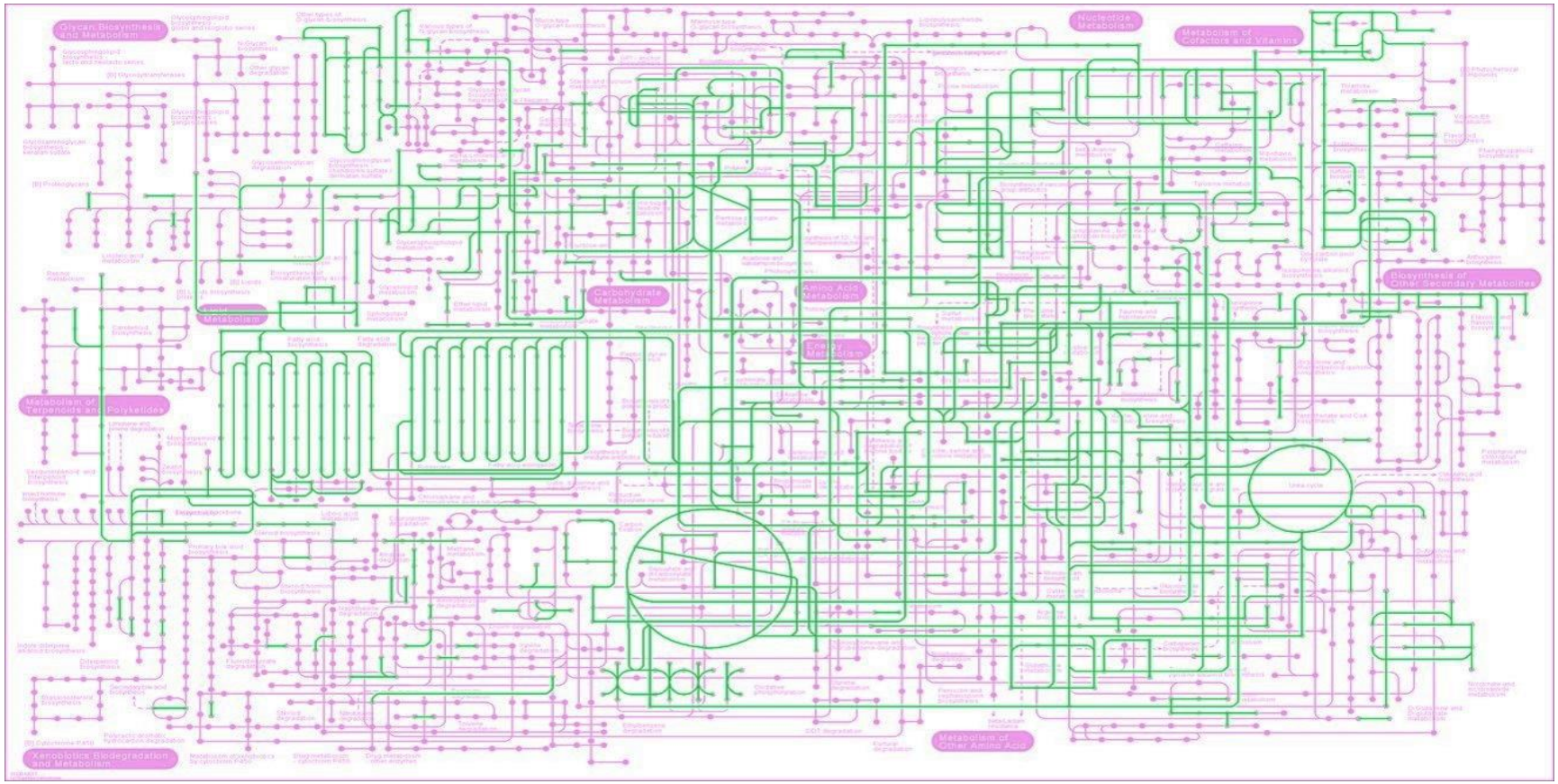

Figure 7 KEGG map of global pathways identified in *C. albidus*. Pathways in green represent the pathways present in *C. albidus*

Nucleotide sequence accession numbers

The draft genome sequence has been deposited to DDBJ/EMBL/GenBank under the accession number LKPZ00000000.

3.3 Metabolism of carbon sources

Recovery and reuse of carbon present in organic waste streams relies upon upcycling of inherent low value carbon products into products of higher value through native or engineered biological pathways. Therefore, current research efforts are increasingly being focused towards developing pathways for bio-based production of chemicals of industrial importance to reduce dependence upon the non-renewable resources. *C. albidus* has the genetic capability to ferment a wide range of hydrophilic (acetate, butyrate, propionate, glucose, sucrose, glycerol, alcohols) and hydrophobic (fatty acids, alkanes, triacylglycerols) carbon sources as well as complex organic waste mixtures such as molasses, bagasse (Singhal, Jaiswal et al. 2015).

3.3.1 Metabolism of carbohydrates

C. albidus can assimilate most common sugars including glucose, fructose, sucrose, mannose, maltose, lactose, xylose, xylitol. The uptake of glucose is mediated by *Snf3* like hexose transporter gene *hxs1*, which is commonly found in other *Cryptococcus* spp. including *C. neoformans* and *C. gattii* (Liu, Wang Y Fau - Baker et al.). Mannose and fructose can directly enter the glycolytic pathway through phosphorylation by the gene *hk*, coding for hexokinase enzyme (EC 2.7.1.1), while the uptake and assimilation of sucrose is meditated through sucrose

transporter gene *sacA*, coding for the enzyme beta-fructofuranosidase (EC 3.2.1.26), which catalyzes its hydrolysis into fructose and glucose. The assimilation of pentose sugars such as ribose is mediated by the gene *rbks*, coding for the enzyme ribokinase (EC 2.7.1.15), which facilitates its entry into pentose phosphate pathway. *C. albidus* can also assimilate sugar alcohols such as glycerol through gene *glpK* and xylitol through gene *sord*, which facilitates its conversion to ribulose to enter the pentose phosphate pathway. *C. albidus* is considered unique among the *Cryptococcus sp.* due to its inability to assimilate galactose. The presence of galactose enzyme activator gene, *galm* and solute carrier family (SLC35) galactose transporter indicates that *C. albidus* is capable of galactose uptake, however, it lacks the galactose kinase gene *galK*, which is essential for conversion of galactose into glucose derivatives to enter the glycolytic pathways(Sellick, Campbell et al. 2008).

3.3.2 Metabolism of alcohols

C. albidus has the capability to assimilate ethanol due to the presence of several copies of NAD^+ and $NADP^+$ dependent alcohol dehydrogenase (EC 1.1.1.1) coding gene *adh* and aldehyde dehydrogenase (EC 1.2.1.3) coding gene *aldh*. The presence of propanol preferring alcohol dehydrogenase gene *adhp* indicates that propanol could also be metabolized for growth, however, it lacks the *bdhA* gene, necessary for butanol uptake. Higher concentrations of ethanol have been reported to be toxic in other oleaginous species (Barth and Gaillardin 1997) and closely related *Cryptococcus neoformans* was severely inhibited by presence of 0.06 mg/mL ethanol in the culture medium (Ouattara, Kra Am Fau - Coulibaly et al.), however, no studies have been conducted thus far to investigate the ethanol toxicity in *C. albidus.*

3.3.3 Metabolism of carboxylic acids

The ability of *C. albidus* to utilize short chain carboxylic acids such as acetate, propionate, butyrate as the sole carbon and energy source has been well documented (Fei, Chang et al. 2011, Vajpeyi and Chandran 2015). The presence of proton-linked monocarboxylate transporter family (*MCT*)(Halestrap and Price 1999) suggests that the transport of monocarboxylic acids across the membrane in *C. albidus* is mediated by the monocarboxylae permease protein. Five putative *mct* genes have been shown to mediate propionate, butyrate, lactate, and acetate transport in *Saccharomyces cerevisiae* and oleaginous yeast *Yarrowia lipolytica* (Christophe, Kumar et al. 2012). However, the role of *MCT* gene in monocarboxylate transport is inconclusive and it was reported in another study (Makuc, Paiva S Fau - Schauen et al.) that a *S. cerevisiae* mutant strain lacking all five *mct* genes exhibited no growth defects on monocarboxylic acids as the sole carbon and energy sources and the uptake rates were indistinguishable from the wild-type. *C. albidus* was also found to have a *gpr1* gene homologue which codes for a plasma membrane protein and has been reported to make *Y. lipolytica* sensitive to the low concentrations of acetate and inhibit its uptake (Barth and Gaillardin 1997). However, we found in our earlier study (Vajpeyi and Chandran 2015) that growth in *C. albidus* was not inhibited even by acetate concentrations as high as 3250 mg COD/L. This suggests that the uptake of monocarboxylic acids in *C. albidus* might be transporter independent and accomplished solely by passive diffusion. Yet another possibility is that the transport of the short chain carboxylic acids might be pH dependent wherein acids, when present in their undissociated form, are soluble in the lipid bilayer and can therefore passively diffuse through the cell membrane and through proton

symport catalyzed by monocarboxylate transporters, when present in dissociated form (Makuc, Paiva S Fau - Schauen et al.)

As for transport of dicarboxylic acids such as succinate and fumarate, *C. albidus* contains a putative *dic1* gene homologue, which codes for a dicarboxylate transport protein (Casal, Paiva et al. 2008).

The presence of these genes indicate that *C. albidus* is able to metabolize a wide variety of alternate and cheap carbon substrates, making it a potential candidate for diverse biotechnological applications.

3.4 Nitrogen metabolism

C. albidus has been reported to metabolize various organic and inorganic nitrogen sources including ammonia (Vajpeyi and Chandran 2015), nitrate, urea, glycine (Fei, Chang et al. 2011) and was found to possess genes to metabolize several other amino acids such as glutamate and aspartate. In all cases, the nitrogen uptake is strictly assimilatory and *C. albidus* lacks the genes to perform nitrification, denitrification or nitrogen fixation. Major facilitator superfamily (*MFS*) class gene *nrt* facilitates the uptake of nitrate and nitrite, after which they are converted into ammonium for assimilation through genes *nr* and *nit-6* coding for NADPH dependent enzymes nitrate reductase (EC 1.7.1.1) and nitrite reductase (EC 1.7.1.4) respectively. Additionally, the presence of *ncd2* gene coding for nitronate monooxygenase (EC 1.13.12.16) suggests that nitroalkanes could also be used as nitrogen source.

Cryptococcus sp. is generally regarded as nitrate negative and most common species including *C. neoformans, C. gattii and, C. laurenti* are unable to utilize nitrate as the sole nitrogen source for growth and even other ascomycetes yeasts including *S. cerevisiae* and oleaginous yeast *Y. lipolytica* are reported to be nitrate negative (Siverio 2002). Therefore, the ability of *C. albidus* to perform assimilatory reduction of nitrate and nitrite is quite unique and suggests that the overall nitrogen metabolism in *C. albidus* has diverged considerably from other basidiomycetes yeast. A phylogenetic study of the eukaryotic *nr* gene reported that certain basidiomycetes and ascomycetes yeast may have acquired the gene through horizontal gene transfer from a common ancestor (Gorfer, Blumhoff et al. 2011).

In addition to inorganic nitrogen, *C. albidus* can also metabolize various organic nitrogen sources such as urea and amino acids including glycine and glutamate through genes *gdhA* and *glc* coding for enzymes glutamate dehydrogenase (EC 1.4.1.4) and glycine dehydrogenase (E.C 1.4.4.2) respectively.

3.5 Sulfur metabolism

Sulfur uptake in *C. albidus* is mediated by the *met3* gene, which is typical in Cryptococcus sp. (Yang, Pascon et al. 2002). Gene *met3* encodes the sulfate adenylyltransferase enzyme (EC 2.7.7.4), which is the first step of the sulfate assimilation in the cysteine/methionine pathway. *met3* gene has also been reported to play a crucial role in cell growth and thermal stability and a *C. neoformans met3* mutant was reported to have lower growth rates and decreased thermotolerance (Yang, Pascon et al. 2002). *C. albidus* performs assimilatory reduction of sulfate

to sulfide for amino acid synthesis. The presence of *sox* and *tst* gene orthologs suggests that it can also utilize thiosulfate by converting it either to sulfate or directly to sulfite.

3.6 Potential biotechnological applications of *C. albidus*

C. albidus has been exploited for its extracellular xylanase production to degrade the hemicellulosic material (Beg, Kapoor M Fau - Mahajan et al.), biopulping of bagasse for ethanol production (Arrizon, Morel et al. 2012, Singhal, Jaiswal et al. 2015) and, to prevent spoilage of post harvest fruit due to blue and green mold (Fan and Tian 2001, Chen, Li et al. 2012). Research efforts have also been focused upon utilization of the oleaginity of *C. albidus* to convert cheap carbon sources into commercial lipids. As discussed in previous sections, *C. albidus* can utilize a diverse array of carbon and nitrogen sources for growth, which makes it a lucrative candidate for further metabolic manipulation for production of various chemicals. Herein, we discuss the potential commercial and biotechnological application of *C. albidus.*

3.6.1 Secretion of proteins

Recent secretome profiling of *C. neoformans* identified 61 extracellular secreted proteins (Geddes, Croll et al. 2015), several of which are industrially important. *C. albidus* also shares several genes for secretion of various enzymes including proteases, phosphatases, lipases and esterases such as alpha amylase (EC 3.2.1.1) coded by gene *amyA*, urease (EC 3.5.1.5) enzyme coded by gene *ure*, several phospholipases coded by genes *pla1*, *plb* and *plb2*, cysteine proteases coded by gene *atg4*. The secretome profile in *Cryptococcus neoformans* has been reported to be modulated by the *pka1* gene encoding for cAMP-dependent protein kinase A (EC 2.7.11.11) (Geddes, Caza et al. 2016). The influence of the cAMP/PKA pathway on transcription has been investigated in several fungi and has been reported to regulate variety of processes including

growth, response to stress, nutrient sensing and secretory pathway components (Hu, Steen Br Fau - Lian et al. , Lee, D'Souza Ca Fau - Kronstad et al.). The presence of the *pka* gene in *C. albidus* suggests a similar mechanism of control.

3.6.2 Organic acids and alcohols

Citric acid

Citric acid, an industrially important chemical finds wide application as a food additive, medical, metallurgy, and textile industries. It is currently produced by molasses fermentation by *Aspergillus niger*, which is expensive and environmentally detrimental (Morgunov, Kamzolova et al. 2013) including the treatment of molasses with ferrocyanides (Kamzolova, Vinokurova et al. 2015). *C. albidus* has the genetic potential to replace molasses with other cheaper carbon substrates, given its ability to metabolize a wide variety of carbon sources including volatile fatty acids and glycerol, which is a by-product of biodiesel production and is easily available as an alternate, low cost carbon source (Kamzolova, Vinokurova et al. 2015).

Citric acid is produced as an intermediate in the citric acid cycle through reactions catalyzed by the enzymes *cs* citrate synthase, *icl* isocitrate lyase, *ms* malate synthase and *NAD-ICDH* and *NADP-ICDH*, NAD^+ and $NADP^+$ dependent isocitrate dehydrogenase, all of which are present in *C. albidus*. *Cryptococcus curvatus* and oleaginous yeast *Y. lipolytica*, with similar genes have been shown to efficiently metabolize crude glycerol for growth (Souza, Schwan Rf Fau - Dias et al.). Additionally, the ability of *Y. lipolytica* to convert glycerol into extracellular citric acid mediated by genes *gk* and *gdh2*, has also been demonstrated. In *C. albidus*, glycerol enters the cell through facilitated diffusion and is converted to 3-*P*-glycerol and then to 3-*P*-

dihydroxyacetone, reactions catalyzed by the enzymes glycerol kinase (EC 2.7.1.30) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), thus entering the glycolytic pathway to yield pyruvate(Papanikolaou, Fakas et al. 2008). Subsequent metabolic steps to lipid or citric acid biosynthesis are the same as for glucose. Pyruvate further yields acetyl-CoA and oxaloacetate, which then enter citric acid cycle. High titer concentrations (~115 g/L)(Morgunov, Kamzolova et al. 2013) of citric acid have been achieved using glycerol as the sole carbon source in both batch(Kamzolova, Vinokurova et al. 2015) and continuous(Rywińska, Juszczyk et al. 2011) cultures of *Y. lipolytica*. Even though no studies have been undertaken thus far to assess the feasibility of *C. albidus* for citric acid production, the presence of identical genes and pathways in other oleaginous and Cryptococcus species, which have been shown to produce citric acid suggests that *C. albidus* might also be a good candidate for citric acid production.

Secretion of dicarboxylic acids

Organic dicarboxylic acids such as succinic and malic acids have great potential as building block chemicals. Succinic acid, a member of the C_4-dicarboxylic acid family, is widely used in the production of foods, pharmaceuticals, and biodegradable plastics. Traditionally, it is produced via chemical synthesis from petrochemical feedstocks that are nonrenewable, and these chemical processes cause environment pollution (Song and Lee 2006). In microorganisms, both malic acid and succinic acid can be produced via three main routes: the tricarboxylic acid (TCA) cycle, the reductive TCA branch, and the glyoxylate pathway. So far, several microorganisms have been found to naturally produce promising succinic acid during fermentation, however, most of the research has focused upon anaerobic fermentation by bacteria, which utilizes the TCA branch. In *C. albidus,* in addition to TCA, we identified all but one gene required for

succinic acid production via the reductive TCA branch including *pck*, *mdh* and *fum* encoding for phosphoenol pyruvate carboxykinase (EC 4.1.1.49), malate dehydrogenase (EC 1.1.1.38), and fumarase (EC 4.2.1.2) (Yang, Lübeck et al. 2016). The missing fumarase reductase encoding *frd* gene has never been identified in any yeast species (Knuf, Nookaew I Fau - Remmers et al.). However, the *frd* gene was successfully engineered and expressed in yeast *Aspergillus sacchatolyticus* for production of succinic acid (Yang, Lübeck et al. 2016). This leads to the possiblity of engineering *C. albidus* to produce succinic and malic acids from a wide variety or carbon sources.

3.6.4 Lipid production

Lipids are perhaps the most versatile manufacturing commodity and find applications in nearly all industries resulting in a global market of USD 80 billion annually. Lipids find various industrial and technology applications in coatings and polymers, printing inks, lubricants, cosmetics/pharmaceuticals, leather processing, surfactants, solvents, hydraulic fluids, pesticide/herbicide adjuvants, and as fuels to name a few.

However, all lipids used today are of plant origin and therefore, their production exerts significant stress upon arable lands, biodiversity and local communities. There are critical concerns regarding the environmental degradation and the socio-economic impact of rainforest destruction for large palm oil plantation in Southeast Asia, which have numerous detrimental consequences including loss of biodiversity, extinction of endangered species, and massive greenhouse gas emissions. Therefore, research efforts are being directed towards development of a sustainable bio-based alternative to at least partially meet the ever increasing lipid demand. Most of these efforts have focused upon the use of oleaginous or oil-producing microbes such as

algae or yeast for conversion of cheap carbon sources into lipids. Oleaginity in *C. albidus* has been well documented with the maximum lipid accumulation reaching 29% w/w while utilizing waste derived volatile fatty acids as the carbon and energy source (Fei, Chang et al. 2011, Vajpeyi and Chandran 2015) and 43% w/w when using glucose for growth (Hansson and Dostálek 1986).

Specifically pertaining to oleaginity in *C. albidus*, we identified two copies of the *acly* gene and nineteen copies of the *me2* and *mdh2* genes coding for enzymes ATP:citrate lyase and malate dehydrogenase respectively, which ensure the continuous cytosolic availability of acetyl-CoA and NADPH for lipid biosynthesis (Ratledge 2004). We also identified seven copies of genes *idh1*, *idh2* and *idh3* coding for NAD^+ and $NADP^+$ dependent isocitrate dehydrogenase, which diverts carbon flow away from the citric acid cycle to lipid biosynthesis under nitrogen limitation (Tang, Zhang et al. 2009) and eight copies of *acc1*, coding for acetyl-coA carboxylase, which catalyzes carboxylation of acetyl-coA to malonyl-coA, the rate limiting step in lipid biosynthesis. *C. albidus* contains both *fas2* and *fas1* genes coding for α- and β- subunits of cytosolic type I fatty acid synthase (FAS), typical in yeasts (Tehlivets, Scheuringer et al. 2007).

3.6.5 Arsenic and heavy metal bioremediation

Heavy metals can accumulate in the food chain through contaminated water and soil and pose significant risks to public health and ecosystem. Several soil-related rhizospheric yeasts have been associated with bioaugmentation of contaminants such as heavy metals and aresenic(Vadkertiová and Sláviková 2006). In general, basidiomycetes yeasts including *C. albidus* are quite sensitive to heavy metals with reported minimum inhibitory concentration

(MIC) of 2mM, 1 mM, 0.5 mM, 1mM for metals zinc, copper, nickel and cadmium respectively (Vadkertiová and Sláviková 2006, Ramos-Garza, Bustamante-Brito et al. 2015). However, in contrast to the isolated monocultures, *Cryptococcus sp.* was reported to tolerate much higher concentrations of heavy metals when growing symbiotically on plant roots. *Cryptococcus* sp. strain CBSB78 was reported to be resistant to 20 mM Cd^{2+}, 20 mM Pb^{2+}, 10 mM Zn^{2+}, and 7 mM Cu^{2+}, and could improve the survival rate of inoculated *Brassica* plants by 20% (Deng, Wang et al. 2012).

In case of arsenic however, *C. albidus* exhibited a much higher resistance compared to other yeasts, with reported MIC of 30 mM for arsenate (As^{5+}) and 100 mM for arsenite (As^{3+}). In general, arsenate is less toxic than arsenite and most bacteria and fungi exhibit a higher tolerance to arsenate(Ramos-Garza, Bustamante-Brito et al. 2015). The reversal of toxicity in *C. albidus* suggests that it may possess a different mechanism for arsenic uptake and remediation. However, the mechanisms governing arsenate uptake and transformation by yeasts are poorly understood and to date, no studies have been performed on *C. albidus.* Most known bacterial and fungal mechanisms for arsenic remediation rely either upon reduction of pentavalent arsenate to trivalent arsenite and expelling the latter form the cell (Čerňanský, Kolenčík et al. 2009), methylation (Qin, Rosen Bp Fau - Zhang et al.) or complexation of arsenic with other molecules to less toxic forms (Rosen 2002). In eukaryotes, heavy metal resistance in conferred by members of multidrug resistance associated protein (MRP) group; we identified several copies of MRP family ATP-binding protein in *C. albidus.* In addition, we also found copies of *ycf1, arsH* and *gst* genes coding for metal resistance protein, arsenic resistance protein and glutathione-S-transferase protein (EC 2.5.1.18) respectively. The function and mechanism of *arsH* gene is not

very well understood, however, it is thought to be homologous to the arsenic resistance gene (*arsB, arsC, arsR)* family present in bacteria, which codes for arsenic reductase enzyme responsible for reduction of arsenate to arsenite (Leung, Wu et al. 2010). The gene *ycf1p*, an MRP homolog, has been shown to confer cadmium (Cd^{+2}) resistance in *S. cerevisiae* (Li, Szczypka et al. 1996). The role of glutathione-S-transferase product of *ure2* gene in resistance to a broad range of heavy metals has been well studied (Rosen 2002, Todorova, Kujumdzieva et al. 2010) and it is understood that the detoxification of arsenate through reduction to arsenite requires the reducing power of glutathione.

In addition to arsenic and heavy metal bioremediation, *C. albidus* has been found to naturally exist in environments contaminated with crude petroleum along with other lipase secreting *Cryptococcus* spp. such as *C. diffluens and C. uzbekistanesis* (Yalçın, Çorbacı et al. 2014). *C. albidus* strains isolated from such environments exhibited extracellular lipase secretion in response to various lipids such as tributyrin, fish oil and olive oil, with maximum lipase activity of 47.26 U/mg. We identified several genes encoding various lipase enzymes in *C. albidus*, including two copies of *pla1* and *plb2* gene coding for phospholipase A and B (EC 3.1.4.4) and two copies of *mgll* gene encoding acylglycerol lipase (EC 3.1.1.23). *C. albidus* also contains a *lac3* gene homolog coding for multicopper oxidase laccase enzyme (EC 1.10.3.2), which has been used for detoxification of industrial effluents from paper and pulp, textile and petrochemical industries(Riva 2006) and as a bioremediation agent for pesticides and certain explosives such as TNT in soil (Cheong, Yeo et al. 2006). Laccase has long been implicated as the virulence agent in some *Cryptococcus* spp. including *C. neoformans* (Waterman, Hacham M Fau - Panepinto et al.), however, the laccase from *C. albidus*, with no known pathogenicity, has

been successfully utilized for reduction of dye content from paper mill effluent, where extracellular laccase activity reached 2 U/mL.

3.6.6 Ethylene production

Ethylene is the most produced organic compound worldwide. The global demand for ethylene reached 150 million tons in 2016 with an economic impact of US$200 billion per year (Eckert C Fau - Xu, Xu W Fau - Xiong et al.). Ethylene is the most widely used feedstock in several industries including plastics, textiles, and solvents. In addition, ethylene can also be catalytically polymerized to gasoline-rich hydrocarbons in the C5-C10 range. Ethylene is currently produced from steam cracking of fossil fuels or from dehydrogenation of ethane, representing the largest CO_2-emitting process in the chemical industry (Ipatieff and Corson 1936, Kusmiyati 2005). Given the ethylene industry's massive size, it alone accounts for 1.5% of United States' carbon footprint (Worrell, Phylipsen et al. 2000). A renewable route to ethylene production would therefore fulfill an enormous energy and chemical market while helping to preserve the environment.

