Isolation of Marine Organisms for the Sustainable Production of Squalene

by

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Abstract

The aim of this research was to isolate and characterize marine thraustochytrids to find a strain that could potentially be used to produce squalene in industrial fermentations. To support this goal, a method for laboratory-scale high throughput squalene extraction was developed to aid in screening the large number of isolates discovered in this work. Four sampling trips were conducted in Canada between Quebec, New Brunswick, Nova Scotia and Prince Edward Island. A total of 406 marine isolates were found from these sampling trips, 270 of which were confirmed as thraustochytrids, based on their morphology. Through a series of flask level screening steps, the best strain for squalene production was selected and used for media development. Media factor screening using a Plackett-Burman design produced a maximum squalene content and concentration of 63.94 mg g^{-1} and 665.96 mg L^{-1}, respectively. The three media factors that had the largest positive effect on squalene production were glycerol, sodium chloride and temperature. These results show that there is potential for this strain to be used commercially for squalene production and further improvements could be achieved by focusing on media and growth optimization.

List of Abbreviations and Symbols Used

-	Low
%	Percent
+	High
≤	Less than or equal to
° C	Degree Celsius
µm	Micrometer
Adj	Adjusted
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
ASW	Artificial Seawater
ATP	Adenosine Triphosphate
cm	Centimetre
CoA	Coenzyme-A
DCW	Dry Cell Weight
DF	Degrees of Freedom
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EPA	Eicosapentaenoic acid
et al.	Et alia
FPP	Farnesyl Pyrophosphate
g	Gram
Glu	Glucose
GM	Genetically Modified

Gly	Glycerol
h	Hour
HMG	3-hydroxy-3-methylglutaryl
IMP	Inosine monophosphate
kb	Kilobase
kGY	Kilogray
km	Kilometer
L	Litre
MEP	Non-mevalonate
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimeter
MSG	Monosodium Glutamate
MVA	Mevalonate
NaCl	Sodium Chloride
PB	Plackett-Burman
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
PUFA	Polyunsaturated Fatty Acid
rDNA	Ribosomal Deoxyribonucleic Acid
RPM	Rotations Per Minute
sec	Second
sp	Species
SP	Soy Peptone
SQ	Squalene
T	Thraustochytrid

Terb	Terbinafine
v/v	Volume per Volume
w/v	Weight per Volume
Y	Yeast
YE	Yeast Extract
β	Beta
ϒ	Gamma

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Chapter 1 : Introduction

Squalene holds significant importance in animals and plants, as it is a key intermediate and precursor to all steroid synthesis as well as cholesterol biosynthesis (Kelly, 1999; Xu et al., 2016). Squalene is a strong natural antioxidant agent which is known to protect cells from harmful free radicals and reactive oxidative species via lipid peroxidation of liposomes (Nakazawa et al., 2012). It has been demonstrated to effectively inhibit tumorigenesis in the colon, lung and skin as well as having chemopreventative actions. These and other immune boosting properties make it highly sought after in medical and pharmaceutical industries (Newmark, 1997; Smith, 2000). Squalene is the most prominent component in skin surface polyunsaturated fatty acids and is commonly used in the cosmetic industry for its emollient, antioxidant and hydrating capabilities in various skin care products (Huang et al., 2009). According to Allied Market Research (www.alliedmarketresearch.com), in 2015 the global squalene market was $110 million USD and is expected to reach $214 million USD by 2022. The already existing uses in cosmetic, food and pharmaceutical markets makes squalene a sought-after commodity with biotechnological and industrial potential.

Currently the primary commercial source of squalene is obtained by harvesting the liver of deep-sea sharks. This source is extremely rich in squalene, and typically 40-60 % of the shark liver mass is squalene (Nakazawa et al., 2012). For reasons of commercial development, cost reduction and sustainability, there is a need to develop alternative sources of this high value product. Squalene content in most food products is minimal, however there are some plants that are notable squalene resources. Olive oil is a plant source which contains a high squalene content of 200 – 700 mg 100 g^{-1} in comparison to other agricultural sources. Pumpkin seeds,

quinoa, amaranth and pseudo-cereal grains all contain variable amounts of squalene (between 58 and 400 mg $100g^{-1}$) and have been considered potential sources of dietary squalene (Martirosyan et al*.,* 2007; Ryan et al., 2007). Although these plant sources are relatively high in terms of food sources, they do not provide enough squalene to be a replacement for squalene obtained from shark liver. Additionally, plant sources are subject to seasonal and local variability, leading to inconsistency with respect to harvesting content and biomass (Kaya et al., 2011).