Ethylene can also be produced biologically. It is a plant hormone that modulates growth and development, and functions in the defense response to abiotic or biotic stress including pathogen attack. *C. albidus* has been shown to spontaneously produce ethylene via oxidation of 2-keto-4-methylthiobutyric acid (KMBA), a transaminated derivative of L-methionine (Fukuda, Takahashi et al. 1989). The conversion of KMBA to ethylene by *C. albidus* is catalyzed by the enzyme NADH–Fe(III)EDTA oxidoreductase. This enzyme catalyzes the release of hydroxyl radicals, which react with KMBA to produce ethylene. Similar enzyme, which required NADH,

Fe(III)EDTA, and oxygen as cofactors to produce ethylene from KMBA, was isolated from the cell-free extracts of *E. coli* (Fukuda, Takahashi et al. 1989, Ladygina, Dedyukhina et al. 2006). However, the ethylene production rate (25.60 nL/mL/h) of *C. albidus* was highest among various ethylene producing bacteria and fungi and even within the *Cryptococcus sp.* with almost a 20-fold increase over *C. laurentii* (Fukuda, Takahashi et al. 1989).

3.6.7 Bio-control of pathogens

Biological control of the post harvest decay of produce through antimicrobial compounds produced by yeasts has been studied extensively and developed commercially as an alternative to the chemical means of control (Fan and Tian 2001, Chen, Li et al. 2012). *Cryptococcus* spp. are especially interesting since they produce a vast number of secondary metabolites, such as antibacterial, antifungal, antiviral, cytotoxic and hallucinogenic compounds (Oro, Ciani et al. 2016). The antimicrobial potency of *C. albidus* against several pathogenic species including *Alternaria* spp., *Fusarium* spp. (Chen, Li et al. 2012), *Penicillium digitatum* (Oro, Ciani et al. 2016), *Penicillium expansum* (Lutz, Lopes et al. 2013) and *Borytis cinerea* (Lutz, Lopes et al. 2013, Oro, Ciani et al. 2016) has been successfully demonstrated. The putative mechanism of action is thought to be a cumulative effect of several factors including biofilm formation, production of toxins, production of hydrolytic enzymes and competition for nutrients. The extracellular proteinase activity of *Cryptococcus* spp. and other basidiomycetes yeast is well documented (Chen, Blank et al. 1996, Kudryavtseva, Dunaevsky et al. 2008). In *C. albidus*, we identified several genes for production of extracellular proteolytic enzymes such as *yme1, atg4, cts* encoding for metalloproteinase, cysteine protease (EC 3.4.22.-), serine protease (EC 3.4.23.5) respectively. In addition to the proteolytic activity, *C. albidus* also contains genes encoding for

several other hydrolytic enzymes including *glu* for β-1-3-glucanase (EC 3.2.1.39) and *sgB* homolog for chitinase (EC 3.2.1.14). *C. albidus* was also reported to employ biofilm formation as an antimicrobial mechanism (Lutz, Lopes et al. 2013). Biofilm formation is a characteristic virulence mechanism of several pathogenic *Cryptococcus* spp. including *C. neoformans* and *C. gattii*. While there are no studies on the mechanism of biofim formation in *C. albidus,* we identified several genes, which have been reported to play a role in biofilm formation in other fungal species. *C. albidus* contains transcription factor *Efg1*, which is a global regulator of the surface protein genes and hyphal formation in *Candida albicans*. We also identified other cell wall biogenesis genes such as *fks1, bglx* encoding 1,3-β-glucan synthase (EC 2.4.1.34) and β-glucosidase (EC 3.2.1.21) enzymes, which are reported to be upregulated during biofilm formation (Fanning and Mitchell 2012) and have been reported to be essential to maintaining viability and shape in *C. neoformans* (Selvakumar, Miyamoto et al. 2006).

3.6.8 Xylanase production

Xylanase (EC 3.2.1.8) is a commercially important enzyme, which degrades hemicellulose (β - 1,4-xylan) into xylose and is used for converting cellulosic matter such as plant cell walls into useful nutrients. Endo-1 ,4- β-xylanases find applications in paper and pulp industry for bleaching, in wheat flour for improving dough handling and quality of baked products, for the extraction of coffee, plant oils, and starch and, in the improvement of nutritional properties of agricultural silage and grain feed (Beg, Kapoor M Fau - Mahajan et al.). Xylanase enzymes are usually present in bacteria and certain fungi but are very rarely reported in yeasts. However, xylanase encoding *xln* gene is present in *C. albidus* and several studies in the past have demonstrated the extracellular secretion of *C. albidus* xylanase for hemicellulose degradation. *C.*

albidus was able to grow on wood by converting β -1,4-xylan into xylose, which was then catabolized by the cells. The enzyme was reported to be almost entirely extracellular (Biely P Fau - Vrsanska, Vrsanska M Fau - Kratky et al.). The lignocellulose degradation capacity of *C. albidus,* in conjuction with *S. cerevisiae,* has also been utilized for production of ethanol from agricultural waste residues (Thakur 2009). Other reported uses include biopulping of eucalyptus, where xylanase from *C. albidus* was found to effectively reduce the lignin content of wood chips for use in the pulp and paper industry (Singhal, Jaiswal Pk Fau - Jha et al.).

3.7 Pathogenicity

Among the 39 basidiomycetous yeasts characterized under the genus *Cryptococcus,* only *C. neoformans* and *C. gattii* are considered to be human pathogens, responsible for causing cryptococcosis in immunocompromised and immunocompetent patients respectively (Ma and May 2009). The cases of *C. albidus* exhibiting pathogenicity, however, are exceedingly rare. A systematic review of non-neoformans associated cases of cryptococcosis (Khawcharoenporn, Apisarnthanarak et al. 2007) reported that there have been about 18 cases where *C. albidus* has been isolated as the pathogen. The possible explanations for the lack of pathogenicity in *C. albidus* include its inability to grow at relatively high body temperature of the host (37^{o}C) and the lack of melanin production due to comparatively lower laccase (EC 1.10.3.2) activity (Ikeda, Sugita et al. 2002, Ma and May 2009). The inability of *C. albidus* to grow at temperatures above 30^{o}C is uncommon in genus *Cryptococcus* and the reason is not yet very well understood. A study found a *C. neoformans* mutant lacking calcenurin encoding *cna1* gene to be viable at 24^{o}C

but not at mammalian physiological temperature and therefore, a mechanism of regulation of growth at elevated temperatures by signaling cascades involving calcineurin was proposed (Perfect 2005). Similarly, another study found an *ras1* gene mutant of *C. neoformans* to be viable but unable to grow at 37^{o}C. However, in *C. albidus*, we found copies of *cna1*, *ste20* and *ras* related *rac1* gene, which acts downstream of *ras* and coordinately with *ste20* to control high-temperature growth in *C. neoformans* (Vallim, Nichols et al. 2005), which points to an alternate mechanism of temperature sensitivity in *C. albidus.* The restriction of RNA synthesis at 37^{o}C due to a temperature sensitive RNA polymerase in *C. albidus* has also been proposed to account for cessation of growth (Stetler, Boguslawski et al. 1978). However, due to several conflicting studies and observations, the effect of high temperatures on growth of *C. albidus* remains unclear and further studies are warranted.

The other factor responsible for the lack of virulence in *C. albidus* is the absence of Melanin production. Melanin is produced by oxidative polymerization of catechols or aminophenols by the laccase enzyme and protects the cells from high temperatures, freezing and thawing, and compared to non-melanized *C. neoformans* cells, melanized cells are less susceptible to oxidants, contributing to increased virulence(Ma and May 2009). We identified a laccase encoding *lac1* gene in *C. albidus*, which suggests that it should also be able to produce melanin. A study on melanin production in non-neoformans *Cryptococcus* spp. (Ikeda, Sugita et al. 2002)reported laccase activity and positive oxidation of laccase substrate by *C. albidus*, however, the activity was found to be much lower compared to *C. neoformans.* Electrophoresis of laccase enzymes from different species showed qualitative differences between *C. neoformans* and other

Cryptococcus spp., suggesting that the species other than *C. neoformans* produce laccase with different molecular structures, which points to possible differences in their evolutionary origin.

3.8 Conclusions

A number of studies on characterization of pathways of *Cryptococcus* genus have been focused upon *C. neoformans* to study their role in its pathogenicity. However, the potential for commercial biotechnological applications of various *Cryptococcus* species have so far been largely ignored. An evaluation of biochemical pathways and the genetic capabilities of the 'non-conventional' yeast *Cryptococcus albidus* presented herein based on its newly sequenced genome identified several genes and pathways of immense industrial and commercial potential. The application portfolio of *C. albidus* includes bioremediation, secretion of several extracellular proteins of commercial significance including proteases, RNAases, laccase, xylanase, pectinase, lipases, and commercial for production of industrially important chemicals such as lipids, organic acids and alcohols. Several studies in the past have attempted to utilize *C. albidus* for these applications. However, such attempts have been impeded by the unavaialbility of the genome and a general lack of knowledge pertaining to the underlying metabolic pathways. We overcome such limitations using the *C. albidus* genome sequence and also highlight various pathways and specific genes involved in the uptake and assimilation for different carbon, nitrogen and sulfur sources. Further research should now concentrate on fundamental mechanisms of gene expression and protein secretion in *C. albidus* for its biotechnological applications.

CHAPTER 4

GLOBAL TRANSCRIPTOMIC AND PROTEOMIC RESPONSES OF THE LIPID PRODUCING YEAST *Cryptococcus albidus* TO NITROGEN LIMITATION

4.1 Introduction

Lipids are among the most versatile manufacturing commodity with applications in nearly all industries resulting in a global market of USD 80 billion annually. However, all lipids used presently are of plant origin and the agricultural expansion necessitated by the increasing global demand for vegetable oils has become the primary cause of deforestation. As forests are turned into croplands, combustion and decay of biomass contribute to the accumulation of greenhouse gases in the atmosphere. By acquiring extensive arable lands, plantations also affect land availability for smallholder farmers and communities, potentially altering local livelihood options (Austin, Kasibhatla et al. 2015). Consequently, there are critical concerns regarding the environmental degradation and the socio-economic impact of rainforest destruction for large oil plantations in Southeast Asia, which have numerous detrimental consequences including loss of biodiversity, extinction of endangered species, and massive greenhouse gas emissions. Therefore, the use of oleaginous or oil-producing microbes such as algae or yeast for conversion of inexpensive carbon sources into lipids to supplement the lipid demand has been of great industrial and research interest. To improve the cost competitiveness of microbial lipids, a large variety of low-cost substrates have been used as carbon sources (Papanikolaou and Aggelis 2011) for the oleaginous microorganisms such as sugar-enriched wastes or residues, polysaccharides, N-acetylglucosamine (Wu 2010), hydrolysates of various products or by-products (Papanikolaou 2010), n-alkanes, ethanol, glycerol (Papanikolaou, Fakas et al. 2008), sewage sludge (Angerbauer, Siebenhofer et al. 2008) and organic acids generated through fermentation of organic wastes (Vajpeyi and Chandran 2015).

Oleaginous yeasts *Cryptococcus albidus* has been reported to efficiently convert a variety of carbon sources including volatile fatty acids (VFA) generated from anaerobic fermentation of

organic waste streams into lipids (Vajpeyi and Chandran 2015). However, the underlying metabolic pathways and factors affecting the lipid productivity (oleaginity) of *C. albidus* and other oleaginous microorganism are poorly understood. Relative carbon to nitrogen ratio (COD:N) has long been implicated to play a role in oleaginity and based primarily on empirical evidence, it is assumed that nitrogen acts as a metabolic 'switch' to divert carbon away from catabolic tricarboxylic acid (TCA) cycle towards anabolic fatty acid biosynthesis under nitrogen limited growth conditions. This phenomena has been reported in several studies utilizing a diverse array of feed stocks and microorganisms including *Candida utilis* using glucose (Botham and Ratledge 1979), *Rhodosporidium toruloides* using glucose (EVANS and RATLEDGE 1984), *Rhodosporidium glutinis* using monosodium glutamate wastewater (Xue, Miao et al. 2008) , *Yarrowia lipolytica* using glycerol (Papanikolaou and Aggelis 2002), *Cunninghamella echinulate* using xylose (Fakas, Papanikolaou et al. 2009) and including our previous study (Vajpeyi and Chandran 2015) using VFA, where the intracellular lipid content of *C. albidus* cultures subjected to nitrogen limitation increased by 52% w/w (from 19% to 29%) (Vajpeyi and Chandran 2015). Furthermore, several studies(Morin, Cescut et al. 2011, Pan and Hua 2012, Zhang, Zhang et al. 2014, Kerkhoven, Pomraning et al. 2016) have attempted to elucidate the underlying biochemical response of oleaginous microorganisms to nitrogen limitation induced stress. The classical belief is that under limiting nitrogen concentrations, the flux through the tricarboxylic acid (TCA) cycle slows down, resulting in buildup of citrate inside the mitochondrion(Liu, Zhao X Fau - Wang et al. 2011). Consequently, citrate is transported outside the mitochondria and is cleaved by the enzyme ATP: Citrate lyase (ACL, EC 2.3.3.8) to form acetyl co-A and the excess acetyl co-A is directed towards fatty acid synthesis for storage (Ratledge 2004).

Even though this general metabolic framework for lipid metabolism in oleaginous species is now available, the understanding of the molecular basis of microbial oleaginity remains limited, largely because most systems biology studies conducted thus far have been targeted studies which exclusively study the expression changes in a subset of genes or proteins known *a priori* to be associated with lipid and nitrogen metabolism. However, cell cycles are very tightly integrated and therefore, it is difficult to elucidate the full complement of factors affecting oleaginity while studying genes or proteins or even transcriptomes and proteomes in isolation. Furthermore, to our knowledge, no literature pertaining to -omics of oleaginity in microbes utilizing VFA or other less ideal carbon sources currently exists. Further, nearly all attempts at such characterization have utilized glucose as the carbon and energy source and therefore, the current understanding of lipogenesis is heavily skewed towards understanding carbohydrate metabolism. However, from a practical perspective, glucose is far too expensive to support any large-scale application of such platforms and therefore, it is necessary to understand the conversion of alternate less expensive carbon sources to lipids at the biological systems level.

Therefore, our primary objective herein was to study the comprehensive metabolic response of oleaginous yeast *C. albidus* utilizing VFA as the assimilative carbon source, to nitrogen limitation by studying the global transcription and translation level changes in conjunction. We demonstrate that the lipid accumulation under nitrogen limitation is tightly integrated with other cellular processes related to lipogenesis, autophagy, macromolecule biosynthesis, and especially, the amino acid metabolism. We also highlight the interconnectedness of the cellular carbon and nitrogen cycles and the overexpression of genes influencing the distribution of carbon flux between energetic, assimilative and the storage pathways under nitrogen limitation.

4.2 Materials and Methods

4.2.1 Culture conditions

Cryptococcus albidus var. albidus (ATCC 10672) was obtained from American Type Culture Collection (ATCC, Manassas, VA). *C. albidus* cultures were propagated through a monthly subculture on liquid yeast mold (YM) broth (BD Difco, Sparks, MD). The nutrient medium for all the experiments contained, per liter: 3g KH_2PO_4, 1g $MgSO_4 \cdot 7H_2O$, 15 mg $FeCl_3 \cdot 6H_2O$, and 7.5 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $CuSO_4.5H_2O$ (ACS grade chemicals, Fisher Scientific, Pittsburgh, PA). Ammonium chloride (NH_4Cl) was used as the nitrogen source and VFA were used as the sole carbon and energy source. All the equipment and solutions for microbial cultivation were steam sterilized at 121°C and 15 psi for 15 min. VFA were sterilized using a 0.2 μm syringe filter (VWR scientific, Bridgeport, NJ) and added to the autoclaved medium upon cooling to prevent losses by volatilization. 24-hour-old *C. albidus* cultures in the late log phase (data not shown) grown on YM broth were used as the inoculum (10% v/v). Reactor pH was measured using an autoclavable pH electrode (Cole Parmer, Vernon hills, IL) and automatically controlled (Jenco 3676, San Diego, CA) at 6.0 ± 0.1 through addition of 2.0 mol/L HCl or NaOH solutions. Sterile lab air (filtered through 0.2 um filter, Millipore MTGR 05010 Bedford, MA) was supplied at 0.2 scfm (360 L/h) to keep the dissolved oxygen concentration near saturation. *C. albidus* cells were cultured in duplicate chemostats (V = 6 L), operated at a hydraulic retention time (HRT) of 3 days. The influent VFA concentration was 6500 mg COD/L in all experiments and three different nitrogen concentrations of 1300, 260 and 26 mg NH_4^+-N/L were chosen such that the final influent COD:N ratios were 5:1 w/w, 25:1 w/w and 250:1 w/w respectively. These ratios

represent nitrogen in relative excess, mild limitation and severe limitation respectively, based on our previous study(Vajpeyi and Chandran 2015).

4.2.2 Chemostat performance monitoring

20 mL samples were withdrawn once a day, using a sterile sampling port assembly. Cell concentrations were measured as absorbance of the culture broth at 600 nm (OD_{600}, Genesys-20 spectrophotometer, Thermoscientific, Waltham, MA). Culture broth was centrifuged (10,000 rpm, 9133 x g, 10 min) and filtered using a 0.2 μm syringe filter (VWR scientific, Bridgeport, NJ) for VFA and nitrogen measurements. Total VFA concentrations were determined using colorimetry (Hach volatile acids reagent set (2244700). Ammonia concentrations were measured using an ion-selective electrode (Thermofisher scientific, Waltham, MA).

4.2.3 Transcriptome analysis

Cells were harvested from 100 μL culture broth (~$2x10^7$ cells) by centrifuging at 16,000xg for 10 minutes at 4^{o}C followed by extraction of RNA from the resulting pellet using Qiacube^{TM} (Qiagen, Valencia, CA). A 200 bp RNA library of *C. albidus* was prepared and sequenced using Ion-torrentPGM^{TM} (Thermofisher scientific, Waltham, MA) as per the manufacturer protocol and all the quality control steps were performed using Bioanalyzer 2100 (Agilent, Santa Clara, CA). The raw reads were quality checked and filtered using MOTHUR (Schloss, Westcott et al. 2009), following which, ribosomal RNA (rRNA) was removed by aligning the reads to Silva rRNA databases using Bowtie2 (Langmead and Salzberg 2012). The filtered and rRNA removed reads were annotated by aligning to the reference *C. albidus* genome, recently sequenced by us

(Vajpeyi and Chandran 2016). Finally, a gene read count table was prepared, normalized to the relative library sizes and analyzed for differential expression using edgeR package (Robinson, McCarthy et al. 2010) in R.

4.2.4 Proteome analysis

Cells were harvested from 5 mL culture broth (~$2x10^7$ cells) by centrifuging at 16,000xg for 10 minutes at 4^{o}C. The resulting pellet was washed twice with 1 mL cold 1X PBS buffer and suspended in 100 μL ChIP lysis buffer containing 1 mM fresh PMSF. The cells were lysed by bead beating for 2 minutes (4 x 30s cycles; 1 min. on ice between cycles). Whole protein lysate was collected and proteins were separated using Novex (Life Technologies, Carlsbad CA) Tris-Glycince SDS gel kit. Protein extracts were heated to 80°C for 15 min, Cysteines were reduced in 2.8 mM dithiothreitol at 60°C for 30 min and alkylated in 8.1 mM iodoacetamide for 30 min at 25°C in the dark. Proteins were digested with trypsin (6 ng ml-1, PromegaCorp.) in 50 mM NH_4HCO_3 for 16 h at 37°C, and 75 fmol of a digest of yeast alcohol dehydrogenase were added to all protein samples (injection vol = 3 ml) as internal standards. Prior to analytical separation on a NanoAcquity UPLC(Waters Corp.), peptides were trapped on a Symmetry C18Trap column (5 mm, 180 mm x 20 mm, Waters Corp.) for 2 min at 10 ml min^{-1} in 1% solvent B (0.3% formic acid in acetonitrile) and 99% solvent A (0.3% formic acid, aqueous). Peptides were separated by a 34 min chromatographic separation with a 5–40% linear gradient of solvent B on a NanoAcquity UPLC at 300 nl min^{-1} using HSS T3 1.8 mm,75 mm x 25 cm reversed phase column operated at 45°C. Mass spectra were recorded with a Synapt G2 QTOF mass spectrometer equipped with an ion mobility separation (IMS) cell (Waters Corp.), which alternated low- and high-energy scans (MSE data-independent acquisition) with a scan time of 0.6 s. A reference mass of [Glu1]-fibrino peptide was infused at 500 nl min^{-1} and sampled every

30 s. All spectra were collected in resolution IMS mode and analyzed against *C. albidus* reference database using Protein Lynx Global Server (PLGS, Waters Corporation, Milford MA). The main settings for the PLGS analysis were as follows: (i) carbamidomethylation of cysteine, (ii) amaximum false identification rate of 4%, (iii) minimum fragment ion matches of 3 per peptide and 7 per protein and (iv)minimum peptide matches per protein of 2. The resulting protein concentrations were normalized to the total protein content and expressed as nmol/ng total protein.

4.2.5 Statistical analysis

Transcriptome data were collected from biological duplicates but a single analytical measurement (RNAseq) resulting in two measurements per gene for each COD:N ratio. Only genes that were detected in both the samples were used for further analysis. While, proteome data presented here were obtained from both biological and analytical duplicates, resulting in four measurements per protein for each COD:N condition. Furthermore, protein measurements not detected at least once in both the biological duplicates were deemed below the quality threshold and discarded. Differential gene and protein expression analysis was performed between the control (COD:N =5:1) and the limitation condition (COD:N = 250:1).

Differential gene expression analysis on the filtered gene count data was performed using the generalized linear model (glm) fit in edgeR. Genes with the false discovery rate (FDR) values =< 0.05 were considered differentially expressed. Up- and down- regulation was determined using the log fold change (log_2FC) value, which is the base 2 logarithm of the ratio of the normalized gene counts. Proteome data were compared using Student's t-test. To minimize the Type-I errors (false positives), arising from multiple testing, the resulting p-values were adjusted

using Benjamini-Hoch correction in R and protein comparisons with FDR =< 0.05 were considered statistically significant.

A table containing log fold changes (log_2FC) and corresponding FDR values for all the genes and proteins discussed in the text has been included in the appendix (Table 9) and a complete list of the log fold change and FDR values are provided as a Microsoft Excel spreadsheet in supplementary information.

4.3 Results and discussion

4.3.1 Chemostat performance

Under progressively nitrogen limiting conditions (increasing initial COD:N ratios), the cells exhibited an increase in the relative COD to N uptake and similar to our previous batch studies, the ΔCOD/ΔN uptake ratio increased from 13.41±1.39 at non limiting condition (COD:N = 5:1) to 511.56±136.66 at extreme limitation (COD:N =250:1). These results show that the nitrogen limitation resulted in disproportionately higher uptake of carbon per gram nitrogen consumed. This finding is further bolstered by the carbon and nitrogen based cell yield coefficients $Y_{X/COD}$ and $Y_{X/N}$. In accordance with our previous batch study, the COD based biomass yield ($Y_{X/COD}$) remained constant at 0.19±0.02 mg X/mgCOD but the nitrogen based yield coefficient ($Y_{X/N}$) increased from 2.85±0.29 to 88.40±22.81 mg X/mg NH_4^+- N. This invariability in $Y_{X/COD}$ can be attributed to the fact that the cells were consuming same amount of COD per gram cell equivalent regardless of the exogenous nitrogen concentration. However, the increase in $Y_{X/N}$ indicates that this additional COD uptake was being channeled towards carbonaceous storage compounds rather than growth. Indeed, the intracellular lipid content of *C. albidus* increased from 17.7±1.6% to 27.7±3.3% under nitrogen limitation, which is also in agreement with our

previous study and suggests that the excessive carbon uptake under nitrogen limitation was indeed channeled towards lipid biosynthesis.

4.3.2 Carbon Metabolism of Cryptococcus albidus

Almost all studies investigating the transcriptomic or proteomic basis of enhanced lipid production by the oleaginous microorganisms have been performed with glucose as the sole carbon and energy source. Therefore, the current understanding of the genes and factors influencing the fatty acid biosynthesis in oleaginous microorganism is rather skewed towards carbohydrate metabolism. For example, a proteomic study on another basidiomycetic yeast *Rhodosporidium toruloides* cultured on glucose observed carbon catabolism repression and reported a downshift in proteins involved in the utilization of alternative carbon sources (Liu, Zhao X Fau - Wang et al.). Similarly, several other studies using glucose as the carbon source and various oleaginous microorganisms such as *Rhodosporidium toruloides* (Zhu, Zhang et al. 2012), *Y. lipolytica (Morin, Cescut et al. 2011), Aspergillus oryzae (Tamano, Bruno et al. 2013)* reasserted the classical opinion of citrate buildup in mitochondria resulting in lipid production. We found significant differences both in the central carbon metabolism and the pathways through which VFA could be channeled into fatty acid biosynthesis owing to the differences in the mechanism of transport of VFA and various intermediates both from outside the cell and within different subcellular organelles. Herein we summarize the VFA uptake and transport mechanisms and the differences with carbohydrate metabolism.