The future and potential use of squalene is suppressed by resource limitations caused by decreasing shark populations and the natural variation present in many plant species. Thus, there has been increasing interest in the cultivation of microalgae and other microorganisms, such as yeasts, as an alternative method of squalene production (Song et al., 2015). Heterotrophic microalgal sources such as thraustochytrids, have been used in industrial fermentations to produce high value lipid products such as docosahexaenoic (DHA), an omega-3 fatty acid, since the 1990s (Burja et al., 2006). Thraustochytrids have the potential of producing more than 80% of their dry cell weight as lipids and more than 50% of their fatty acid profile as DHA (Burja *et al*., 2006; Gupta *et al*., 2016). Studies by Song et al. (2015) and Nakazawa et al. (2014) have explored the use of fungi and thraustochytrids for the production of squalene. In these studies, a variety of geographical locations were used to obtain the strain isolates *Pseudozyma* sp. SD301 (Song et al., 2015)) and Yonez5-1 (Nakazawa et al., 2014), which produced squalene concentrations of 2.45 g L^{-1} and 1.08 g L^{-1}, respectively. Although these strains are not productive enough for commercial development unless further optimized, there is promise in this type of microbial production technology (Nakazawa et al., 2014). Amyris, an innovated and sustainable biotech company, have genetically modified (GM) a strain of yeast which is used to produced squalene for the cosmetics industry. This yeast strain uses fermentation technology to

industrially and sustainably produce squalene (July & Platt, 2015). GM derived products are a point of contention in many European markets however, markets in Canadian, United States and Argentina tend to be more accepting of GM derived products which lead to sustainability (Library of Congress, 2015; Tosun & Schaub, 2017). Large scale heterotrophic microalgal fermentation could potentially provide a sustainable, renewable source of squalene, however more research is needed to make this a commercially viable process.

1.1 Objectives and Hypothesis

The goal of this study was to explore squalene production from marine microorganisms by isolating and screening suitable strains for media development and potential large-scale industrial processing.

1.1.1 Objectives

The specific objectives of this work were to:

1. Isolate microbial species capable of naturally producing squalene from Canadian marine environments;
2. Develop high throughput screening methods to select the best squalene producing strains
3. Select the most suitable strain for industrial development by screening isolates for biomass and squalene production
4. Develop a culture media for the selected strain to promote both biomass and squalene production using Plackett-Burman experimental design

1.1.2 Hypotheses

The following hypotheses are proposed:

- Thraustochytrids that have the ability to produce significant quantities of squalene can be isolated from Canadian marine environments.
- Specialized cultivation media can be developed to promote biomass and squalene production by thraustochytrids during flask fermentations.

Chapter 2 : Literature Review

2.1 Overview of Thraustochytriaceae Family

Thraustochytrids are biflagellate obligate heterotrophs found in a wide array of marine habitats and are known for their ability to produce high amounts of biomass in industrial applications (Burja et al., 2006). They are well-established in the field of single cell oil production for their ability to produce large quantities of long-chain omega-3 fatty acids (e.g. DHA) and have also been exploited for their commercial production capabilities (Fossier et al., 2018). "Thraustochytrid" is a colloquial terminology for the genera within the *Thraustochytriaceae* family. They are commonly, and incorrectly, referred to as microalgae when referring to their biotechnological applications (Armenta & Valentine, 2013; Leyland et al., 2017) however, microalgae refer to a group of photosynthetic algae from which members of the *Thraustochytriaceae* family are derived, and the thraustochytrids lack structures such as chloroplasts that are needed to perform photosynthesis (Cavalier-Smith, 2002).