Short chain carboxylic acids are soluble in the lipid bilayer and can therefore passively diffuse through the cell membrane when present in un-dissociated form and through proton symport catalyzed by monocarboxylate transporters, when present in dissociated form(Casal, Paiva S Fau

- Queiros et al.). At our operational pH of 6.0±0.1, the VFA would have been present in dissociated ionized form (pKa = 4.76, 4.88 and 4.82 for acetate, propionate and butyrate respectively). In order to participate in the cellular metabolism, VFA must be first activated in the cytosol by the addition of coenzyme A (-CoA) by the enzyme acetyl-CoA synthetase (ACS, EC 6.2.1.1), encoded by the gene *acs* and transported to mitochondrion to participate in the TCA cycle In contrast, glucose cannot passively diffuse inside the cell and enters via facilitated diffusion, mediated by ATP-dependent hexose transporters (HXTs)(Kim, Roy et al. 2013). Upon entry, glucose participates in the cellular metabolism through glycolysis in the cytosol, where it is converted to pyruvate, which is then transported to mitochondria and converted to acetyl-CoA by the action of pyruvate dehydrogenase complex (EC 1.2.4.1) to participate in the TCA for energy production(Zhu, Zhang et al. 2012). The TCA cycle is indispensible for providing carbon backbones for synthesis of cellular macromolecules. However, it is strictly oxidative in terms of carbon and generates reduced electron carriers. From an overall carbon balance perspective, it offers no net carbon assimilation, since the two carbon molecules entering the cycle as acetyl-CoA are lost as carbon dioxide, and therefore, the assimilative acetate metabolism differs significantly from the carbohydrate metabolism. Carbohydrates are indispensable for microbial growth and are necessary for biosynthesis of several important structural and functional macromolecules such as N-acetylglucosamine for cell wall synthesis, ribose sugars for nucleotide synthesis,among others (Gancedo 1986). While glycolytic intermediates can provide precursors for their biosynthesis when glucose is available, acetyl-CoA assimilation in case of acetate can be quite complex as depicted in Figure 10. Acetyl-CoA must enter the glyoxylate cycle in peroxisomes, which bypasses the second decarboxylation step in the TCA to synthesize malate by the action of enzymes isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MLS,

EC 2.3.3.9) encoded by *aceA* and *aceB* orhthologs respectively, which cn then form phosphoenolpyruvate (PEP) and enter gluconeogenesis(Kornberg Hl Fau - Krebs and Krebs)(Figure 10). A study on the metabolic flux of ^{13}C-labeled acetate in *S. cerevisiae* (dos Santos, Gombert Ak Fau - Christensen et al.) showed that the glyoxylate bypass pathway was the sole route for carbon assimilation. Thus, the glyoxylate cycle serves as a link between catabolic activities and biosynthetic capacities, and enables the cells to utilize acetate as the sole carbon source.

Therefore, upon uptake and activation, metabolic pools of acetyl-CoA in cytosol must be partitioned into peroxisomes and mitochondrion depending on the assimilative and energetic requirements of the cell, while cytosolic acetyl-CoA is used for biosynthesis of lipids (Nielsen 2014). Another complication arises due to the fact that mitochondrion membranes are impermeable to acetyl-CoA due to its amphiphilic nature and bulkiness of coenzyme-A; (Strijbis and Distel 2010) and due to a lack of studies pertaining to acetate metabolism, it is unclear how acetyl-CoA is transported to mitochondrion to participate in the TCA. Two possible routes have been identified. In one route, the malate produced through the glyoxylate cycle in peroxisomes can be transported into mitochondria (Chen, Zhang et al. 2015), facilitated by the mitochondrial solute carrier family (SLC25A11) transporter protein OGC, which pumps out α-ketoglutarate in exchange for malate entry into the mitochondrion(Palmieri 2013). In that case, all acetyl-CoA must be first channeled to peroxisomes without regards to its eventual assimilative or energetic destiny. Another possible route is to transport it in the form of acetyl-carnitine through the carnitine shuttle, however, this route is exclusively used for transport of large chain fatty acids into mitochondria for β-oxidation (Strijbis and Distel 2010).

Effect on oxidative carbon metabolism

Differential gene expression data revealed near complete suppression of the TCA cycle (Figure 10) under nitrogen limited conditions with nearly all the related genes (*cs, idh3, aco, aco2, ogdh, sdh2, sdh3* and, *mdh2*) (Table 8) showing a negative fold change under both nitrogen limited conditions (COD:N≥ 25:1) compared to sufficient nitrogen condition (COD:N = 5:1). On a proteome level however, we found the concentrations of all related enzymes except IDH3 to be invariant (Figure 10). TCA cycle is allosterically controlled by the intracellular ADP/ATP ratio, wherein high ATP concentration increases the K_M of citrate synthase enzyme for acetyl-CoA, resulting in reduced flux through the cycle. Therefore, it is possible that the overall flux through the TCA cycle may have been lowered through post-translational control, while the protein levels remained unchanged.

Table 8 Differential gene expression and corresponding FDR values for TCA cycle genes under various nitrogen limiting conditions.

Gene	COD:N =25:1		COD:N = 250:1	
	log_2FC	**FDR**	**log_2FC**	**FDR**
cs	-1.51	1.97e-09	-1.01	2.45e-04
idh3	-0.91	0.041	-0.75	0.045
aco	-1.31	6.52e-07	-0.39	0.357
sdh2	-1.64	0.002	-0.45	0.609
sdh3	-3.44	1.70e-07	-1.60	0.0259
mdh2	-1.78	2.90e-19	-1.09	2.70e-07

Gene *idh3* encodes the enzyme NAD^+- isocitrate dehydrogenase (IDH3, EC 1.1.1.41), and is especially important since it catalyzes a key step of TCA cycle during acetate metabolism, namely, the oxidative decarboxylation of isocitrate to α-ketoglutarate(Ratledge 2004). The substrate, isocitrate, is the precursor for the glyoxylate bypass, which is the only carbon assimilative pathway during growth on acetate and provides dicarboxylate skeletons for carbohydrate and amino acid synthesis (Cronan and Laporte 2006). The product, α-ketoglutarate participates in glutamate formation, the key step in cellular nitrogen assimilation. Since the flux through the IDH3 enzyme impacts both the carbon and nitrogen assimilation, we propose that this enzyme is an important control point for the overall metabolic rate of cell growth when acetate is the carbon source, and therefore, the a decrease in the expression level of this gene and the corresponding decrease in the protein concentration of IDH3 must indicate that the cells enter a lower metabolic state as a consequence of nitrogen limitation. Indeed, in our previous study on batch cultures of *C. albidus* using VFA as the carbon and energy source, we reported a decrease in the maximum specific cell growth rate (μ_{max}) of the cells in response to nitrogen limitation (Vajpeyi and Chandran 2015).

Under growth on glucose, the supply of acetyl-CoA into TCA cycle is controlled by the activity of mitochondrial pyruvate transporter and the pyruvate dehydrogenase complex(Vacanti, Divakaruni et al. 2014). However, when acetate is present as the carbon source, the malate route controls the entry into TCA rather than the pyruvate route. In accordance, we found the expression levels of cytosolic malate synthase encoding *aceB* gene and the gene encoding the mitochondrial OGC transport protein, which facilitates the entry of malate into mitochondrion, to be nearly two-fold lower in *C. albidus* cultures subjected to severe nitrogen limitation (COD:N = 250:1) (Figure 10). On a proteome level however, the concentration of malate synthase remained

unchanged under nitrogen limitation while the OGC transport protein was not detected by us. Malate synthase enzyme is involved in glyoxylate bypass and its activity has been reported to be post-transnationally controlled through reversible phosphorylation of the enzyme by a specific kinase (Cronan and Laporte 2006). The gene expression levels under mild limitation (COD = 25:1), while numerically lower were not statistically significant. This suggests that TCA cycle suppression under nitrogen limitation is progressive and increases with increase in the degree of nitrogen limitation and that under nitrogen limitation, the transport of acetyl-CoA to mitochondrion is also progressively reduced due to a lower flux through TCA cycle, resulting in its buildup in cytosol. This is an important distinction from the glucose metabolism, where excess carbon builds up inside the mitochondrion in the form of citrate.

Several studies have reported that in oleaginous organisms under nitrogen limitation, the flux through TCA cycle slows down (Liu, Zhao X Fau - Wang et al. , Zhu, Zhang et al. 2012, Liang and Jiang 2013). As a result of decrease in TCA flux, non-oleaginous species show a concomitant decrease in carbon uptake and metabolism or channel it towards carbohydrate storage, while, in oleaginous microorganism, carbon uptake continues unhindered and excess carbon is channeled towards lipid storage (Morin, Cescut et al. 2011). In this study, we found the genes encoding for several monocarboxylate transporters to be statistically invariant (Figure 10) which suggests that nitrogen limitation did not have an effect on carbon uptake. Indeed, we found that the COD consumed per gram cell equivalent remained constant and was thus unaffected by the nitrogen limitation. This excess carbon uptake was presumably channeled toward fatty acid biosynthesis and other forms of carbon storage. At this juncture however, the VFA metabolism again diverges from the glucose metabolism. It has been reported that in the cells metabolizing glucose, citrate builds up in mitochondria due to lower flux through TCA

cycle and this excess citrate is transported outside the mitochondria by the citrate transporter protein (CTP) belonging to solute carrier family (SLC25) (Sun, Aluvila et al. 2010) and converted back to acetyl-coA by the action of *acly* encoded enzyme ATP:Citrate lyase (ACLY, EC 2.3.3.8), which is further channeled towards fatty acid biosynthesis. Several studies have reported the prominent role of *acly* gene in fatty acid biosynthesis and an increase in *acly* expression in various oleaginous species under nitrogen limitation(Morin, Cescut et al. 2011, Zhu, Zhang et al. 2012, Liang and Jiang 2013, Dulermo, Lazar et al. 2015, Tang, Zan et al. 2016). However, we found the gene expression of *acly* and the corresponding protein concentration of ACLY to be 2-fold lower (Figure 10) under both nitrogen limitation conditions. Additionally, while the expression levels of the gene encoding the mitochondrial citrate transporter protein (CTP, SLC25A1) remained unchanged under nitrogen limitation, the protein concentration was nearly 60-fold lower (log_2FC = -5.91) under severe nitrogen limitation (COD:N =250:1) but remained invariant under mild limitation (COD:N = 25:1). This indicates that in case of growth on VFA, citrate is perhaps not the primary supplier of acetyl-CoA for fatty acid biosynthesis. To further understand difference, it is important to first understand the transport of acetyl-CoA between subcellular locations of various metabolic pathways, since the essential metabolic pathways such as fatty acid biosynthesis, the TCA cycle, and the glyoxylate cycle are physically separated into different organelles and the mechanism for shuttling of carbon between them can be markedly different during growth on different carbon sources. In eukaryotes, TCA cycle occurs inside the mitochondrion whereas, the fatty acid biosynthesis occurs in cytosol, and acetyl-CoA, the precursor for both these pathways cannot freely diffuse between cytosol and mitochondrion. Therefore, we propose that once the flux through TCA slows down under nitrogen limitation and when VFA are the sole carbon and energy source,

excess acetyl-CoA would build up in the cytosol and could be directly channeled into the fatty acid biosynthesis rather than being cycled through mitochondrion as citrate, which is perhaps the reason that we observed a decrease in the expression level of *acly* gene and the concentration of citrate transporter protein (SLC25A1). In fact, the ACLY enzyme has been reported to control the glucose to acetate metabolic switch in the cells wherein a deletion of *acly* gene did not have an impact on cell viability as cells were able to upregulate acetyl-coA synthetase (ACSS) encoding *acs1* gene and utilize exogenous acetate instead of glucose to provide acetyl-CoA for *de novo* lipogenesis (Zhao, Torres et al. 2016). Therefore, in absence of glucose, ACSS and not ACLY provides acetyl-CoA needed for lipid biosynthesis. This finding is further bolstered by the fact that we observed the expression levels of genes encoding monocarboxylate transporters, the gene *acs1* and the corresponding protein acetyl-CoA synthetase levels to be statistically invariant under nitrogen limitation (Figure 10), suggesting that the acetate uptake and its entry into cellular metabolism was not impacted by nitrogen limitation. All these findings consistently suggest that nitrogen limited cells were consuming the COD at the same rate but channeling it to intracellular storage rather than energy metabolism.

Metabolism of other VFA

It is also interesting to discuss the metabolism of VFA other than acetate since the metabolic pathways they enter upon uptake are also quite different. Butyrate, a linear four-carbon carboxylic acid undergoes one cycle of β-oxidation to yield two acetyl-CoA molecules. However, β-oxidation takes place inside the mitochondrion and therefore, butyrate, upon conversion to butyryl-CoA, must be transported inside the mitochondrion. This transport is accomplished through the acylcarnitine shuttle enzymes acylcarnitine transferase I and II located

on outer and inner mitochondrial membrane respectively. We found the genes and the corresponding proteins associated with the acyl-carnitine shuttle and β-oxidation to be invariant among the different nitrogen concentration, which suggests that butyrate uptake was also unaffected by the nitrogen limitation (Figure 10). Even though the uptake and entry of butyrate into cellular metabolism was not impacted by the nitrogen limitation, it is still unclear where the butyrate-associated carbon was channeled and to what extent did it participate in the lipid metabolism. Since butyrate can only be converted to acetyl-CoA inside the mitochondria, it must be transported back to cytosol as citrate and cleaved back to acetyl-CoA in order to participate in the fatty acid biosynthesis. However, since the concentration of mitochondrial citrate transporter protein (*ctp1*, SLC25A1, $\log_2$FC = -5.91) (Figure 10) was about 60-fold lower under nitrogen limitation, it remains unclear whether butyrate participated in fatty acid biosynthesis or if it was strictly confined to energy metabolism.

Propionate however, being an odd numbered, 3 carbon acid cannot undergo β-oxidation and instead enters the cellular metabolism through TCA cycle wherein it is first converted to succinate via carboxylation to a methyl-malonyl intermediate (Pronk, van der Linden-Beuman A Fau - Verduyn et al.). However, in *C. albidus,* we did not detect the gene encoding the propionate-CoA carboxylase enzyme, which catalyzes the conversion of propionate to succinate for its entry into TCA cycle. Some other studies have also reported the absence of methyl-malonyl pathway in several yeasts including *S. cerevisiae* (Pronk, van der Linden-Beuman A Fau - Verduyn et al.) and oleaginous yeast *Yarrowia lipolytica* (Uchiyama H Fau - Ando, Ando M Fau - Toyonaka et al.). In *C. albidus*, we found an alternative pathway for propionate assimilation, which has also been also reported in *Y. lipolytica* (Papanikolaou, Beopoulos et al. 2013) wherein propionyl-CoA is converted into 2-methyl citrate by 2-methylcitrate synthase (EC

2.3.3.5), and eventually to succinate through a multistep conversion, which can then enter the TCA cycle through succinate dehydrogenase enzyme. However, under nitrogen limitation, a decrease in the expression levels of the genes encoding for 2-methylisocitrate dehydratase and succinate dehydrogenase (Figure 10) indicates that the assimilation of propionate must slow down under nitrogen limitation. Interestingly, we also detected an acetylating malonate semialdehyde dehydrogenase (EC 1.2.1.18) encoding *mmSa* gene in *C. albidus,* which can decarboxylate propionyl-CoA to produce acetyl-CoA in the mitochondrial matrix, which can then enter the TCA cycle. We found that the *mmSa* gene expression level was also lower (log_2FC = -0.79, FDR= 0.03) under mild limitation, while the gene expression level under severe limitation and protein concentration under both nitrogen limited conditions remained unchanged. Since the TCA cycle is the only possible route for entry of propionate into cellular metabolism, propionate uptake would be expected to decrease concomitantly with decrease in TCA flux under nitrogen limitation. Indeed, in our previous study, we reported preferential consumption of acetate and butyrate over propionate by *C. albidus,* with acetate and butyrate exhibiting much higher specific substrate utilization rates than propionate.

Therefore, out of the three VFA supplied for growth, propionate does not seem to participate in either the energy or the lipid metabolism under nitrogen limitation and is perhaps not an ideal VFA for enhancing lipid accumulation in *C. albidus*. It is evident that in mixed substrate systems, acetate is preferably used for energy and growth over other VFA. In case of VFA derived from anaerobic fermentation of organic wastes, manipulation of fermentation parameters to yield a predominantly acetate product might lead to improved lipid yield downstream.

Effect on assimilative carbon metabolism

Under stoichiometrically balanced (nitrogen-sufficient) growth conditions, acetyl-CoA, the precursor for lipogenesis is partitioned into four major cellular compartments (Figure 10), namely, mitochondrion for energy production, peroxisomes for synthesis of structural macromolecules through glyoxylate bypass, nucleus for synthesis of nuclear macromolecules and finally, into cytosol for lipid and protein biosynthesis. However, under nitrogen limitation, even though the carbon uptake and activation by coenzyme A in cytosol continued unhindered, there is evidence that its flux into different cellular compartments and processes might be redistributed (Morin, Cescut et al. 2011). The decrease in oxidative carbon metabolism due to reduced flux into TCA cycle resulted in decreased transport of acetyl-CoA into the mitochondrion and is evident by the decrease in the expression levels of the mitochondrial OGC transporter protein (SLC25A11) (Figure 10) and the encoding gene.

As regards to the assimilative carbon flux; in peroxisomes, we found that the two critical glyoxylate bypass genes, *aceA* and *aceB* and the corresponding proteins isocitrate lyase and malate synthase were unaffected by the nitrogen limitation. Similarly, *pckA* gene and the corresponding phosphoenolpyruvate carboxykinase enzyme (EC 4.1.149), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvayte (PEP), also remained unaffected. PEP is the precursor for gluconeogenesis pathway, which is responsible for providing carbohydrate skeletons for synthesis of structural and nuclear macromolecules (Turcotte, Liang et al. 2010). During growth on acetate, gluconeogenesis results in the production of glucose-6-phosphate, which is critical for cell growth. For instance, glucose-6-phosphate is required for nucleotide metabolism, glycosylation, cell wall biosynthesis, and storage of carbohydrates (Turcotte, Liang et al. 2010) We found that the expression levels of several genes (*fbp, gapA, tpiA*) involved in gluconeogenesis were invariant under mild nitrogen limitation(COD:N = 25:1) but were

statistically lower under severe nitrogen limitation (COD:N = 250:1) (Figure 10). The enzyme fructose-1,6-bisphosphatase I (EC 3.1.3.11) encoded by the gene *fbp* catalyzes the highly exergonic ($\Delta G'^{o}$ = -16.3 KJ/mol) conversion of fructose-1,6-bisphosphate to fructose-6-phosphate and confers gluconeogenesis its irreversibility(Lehninger, Nelson et al. 2008) and a decrease in its relative gene expression level suggests that carbon assimilation into cellular macromolecules essential for cell proliferation and growth was progressively inhibited by nitrogen availability. Furthermore, transcription of the *gnl* gene of the pentose phosphate pathway, encoding for gluconolactonase enzyme (EC 3.1.1.17) was also lowered both at the transcriptional ($\log_2$FC = -0.99) and proteome level ($\log_2$FC = -4.44) under severe nitrogen limitation. The pentose phosphate pathway furnishes ribose-5-phosphate, a precursor for nucleotide biosynthesis. Furthermore, transcription level of several genes (*purF, pyrE, ura2*) (Table 9) pertaining to nucleotide biosynthesis, purine and pyrimidine metabolism was also lowered. Interestingly, cellular response to nitrogen limitation was not limited to cessation of nucleotide biosynthesis; rather there was a concurrent increase in the expression levels of several genes and proteins pertaining to nucleic acid degradation metabolism under severe nitrogen limitation. For instance, the relative concentration of enzymes ribonuclease II ($\log_2$FC = 8.91), ribonuclease T2 ($\log_2$FC = 7.15), which catalyze the degradation of the RNA polymer was much higher under nitrogen limitation even though the corresponding transcript level remained invariant.. The expression level of *ncs1* gene, which is dedicated to scavenging nucleotide bases to serve as nitrogen sources (Schein, Hunt et al. 2013), was nearly 4-fold higher. Furthermore, transcription of several genes including 5'-nucleotidase (EC 3.1.3.5) encoding *surE*, guanine deaminase (EC 3.5.4.3) encoding *guaD* and urate oxidase (EC 1.7.3.3) encoding *uaZ*, which catalyze the release of ammonium through degradation of nucleotides, were also increased(Table

9). It is evident that nitrogen limitation resulted in nucleotide catabolism and autophagy of nucleic acids, perhaps in an attempt to salvage intracellular nitrogen.

Nutrient limitation has been reported to suppress ribosome biogenesis (Zhu, Zhang et al. 2012) and we found the expression levels of nearly all genes and corresponding proteins pertaining to ribosome synthesis to be comparatively lower under nitrogen limitation (Figure 8). Furthermore, under extreme nitrogen limitation, transcription levels of genes encoding translation initiation factors and nearly all aminoacyl tRNA synthetases, which are responsible for initiating translation and transferring the amino acids to the growing polypeptide chain respectively, were also found to be lower , both at the transcriptional and translational level, which points to suppression of protein synthesis (Figure 9). Another oleaginous yeast *Y. lipolytica* also exhibited similar repression of nucleic acid and protein biosynthesis under nitrogen limitation (Morin, Cescut et al. 2011).

Therefore, it is abundantly clear that under nitrogen limitation, the carbon flux into nearly all assimilative and energetic processes was negatively impacted.

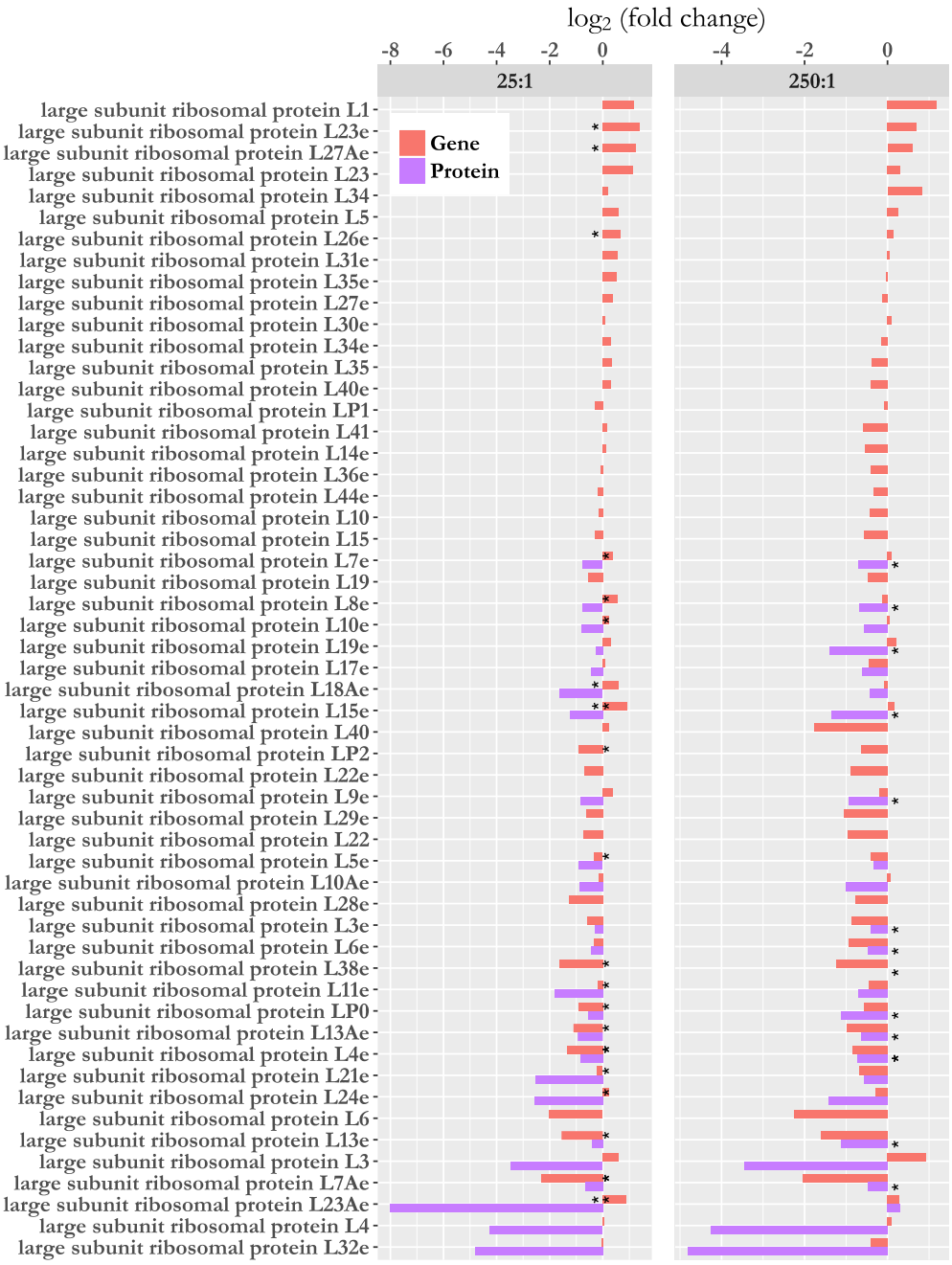

Figure 8 Differential expression of genes encoding ribosomal units and corresponding protein concentrations under nitrogen limitation. Down-regulation indicates cessation of protein biosynthesis under nitrogen limitation. "*" indicates statistically significant difference

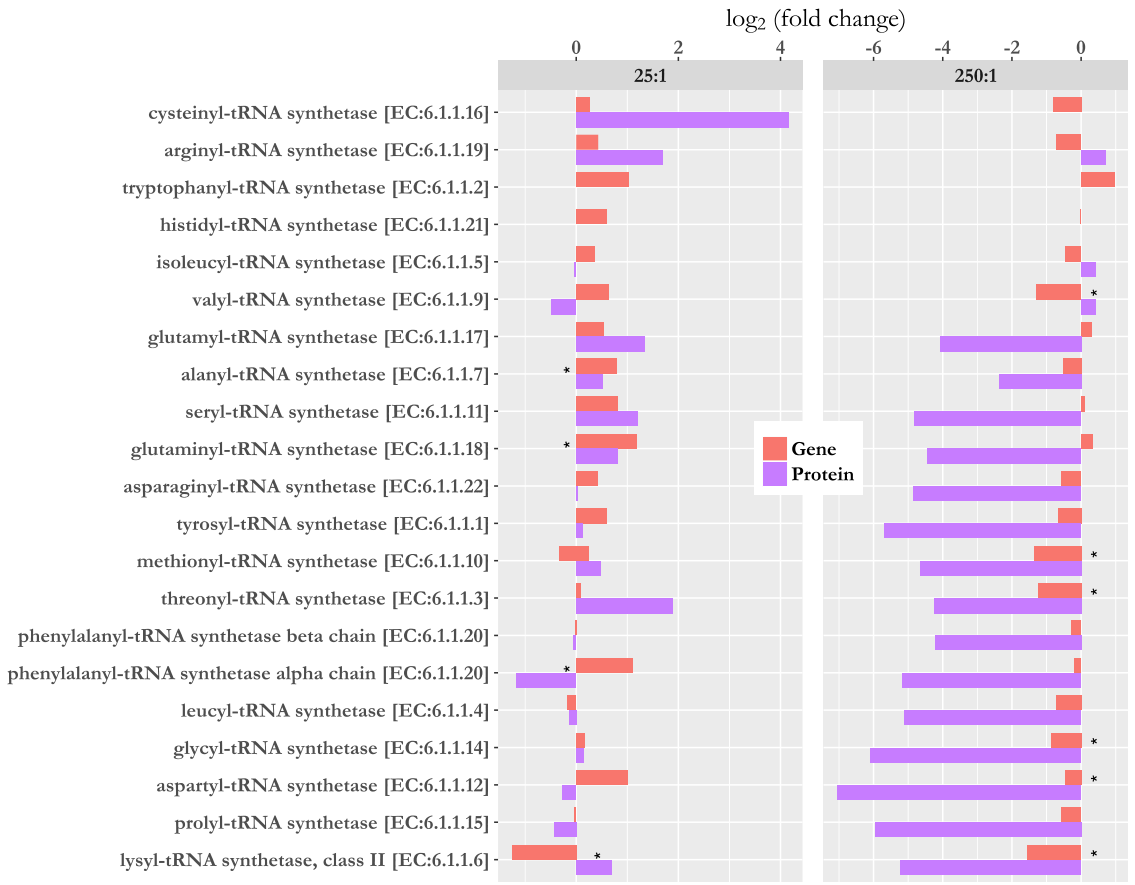

Figure 9 Differential expression of genes encoding aminoacyl tRNA synthetases (aaRS) and corresponding protein concentrations under nitrogen limitation. aaRS attaches amino acids to the growing polypeptide chain during translation. Down-regulation indicates cessation of protein biosynthesis under nitrogen limitation. "*" indicates statistically significant differences.

Redistribution of carbon flux and the fatty acid metabolism under nitrogen limitation

The primary consequence of the reduced carbon flux into cell organelles and continued carbon uptake would be an increase in the cytosolic pools of acetyl-CoA. The fatty acid biosynthesis in *C. albidus* also occurs in the cytosol and proceeds through carboxylation of acetyl-CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) encoded by the *acc* gene. Malonyl-CoA is the substrate for the fatty acid synthase enzyme (FAS I) and its formation is considered the first committed step towards lipid biosynthesis. Fungal fatty acid synthase (FAS I) is composed of two subunits, *fas1* (β subunit) and *fas2* (α subunit); *fas1* harbors acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH), malonyl-palmitoyl transferase (MPT) activities, while *fas2* contains acyl carrier protein (ACP), 3-ketoreductase (KR) and the 3- ketosynthase (KS) activities (Tehlivets, Scheuringer K Fau - Kohlwein et al.). In *C. albidus,* despite an increase in the intracellular lipid content of the cells, transcript levels of the *acc* gene and of the detected domains of the *fas1* and *fas2* genes did not display a significant change in response to nitrogen limitation (Figure 10). At the protein level however, we were unable to detect ACC, while for FAS I, only the ER domain was detected, which also remained statistically invariant. A proteomics study on oleaginous yeast *Rhodosporidium toruloides*, also only found a few proteins pertaining to lipid metabolism. Presumably, the proteins related to lipid metabolism are hydrophobic and are generally underrepresented in proteomic analyses due to their poor solubility and unpredictable separation behavior (Liu, Zhao X Fau - Wang et al.). Nevertheless, since all the domains of the FAS I enzyme are fused together into a single multifunctional unit, we concluded that the FAS I level must also be unchanged. Similar findings have been reported for other oleaginous yeasts such as *Y. lipolytica*, which did not show any significant transcriptome level changes in the lipid metabolism upon nitrogen limitation (Morin, Cescut et

al. 2011) and *Rhodosporidium toruloides*, in which the key genes of lipogenesis including *acc1*, *acl* and *me1* were not found to transcriptionally regulated (Zhu, Zhang et al. 2012).

Due to this apparent absence of a transcriptome level control over the genes governing fatty acid biosynthesis, we propose that in *C. albidus*, the lipid accumulation might be a consequence of the carbon flux redistribution, resulting in enhanced lipid production through increased enzyme activity. We further propose that the activity of FAS I enzyme in *C. albidus* is controlled at a posttranslational level and that the higher lipid accumulation under nitrogen limitation is due to an increased flux through the FAS I enzyme system as a consequence of increased cytosolic availability of its substrate, acetyl-CoA. It has been reported in yeasts that the intracellular levels of the fatty acid synthase enzyme are very tightly controlled and excess FAS protein is rapidly degraded by vacuolar proteases (Tehlivets, Scheuringer et al. 2007). This points to the possibility that the increased lipid accumulation might be a consequence of increased enzyme activity rather than increased transcription or translation. This possibility is also further bolstered by an examination of the factors governing FAS I activity. As such, fatty acid biosynthesis is an endergonic process and requires 14 moles of NADPH per mole of palmitate synthesized (Lehninger, Nelson et al. 2008) and it has been reported that these NADPH are not drawn from the general cellular pool but exclusively and directly from a dedicated enzymatic conversion (Ratledge 2002). It has been postulated that this NADPH is furnished by the reaction of malic enzyme (EC 1.1.1.40, MAE), which converts malate to pyruvate and CO_2, providing NADPH for lipid biosynthesis and has thus been proposed to be a rate limiting step of lipid biosynthesis (Rodríguez-Frómeta, Gutiérrez et al. 2013). However, the significance of MAE has been debated recently and several alternate mechanisms for NADPH supply have also been proposed, while the role of MAE has been shown to be dispensable in some oleaginous yeast (Zhang, Zhang et al.

2013, Zhang, Wu et al. 2016). The annotated genome sequence of *C. albidus* revealed that it lacks the cytosolic malic enzyme and similar to *Y. lipolytica* (Dujon, Sherman et al. 2004), it only contains one malic enzyme gene, which encodes the mitochondrial malic enzyme. The overexpression of the mitochondrial malic enzyme was not found to alter the lipid content in *Y. lipolytica* (Beopoulos, Nicaud et al. 2011), while in another oleaginous yeast *Lipomyces starkeyi*, malic enzyme is NAD^+- dependent (Tang, Zhang et al. 2010) and unlikely to participate in lipid accumulation. Therefore, the role of malic enzyme and the source of NADPH for fatty acid biosynthesis in oleaginous yeasts remain unclear. In *C. albidus*, the expression of the mitochondrial MAE encoding *me2* gene and the corresponding protein remained unchanged under nitrogen limitation. However, pyruvate produced by the activity of MAE can be converted to acetaldehyde and further to acetyl-CoA in cytosol by the action of *pdc* encoded pyruvate decarboxylase enzyme (EC 4.1.1.1). We found the gene expression of *pdc* to be 3-fold higher (Figure 10) under both limiting conditions, perhaps as a consequence of an increase in cytosolic pyruvate concentration, which suggests that the activity of MAE under nitrogen limitation might have been increased.

Among other possible source of NADPH for lipid biosynthesis, the pentose phosphate pathway (PPP), cytosolic $NADP^+$- dependednt isocitrate dehydrogenase (IDH) enzyme and the mannitol metabolism have been proposed. A study on comparison of various NADPH sources in *Y. lipolytica* concluded that both the mannitol pathway and the IDH were able to provide NADPH for FA synthesis, with a similar lipid yield, but lower than in the simulation with the PPP as source for NADPH (Kavšček, Bhutada et al. 2015). In case of *C. albidus*, we reported a decrease in the expression of PPP related genes, while the expression of the cytosolic IDH enzyme and the encoding *idh1* gene were unaffected by the nitrogen limitation. However, we found the

expression of the $NADP^+$- dependent mannitol dehydrogenase enzyme encoding *mtdH* to be 3-fold higher under both nitrogen-limiting conditions. Similar observations were reported for *Y. lipolytica*, which suggests that in *C. albidus*, mannitol metabolism might be the source of NADPH for fatty acid biosynthesis.

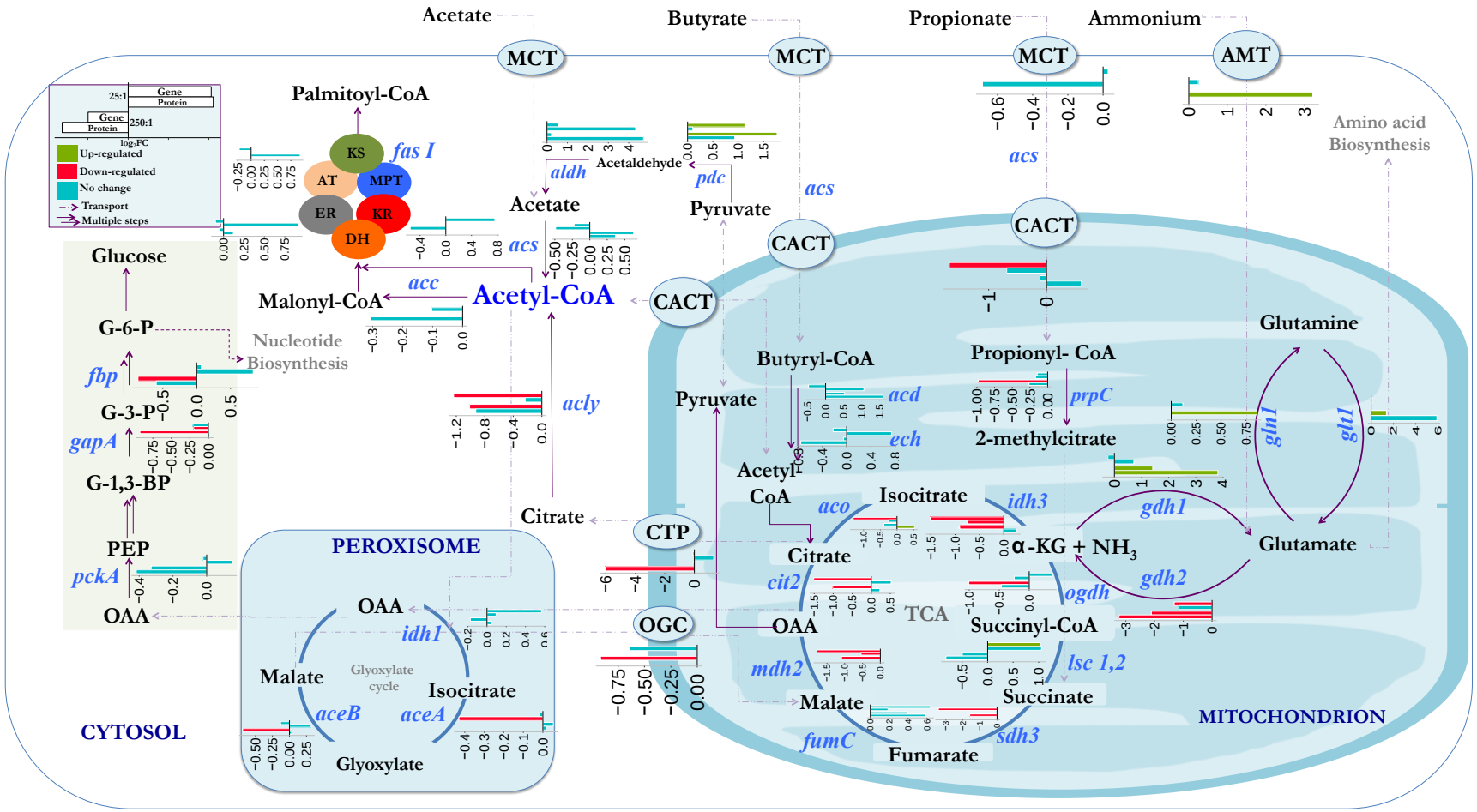

Figure 10 : Metabolic reconstruction of major carbon and nitrogen pathways in *C. albidus* **under nitrogen limitation. Plots next to each gene show the relative change in the gene expression and protein concentration and the colors indicate statistical significance. The top bar indicates the gene expression and bottom bar indicates the concentration of corresponding protein. Overall the genes pertaining to oxidative carbon metabolism were down-regulated while the genes pertaining to assimilative pathways were up-regulated or remained unchanged under nitrogen limitation.**

4.3.3 Nitrogen metabolism

The overall picture obtained was that under nitrogen limitation, *C. albidus* attempts to minimize its nitrogen usage and conserve its ammonium inventory by salvaging it from any non-essential metabolites. In purview of the global –omic response, the most highly expressed genes and proteins were involved in nitrogen uptake, protein turnover and autophagy to utilize assimilated nitrogen (Figure 11), while genes and corresponding proteins pertaining to catabolic nitrogen processes, protein synthesis and amino acid metabolism were down-regulated with some exceptions.

Nitrogen catabolite repression (NCR) is the physiological response reported in several yeast, whereby genes encoding permeases and enzymes for catabolism of poor nitrogen sources are repressed in the presence of readily utilizable nitrogen sources and activated in their absence (Kingsbury, Goldstein et al. 2006). In another basidiomycetic yeast *Cryptococcus neoformans*, NCR was found to be activated by *gat1*, which belongs to a conserved family of zinc finger containing transcriptional regulators known as GATA-factors(Kmetzsch, Staats et al. 2011). The same GATA transcriptional regulator was reported to control NCR in *S. cerevisiae* (Kerkhoven, Pomraning et al. 2016). In *C. albidus* under nitrogen limitation, we found the protein level of a putative GATA-type transcription factor to be over 200-fold lower under severe nitrogen limitation condition (COD:N = 250:1). Consequently, NCR was relieved and we found several NCR associated genes including *mep* dependent ammonium transporters, *yat* and *can1* encoding for permeases for amino acid transport, genes encoding nitrate reductase (*nr*), nitrite reductase (*nit-6*), and several nitrate, nitrite and ammonium transporters (*amt*, *nrt*, *narK*, *nrtP*, *nasA*) to be highly up-regulated under nitrogen limitation with several genes exhibiting over a 50 fold increase (Figure 11). On a proteome level however, we did not observe a strong correlation with

the transcriptomic response and the expression levels of proteins corresponding to the most highly expressed genes remained statistically invariant. Specifically pertaining to proteins corresponding to NCR related genes, the intracellular levels of ammonium transporters and proteins corresponding to metabolism of alternate nitrogen sources such as nitrate, nitrite, amino acids *etc.* were not significantly altered, while the levels of proteins catalyzing the autophagy and ammonium assimilation in the cell were significantly higher. This indicates a strong post transcriptional control of NCR genes. Since, the expression of NCR genes in yeasts is regulated by the same GATA factor promoter (Georis, Feller A Fau - Vierendeels et al.), it is possible that the genes are transcribed at an equal level in response to nitrogen limitation but the translation of genes is post-transcriptionally controlled based upon the intracellular conditions. Similar responses were observed in a study (Kolkman, Daran-Lapujade et al. 2006) on the proteomic responses of *S. cerevisiae* to nitrogen limitation wherein translation of proteins under nitrogen limitation did not show a concomitant increase with increase in their corresponding mRNA levels and the protein concentration was controlled through increased translational efficiency and/or decreased protein degradation.

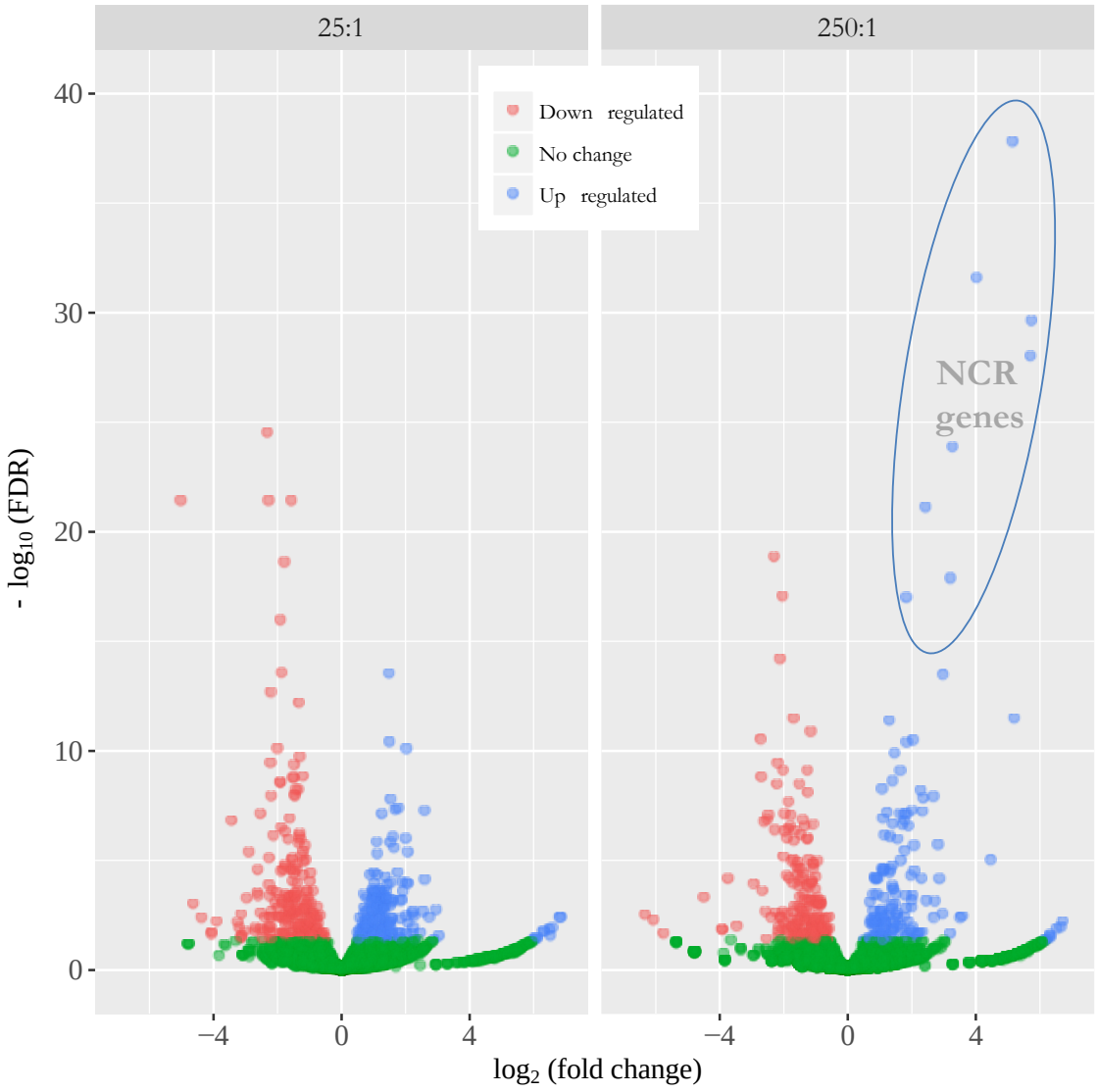

Figure 11 Volcano plot representing the overall differential expression of various genes. Values along the x-axis indicate the relative change in expression and y-axis indicates statistical confidence. Overall, the genes pertaining to nitrogen catabolite repression (NCR) were among the most highly up-regulated and formed a separate cluster.

However, mild limitation (COD:N = 25:1) did not elicit the same response and we did not find any significant changes in expression levels of the NCR related genes or proteins. This suggests

that the intracellular nitrogen availability might be uncoupled or only loosely coupled with the extracellular environment since the COD:N ratio of 25:1, while stoichiometrically limiting, does not seem to be limiting at a metabolic level. A study on the *in-vivo* ammonium concentration in *S. cerevisiae* (Cueto-Rojas, Maleki Seifar et al. 2016) showed that cells attempted to maintain a constant intracellular ammonium concentration under various nitrogen sources and concentrations. Therefore, it is possible that the stoichiometric nitrogen limitation at COD:N ratio of 25:1 resulted only in partial metabolic nitrogen limitation in *C. albidus.*

Under favorable growth conditions, GATA based TOR signaling cascade is active and yeast cells maintain a robust rate of ribosome biogenesis, translation initiation, and nutrient import. However, under nitrogen starvation, TOR targets a Ser/Thr kinase (EC 2.7.1.1) that regulates cell growth and metabolism in response to environmental cues (Wullschleger, Loewith et al. 2006). We found the transcription level gene encoding Ser/Thr kinase to be statistically lower (log_2FC = -1.55 and -1.36 respectively) under both limiting conditions and we were unable to detect the corresponding protein. Consequently, ribosome biogenesis and protein translation were repressed, thus blocking the biosynthesis of nitrogenous macromolecules (nucleic acids and proteins). However, again under mild limitation, the effect was much less pronounced and bulk of the related genes remained unaffected (Figure 8,9). Inhibited TOR signaling can also activate autophagy in cells (Rodriguez-Navarro and Cuervo 2010) and a number of autophagy-related genes were up-regulated on nitrogen starvation (Figure 12). Interestingly, autophagy has also been reported to play a role in lipogenesis, wherein under nutrient limitation, autophagy may play an important role in freeing up the cellular real estate to make more room for lipid-droplet formation and act as an additional energy source through degradation of other cellular

components, thus preventing the oxidation of the lipids already transformed into triglycerides (Rodriguez-Navarro and Cuervo 2010).

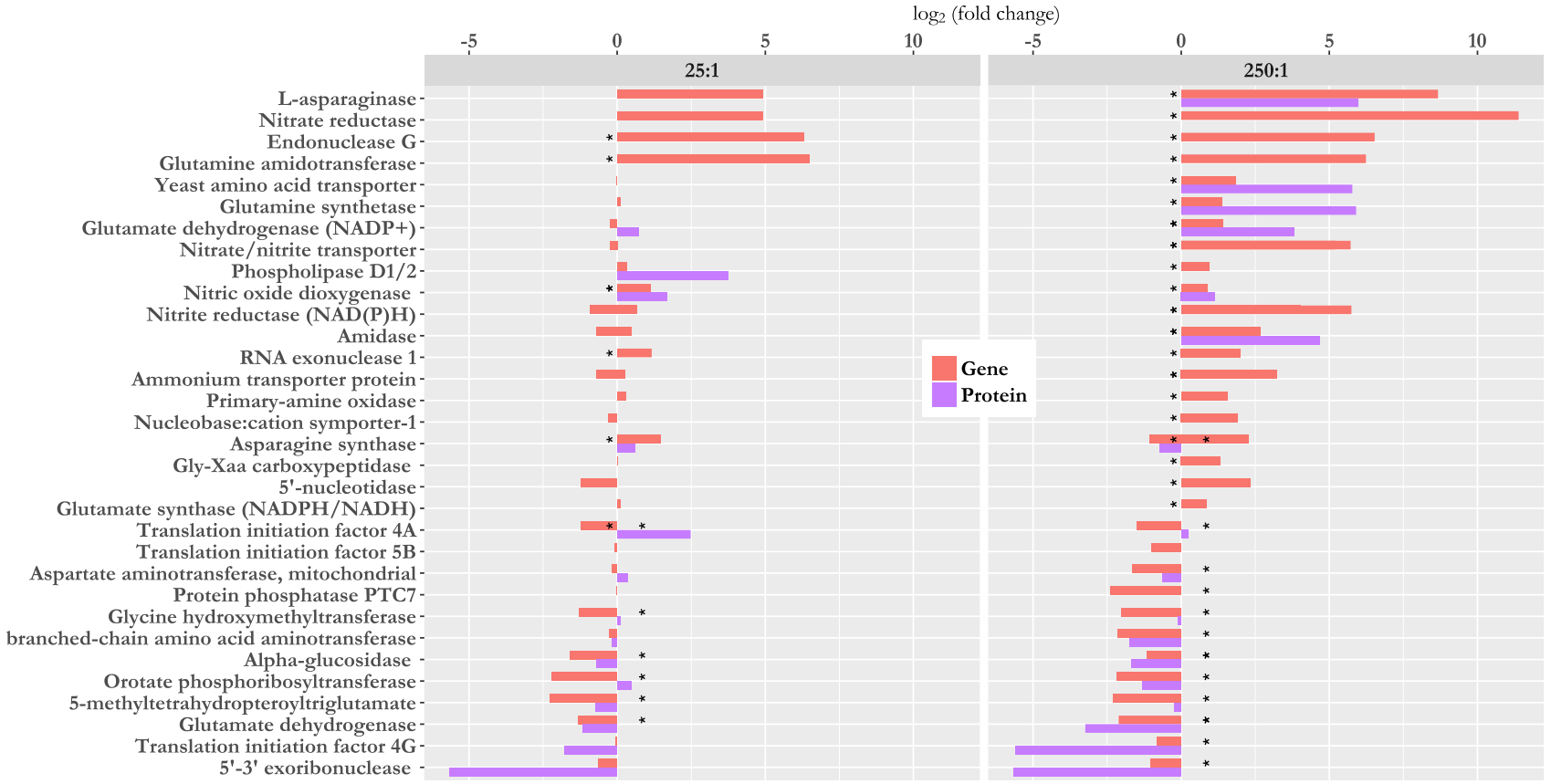

Figure 12 Differential expression of major genes and corresponding proteins involved in nitrogen catabolite repression and autophagy under nitrogen limitation. Genes involved in autophagy and nitrogen transport were highly up-regulated and the genes pertaining to translation initiation factors and amino acid and protein biosynthesis were down-regulated. " * " indicates statistically significant differences.