2.1.1 Phylogeny

Thraustochytrids are classified under the phylum *Heterokonta,* class *Labytinthulomycetes,* order *Labyrithulales* and the family *Thraustochytriaceae* (Dick, 2001; Fossier et al., 2018). Currently ten genera have been identified within the thraustochytrid family based on their life cycle, morphology, ultrastructure and biochemical markers such as fatty acid and carotenoid profiles. The genre includes, *Thraustochytrium* (Sparrow, 1936), *Japonochytrium* (Kobayashi and Ookubo, 1953), *Schizochytrium* (Solomon & Belsky, 1964), *Ulkenia* (Gaertner, 1968), *Aurantiochytrium* (Yokoyama & Honda, 2007), *Sicyoidochytrium, Parietichytrium, Botryochytrium, Oblongichytrium* (Yokoyama et al., 2007), and *Monorhizochytrium* (Doi &

Honda, 2017). All thraustochytrids should share three common phenotypic features: a non-cellulosic cell wall, presence of an ectoplasmic net emerging from the bothrosome or sagenogenetosome, and a biflagellate zoospore (with short posterior and long anterior flaggellum) (Fossier et al., 2018). Additionally, there have also been efforts to classify and identify thraustochytrids based on their polyunsaturated fatty acid (PUFA) profile; however, this strategy does lack resolution with respect to classifying genre (Huang et al., 2003; Nagano, Taoka et al., 2009).

2.2.2 Ecology

Thraustochytrids are eukaryotic, unicellular and heterotrophic obligate marine protists that play an important role in the decomposition of detritus in mangrove forests and other nutrient rich marine ecosystems (Aasen et al., 2016; Singh et al., 2014). Thraustochytrids have been isolated from a vast array of coastal environments ranging from tropical waters in Mediterranean and Arabian waters (Bongiorni & Dini, 2002; Ramaiah, et al., 2005) to colder climates such as Subantarctic waters (Bahnweg & Sparrow, 1974; Caamaño et al., 2017). They are a robust group of organisms found ubiquitously through marine environments and have an absolute sodium requirement that cannot be replaced by potassium salt compounds (Fossier et al., 2018). Typical marine waters have a salt content of 30% - 35% and thraustochytrids tend to grow best within the 20% - 34% range (Raghukumar, 2002). Thraustochytrids are generally known to inhabit the surface of decomposing organic material. They may also be capable of necrotrophic growth on select marine invertebrates (Porter, 1990). As primary decomposers, they play an important role in enriching mangrove and estuarian ecosystems by decaying mainly vegetative litter (Burja et al., 2006).

2.2.3 Life Cycle

Thraustochytrids undergo an asexual life cycle that varies between genres. As discussed, the phylogeny and identification of thraustochytrids has changed over time as they have been increasingly studied. Originally, Sparrow (1973) identified these organisms under the Class *Oomycetes* because they have a life cycle that is reminiscent of zoophoric fungi, which belong to this group. Common among thraustochytrids is the production of ovoid or spherical thalli with rhizoid-like outgrowths (Figure 2.1). The rhizoid-like outgrowths connect to the thallus via the bothrosome or sagenogenetosome (Raghukumar, 2002). *Thraustochytrium* spp. proliferate zoospores internally by direct division of cytoplasmic content, whereas *Schizochytrium* spp. and *Aurantiochytrium* spp. divide by successive bipartitions (Beakes et al., 2015). This successive bipartitioning is a distinct life cycle parameter that differentiates these groups. Proliferation results in a cluster of cells, where each cell gives rise to zoosporangium that will produce zoospores (Beakes et al., 2015; Porter, 1990; Yokoyama & Honda, 2007). The size and shape of the proliferating bodies within a genus varies between 4 – 20 μm in diameter. Figure 2.1 is a simple representation of the two main methods of zoospore production (direct division and bipartition) seen within this group of organisms.