In yeasts, the cell cycle is tightly controlled by the environmental conditions including nutrient availability and before engaging in a subsequent round of division, cells must sense and integrate both intracellular and extracellular signals (Parrou, Enjalbert B Fau - Plourde et al.). Additionally, several recent studies have reported 'cross-talk' between carbon and nitrogen signaling pathways (Schneper, Düvel et al. 2004). In one study (Bertram, Choi et al. 2002) glucose concentration was shown to directly influence the *gln3* transcriptional regulator for nitrogen uptake in *S. cerevisiae*, showing that glucose and nitrogen signaling pathways converge onto *gln3*, which may be critical for both nutrient sensing and starvation responses. Another study on dynamic transcriptional response of nitrogen-starved cultures of *S. cerevisiae* reported up-regulation of genes related to central carbon metabolism including gluconeogenesis and oxidative phosphorylation in response to pulses of ammonium as cells switched from fermentative to oxidative metabolism within seconds (Dikicioglu, Karabekmez E Fau - Rash et al.). This points to a tight co-regulation of nitrogen and carbon metabolism in yeast cells. The fact that nitrogen limitation could impact carbon uptake makes it important to understand the global cellular response to nitrogen limitation and its implications to carbon sensing and uptake, intended or otherwise.

Effect on nitrogen assimilation

Two pathways for ammonium assimilation have been reported in yeasts(Avendaño, Deluna et al. 1997). The first pathway involves the combined action of enzymes glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.13) to catalyze the ATP dependent conversion of ammonium and α-ketoglutarate into glutamate through following reactions:

$$NH_3 + \text{glutamate} + ATP \rightarrow \text{glutamine} + ADP + Pi$$

$$\text{glutamine} + \text{2-oxoglutarate} + NADPH + H^+ \rightarrow 2\ \text{glutamate} + NADP^+$$

The second pathway utilizes the NAD^+- and the $NADP^+$- dependent glutamate dehydrogenase enzymes [EC 1.4.1.2, EC 1.4.1.4] encoded by genes *GDH2* and *GDHA* respectively, which catalyze the reversible oxidative deamination of glutamate into α-ketoglutarate and ammonium. $NADP^+$ is utilized in the forward reaction of α-ketoglutarate and free ammonium, which are converted to glutamate and NAD^+ is utilized in the reverse reaction, which involves glutamate being converted to free ammonium and α-ketoglutarate, which can then enter either the catabolic TCA cycle for energy production or the glyoxylate cycle to be channeled towards carbon anabolism. The $NADP^+$ -GDH enzyme catalyzes the following reaction:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NAD(P)H + H^+ \rightarrow \text{Glutamate} + H_2O + NAD(P)^+$$

Together, these two pathways account for roughly 80-85% of nitrogen assimilation in the cells (Dikicioglu, Karabekmez E Fau - Rash et al. , Kim and Kim 2016) since all other amino acids can be synthesized from glutamate through various reactions catalyzed by transaminases (Cueto-Rojas, Maleki Seifar et al.). The presence of two pathways for nitrogen assimilation in several microorganisms has stimulated discussion on the need for two routes for the biosynthesis of the same end product. Both the GDH and GS-GOGAT pathways produce 1 mole of glutamate from 1 mole each of NH_3, α-ketoglutarate and NADPH. The GS-GOGAT pathway is energetically more costly than the GDH pathway, consuming 1 additional ATP but the GDH enzyme from several organisms has been reported to have very low affinity (high K_m) for ammonium. Therefore, the relative importance of the two pathways is quite unclear with different organisms utilizing either pathway under different growth conditions. In *S. cerevisiae*, for instance, the *GDH* pathway is considered to be the major nitrogen assimilator (Cueto-Rojas, Maleki Seifar et al.), while in *Klebsiella aerogenes*, the *GS-GOGAT* pathway functions to assimilate ammonium

and synthesize glutamate when the ammonium concentration is low and *GDH* plays this role when the cells are cultivated under ammonium excess (Avendaño, Deluna et al. 1997). Yet another study reports that in *Escherichia coli*, glutamate biosynthesis proceeds through $NADP^+$ -GDH when the cell is carbon and energy limited, while the *GS-GOGAT* pathway functions when the cell is not under energy limitation (Helling).

In *C. albidus*, we reported (Vajpeyi and Chandran 2016) the presence of both the pathways and therefore, it is important to understand the specific mechanism of nitrogen uptake in *C. albidus* and its impact on carbon uptake. These nitrogen assimilation pathways are not directly involved in fatty acid biosynthesis and therefore, thus far they have not been considered to have an effect on the lipid metabolism of oleaginous microorganisms. However, glutamate dehydrogenase (GDH), in conjunction with isocitrate dehydrogenase (IDH), operates at a crucial intersection of carbon and nitrogen metabolisms and deserves special attention because of its amphibolic nature and the fact that it is one of the major branch points for metabolic fluxes of carbon and nitrogen in yeast cells (Stillman, Baker et al. 1993). In our experiments, we found that for under severe limitation, the expression level of $NADP^+$- dependent GDH enzyme was much higher at both transcriptional (log_2FC =1.42) and protein levels (log_2FC = 3.84), while the expression level of NAD^+- dependent GDH enzyme, which catalyzes the reverse reaction, was statistically lower at both transcriptomic (log_2FC = -2.11) and proteomic level (log_2FC = -3.21). However, for the *GS-GOGAT* pathway, only the expression of *glnA* encoded glutamine synthetase enzyme was increased at both transcriptional (logFC = 1.37) and translational level (log_2FC = 3.18) but glutamate synthase enzyme, which catalyzes the second step, was not detected in the proteome and the increase in gene expression, while statistically significant, was less than 2-fold (log_2FC = 0.86) (Figure 10). This seems to suggest that the $NADP^+$-GDH mediated ammonium uptake is

the dominant route of nitrogen assimilation in *C. albidus.* This apparent increase in the nitrogen anabolism under nitrogen limitation seems counterintuitive, however, since GDH is at the intersection of several important metabolic pathways, its activity is very tightly regulated by several allosteric compounds that are metabolic intermediates and even though the molecular mechanisms involved in its allostery remain only partially understood (Smith and Stanley 2008), chemically diverse compounds, such as purine nucleotides (ADP, ATP, GTP and NADH), leucine and even palmitoyl-CoA have been shown to influence GDH expression and activity (Spanaki and Plaitakis 2012). Given the sheer allosteric complexity involved in GDH regulation, the increase in the $NADP^+$-GDH concentration could have occurred due to several possible reasons.

First, the reaction equilibrium for $NADP^+$-GDH favors the reactants under balanced conditions since the Michaelis-Menten coefficient (K_m) for ammonium in the reductive amination reaction is so high that it seems to be unfavorable to the formation of glutamate. The K_m value for GDH for another oleaginous yeast *Rhodosporidium toruloides* was reported to be 13 mM (EVANS and RATLEDGE 1984), which means that the enzyme has a very low affinity for ammonium and under limited nitrogen conditions, the reaction probably only makes a very modest contribution to overall ammonium assimilation. Although, we did not measure the intracellular ammonium concentrations in our experiments, some inferences could be drawn from the extracellular NH_4^+-N concentration nevertheless. The average effluent ammonium concentration in the nitrogen-limited chemostats was 1mM and 12 mM respectively, compared to 60 mM during excess. For growth under nitrogen-sufficient conditions, the ammonium concentration (S_{NH4}) was much higher than K_m ($S_{NH} \gg K_m$) and the enzyme activity in that case would have followed zero order kinetics and would have been independent of ammonium concentration. However, under severe

limitation, intracellular ammonium concentration (S_{NH4}) would have been much lower than the K_m ($S_{NH4} \ll K_m$), which means that the enzyme kinetics was first order with respect to ammonium and can be expressed as:

$$v = k_2 * [E_o] * [S_{NH4}]/K_m$$

where, E_o is the total enzyme concentration and k_2 is the rate constant for glutamate formation. Furthermore, α-ketoglutarate required for this reaction is provided by the TCA cycle. Under nitrogen limitation, when the flux through the TCA cycle slows down, the lack of α-ketoglutarate could further lower the activity of GDH enzyme. We propose that under such conditions, the combined effect of low substrate availability and a high K_m would necessitate high enzyme concentrations for the $NADP^+$-GDH enzyme in order for it to have any meaningful impact on nitrogen assimilation.

Another possible mechanism of $NADP^+$-GDH up-regulation arises when the metabolic control of GDH is taken into account. Several studies have shown the activity of $NADP^+$-GDH enzyme to be strongly inhibited by GTP and activated by high levels of GDP (Li, Li et al. 2012), which in turn suggests that the activity of the GDH1 enzyme is controlled by the carbon metabolism rather than the ammonium availability since GTP/GDP ratio in cells is a function of carbon and energy metabolism and is primarily reliant on the flux through TCA cycle. The control of GDH activity through GTP level rather than the ATP level is probably not accidental; the TCA cycle generates nearly 30 ATP per turn but only one GTP. Therefore, mitochondrial GTP:GDP ratio is far more sensitive gauge of TCA activity and consequently, of the oxidative carbon flux (Smith and Stanley 2008). This further stresses the tight co-regulation of carbon and nitrogen fluxes through the activity of GDH. The complicated allosteric control of a seemingly mundane amination reaction may seem unnecessarily cumbersome, however, it is remarkable that a single enzyme

can influence oxidative and assimilative carbon flux, amino acid biosynthesis, nucleic acid metabolism and overall cell growth rate and activity.

4.4 Conclusions

This study highlights the role of nitrogen availability upon carbon uptake and lipid biosynthesis in *C. albidus* based upon its transcriptomic and proteomic responses to nitrogen limitation. The expression of genes pertaining to energetic and assimilative carbon cycles (TCA, gluconeogenesis, nucleic acid biosynthesis) was statistically lower under nitrogen limitation, while the genes pertaining to carbon uptake and lipid accumulation were unaffected. This apparent redistribution of carbon in cellular metabolism caused acetyl-CoA to accumulate in the cytosol and be channeled towards lipid storage. Among the different VFA utilized, acetate and butyrate were found most suitable for lipid accumulation. During nitrogen metabolism, most highly expressed genes were involved in nitrogen uptake, protein turnover and autophagy to scavenge and redirect assimilated nitrogen, while transcription of genes pertaining to catabolic nitrogen processes, protein synthesis and amino acid metabolism was decreased.

Furthermore, in *C. albidus,* we did not observe a strong correlation between transcriptomic and proteomic responses and several proteins of importance were found to be controlled either at expression (post-transcriptional) level or at activity (post-translational) level.

Although, this study reaffirms the classical opinion of nitrogen-mediated lipid biogenesis, it also highlights the differences in carbon metabolism under growth on alternate carbon sources and, by studying the global transcriptomic and proteomic responses in conjunction, it presents a much broader and clearer view of oleaginity of *C. albidus.* In sum, this comparative transcriptome and proteome data will help elucidate factors driving lipid accumulation in *C. albidus* and contribute

toward bioprocess development and optimization for production of sustainable lipid alternatives from ‘waste’ streams.

Appendix

Table 9 List of genes and corresponding proteins and their differnetial expression (log_2FC) and significance (FDR) under differnt nitrogen limiting conditions. NA indicates that the corresponding gene or protein was not found.

KEGG ID	GENE SYMBOL	PROTEIN NAME	5:1 VS 25:1				5:1 VS 250:1			
			GENE log_2FC	FDR	PROTEIN log_2FC	FDR	GENE log_2FC	FDR	PROTEIN log_2FC	FDR
K07513	*acaa1*	acetyl-CoA acyltransferase 1 [EC:2.3.1.16]	-1.08E+00	3.05E-01	-1.42E+00	5.37E-01	2.65E-01	8.82E-01	6.24E-01	7.81E-01
K07513	*acaa1*	acetyl-CoA acyltransferase 1 [EC:2.3.1.16]	-1.53E-01	9.54E-01	NA	NA	7.07E-01	7.06E-01	NA	NA
K11262	*acaca*	acetyl-CoA carboxylase / biotin carboxylase 1 [EC:6.4.1.2 6.3.4.14]	-1.03E-01	9.06E-01	NA	NA	-3.08E-01	6.53E-01	NA	NA
K01638	*aceb, glcb*	malate synthase [EC:2.3.3.9]	-1.31E-01	8.11E-01	3.20E-01	2.54E-01	-6.89E-01	3.11E-02	3.29E-02	9.07E-01
K01681	*aco, acna*	aconitate hydratase [EC:4.2.1.3]	-1.31E+00	6.52E-07	-2.49E-01	7.49E-02	-3.91E-01	3.57E-01	5.25E-01	6.15E-05
K00997	*acps*	holo-[acyl-carrier protein] synthase [EC:2.7.8.7]	4.68E+00	2.94E-01	NA	NA	4.98E+00	2.89E-01	NA	NA
K15437	*aimp1, arc1*	aminoacyl tRNA synthase complex-interacting multifunctional protein 1	1.29E+00	1.36E-01	4.79E+00	2.12E-01	7.99E-01	5.31E-01	NA	1.00E+00
K01490	*ampd*	AMP deaminase [EC:3.5.4.6]	3.89E-01	5.10E-01	NA	NA	2.68E-01	7.50E-01	NA	NA
K00620	*argj*	glutamate N-acetyltransferase / amino-acid N-acetyltransferase [EC:2.3.1.35 2.3.1.1]	4.82E-01	5.34E-01	-1.29E+00	3.78E-01	-1.23E-01	9.40E-01	2.57E-01	8.56E-01
K01953	*asnb, asns*	asparagine synthase (glutamine-hydrolysing) [EC:6.3.5.4]	2.99E-01	7.78E-01	NA	NA	2.27E+00	7.37E-09	NA	NA
K01953	*asnb, asns*	asparagine synthase (glutamine-hydrolysing) [EC:6.3.5.4]	1.48E+00	3.35E-14	6.04E-01	3.41E-01	-1.08E+00	1.62E-05	-7.19E-01	2.73E-01
K00685	*ate1, ate1*	arginine-tRNA-protein transferase [EC:2.3.2.8]	1.43E-02	9.95E-01	NA	NA	6.36E-01	7.58E-01	NA	NA
K06640	*atr*	serine/threonine-protein kinase ATR [EC:2.7.11.1]	-4.01E-01	3.25E-01	NA	NA	-5.55E-01	1.90E-01	NA	NA
K15192	*btaf1, mot1*	TATA-binding protein-associated factor [EC:3.6.4.-]	4.71E-01	2.94E-01	NA	NA	-2.45E-01	7.52E-01	NA	NA
K01647	*cs, glta*	citrate synthase [EC:2.3.3.1]	-1.51E+00	1.97E-09	5.23E-01	7.57E-02	-1.01E+00	2.45E-04	2.26E-01	4.49E-01
K01876	*dars, asps*	aspartyl-tRNA synthetase [EC:6.1.1.12]	1.00E+00	5.48E-02	-2.73E-01	9.08E-01	-4.64E-01	6.42E-01	-7.05E+00	2.42E-02
K00627	*dlat, acef, pdhc*	pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase)	4.40E-01	1.45E-01	-4.87E-01	3.10E-01	-1.69E-01	7.54E-01	3.57E-01	4.50E-01

		[EC:2.3.1.12]								
K00658	*dlst, sucb*	2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase) [EC:2.3.1.61]	-4.30E-01	5.01E-01	-2.33E-01	3.26E-01	-1.53E+00	1.48E-03	-1.49E-01	5.28E-01
K00262	*e1.4.1.4, gdha*	glutamate dehydrogenase (NADP+) [EC:1.4.1.4]	-2.38E-01	6.31E-01	7.15E-01	1.37E-01	1.41E+00	2.82E-09	3.81E+00	4.94E-16
K00624	*e2.3.1.7*	carnitine O-acetyltransferase [EC:2.3.1.7]	-1.68E+00	4.83E-04	-6.89E-01	5.41E-01	-1.14E-01	9.30E-01	6.02E-01	5.84E-01
K00624	*e2.3.1.7*	carnitine O-acetyltransferase [EC:2.3.1.7]	-4.01E-01	3.13E-01	2.30E-02	9.71E-01	2.45E-01	6.36E-01	4.32E-01	4.92E-01
K00851	*e2.7.1.12, gntk, idnk*	gluconokinase [EC:2.7.1.12]	7.46E-01	6.34E-01	NA	NA	1.24E+00	3.64E-01	NA	NA
K01424	*e3.5.1.1, ansa, ansb*	L-asparaginase [EC:3.5.1.1]	4.92E+00	2.41E-01	NA	1.00E+00	8.67E+00	4.25E-10	5.98E+00	5.23E-02
K01426	*e3.5.1.4, amie*	amidase [EC:3.5.1.4]	4.25E+00	3.96E-01	NA	NA	5.33E+00	1.94E-01	NA	NA
K01610	*e4.1.1.49, pcka*	phosphoenolpyruvate carboxykinase (ATP) [EC:4.1.1.49]	-2.17E-02	9.66E-01	1.50E-01	6.51E-01	-3.29E-01	2.90E-01	-4.17E-01	2.17E-01
K01637	*e4.1.3.1, acea*	isocitrate lyase [EC:4.1.3.1]	-1.66E-02	9.85E-01	-4.30E-01	3.53E-02	5.40E-02	9.53E-01	1.65E-02	9.35E-01
K01885	*ears, gltx*	glutamyl-tRNA synthetase [EC:6.1.1.17]	5.45E-01	2.23E-01	1.35E+00	6.92E-01	3.09E-01	6.42E-01	-4.08E+00	2.17E-01
K03239	*eif2b1*	translation initiation factor eIF-2B subunit alpha	7.75E-01	3.43E-01	NA	NA	2.41E-01	8.87E-01	NA	NA
K03754	*eif2b2*	translation initiation factor eIF-2B subunit beta	2.07E-01	8.03E-01	NA	NA	2.77E-01	7.57E-01	NA	NA
K03241	*eif2b3*	translation initiation factor eIF-2B subunit gamma	9.55E-01	1.31E-01	NA	NA	6.22E-01	4.79E-01	NA	NA
K03680	*eif2b4*	translation initiation factor eIF-2B subunit delta	3.41E-01	8.40E-01	NA	NA	8.41E-01	5.07E-01	NA	NA
K03240	*eif2b5*	translation initiation factor eIF-2B subunit epsilon	8.54E-02	9.31E-01	NA	NA	-5.29E-01	4.85E-01	NA	NA
K15027	*eif2d*	translation initiation factor 2D	9.78E-01	5.52E-01	NA	NA	1.31E+00	4.12E-01	NA	NA
K03237	*eif2s1*	translation initiation factor 2 subunit 1	4.48E-01	6.29E-01	NA	NA	-6.27E-02	9.75E-01	NA	NA
K03238	*eif2s2*	translation initiation factor 2 subunit 2	1.10E+00	1.43E-01	NA	NA	-2.25E-01	9.12E-01	NA	NA
K03242	*eif2s3*	translation initiation factor 2 subunit 3	6.33E-01	1.07E-01	4.48E+00	2.33E-01	-8.69E-01	7.60E-02	NA	1.00E+00
K03254	*eif3a*	translation initiation factor 3 subunit A	2.85E-01	5.93E-01	5.22E+00	1.68E-01	-1.22E-01	8.94E-01	NA	1.00E+00
K03253	*eif3b*	translation initiation factor 3 subunit B	9.61E-01	7.89E-04	1.08E+00	6.88E-01	3.27E-01	5.34E-01	-4.43E+00	1.32E-01

K03252	*eif3c*	translation initiation factor 3 subunit C	6.11E-01	1.09E-01	4.56E+00	2.29E-01	2.04E-01	7.63E-01	NA	1.00E+00
K03251	*eif3d*	translation initiation factor 3 subunit D	6.88E-01	5.08E-02	NA	NA	-1.11E-01	9.09E-01	NA	NA
K03250	*eif3e, int6*	translation initiation factor 3 subunit E	9.79E-01	3.40E-02	4.68E+00	2.18E-01	5.00E-01	4.78E-01	NA	1.00E+00
K03249	*eif3f*	translation initiation factor 3 subunit F	-4.09E-01	8.05E-01	NA	NA	3.38E-01	8.63E-01	NA	NA
K03248	*eif3g*	translation initiation factor 3 subunit G	3.26E-01	7.82E-01	-3.83E-02	9.90E-01	-1.59E+00	1.17E-01	-1.07E+00	7.19E-01
K03247	*eif3h*	translation initiation factor 3 subunit H	1.35E-01	8.80E-01	4.63E+00	2.26E-01	-3.84E-01	6.49E-01	NA	1.00E+00
K03246	*eif3i*	translation initiation factor 3 subunit I	7.74E-01	2.51E-01	5.65E-01	7.03E-01	-2.03E-01	9.00E-01	-1.85E-01	9.02E-01
K03245	*eif3j*	translation initiation factor 3 subunit J	4.00E-01	7.31E-01	NA	NA	-8.07E-01	5.05E-01	NA	NA
K15028	*eif3k*	translation initiation factor 3 subunit K	-4.64E-01	6.56E-01	NA	NA	-1.23E+00	1.73E-01	NA	NA
K15029	*eif3l*	translation initiation factor 3 subunit L	-7.22E-01	2.32E-01	4.23E+00	1.59E-01	-1.06E+00	9.63E-02	NA	1.00E+00
K15030	*eif3m*	translation initiation factor 3 subunit M	3.20E-01	4.84E-01	5.59E+00	7.03E-02	-2.86E-01	6.42E-01	NA	1.00E+00
K03257	*eif4a*	translation initiation factor 4A	-1.21E+00	2.72E-03	2.48E+00	1.81E-02	-1.50E+00	5.61E-04	2.47E-01	8.14E-01
K03258	*eif4b*	translation initiation factor 4B	6.11E-01	1.29E-01	6.62E-01	8.40E-01	5.91E-02	9.55E-01	-5.14E+00	1.45E-01
K03259	*eif4e*	translation initiation factor 4E	4.82E-01	6.12E-01	3.97E+00	1.76E-01	1.42E-01	9.41E-01	NA	1.00E+00
K03259	*eif4e*	translation initiation factor 4E	-1.37E+00	2.14E-01	3.90E-01	8.78E-01	-1.01E+00	4.61E-01	-5.73E+00	6.32E-02
K03260	*eif4g*	translation initiation factor 4G	-5.07E-02	9.25E-01	-1.78E+00	5.99E-01	-8.31E-01	1.09E-03	-5.60E+00	1.28E-01
K03260	*eif4g*	translation initiation factor 4G	3.68E-01	4.62E-01	NA	NA	-1.03E-01	9.25E-01	NA	NA
K03262	*eif5*	translation initiation factor 5	1.55E-01	8.41E-01	NA	NA	7.96E-02	9.46E-01	NA	NA
K03243	*eif5b*	translation initiation factor 5B	-8.64E-02	9.21E-01	NA	NA	-9.87E-01	5.45E-02	NA	NA
K03264	*eif6*	translation initiation factor 6	6.63E-01	3.13E-01	-3.13E-01	7.48E-01	-1.17E-01	9.44E-01	-1.88E+00	6.94E-02
K08852	*ern1*	serine/threonine-protein kinase/endoribonuclease IRE1 [EC:2.7.11.1 3.1.26.-]	-7.79E-01	3.13E-01	NA	NA	-7.44E-01	4.20E-01	NA	NA
K01624	*fba, fbaa*	fructose-bisphosphate aldolase, class II [EC:4.1.2.13]	-9.25E-01	3.21E-02	2.27E-01	5.51E-01	-1.86E-01	8.31E-01	3.94E-01	3.00E-01

K01915	*glna, glul*	glutamine synthetase [EC:6.3.1.2]	9.81E-02	9.31E-01	NA	1.00E+00	1.38E+00	1.32E-03	5.90E+00	5.69E-02
K00264	*glt1*	glutamate synthase (NADPH/NADH) [EC:1.4.1.13 1.4.1.14]	1.16E-01	8.00E-01	NA	NA	8.61E-01	6.43E-05	NA	NA
K00006	*gpd1*	glycerol-3-phosphate dehydrogenase (NAD+) [EC:1.1.1.8]	3.42E-01	8.54E-01	NA	NA	-9.09E-01	6.45E-01	NA	NA
K00006	*gpd1*	glycerol-3-phosphate dehydrogenase (NAD+) [EC:1.1.1.8]	-3.06E-01	6.23E-01	4.94E-01	5.09E-01	-2.71E-01	7.20E-01	6.02E-01	4.21E-01
NA	*kr*	3-oxoacyl-ACP reductase	7.63E-01	8.41E-01	NA	NA	-5.54E-01	9.30E-01	NA	NA
K06961	*krr1*	ribosomal RNA assembly protein	1.56E+00	1.96E-02	NA	NA	6.24E-01	6.42E-01	NA	NA
K15456	*kti12*	protein KTI12	2.60E-01	9.10E-01	NA	NA	-4.24E-01	8.87E-01	NA	NA
K01899	*lsc1*	succinyl-CoA synthetase alpha subunit [EC:6.2.1.4 6.2.1.5]	7.87E-01	2.39E-01	-3.94E-01	5.15E-01	-3.43E-01	7.91E-01	-7.63E-01	2.13E-01
K01900	*lsc2*	succinyl-CoA synthetase beta subunit [EC:6.2.1.4 6.2.1.5]	1.03E+00	6.61E-04	1.05E+00	8.40E-02	-4.97E-01	3.88E-01	-8.19E-01	2.03E-01
K01655	*lys21, lys20*	homocitrate synthase [EC:2.3.3.14]	-1.17E+00	1.70E-03	9.45E-01	1.32E-01	-2.21E+00	3.92E-09	-1.02E+00	1.25E-01
K01705	*lys4*	homoaconitate hydratase [EC:4.2.1.36]	-1.47E-01	8.45E-01	-1.71E-01	8.55E-01	-9.80E-01	4.79E-02	-2.63E-01	7.79E-01
K00026	*mdh2*	malate dehydrogenase [EC:1.1.1.37]	-1.78E+00	2.90E-19	-5.43E-01	2.17E-03	-1.09E+00	2.70E-07	2.62E-02	8.80E-01
K00027	*me2, sfca, maea*	malate dehydrogenase (oxaloacetate-decarboxylating) [EC:1.1.1.38]	4.00E-01	7.62E-01	NA	NA	7.06E-01	5.42E-01	NA	NA
K00789	*metk*	S-adenosylmethionine synthetase [EC:2.5.1.6]	-1.42E+00	2.81E-01	4.32E+00	2.16E-01	-8.56E-01	6.27E-01	3.90E+00	2.44E-01
K00140	*mmsa, iola, aldh6a1*	malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase [EC:1.2.1.18 1.2.1.27]	-7.92E-01	2.92E-02	2.02E-02	9.51E-01	8.62E-02	9.26E-01	1.14E-01	7.30E-01
K13124	*morg1*	mitogen-activated protein kinase organizer 1	-1.54E+00	1.00E-01	NA	NA	-2.09E+00	5.18E-02	NA	NA
K17439	*mrpl35*	large subunit ribosomal protein L35	3.27E-01	8.00E-01	NA	NA	-3.82E-01	8.22E-01	NA	NA
K17421	*mrpl40*	large subunit ribosomal protein L40	2.35E-01	9.03E-01	NA	NA	-1.77E+00	2.27E-01	NA	NA
K17422	*mrpl41*	large subunit ribosomal protein L41	1.69E-01	9.53E-01	NA	NA	-5.89E-01	8.54E-01	NA	NA
K17424	*mrpl43*	large subunit ribosomal protein L43	2.95E+00	6.72E-01	NA	NA	4.76E+00	3.41E-01	NA	NA
K17425	*mrpl44*	large subunit ribosomal protein L44 [EC:3.1.26.-]	4.47E-01	6.12E-01	NA	NA	2.76E-01	8.36E-01	NA	NA
K17427	*mrpl46*	large subunit ribosomal protein L46	5.72E+00	8.36E-02	NA	NA	4.51E+00	3.98E-01	NA	NA

K17402	*mrps23*	small subunit ribosomal protein S23	-1.01E-01	9.78E-01	3.71E+00	1.96E-01	3.80E-01	9.25E-01	NA	1.00E+00
K17413	*mrps35*	small subunit ribosomal protein S35	2.12E+00	8.66E-02	NA	NA	1.07E+00	6.42E-01	NA	NA
K07203	*mtor, frap, tor*	serine/threonine-protein kinase mTOR [EC:2.7.11.1]	7.24E-01	3.78E-01	NA	NA	-6.75E-01	6.08E-01	NA	NA
K01893	*nars, asns*	asparaginyl-tRNA synthetase [EC:6.1.1.22]	4.15E-01	3.89E-01	2.10E-02	9.95E-01	-5.71E-01	3.00E-01	-4.85E+00	1.71E-01
K17871	*ndh1*	NADH:ubiquinone reductase (non-electrogenic) [EC:1.6.5.9]	-1.15E+00	8.10E-02	6.28E+00	4.36E-02	-9.42E-01	2.38E-01	NA	1.00E+00
K17877	*nit-6*	nitrite reductase (NAD(P)H) [EC:1.7.1.4]	6.77E-01	7.89E-01	NA	NA	5.75E+00	2.72E-30	NA	NA
K17877	*nit-6*	nitrite reductase (NAD(P)H) [EC:1.7.1.4]	-9.18E-01	4.02E-01	NA	NA	4.02E+00	2.96E-32	NA	NA
K10534	*nr*	nitrate reductase (NAD(P)H) [EC:1.7.1.1 1.7.1.2 1.7.1.3]	4.91E+00	2.42E-01	NA	NA	1.14E+01	6.20E-59	NA	NA
K02575	*nrt, nark, nrtp, nasa*	MFS transporter, NNP family, nitrate/nitrite transporter	1.94E-02	9.96E-01	NA	NA	5.21E+00	3.91E-12	NA	NA
K02575	*nrt, nark, nrtp, nasa*	MFS transporter, NNP family, nitrate/nitrite transporter	-2.23E-01	9.52E-01	NA	NA	5.72E+00	1.14E-28	NA	NA
K01027	*oxct*	3-oxoacid CoA-transferase [EC:2.8.3.5]	-5.43E-01	2.27E-02	-9.66E-02	8.15E-01	4.44E-02	9.46E-01	8.20E-01	4.45E-02
K01958	*pc, pyc*	pyruvate carboxylase [EC:6.4.1.1]	-5.99E-01	1.09E-01	-5.85E-01	7.29E-02	-8.39E-02	9.25E-01	4.83E-01	1.28E-01
K00161	*pdha, pdha*	pyruvate dehydrogenase E1 component alpha subunit [EC:1.2.4.1]	5.92E-01	1.64E-01	5.03E-01	6.37E-01	3.82E-01	5.08E-01	7.62E-01	4.74E-01
K00162	*pdhb, pdhb*	pyruvate dehydrogenase E1 component beta subunit [EC:1.2.4.1]	-9.25E-02	9.21E-01	4.12E-01	5.89E-01	-7.60E-02	9.53E-01	1.84E+00	1.65E-02
K00898	*pdk2_3_4*	pyruvate dehydrogenase kinase 2/3/4 [EC:2.7.11.2]	-1.58E-01	7.39E-01	6.10E+00	8.11E-02	-1.21E+00	1.07E-05	5.95E+00	8.62E-02
K19030	*pfkfb4*	6-phosphofructo-2-kinase / fructose-2,6-biphosphatase 4 [EC:2.7.1.105 3.1.3.46]	4.24E-01	6.66E-01	NA	NA	1.18E+00	6.58E-02	NA	NA
K00927	*pgk, pgk*	phosphoglycerate kinase [EC:2.7.2.3]	3.51E-01	4.38E-01	-7.06E-02	8.80E-01	2.75E-01	6.36E-01	6.36E-01	1.70E-01
K00873	*pk, pyk*	pyruvate kinase [EC:2.7.1.40]	-7.97E-01	1.55E-01	1.60E-01	8.20E-01	-7.35E-01	2.67E-01	8.80E-01	2.05E-01
K01887	*rars, args*	arginyl-tRNA synthetase [EC:6.1.1.19]	4.31E-01	3.51E-01	1.70E+00	3.19E-01	-7.05E-01	1.92E-01	7.04E-01	6.76E-01
K02864	*rp-l10, mrpl10, rplj*	large subunit ribosomal protein L10	-1.68E-01	9.48E-01	NA	NA	-4.28E-01	8.83E-01	NA	NA
K02865	*rp-l10ae, rpl10a*	large subunit ribosomal protein L10Ae	-1.49E-01	7.54E-01	-8.65E-01	1.29E-01	6.74E-02	9.28E-01	-1.01E+00	7.84E-02
K02866	*rp-l10e, rpl10*	large subunit ribosomal protein L10e	2.34E-01	5.39E-01	-8.14E-01	1.03E-02	4.69E-02	9.53E-01	-5.64E-01	7.32E-02
K02868	*rp-l11e, rpl11*	large subunit ribosomal protein L11e	-1.89E-01	6.34E-01	-1.82E+00	1.55E-02	-4.52E-01	1.67E-01	-6.99E-01	3.37E-01

K02871	*rp-l13, mrpl13, rplm*	large subunit ribosomal protein L13	5.83E+00	7.22E-02	NA	NA	5.33E+00	1.98E-01	NA	NA
K02872	*rp-l13ae, rpl13a*	large subunit ribosomal protein L13Ae	-1.10E+00	1.59E-03	-9.42E-01	8.11E-02	-9.90E-01	1.29E-02	-6.39E-01	2.33E-01
K02873	*rp-l13e, rpl13*	large subunit ribosomal protein L13e	-1.55E+00	1.16E-05	-4.21E-01	4.55E-01	-1.59E+00	2.15E-05	-1.11E+00	5.48E-02
K02875	*rp-l14e, rpl14*	large subunit ribosomal protein L14e	1.18E-01	8.50E-01	NA	NA	-5.41E-01	1.95E-01	NA	NA
K02876	*rp-l15, mrpl15, rplo*	large subunit ribosomal protein L15	-2.89E-01	9.23E-01	NA	NA	-5.65E-01	8.76E-01	NA	NA
K02877	*rp-l15e, rpl15*	large subunit ribosomal protein L15e	9.28E-01	1.60E-02	-1.25E+00	1.95E-02	1.52E-01	8.70E-01	-1.36E+00	1.12E-02
K02880	*rp-l17e, rpl17*	large subunit ribosomal protein L17e	1.04E-01	8.63E-01	-4.58E-01	2.67E-01	-4.54E-01	3.44E-01	-6.07E-01	1.44E-01
K02882	*rp-l18ae, rpl18a*	large subunit ribosomal protein L18Ae	5.97E-01	3.14E-02	-1.62E+00	9.04E-02	-8.06E-02	9.16E-01	-4.30E-01	6.43E-01
K02883	*rp-l18e, rpl18*	large subunit ribosomal protein L18e	7.41E-01	8.24E-02	-8.00E-01	2.50E-02	2.31E-01	7.57E-01	-5.75E-01	1.05E-01
K02884	*rp-l19, mrpl19, rpls*	large subunit ribosomal protein L19	-5.39E-01	7.89E-01	NA	NA	-4.76E-01	8.54E-01	NA	NA
K02885	*rp-l19e, rpl19*	large subunit ribosomal protein L19e	3.11E-01	4.55E-01	-2.61E-01	6.34E-01	2.08E-01	7.01E-01	-1.41E+00	1.33E-02
K02889	*rp-l21e, rpl21*	large subunit ribosomal protein L21e	-2.10E-01	7.53E-01	-2.55E+00	1.36E-02	-6.85E-01	2.00E-01	-5.54E-01	5.67E-01
K02890	*rp-l22, mrpl22, rplv*	large subunit ribosomal protein L22	-7.25E-01	6.58E-01	NA	NA	-9.53E-01	5.86E-01	NA	NA
K02891	*rp-l22e, rpl22*	large subunit ribosomal protein L22e	-6.99E-01	2.08E-01	NA	NA	-8.88E-01	1.43E-01	NA	NA
K02892	*rp-l23, mrpl23, rplw*	large subunit ribosomal protein L23	1.13E+00	3.10E-01	NA	NA	3.05E-01	9.03E-01	NA	NA
K02893	*rp-l23ae, rpl23a*	large subunit ribosomal protein L23Ae	9.00E-01	9.70E-04	-8.01E+00	1.45E-09	2.84E-01	5.53E-01	3.04E-01	6.37E-01
K02894	*rp-l23e, rpl23*	large subunit ribosomal protein L23e	1.38E+00	6.45E-05	NA	NA	7.02E-01	1.84E-01	NA	NA
K02896	*rp-l24e, rpl24*	large subunit ribosomal protein L24e	2.37E-01	5.34E-01	-2.58E+00	7.70E-03	-2.86E-01	5.04E-01	-1.43E+00	1.20E-01
K02896	*rp-l24e, rpl24*	large subunit ribosomal protein L24e	1.01E+00	1.29E-01	NA	NA	7.62E-01	3.93E-01	NA	NA
K02898	*rp-l26e, rpl26*	large subunit ribosomal protein L26e	6.72E-01	7.31E-03	NA	NA	1.38E-01	8.11E-01	NA	NA
K02899	*rp-l27, mrpl27, rpma*	large subunit ribosomal protein L27	2.00E+00	3.45E-01	NA	NA	1.15E+00	7.54E-01	NA	NA
K02900	*rp-l27ae, rpl27a*	large subunit ribosomal protein L27Ae	1.26E+00	1.85E-03	NA	NA	5.93E-01	3.54E-01	NA	NA
K02901	*rp-l27e, rpl27*	large subunit ribosomal protein L27e	3.71E-01	1.60E-01	NA	NA	-1.18E-01	8.15E-01	NA	NA

K02903	*rp-l28e, rpl28*	large subunit ribosomal protein L28e	-1.27E+00	5.01E-01	NA	NA	-7.82E-01	7.63E-01	NA	NA
K02905	*rp-l29e, rpl29*	large subunit ribosomal protein L29e	-6.16E-01	5.05E-01	NA	NA	-1.05E+00	2.95E-01	NA	NA
K02906	*rp-l3, mrpl3, rplc*	large subunit ribosomal protein L3	5.77E-01	6.83E-01	-3.46E+00	2.18E-01	9.24E-01	4.62E-01	-3.46E+00	2.25E-01
K02908	*rp-l30e, rpl30*	large subunit ribosomal protein L30e	8.12E-02	9.64E-01	NA	NA	9.26E-02	9.69E-01	NA	NA
K02910	*rp-l31e, rpl31*	large subunit ribosomal protein L31e	5.63E-01	1.94E-01	NA	NA	5.69E-02	9.56E-01	NA	NA
K02912	*rp-l32e, rpl32*	large subunit ribosomal protein L32e	-5.09E-02	9.51E-01	-4.82E+00	2.08E-01	-4.08E-01	4.50E-01	-4.82E+00	2.12E-01
K02914	*rp-l34, mrpl34, rpmh*	large subunit ribosomal protein L34	2.09E-01	9.53E-01	NA	NA	8.26E-01	7.64E-01	NA	NA
K02915	*rp-l34e, rpl34*	large subunit ribosomal protein L34e	2.95E-01	3.83E-01	NA	NA	-1.51E-01	7.71E-01	NA	NA
K02918	*rp-l35e, rpl35*	large subunit ribosomal protein L35e	5.14E-01	6.85E-02	NA	NA	-2.97E-02	9.74E-01	NA	NA
K02920	*rp-l36e, rpl36*	large subunit ribosomal protein L36e	-7.39E-02	9.10E-01	NA	NA	-4.11E-01	3.86E-01	NA	NA
K02923	*rp-l38e, rpl38*	large subunit ribosomal protein L38e	-1.62E+00	5.63E-04	NA	NA	-1.23E+00	2.58E-02	NA	NA
K02925	*rp-l3e, rpl3*	large subunit ribosomal protein L3e	-5.73E-01	5.60E-02	-2.90E-01	3.34E-01	-8.71E-01	1.12E-03	-4.13E-01	1.73E-01
K02926	*rp-l4, mrpl4, rpld*	large subunit ribosomal protein L4	3.91E-02	9.69E-01	-4.26E+00	1.58E-01	9.42E-02	9.41E-01	-4.26E+00	1.63E-01
K02927	*rp-l40e, rpl40*	large subunit ribosomal protein L40e	3.12E-01	4.70E-01	NA	NA	-4.01E-01	3.92E-01	NA	NA
K02929	*rp-l44e, rpl44*	large subunit ribosomal protein L44e	-2.07E-01	7.89E-01	NA	NA	-3.41E-01	6.49E-01	NA	NA
K02930	*rp-l4e, rpl4*	large subunit ribosomal protein L4e	-1.35E+00	7.18E-09	-8.44E-01	1.03E-02	-8.49E-01	1.37E-03	-7.26E-01	2.71E-02
K02931	*rp-l5, mrpl5, rple*	large subunit ribosomal protein L5	6.09E-01	7.85E-01	NA	NA	2.66E-01	9.42E-01	NA	NA
K02932	*rp-l5e, rpl5*	large subunit ribosomal protein L5e	-3.18E-01	2.66E-01	-8.96E-01	2.04E-02	-4.03E-01	1.66E-01	-3.35E-01	3.78E-01
K02933	*rp-l6, mrpl6, rplf*	large subunit ribosomal protein L6	-2.01E+00	1.68E-01	NA	NA	-2.26E+00	1.95E-01	NA	NA
K02934	*rp-l6e, rpl6*	large subunit ribosomal protein L6e	-3.28E-01	5.00E-01	-4.57E-01	2.72E-01	-9.33E-01	2.03E-02	-4.64E-01	2.66E-01
K02936	*rp-l7ae, rpl7a*	large subunit ribosomal protein L7Ae	-2.32E+00	3.35E-25	-6.61E-01	3.38E-02	-2.04E+00	1.01E-17	-4.81E-01	1.20E-01
K02937	*rp-l7e, rpl7*	large subunit ribosomal protein L7e	3.68E-01	2.66E-01	-7.53E-01	2.00E-02	9.53E-02	8.83E-01	-7.01E-01	3.04E-02
K02938	*rp-l8e, rpl8*	large subunit ribosomal protein L8e	5.57E-01	1.33E-01	-7.51E-01	1.55E-02	-1.38E-01	8.48E-01	-6.89E-01	2.64E-02

K02940	*rp-l9e, rpl9*	large subunit ribosomal protein L9e	3.75E-01	3.27E-01	-8.51E-01	6.54E-02	-1.98E-01	7.34E-01	-9.45E-01	4.18E-02
K02941	*rp-lp0, rplp0*	large subunit ribosomal protein LP0	-9.25E-01	3.50E-02	-5.52E-01	3.08E-01	-5.60E-01	3.31E-01	-1.13E+00	4.16E-02
K02942	*rp-lp1, rplp1*	large subunit ribosomal protein LP1	-3.14E-01	5.56E-01	NA	NA	-7.70E-02	9.41E-01	NA	NA
K02943	*rp-lp2, rplp2*	large subunit ribosomal protein LP2	-9.03E-01	2.69E-03	NA	NA	-6.31E-01	9.33E-02	NA	NA
K02946	*rp-s10, mrps10, rpsj*	small subunit ribosomal protein S10	1.02E+00	2.11E-01	NA	NA	3.54E-01	8.18E-01	NA	NA
K02948	*rp-s11, mrps11, rpsk*	small subunit ribosomal protein S11	1.10E+00	6.34E-01	NA	NA	-6.02E-01	8.89E-01	NA	NA
K02949	*rp-s11e, rps11*	small subunit ribosomal protein S11e	-1.98E-03	9.97E-01	-8.20E+00	1.81E-09	-3.38E-01	5.41E-01	-2.06E-01	7.64E-01
K02951	*rp-s12e, rps12*	small subunit ribosomal protein S12e	1.02E-01	8.72E-01	NA	NA	-4.17E-01	3.85E-01	NA	NA
K02952	*rp-s13, rpsm*	small subunit ribosomal protein S13	1.61E-01	9.25E-01	NA	NA	-5.99E-01	7.16E-01	NA	NA
K02953	*rp-s13e, rps13*	small subunit ribosomal protein S13e	-2.00E+00	9.27E-11	-4.84E+00	2.08E-01	-1.65E+00	5.13E-07	-4.84E+00	2.13E-01
K02955	*rp-s14e, rps14*	small subunit ribosomal protein S14e	-5.38E-01	3.02E-01	NA	NA	-4.27E-01	5.03E-01	NA	NA
K02956	*rp-s15, mrps15, rpso*	small subunit ribosomal protein S15	1.03E+00	2.22E-02	NA	NA	4.94E-01	4.83E-01	NA	NA
K02957	*rp-s15ae, rps15a*	small subunit ribosomal protein S15Ae	-5.31E-01	3.98E-02	NA	NA	-9.48E-01	1.28E-05	NA	NA
K02958	*rp-s15e, rps15*	small subunit ribosomal protein S15e	1.69E-01	6.71E-01	-2.73E+00	6.47E-03	-2.82E-01	4.56E-01	-2.72E-01	7.68E-01
K02959	*rp-s16, mrps16, rpsp*	small subunit ribosomal protein S16	7.81E-02	9.73E-01	NA	NA	-9.09E-01	6.44E-01	NA	NA
K02960	*rp-s16e, rps16*	small subunit ribosomal protein S16e	2.18E-01	5.48E-01	NA	NA	-3.44E-01	3.49E-01	NA	NA
K02961	*rp-s17, mrps17, rpsq*	small subunit ribosomal protein S17	6.59E-01	7.95E-01	NA	NA	-9.69E-01	7.86E-01	NA	NA
K02962	*rp-s17e, rps17*	small subunit ribosomal protein S17e	1.48E-01	8.12E-01	-5.94E+00	9.81E-02	-4.78E-01	3.66E-01	-6.60E-01	8.34E-01
K02963	*rp-s18, mrps18, rpsr*	small subunit ribosomal protein S18	-7.15E-02	9.68E-01	NA	NA	-6.92E-01	6.45E-01	NA	NA
K02964	*rp-s18e, rps18*	small subunit ribosomal protein S18e	-2.79E-01	3.38E-01	-5.54E+00	1.26E-01	-5.92E-01	1.51E-02	-8.42E-01	7.99E-01
K02966	*rp-s19e, rps19*	small subunit ribosomal protein S19e	2.16E-01	5.97E-01	NA	NA	-2.12E-01	6.49E-01	NA	NA
K02967	*rp-s2, mrps2, rpsb*	small subunit ribosomal protein S2	-1.49E-01	9.08E-01	NA	NA	-5.23E-01	6.36E-01	NA	NA
K02969	*rp-s20e, rps20*	small subunit ribosomal protein S20e	-1.37E+00	1.77E-02	NA	NA	-5.14E-01	5.51E-01	NA	NA

K02971	*rp-s21e, rps21*	small subunit ribosomal protein S21e	2.05E-01	7.84E-01	NA	NA	-1.86E-01	8.56E-01	NA	NA
K02973	*rp-s23e, rps23*	small subunit ribosomal protein S23e	7.13E-01	1.65E-03	NA	NA	7.40E-02	9.15E-01	NA	NA
K02974	*rp-s24e, rps24*	small subunit ribosomal protein S24e	-1.86E-01	7.38E-01	-4.33E+00	1.53E-01	-4.24E-01	3.82E-01	-4.33E+00	1.58E-01
K02975	*rp-s25e, rps25*	small subunit ribosomal protein S25e	-3.45E-01	4.91E-01	NA	NA	-5.92E-01	2.43E-01	NA	NA
K02976	*rp-s26e, rps26*	small subunit ribosomal protein S26e	-1.61E+00	2.14E-03	NA	NA	-1.70E+00	2.67E-03	NA	NA
K02977	*rp-s27ae, rps27a*	small subunit ribosomal protein S27Ae	2.56E-01	3.53E-01	NA	NA	-3.44E-01	2.25E-01	NA	NA
K02978	*rp-s27e, rps27*	small subunit ribosomal protein S27e	-9.59E-02	8.91E-01	NA	NA	-5.66E-01	2.51E-01	NA	NA
K02979	*rp-s28e, rps28*	small subunit ribosomal protein S28e	8.19E-01	6.36E-02	NA	NA	3.24E-01	6.65E-01	NA	NA
K02981	*rp-s2e, rps2*	small subunit ribosomal protein S2e	-1.30E+00	2.20E-10	-1.06E+00	8.76E-03	-6.78E-01	5.17E-03	-4.92E-01	2.16E-01
K02983	*rp-s30e, rps30*	small subunit ribosomal protein S30e	-3.59E-01	3.14E-01	NA	NA	-3.12E-01	4.53E-01	NA	NA
K02984	*rp-s3ae, rps3a*	small subunit ribosomal protein S3Ae	5.83E-01	5.11E-03	-4.00E-01	3.36E-01	6.81E-03	9.94E-01	-8.00E-01	5.81E-02
K02985	*rp-s3e, rps3*	small subunit ribosomal protein S3e	-1.16E+00	7.47E-04	-5.02E-01	1.28E-01	-1.18E+00	1.40E-03	-5.27E-01	1.12E-01
K02987	*rp-s4e, rps4*	small subunit ribosomal protein S4e	7.56E-01	9.83E-02	-3.13E-01	3.51E-01	1.89E-01	8.26E-01	-5.48E-01	1.07E-01
K02989	*rp-s5e, rps5*	small subunit ribosomal protein S5e	-9.34E-01	1.11E-03	-1.38E+00	4.35E-02	-7.66E-01	2.19E-02	-2.28E-01	7.31E-01
K02991	*rp-s6e, rps6*	small subunit ribosomal protein S6e	2.32E-01	6.35E-01	-8.39E-01	3.15E-02	-1.54E-01	8.18E-01	-5.79E-01	1.35E-01
K02992	*rp-s7, mrps7, rpsg*	small subunit ribosomal protein S7	1.21E+00	6.84E-01	NA	NA	2.41E+00	2.55E-01	NA	NA
K02993	*rp-s7e, rps7*	small subunit ribosomal protein S7e	4.73E-01	3.18E-01	-1.05E+00	4.21E-04	-5.28E-02	9.62E-01	-7.32E-01	1.29E-02
K02995	*rp-s8e, rps8*	small subunit ribosomal protein S8e	6.29E-01	1.56E-02	-8.94E-01	8.77E-05	-5.46E-02	9.41E-01	-5.25E-01	1.90E-02
K02996	*rp-s9, mrps9, rpsi*	small subunit ribosomal protein S9	1.93E+00	5.89E-02	NA	NA	4.98E-01	8.56E-01	NA	NA
K02997	*rp-s9e, rps9*	small subunit ribosomal protein S9e	-1.92E-01	6.56E-01	-6.59E-01	1.25E-02	-3.17E-01	4.24E-01	-3.06E-01	2.40E-01
K02998	*rp-sae, rpsa*	small subunit ribosomal protein SAe	-1.28E+00	1.34E-06	-6.08E-01	3.58E-02	-1.12E+00	1.01E-04	-4.14E-01	1.51E-01
K00236	*sdhc, sdh3*	succinate dehydrogenase (ubiquinone) cytochrome b560 subunit	-3.44E+00	1.71E-07	NA	NA	-1.60E+00	2.60E-02	NA	NA
K15100	*slc25a1, ctp*	solute carrier family 25 (mitochondrial citrate transporter), member 1	8.29E-01	5.43E-01	NA	NA	8.31E-01	5.87E-01	NA	NA