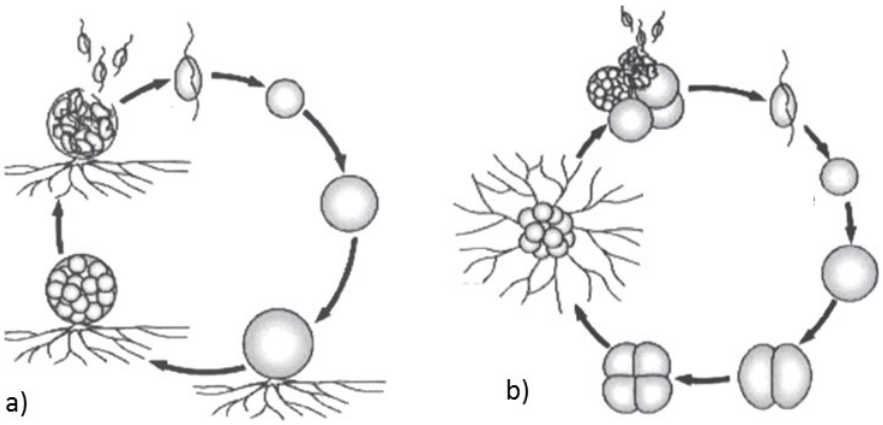

Figure 2.1. Life cycle of a) *Thraustochytrium* spp. and b*) Schizochytrium* spp. and *Aurantiochytrium* spp (Beakes et al., 2015)

2.1.3 High value products derived from Thraustochytrids

Thraustochytrids have been used for the industrial production of docosahexaenoic (DHA), an omega-3 fatty acid, since the 1990s (Aasen et al., 2016). Omega-3 fatty acids are used in health and food industries for their beneficial antioxidant properties related to human health, making them a sought-after high value lipid product. Commonly, fish sources are used for DHA production, however algal alternatives such as thraustochytrids are of high interest as a more sustainable source (Lewis et al., 1999). As previously mentioned, some thraustochytrids have demonstrated the ability to produce more than 80 % of their dry cell weight as lipid and more than 50 % of their fatty acid profile as DHA (Burja et al., 2006; Gupta et al., 2016; Singh et al., 2014). In order to select commercially viable *Thraustochytriaceae,* heterotrophic fermentations are conducted in both research and industrial settings. Research has demonstrated that some thraustochytrid strains can produce a biomass concentration of greater than 100 g/L dry cell weight (DCW) in four days while maintaining a DHA-rich fatty acid profile (Aasen *et al*., 2016).

In the *Thraustochytriaceae* family, *Schizochytrium, Aurantiochytrium* and *Ulkenia* have demonstrated the highest cell densities and lipid productivities, which make them attractive candidates for industrial production. More recently, thraustochytrids have been recognized for their ability to produce other high value lipid-related products such as squalene and carotenoids, which also have established markets in the pharmaceutical, food and animal feed industries (Kelly, 1999; Nakazawa et al., 2012; Song et al., 2015).

2.2 Overview of Squalene

Squalene is a 30 carbon terpenoid compound, which is widely spread throughout nature in both plants and animals and is a key intermediate of cholesterol synthesis. It is produced by the mevalonate pathway (MVA) or the non-mevalonate pathway (MEP) depending on the organism and is an important commodity in the food, pharmaceutical and cosmetic industries (Xu et al., 2016). This section will cover the structure, function, biosynthesis, and sources of squalene, as well as extraction methods.

2.2.1 Squalene structure

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18- 22-hexane) (Figure 2.2) is a polyprenyl polyunsaturated compound containing six isoprene units. As previously mentioned, squalene holds significant importance in animals and plants, as it is a key intermediate and precursor to all steroid synthesis, as well as cholesterol biosynthesis (Aasen et al., 2016; Kelly, 1999).