K15100	*slc25a1, ctp*	solute carrier family 25 (mitochondrial citrate transporter), member 1	-1.01E-01	9.34E-01	1.31E+00	4.23E-01	-8.30E-02	9.62E-01	-6.04E+00	9.61E-03
K15100	*slc25a1, ctp*	solute carrier family 25 (mitochondrial citrate transporter), member 1	3.94E-01	6.68E-01	NA	NA	1.41E-01	9.30E-01	NA	NA
K15104	*slc25a11, ogc*	solute carrier family 25 (mitochondrial oxoglutarate transporter), member 11	-6.38E-01	8.17E-02	NA	NA	-9.17E-01	8.75E-03	NA	NA
K15109	*slc25a20_29, cact, cacl, crc1*	solute carrier family 25 (mitochondrial carnitine/acylcarnitine transporter), member 20/29	4.92E+00	2.40E-01	NA	NA	5.85E+00	8.83E-02	NA	NA
K15109	*slc25a20_29, cact, cacl, crc1*	solute carrier family 25 (mitochondrial carnitine/acylcarnitine transporter), member 20/29	4.58E-01	8.08E-01	NA	NA	1.17E+00	3.98E-01	NA	NA
K03125	*taf1*	transcription initiation factor TFIID subunit 1	1.39E-01	9.28E-01	NA	NA	3.14E-01	8.42E-01	NA	NA
K03128	*taf2*	transcription initiation factor TFIID subunit 2	-2.09E-01	7.53E-01	NA	NA	-2.25E-01	7.64E-01	NA	NA
K03130	*taf5*	transcription initiation factor TFIID subunit 5	-3.97E-01	7.31E-01	NA	NA	-3.88E-02	9.91E-01	NA	NA
K03131	*taf6*	transcription initiation factor TFIID subunit 6	1.66E-01	8.89E-01	NA	NA	-2.58E-02	9.94E-01	NA	NA
K01868	*tars, thrs*	threonyl-tRNA synthetase [EC:6.1.1.3]	8.97E-02	8.41E-01	1.88E+00	5.72E-01	-1.25E+00	9.44E-09	-4.26E+00	1.93E-01
K03457	*tc.ncs1*	nucleobase:cation symporter-1, NCS1 family	-3.02E-01	7.64E-01	NA	NA	1.91E+00	3.15E-07	NA	NA
K03136	*tfiie1, gtf2e1, tfa1, tfe*	transcription initiation factor TFIIE subunit alpha	8.83E-01	5.19E-01	NA	NA	5.54E-01	7.91E-01	NA	NA
K03137	*tfiie2, gtf2e2, tfa2*	transcription initiation factor TFIIE subunit beta	-7.80E-01	3.08E-01	NA	NA	6.48E-02	9.70E-01	NA	NA
K03139	*tfiif2, gtf2f2, tfg2*	transcription initiation factor TFIIF subunit beta [EC:3.6.4.12]	3.78E-01	7.39E-01	NA	NA	-6.02E-01	6.50E-01	NA	NA
K03142	*tfiih2, gtf2h2, ssl1*	transcription initiation factor TFIIH subunit 2	2.42E-01	8.39E-01	NA	NA	1.70E-02	9.94E-01	NA	NA
K03143	*tfiih3, gtf2h3, tfb4*	transcription initiation factor TFIIH subunit 3	-3.17E-01	8.48E-01	NA	NA	2.97E-01	8.83E-01	NA	NA
K03144	*tfiih4, gtf2h4, tfb2*	transcription initiation factor TFIIH subunit 4	1.06E+00	3.39E-01	NA	NA	1.06E+00	4.04E-01	NA	NA
K15430	*trm11, trmt11*	tRNA (guanine10-N2)-methyltransferase [EC:2.1.1.214]	2.41E-01	8.53E-01	NA	NA	1.14E-01	9.55E-01	NA	NA
K15448	*trm112, trmt112*	multifunctional methyltransferase subunit TRM112	4.82E-02	9.87E-01	NA	NA	-1.10E+00	5.81E-01	NA	NA
K15447	*trm44, mettl19*	tRNASer (uridine44-2'-O)-methyltransferase [EC:2.1.1.211]	-1.32E+00	4.67E-01	NA	NA	-9.89E-01	6.63E-01	NA	NA
K15429	*trm5, trmt5*	tRNA (guanine37-N1)-methyltransferase [EC:2.1.1.228]	4.68E+00	2.97E-01	NA	NA	4.20E+00	4.61E-01	NA	NA
K03256	*trm6, gcd10*	tRNA (adenine-N(1)-)-methyltransferase non-catalytic subunit	1.38E-02	9.94E-01	NA	NA	-1.67E-01	9.25E-01	NA	NA

K07442	*trm61, gcd14*	tRNA (adenine57-N1/adenine58-N1)-methyltransferase [EC:2.1.1.219 2.1.1.220]	-7.38E-01	7.73E-01	NA	NA	1.29E-01	9.75E-01	NA	NA
K15443	*trm82, wdr4*	tRNA (guanine-N(7)-)-methyltransferase subunit TRM82	7.84E-01	1.29E-01	NA	NA	-6.32E-02	9.67E-01	NA	NA
K15444	*trm9*	tRNA (uracil-5-)-methyltransferase TRM9 [EC:2.1.1.229]	7.92E-02	9.51E-01	NA	NA	-3.58E-01	7.58E-01	NA	NA
K03439	*trmb, mettl1*	tRNA (guanine-N7-)-methyltransferase [EC:2.1.1.33]	7.51E-01	5.07E-01	NA	NA	5.03E-01	7.63E-01	NA	NA
K03439	*trmb, mettl1*	tRNA (guanine-N7-)-methyltransferase [EC:2.1.1.33]	3.98E-01	8.50E-01	NA	NA	-1.25E+00	5.61E-01	NA	NA
K00555	*trmt1, trm1*	tRNA (guanine26-N2/guanine27-N2)-dimethyltransferase [EC:2.1.1.215 2.1.1.216]	6.85E-01	2.14E-01	NA	NA	2.80E-01	7.77E-01	NA	NA
K15445	*trmt10a, trm10, rg9mtd2*	tRNA (guanine9-N1)-methyltransferase [EC:2.1.1.221]	-2.91E-01	8.94E-01	NA	NA	-3.51E-01	9.00E-01	NA	NA
K15331	*trmt2b, trm2*	tRNA (uracil-5-)-methyltransferase [EC:2.1.1.35]	2.60E-01	8.53E-01	NA	NA	-1.75E-01	9.36E-01	NA	NA
K00365	*uaz*	urate oxidase [EC:1.7.3.3]	1.19E-01	9.73E-01	-1.53E-01	9.02E-01	1.62E+00	3.93E-01	1.72E+00	1.66E-01
K12618	*xrn1, sep1, kem1*	5'-3' exoribonuclease 1 [EC:3.1.13.-]	3.98E-02	9.51E-01	NA	NA	-3.37E-01	4.18E-01	NA	NA
K12619	*xrn2, rat1*	5'-3' exoribonuclease 2 [EC:3.1.13.-]	-6.40E-01	1.63E-01	-5.66E+00	1.37E-01	-1.05E+00	1.92E-02	-5.66E+00	1.40E-01
K01866	*yars, tyrs*	tyrosyl-tRNA synthetase [EC:6.1.1.1]	6.00E-01	4.05E-01	1.20E-01	9.69E-01	-6.51E-01	5.18E-01	-5.69E+00	1.07E-01
K01866	*yars, tyrs*	tyrosyl-tRNA synthetase [EC:6.1.1.1]	1.11E-01	9.40E-01	NA	NA	-3.77E-01	8.00E-01	NA	NA
K16261	*yat*	yeast amino acid transporter	-2.98E-02	9.67E-01	NA	1.00E+00	1.83E+00	1.20E-17	5.78E+00	6.37E-02
K16066	*ydfg*	3-hydroxy acid dehydrogenase / malonic semialdehyde reductase [EC:1.1.1.381 1.1.1.-]	1.24E+00	1.76E-01	-4.15E-01	7.35E-01	1.19E+00	2.64E-01	-1.88E-01	8.78E-01

CHAPTER 5

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This dissertation represents efforts towards development of organic waste fueled bio-refineries, capable of achieving circular economy and resource recovery through conversion of the embedded chemical energy and nutrients present in the organic waste, into biochemical products of commercial value. Specifically, the feasibility of conversion of volatile fatty acids produced through the anaerobic fermentation of organic wastes into lipids by the oleaginous yeast *Cryptococcus albidus* was evaluated. In the first phase, the effect of nitrogen limitation on lipid accumulation by *C. albidus* was evaluated and the optimum process and culture conditions to maximize lipid accumulation were identified. Second phase of this research utilized molecular biology technologies to improve the understanding of the biochemical basis of oleaginity. The complete genome of *C. albidus* was sequenced, annotated and deposited to national center of bioinformatics (NCBI) for public research use. Further the impact of nitrogen limitation on the overall cellular metabolism was evaluated by studying the transcriptome and proteome level changes under nitrogen limitation. The combination of process improvement and utilization of molecular biological techniques led to improved lipid yields and better understanding of microbial oleaginity. Herein, we discuss the broader impact and intellectual merit of this research coupled with potential challenges to its full-scale deployment. An outlook on the future directions and recommendations is also presented at the end.

5.1 Broader impact and intellectual merit

The organics contained in waste streams are primarily responsible for greenhouse gas (GHG) emissions from landfills and, account for 28% of the total municipal solid waste (MSW) in the US (USEPA, 2012). About 571 million metric tons of organic waste is generated per year (Hoornweg & Bhada-Tata, 2012), presenting a significant opportunity for wider application of

the technology presented in this dissertation. Several governments worldwide have committed to zero waste landfilling initiatives in many cities (e.g New York, San Francisco) and countries (France), representing an optimal opportunity.

On the other hand, majority of globally traded lipids are of plant origin, with a global market value estimated to be over 80 billion USD. Vegetable oils, used in a myriad of personal care products such as cosmetics, detergents and packaged foods, are obtained from large palm or soybean plantations, often in tropical regions of the world, where primary forests need to be cleared for their installation. They are then shipped across the world for further refinement and use. Both the deforestation as well as the transport is environmentally detrimental. The main concern comes from the removal of rain forests and peat lands, which act as natural carbon sinks, leading to emissions of over 2 billion tons of CO_2 per year (Strack, 2008). As a matter of fact, 3,300 tons of CO_2 is released into the atmosphere for the clearing each hectare of tropical peatland in Indonesia (Kurnianto & Murdiyarso), the world's biggest palm oil producer. This, as well as the destruction of habitat, and the displacement and even killing of indigenous peoples for illegal palm oil plantations, have caused palpable public damage to big corporations using vegetable oils in their supply chains.

Therefore, the broader impact of this work addresses two major environmental problems facing society: waste management and deforestation due to vegetable oil plantations. There are critical concerns regarding the environmental degradation and the socio-economic impact of rainforest destruction for large palm oil plantation in Southeast Asia, which have numerous detrimental consequences including loss of biodiversity, extinction of endangered species, and massive greenhouse gas emissions. The process described herein is a sustainable alternative for institutions and cities to achieve reduction in quantity, cost and carbon footprint associated with

organic waste disposal. The output is a sustainable vegetable oil replacement that can be used as a manufacturing input for large personal care companies such as Colgate-Palmolive, Procter and Gamble, Johnson & Johnson, and Unilever relieving pressure on the current sources of these lipids.

5.2 Challenges to scale-up

While multiple risk exist in scaling up any technology from lab scale to commercial scale, the specific risks associated with this process include:

1. Scale up of biological system: The factors which may have an impact are: 1) inadequate mixing in the upstream anaerobic reactor, which may lead to non-uniform microbial community, which may reduce the yield of volatile fatty acids by increasing the system solids retention time (SRT) and lead to proliferation of methanogens in the system. 2) Achieving proper mechanical hydrolysis of the influent organic load is critical to maintaining the system SRT, since improper blending will extend the time needed for biological hydrolysis and may again lead to loss of carbon through methane production in the system. 3) Inadequate aeration in the downstream aerobic reactor: Maintaining a non limiting dissolved oxygen (DO) concentration in the downstream is essential for adequate microbial growth and lipid production. However, given the potential challenges with high cell density of yeast cultures, more complex mixing dynamics at the large scale, and the risk of excessive foaming due to high aeration, it may prove challenging to provide and maintain enough oxygen and 4) Finally, maintaining the sterility in the downstream aerobic reactor may provide additional challenges. It is our assumption that given the aerobic environment and the non-ideal carbon source (VFA) in the downstream reactor,

yeast should be able to outcompete any other microbes crossing over from the upstream reactor.

2. Efficient lipid extraction at large scale: Many microbial lipid production attempts thus far have been algae based and have faced significant challenges in large scale lipid extraction. The technology proposed herein has an advantage since it relies on high density yeast culture which is easier to extract from suspended phase and omits the costly drying steps. However, disruption of the yeast cell to extract the lipids may yet prove challenging at the larger scale.
3. Lipid quality control: Maintaining a stable lipid composition is crucial to any potential commercial application of microbial lipids. However, the lipid composition is dependent upon the influent VFA composition and the relative composition of VFA produced from anaerobic fermentation is dependent upon the type of organic waste being fermenter. Therefore, the ability to maintain a consistent quality of output product at larger scale may prove challenging.
4. Waste Access: Most municipal waste streams contain non-organic residues such as plastics, glass and paper waste and therefore, the access to acceptable (quantity and quality) waste streams at larger scales as the process increases in size and replicates across geography may prove challenging.

5.3 Future perspectives: VFA fueled biorefineries

In 2004, a report published by United States Department of Energy (US DOE) (T. Werpy 2004) identified twelve bio based chemicals with the greatest commercial significance in the US. Since publication, this report has spurred widespread research efforts to develop economically feasible

bio-refineries for production of these chemicals. While the potential for conversion of VFA to 'building-block chemicals' has been studied extensively, the metabolic understanding of pathways and molecular mechanisms involved in VFA uptake and conversion remains fairly limited. Herein, we describe the specific metabolic pathways, biocatalyst platforms and mechanisms for the production of a subset of these chemicals, specifically from VFA as precursor molecules.

Currently existing bio-based pipelines depend upon carbohydrates (glucose, sucrose) or food crops making them unsustainable for large scale production. However, most of these production pathways include few key intermediates such as acetyl-CoA and pyruvate, which can be readily produced from VFA. Therefore, research efforts are now being focused to 'retrofit' the existing bio-based pipelines to incorporate VFA as a cheaper and alternate carbon substrate to replace the more expensive carbohydrates or unsustainable food and agricultural commodity based bio-substrates with varying degree of success. For instance, the use of VFA for microbial lipid and polyhydroxyalkanoate (bio-plastics) production has been extensively studied and the costs of such processes have reduced dramatically. The cost of bio-plastics produced through fermentation of wastewater was reported to be 0.75-1.45 $/Kg (Fernández-Dacosta, Posada et al. 2015) which is comparable to the market price of various commercial plastics (0.5-1.2 $/Kg). Similarly, the cost of biodiesel production from food waste fermentation was reported to be 0.81-2.53 $/L(Vajpeyi and Chandran 2015) compared to the average price of gasoline in the US and the EU (0.5-1.5$/L).

5.4 Conversion of VFA to adipic acid (Hexanedioic acid)

Adipic acid is one of the most important commercial aliphatic, straight-chain dicarboxylic acids. Adipic acid is primarily used for manufacturing nylon 6-6 (Table 8) (Figure 12), the annual global market of which is estimated at approximate US$ 6.3 billion. Currently, the only economically feasible option to produce adipic acid is using petroleum carbon. Most adipic acid is produced by oxidation of ketone alcohol (KA) oil (mixture of cyclohexanol and cyclohexanone) catalyzed by nitric acid, which is extremely detrimental to the environment with the massive production of carbon dioxide/nitrous and nitrogen oxide (Deng, Ma et al. 2016).

5.4.1 Metabolic pathways for adipic acid production (Figure 13 (pathway1))

Despite the high commercial interest and the research efforts, so far no economically feasible biological production methods exist, partially due to the absence of any natively occurring adipate synthesis pathways in microorganisms. In cellular metabolism, adipic acid is an intermediate in degradation pathway of cyclohexane, cyclohexanone and ε-caprolactam under aerobic conditions (KEGG pathway 00930)(Figure 12).

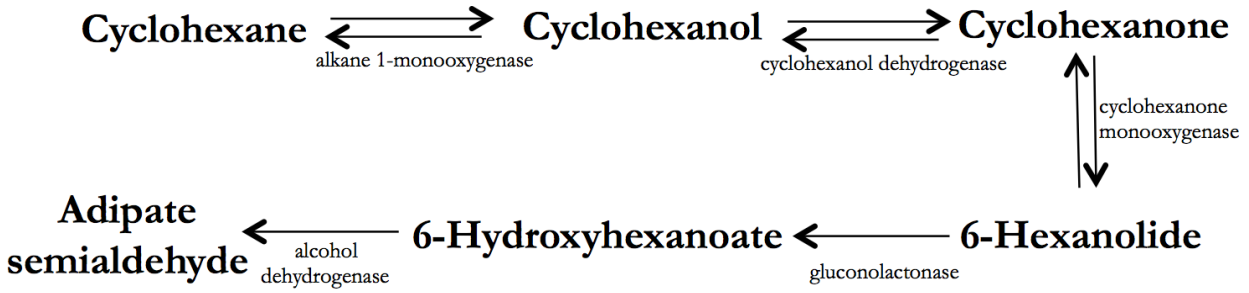

Figure 13 Major reactions in biological conversion of cyclohexane to adipate semialdehyde (KEGG pathway 00930)

The degradation of these compounds results in an intermediate adipate semialdehyde, which is then further oxidized to adipic acid by 6-oxohexanoate dehydrogenase (EC 1.2.1.63) (Polen, Spelberg et al. 2013). Two primary biological routes available for adipate synthesis are: (i) biosynthesis of the precursors for adipic acid (cis, cis-muconic acid or glucaric acid), which can be further converted to adipic acid via a chemo-catalytic conversion (De Guzman 2010) and, (ii) synthesis of adipic acid directly from carbon sources. However, the biological pathways for precursor production (KEGG pathway 00053) again rely upon petroleum derivatives such as catechol or benzoate and therefore cannot be used in a strictly 'waste-to-biorefinery' pipeline, unless aromatic hydrocarbon wastes are considered. The bioconversion of VFA to adipic acid can however, be achieved by engineered yeast strains through α- and/or ω-oxidation of long chain n-alkanes (Smit, Mokgoro et al.), alcohols (Picataggio, Rohrer et al. 1992) or fatty acid substrates (Yang, Lu W Fau - Zhang et al.). In general, the production of dicarboxylic acids from yeasts is impaired by a very effective β–oxidation pathway. Therefore, genetically modified strains must suppress the acyl-CoA oxidase enzyme (EC 1.3.3.6), which blocks the β–oxidation and redirects the substrates to the terminal ω-carbon oxidation. The production of adipic and other dioic acids has been demonstrated in engineered strains of yeasts *Yarrowia lipolytica* (Smit, Mokgoro et al.) and *Candida tropicalis* (Yang, Lu W Fau - Zhang et al.) using C12-C16 alkanes, fatty acids and monocarboxylic acids (VFA) as the carbon substrate, all of which can be produced biologically from organic wastes. The biological conversion (Figure 13) begins with formation of 3-oxoadipyl-CoA from acetate and succinate by the enzyme β–ketothiolase (EC 2.3.1.174), which is further converted to 3-hydroxyadipyl-CoA and then to adipyl-Co-A by the enzymes 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and 3-hydroxyadipyl-CoA

dehydratase (EC 4.2.1.17). Finally coenzyme-A is hydrolyzed by adipyl-CoA synthetase enzyme (EC 6.2.1.5) to form adipic acid (Burgard, Pharkya et al. 2012). The biological capability of even wild-type yeast strains to convert short chain VFA into long chain fatty acids has also been demonstrated (Vajpeyi and Chandran 2015), which can now be exploited further to convert in an integrated fashion those fatty acids into dicarboxylic acids. The development of metabolically engineered strains of oleaginous yeasts with suppressed β–oxidation pathway provides opportunities for the conversion of VFA into dicarboxylic acids previously thought to be biologically inaccessible.

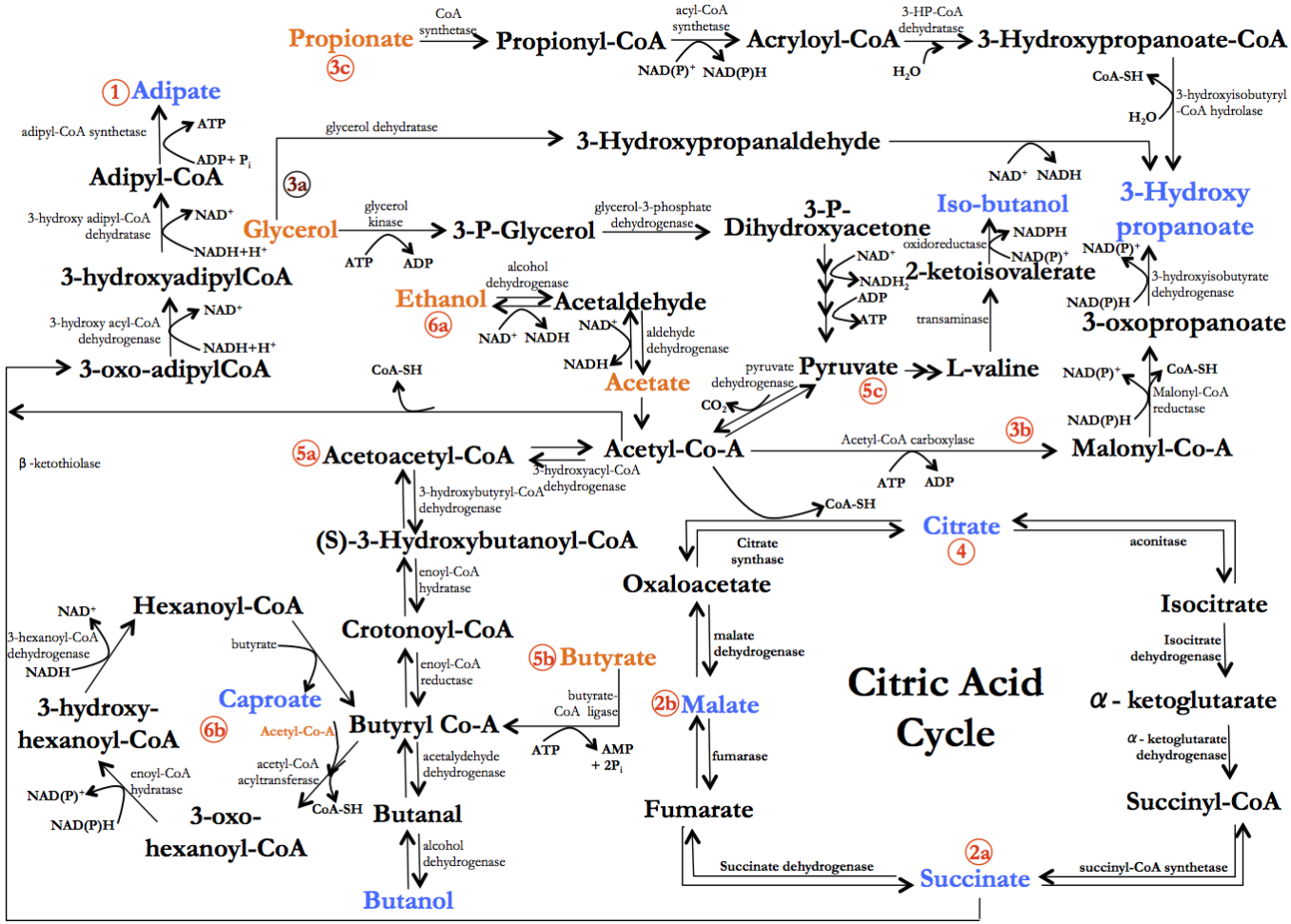

Figure 14 An overview of the major metabolic pathways, substrates (orange) and products (blue) suggested in this review. The numbers represent the pathways for synthesis of 1: adipic acid from acetate and succinate, 2a&b: Succinate and Malate, 3a,b&c: 3Hydroxypropionate (3HP) from glycerol, acetate and propionate, 4: Citric acid from different VFA, 5a&b: butanol from acetate and butyrate, 5c: iso-butanol from acetate and butyrate via pyruvate, 6a&b: ethanol, acetate and butyrate to MCFA.

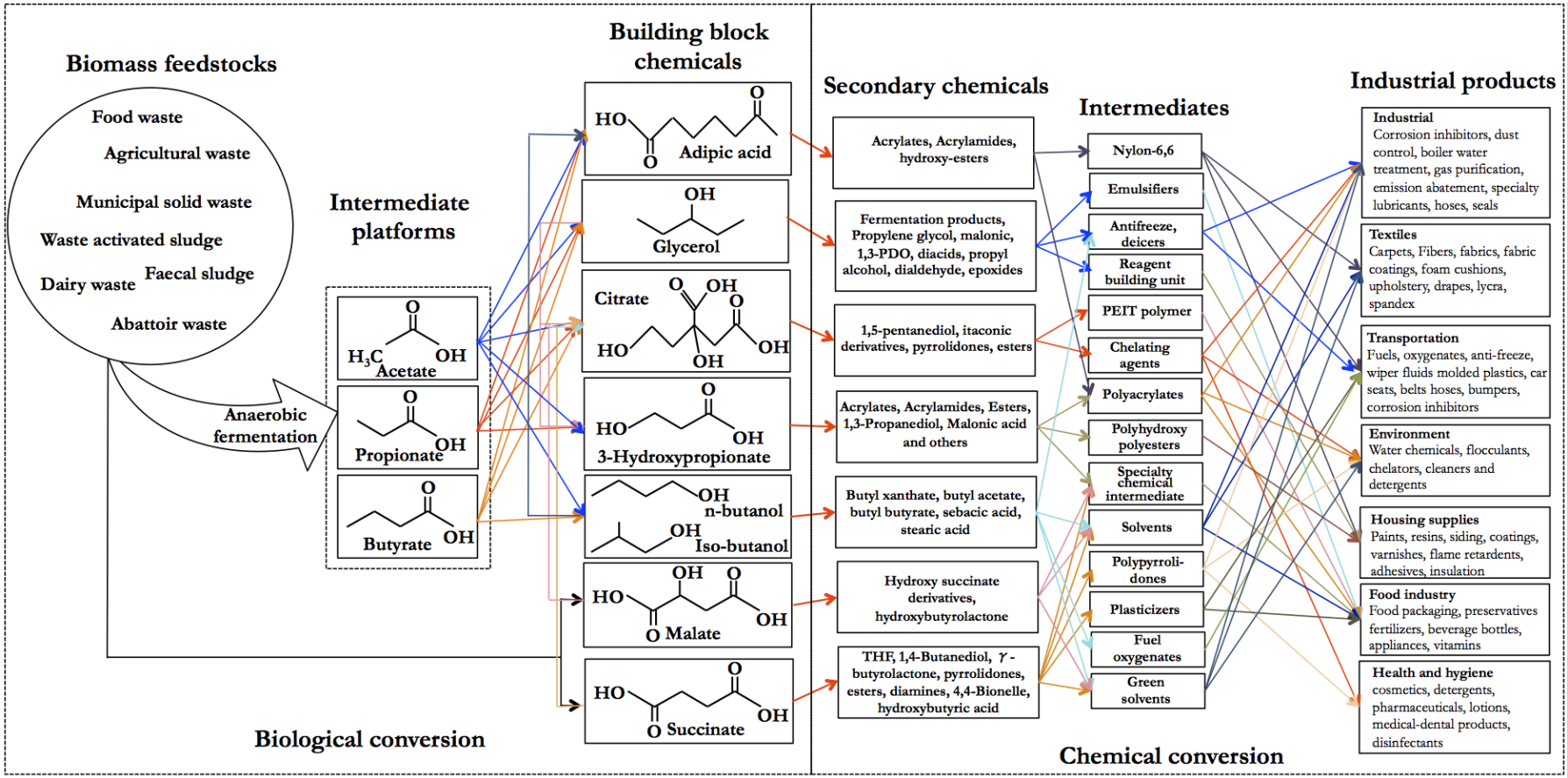

Figure 15 Various possibilities for a "waste-to-bio refinery" pipeline. Organic waste from diverse sources can be linked to various products of industrial significance using volatile fatty acids as the key intermediates. (Partially adapted from USDoE report (T. Werpy 2004))

Table 10 Summary of current and proposed feedstocks, microorganisms available for biological production and the industrial significance of the products.

Building block chemical	Current feedstock	Proposed feedstock	Microorganism(s) available	Aerobic/ Anaerobic	Engineered/ Wild-type	Industrial significance
Adipic acid	Cyclohexane, Cyclohexanone	Monocarboxylic acids, free fatty acids	*Yarrowia lipolytica, Candida tropicalis*	Ae	En	Polyacrylamides, Polyhydroxyesters, Speciality chemicals
Succinic acid	Butane, Maleic anhydride	Diverse organic waste	Mixed anaerobic consortia. Major species: *Actinobacillus succinogenes, Mannheimia succiniciproducens*	Ae, An	Wt	Plasticizers, Polypyrrolidones, Solvents, Speciality chemicals
Malic acid	Maleic, Fumaric acid	Organic waste with high sugar content	*Aspergillus sp.*	An	Wt	Plasticizers, Food additives, Solvents
3-Hydroxypropionate	Propylene	Glycerol	*Lactobacillus sp., Escherichia coli, Klebsiella pneumonia*	Ae, An	Wt, En	Polyhydroxypolyamides, Polyacrylates
Citric acid	Starch/Sucrose	Glycerol,VFA	*Yarrowia lipolytica*	Ae	Wt, En	Phthalate polyesters, Food additives, Chelating agents
Butanol	Propylene/ Acetaldehyde	Acetate, Butyrate	*Clostridium sp.*	Low O_2	Wt	Solvents, Fuel additives, Phthalates, Antibiotics
MCFA	Petroleum based	Mixed VFA, ethanol	*Mixed anaerobic consortia.* Major sp: *Clostridium sp.*	An	Wt.	Food supplements, corrosion inhibitors, antimicrobial

5.5 Four carbon dicarboxylic acids (Succinic acid, malic acid)

Succinic acid, derived from fermentation of agricultural carbohydrates, has a specialty chemical market in industries producing food products, pharmaceuticals, surfactants and detergents, green solvents and biodegradable plastics, and ingredients to stimulate animal and plant growth (Zeikus, Jain et al. 1999) (Table 8) (Figure 14). Succinic acid is currently produced mainly through chemical processes and has a $15 billion dollar market, which includes its derivatives.

Malic acid, with a worldwide consumption of 55,000 tons/year, is mostly used in the beverage (51%) and food (42%) industry and it shares a 10% market of the food and beverage industry mainly as an acidulant. Currently, malic acid is produced mainly by chemical synthesis via hydration of maleic or fumaric acids or via enzymatic conversion of fumaric acid by immobilized bacterial cells (Goldberg, Rokem et al. 2006).

5.5.1 Metabolic pathways for succinic and malic acid production

Succinic and malic acids, which are both four carbon dicarboxylic acids, are intermediates of the citric acid cycle (KEGG pathway 00020). Succinic acid is also an end product of anaerobic fermentation of carbohydrates and is naturally produced along with the VFA (Lee, Han et al. 2011) and fermentative succinic acid production is fairly well established (Zeikus, Jain et al. 1999, Song and Lee 2006). The metabolic pathways leading to the synthesis of succinic acid are diverse. Many different bacterial succinic acid producers have been isolated from various anaerobic environments. However, the best candidates identified are bacteria isolated from rumen including *Actinobacillus succinogenes* and *Mannheimia succiniciproducens*, since they produce succinic acid as a major fermentation product. Although major dicarboxylic acids (malate, fumarate, succinate) are not found in the rumen, large amounts of these acids are

produced by fixation of about 60-70 mol% carbon dioxide produced during anaerobic fermentation, and further converted to propionic acid by succinic acid utilizing bacteria (Song and Lee 2006). Therefore, succinic acid production is highly flexible and can be achieved biologically under aerobic or anaerobic conditions through bacterial or fungal fermentation by utilizing diverse organic feedstocks (Pinazo, Domine et al. 2015).

Malic acid is an intermediate of TCA cycle and has been produced using fungi such as *Aspergillus sp.* through fermentation of carbohydrates. Environmental stressors such as nitrogen limitation (high C:N ratio) and low pH appear to be a common requirement for the unusually high production and accumulation of malic acid by all producing organisms. Nitrogen limited cultures of fungi redirect cellular carbon towards malic acid production through the reductive TCA pathway rather than biomass synthesis. In addition to nitrogen limitation, addition of calcium carbonate ($CaCO_3$) to the growth medium has been shown to result in high production of malic acid. The addition of $CaCO_3$ ensures a pH of about 6 during fumaric and L-malic acid accumulation processes by neutralizing the acids produced during fermentation and also provides CO_2 as a substrate, which can be fixed by pyruvate carboxylase (EC 6.4.1.1) to produce oxaloacetate. The activity of the enzyme pyruvate carboxylase and its location in the cytoplasm of the cells are believed to result in high yield of L-malic acid (Tsao, Cao et al. 1999). Carbohydrates such as glucose enter the TCA pathway through glycolytic conversion to pyruvate, which is further converted to acetyl-CoA and enters the TCA cycle (Goldberg, Rokem et al. 2006). VFA such as acetate and butyrate can be readily converted to acetyl-CoA by the enzymes acetyl-CoA synthetase (EC 6.2.1.1) (Figure 14(5a)) and butyryl-CoA synthetase (EC 6.2.1.2) (Figure 14(5b)) and in turn can be converted to other organic acids through the TCA cycle.

5.5 3- Hydroxypropionic acid (3HP)

3HP is an attractive bio-based chemical, which can be chemically converted into acrylic acids, acrylic esters and amides and thereby it enables sustainable production of plastics, paints etc (Table 8)(Figure 14). The market for acrylic acid and its derivatives is forecasted to grow to US$ 18.8 billion by 2020 (Kildegaard, Wang et al. 2015). Currently, the commercial production of acrylic acid is based on chemical oxidation of non-renewable petroleum based propylene. Because of its high industrial relevance, several commercial enterprises, such as OPX Biotechnologies-Dow Chemical, Cargill-Novozymes-BASF, have also been developing bio-based processes for the production of 3-HP with the objective to produce acrylic acid from different feed-stocks such as glycerol and sugars (Chen and Nielsen 2016).

5.6.1 Metabolic pathways for production of 3HP

Many microorganisms including both prokaryotes and eukaryotes can produce 3HP as an intermediate or an end product through several metabolic pathways. The major pathways for biological 3HP production are either based upon carbohydrates (glucose and xylose) or glycerol. However, from a "waste-to-biorefinery" perspective, glycerol is much more lucrative as a substrate since it is already produced abundantly as a by-product from the production of biodiesel. Since VFA can be sustainably converted to biodiesel through organic waste fermentation (Vajpeyi and Chandran 2015), the conversion of glycerol to 3HP provides an opportunity to salvage the 'residual' carbon from the biodiesel pipeline and thereby improving the overall efficiency of carbon recovery from the waste. The conversion of glycerol to 3HP is a two step enzymatic reaction (Figure 14(3a)) where glycerol is first converted to 3-

hydroxypropanaldehyde (3-HPA) by the enzyme glycerol dehydratase (EC 4.2.1.30) and then 3-HPA is oxidized to yield 3HP by the enzyme aldehyde dehydrogenase (EC 1.2.1.3) (Kumar, Ashok et al. 2013). The conversion of glycerol to 3HP has been demonstrated in several studies using *Lactobacillus sp.* (Garai-Ibabe, Ibarburu et al. 2008, Krauter, Willke et al. 2012), which produces 3HP as an end product of glycerol metabolism. Genetically engineered strains of *Escherichia coli* and *Klebsiella pneumonia* have also been shown to produce 3HP from glycerol (Chen and Nielsen 2016).

Of interest for 3HP production could also be two alternate, as yet unexplored pathways involving direct conversion of some VFA to 3HP. As a part of propanoate metabolism (KEGG pathway 00640) and overall carbon metabolism (KEGG pathway 01200), acetic acid, which is readily converted to acetyl-CoA (Figure 14(3b)) can be further converted to malonyl-CoA by enzyme acetyl-CoA carboxylase (EC 6.4.1.2) and further to 3-oxopropanoate and 3-hydroxypropanoate through the action of enzymes malonyl-CoA reductase (EC 1.2.1.75) and 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31). As an alternative pathway (Figure 14 (3c)), propionic acid can also be converted to propionyl-CoA and then to acryloyl-CoA by enzyme acyl-CoA dehydrogenase (EC 1.3.8.7) and then further to 3-HP by enzyme 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4).

5.7 Conversion of VFA into citric acid

Citric acid has been successfully produced biologically on large scale. Citric acid finds widespread application in food and beverage industry and a range of technical and industrial segments (Table 8, Figure 14) as well and the current demand exceeds 1.75 million tons/year (Chen and Nielsen 2016). Currently, citric acid is produced from submerged fermentation of

Aspergillus niger using starch or sucrose based media. However, newer and cheaper technologies are being explored to enhance competitiveness.

5.7.1 Metabolic pathways for citric acid production

VFA can either be used directly as carbon source for citric acid production or they can be transformed into other more suitable substrates to increase the productivity. The use of succinic acid as a carbon source for citric acid production from *A. niger* cultures has been demonstrated (Xu, Madrid et al.), although with much lower yields (~0.5 g/L) compared to glucose (~200 g/L). However, more recently, much higher titer concentrations (~115 g/L) (Morgunov, Kamzolova et al. 2013) of citric acid have been achieved using glycerol as the sole carbon source in both batch (Kamzolova, Vinokurova et al. 2015) and continuous (Rywińska, Juszczyk et al. 2011) cultures of both wild-type and engineered strains of yeast *Y. lipolytica*. Glycerol, a by-product of biodiesel production has gained prominence as an alternate carbon source for citric acid production due to its low cost and high availability(Kamzolova, Vinokurova et al. 2015). *Y. lipolytica* has demonstrated the capability to convert VFA into lipids (Fontanille, Kumar et al. 2012), which are the precursor to biodiesel. Further, the conversion of glycerol into citric acid and VFA into lipids by *Y. lipolytica* was achieved under similar conditions of nitrogen limitation. Therefore, it might now be conceptually possible to produce lipids and citric acid in conjunction in a single step process through co-fermentation of glycerol and VFA using *Y. lipolytica*. It must be stressed that these are expected to be distinct pathways, since glycerol is used directly without fermentation to VFA (described below).

In the lipid or citric acid producing microorganisms that are cultivated on glycerol as the sole carbon source (Figure 14(4)), glycerol enters the microbial cell by facilitated diffusion and is firstly converted to 3-*P*-glycerol and then to 3-*P*-dihydroxyacetone, reactions catalyzed by the

enzymes glycerol kinase (EC 2.7.1.30) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), thus entering the second branch of the glycolytic pathway (KEGG pathway 00010) to yield pyruvate (Papanikolaou, Fakas et al. 2008). Subsequent metabolic steps to lipid or citric acid biosynthesis are the same as for glucose. Pyruvate further yields acetyl-CoA and oxaloacetate, which then enter citric acid cycle (Figure 14) (KEGG pathway 00020) or fatty acid biosynthesis (KEGG pathway 00061).

5.8 Bio-butanol production from VFA

Typically bioethanol or biodiesel have been used as a substitute to gasoline and diesel. However, butanol has sufficient similarity to gasoline to be used directly in any gasoline engine without any modification to the fuel or the engine (Table 8, Figure 14). It is also a superior fuel additive due to its relatively higher energy content, lower volatility, diminished flammability, low corrosiveness and low hygroscopicity (Mervat I. Foda 2010). Currently, both n- and iso-butanol are produced chemically via carbonylation of propylene or hydrogenation of butyralydehyde. The bio-based production of butanol shares the major common problems: high cost of substrate and low titer volumes. Additionally, the toxicity of butanol and its precursor, butyraldehyde to the fermenting microorganisms is also a challenge (Ezeji, Qureshi et al. 2007).

5.8.1 Metabolic pathways for n-butanol and iso-butanol production

Traditionally, the biological production of n- and iso-butanol has focused upon the integrated acetone-butanol-ethanol (ABE) fermentation system using glucose or starch based feedstocks as the carbon source using *Clostridia sp.* Iso-butanol is also reported to be naturally produced in small amounts by certain yeasts *e.g. Sacchromyces cerevisiae* by degradation of amino acids

such as valine, leucine etc. (Ezeji, Qureshi et al. 2014). While there are no published studies so far on direct use of VFA as the substrate for butanol production, the pathways for such biochemical transformation do exist natively and therefore, it might be plausible to achieve such a bioconversion. The pathway for natural iso-butanol production exists in yeasts, which perform alcohol fermentation from carbohydrates, where, a small amount of pyruvate, which can also be generated from acetate and butyrate (Figure 14 (5a, 5b)), is redirected to iso-butanol production after conversion to L-valine (KEGG pathway 00280). Briefly, valine is converted to 2-ketoisovalerate by transaminase enzymes (EC 2.6.1.42), which is further converted to iso-butanol via iso-butyraldehyde by the enzyme 2-oxoisovalerate oxidoreductase (EC 1.2.7.7). The major fermentative pathway for n-butanol production exists as a part of larger cellular butanoate metabolism (KEGG pathway 00650). Briefly, acetate (Figure 14(5a)) enters the pathway as acetyl-CoA and is further transformed to acetoacetyl-CoA by enzyme 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and eventually to butyryl-CoA through crotonoyl-CoA by enzyme enoyl-CoA reductase (EC 1.3.1.86), while butyrate (Figure 14(5b)) can enter the pathway directly after transformation to butyrly-CoA by the enzyme butyrate-CoA synthetase (EC 6.2.1.2). Butyryl-CoA can then be transformed to butanal (butyraldehyde) and further to butanol by broad specificity acetalydehyde dehydrogenase (EC 1.2.1.10) and alcohol dehydrogenase (EC 1.1.1.1) enzymes. The conversion of acetate to butanol requires oxidation of 2 mol of NADH to NAD^+ per mol of acetate and therefore it is not thermodynamically feasible. However, the pathway from butyric acid to butanol is relatively short from a metabolic point of view and also thermodynamically feasible (Gibbs free energy of formation, $\Delta_f G°$= -171.84 KJ/mol). Indeed, the use of butyric acid in conjunction with other less expensive carbon sources such as glycerol has been shown to double the butanol yield (19% w/w to 38% w/w) in anaerobic cultures of

Clostridium pasteurianum (Regestein, Doerr et al. 2015). In another study, continuous anaerobic co-fermentation of glucose and butyric acid (2:1 w/w) by *Clostridium saccharoperbutylacetonicum* resulted in a very high yield of butanol (68.6 % mol/mol-C, 7.9 g/L titer) (Baba, Tashiro et al. 2012). However, an attempt to use butyrate as the sole carbon source resulted in a much lower titer concentration (~0.8 g/L) despite very high yield (0.95 mol/mol-C) due to the inability of butyric acid to support the cell growth (Al-Shorgani, Ali et al. 2011). Several studies have shown butyrate to be a poor carbon source for cell growth (Vajpeyi and Chandran 2015). However, the fact that co-metabolism of butyric acid results in higher yields of butanol suggests that other less expensive carbon sources such as glycerol or even acetate can be used in conjunction to support cellular growth, as further supported by the genome sequence of *Clostridium saccharoperbutylacetonicum* (Poehlein, Krabben et al.).

5.9 Medium chain fatty acids (MCFA)

MCFA are saturated aliphatic carboxylic acids with six to twelve carbon atoms *e.g.* caproic acid (C6:0), caprylic (C8:0), Lauric acid (C12:0) , which can be used as biochemical building blocks for the production of biofuels and other commodity chemicals. The use of MCFA for production of antimicrobials and corrosion inhibitors has been demonstrated (Grootscholten, Strik et al. 2014). MCFA have also shown potential for some medical applications and use as nutritional supplements (Table 8). Being fairly inert chemically, MCFA can be rapidly absorbed and are easily accessible for metabolic breakdown to serve as a source of fuel calories (Wanten and Naber). The chain elongation of VFA from anaerobic digestion has been lately recognized as a mechanism to facilitate recovery of organic MCFA, which, due to longer carbon molecule chain

become more apolar and their extraction from the fermentation medium is eased (Kleerebezem, Joosse et al. 2015).

5.9.1 Metabolic pathways for MCFA production

MCFA production involves a mixed culture fermentation called chain elongation, where chain elongation bacteria convert shorter chain compounds such as ethanol and VFA to medium chain fatty acids (MCFA). The chain elongation by fermentative bacteria requires the presence of reduced energy-rich compounds such as ethanol, lactate or acetate and a high H_2 partial pressure to prevent the oxidation, and thus removal, of MCFA (Spirito, Richter et al. 2014). The microbial populations dominating the chain elongation process are relatives of *Clostridium kluyveri. C. kluyveri* uses the fermentation pathway of reversed β-oxidation which can be described by three coupled reactions, first (Figure 14(6a)), ethanol is converted to acetaldehyde and then to acetate producing NADH and ATP via substrate level phosphorylation by the enzyme alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3). Second, the fatty acid as acetate is elongated in a cyclic pathway to butyrate using CoA, NADH and $FADH_2$ through the coupling of two acetyl-CoA moieties to butyryl-CoA in a cyclic loop (Figure 14(5a)) catalyzed by enzymes described previously in text. Third, further chain elongation (Figure 14(6b)) (KEGG pathway 00062) to caproate occurs from butyrate and ethanol in a similar loop by coupling butyryl-CoA with acetyl-CoA to form 3-oxo-hexanoyl-CoA by enzyme acetyl-CoA transferase (EC 2.3.1.16) which is further hydrolyzed to 3-hydroxy hexanoyl-CoA and then to hexanoyl-CoA by enzymes enoyl-CoA hydratase (EC 1.1.1.211) and 3-hexanoyl-CoA dehydrogenase (EC 1.3.1.8). At this point another butyrate molecule enters the pathway loop thereby releasing caproic acid. Concluding from this mechanism, in every loop two carbons are added up to the

original carboxylic acid. This results in products with an even number of carbon atoms (Steinbusch, Hamelers et al. 2011). The combined pathways of ethanol oxidation and reverse β oxidation that form chain elongation are thermodynamically feasible at a pH of 7 with a Gibbs free energy change value of about −185 kJ/5 mol for both *n*-butyrate and *n*-caproate production(Spirito, Richter et al. 2014). The predominant MCFA obtained in a system based on dry anaerobic digestion were n-caproate and n-caprylate, (solubility 10.2 and 0.8 g/L respectively), and the production was promoted by the presence of low concentrations of ethanol (Grootscholten, Kinsky dal Borgo et al. 2013). Since acetate is the main product of anaerobic fermentation accompanied by very low concentrations of ethanol, the fermentation effluent can be directly used as the feedstock for MCFA production, thus eliminating the necessity for any expensive pretreatment steps.

5.10 Nutrient removal during waste treatment

Wastewater treatment plants are modifying operations and terminology to Water Resource Recovery Facilities (WRRFs). For WRRFs faced with stringent nutrient (N&P) discharge limits, addition of external organic ccarbon sources is needed, especially when the influent organic carbon (COD) to nitrogen or phosphorous content is limiting. For instance, the City of New York has committed about $100 million per year to enhance nitrogen removal in just four out of the fourteen WRRFs (Allen Deur 2014). The most commonly carbon source for enhancing nitrogenr removal is metanol, although it has been shown that the rate of denitrification on methanol is lower than that on VFA (Baytshtok, Kim et al. 2008 , Baytshtok, Lu et al. 2009, Lu and Chandran 2010, Lu, Nuruzzaman et al. 2011, Lu, Chandran et al. 2014). Additionally, not all microorgainsms can utilize metanol leading to limitations in the extent of nitrogen removal

during transitioning to methanol from a different carbon source or even just the influent organic carbon present in influent sewage. Therefore, production and utilization of VFA for enhanced nutrient removal can be significantly beneficial for water resource recovery facilities.

The organic carbon compounds from anaerobic fermentation, including VFA, might serve as carbon source for nutrient removal from wastewater, due to carbon to nitrogen or phosphorous ratio is in some cases not enough to carry out the complete removal process. An ample collection of recent data from enhanced nitrogen and phosphorous removal from wastewater by adding VFA from diverse waste fermentation has been published recently (Lee, Chua et al. 2014).

5.11 Conclusions

Herein, we present various pathways, microorganisms, culture conditions and current status of bio-based production of certain building-block chemicals such as adipic acid, butanol, organic acids such as citric, malic and succinic acids. These chemicals have the highest potential to be economically produced from VFA since the pathways for their bioconversion either exist natively or have been metabolically engineered. Although many alternatives to use VFA from anaerobic fermentation (as suggested in this review) are not currently cost-effective, in the future, the quantification and inclusion of environmental cost or footprint to use petroleum-based products and commodities might provide competitiveness to resource recovery from organic waste. Nevertheless further research is needed to reduce the costs and enhance productivity. Certain other important chemicals of biological origin, including furans, mixed alcohols, long chain sugars (sorbitol, xylitol), solvents (acetone, formaldehyde) and organic acids (glucaric, levulinic, itaconic acids), although not discussed in this review, could also potentially be produced from VFA. However, the understanding of metabolic pathways for such

transformations remains fairly limited and more research efforts are needed to identify the appropriate pathways, platforms and microorganisms.

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