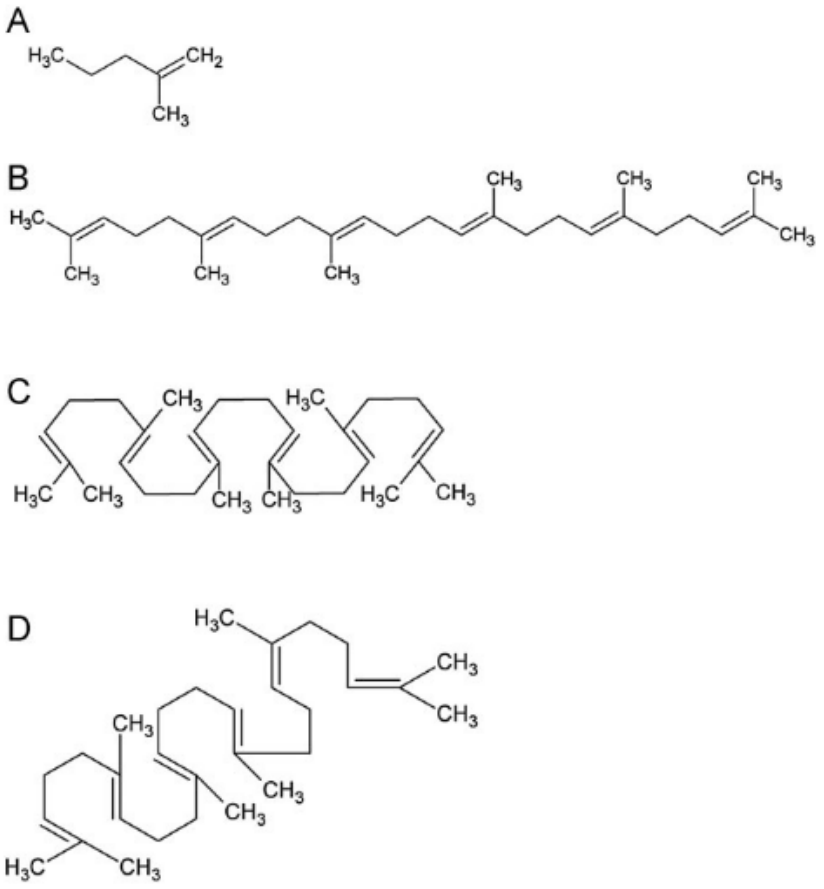

Figure 2.2. Chemical structures of squalene: a) single isoprene unit, b) stretched form, c) coiled form, and d) steroid-like form (Spanova & Daum, 2011).

Terpenes are classified according to the number of isoprene units and carbon atoms that are present. Squalene is a triterpene since it contains 6 isoprene units and 30 carbons. Terpenes are a dynamic group of compounds that may be lipophilic or hydrophilic, volatile or non-volatile, cyclic or acyclic and are capable of many bioactive properties (Reddy & Couvreur, 2009). Many vitamins and carotenoids are also classified as terpenes, such as β-carotene and lutein. These types of compounds are common intermediates in the biosynthesis of bioactive compounds in plants and animals (Xie et al., 2017)

2.2.2 Squalene synthesis and regulation

The synthesis of squalene (Figure 2.3) is initialized by a condensation reaction catalyzed by the enzyme HMG-CoA synthase to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG CoA is then reduced to produce mevalonate by the reductive action of the enzyme HMG-CoA reductase. Next isopentenyl pyrophosphate is formed by two sequential phosphorylation reactions followed by a decarboxylation reaction. Isopentenyl pyrophosphate is the first intermediate product in the pathway involved in making isoprenoids. Isopentenyl pyrophosphate is then catalyzed by farnesyl-pyrophosphate to give rise to the 15-carbon farnesyl pyrophosphate (FPP) involved in squalene formation. Two molecules of FPP are attached via a niacin dependent reduction reaction via squalene synthase (SQS) to produce squalene (Brown & Sharpe 2015; Kelly, 1999; Xie et al., 2017).

In microalgae, the production of carotenoids such as β-carotene can also be synthesized from FPP (Xie et al., 2017). Nakazawa et al. (2014) studied 176 isolated thraustochytrid strains obtained from various marine environments. For *Aurantiochytrium* spp*.,* a high correlation was found between orange colonies and increased squalene content by both qualitative and quantitative methods. These results suggest that a relationship exists between the metabolic synthesis of squalene and FPP derived carotenoids, and that it influences the color of thraustochytrid colonies, as discussed by Furubayashi et al. (2014). Furubayashi et al. 2014 hypothesize that colony color may be an indicator of high squalene production, and therefore potentially helpful in strain isolation and selection.

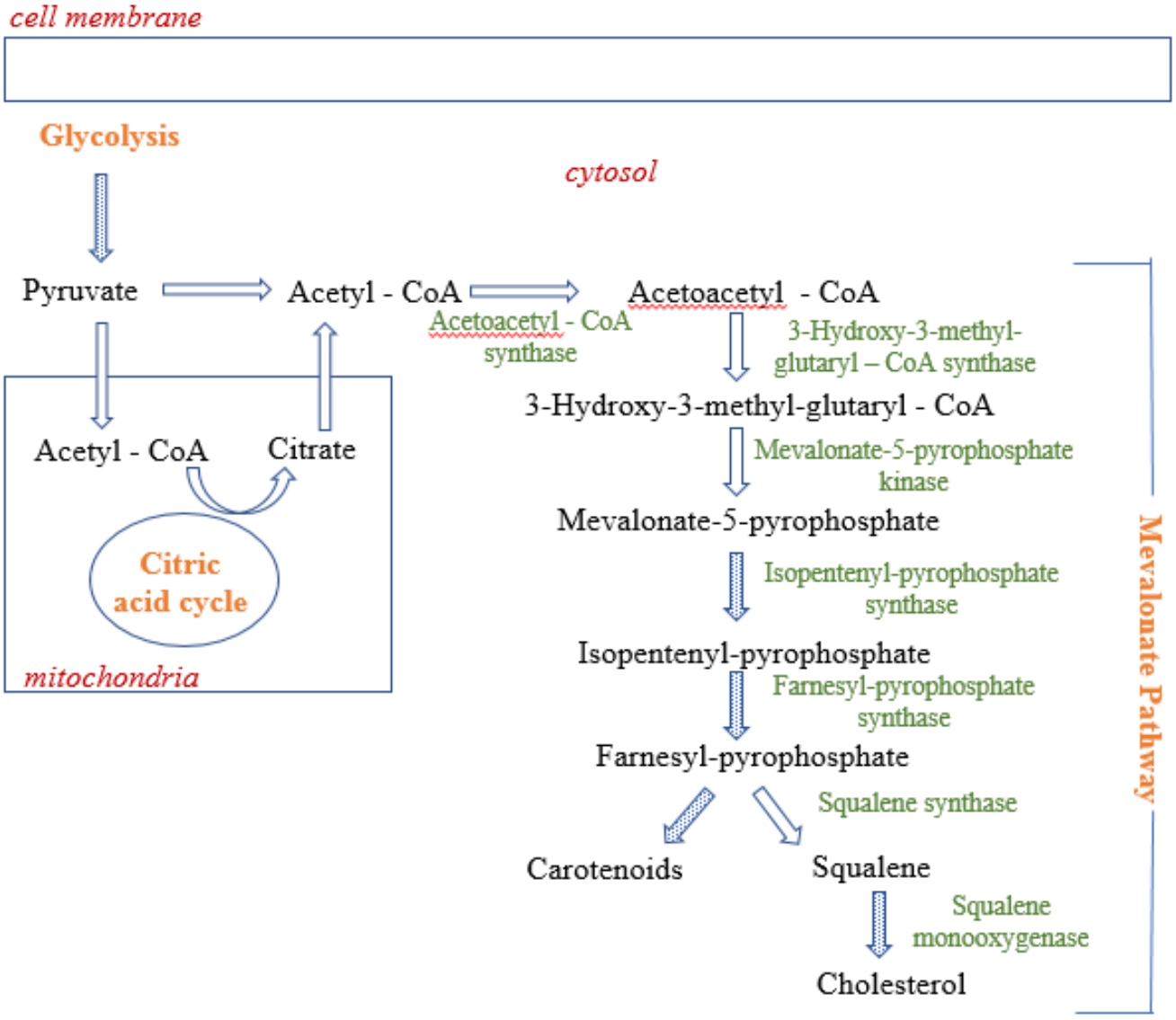

Figure 2.3. Squalene synthesis via the MVA pathway beginning with acetyl-CoA derived from either glycolysis or the citric acid cycle. Dotted arrows indicate that there has been an abbreviation in steps between the biological intermediates.

Regulatory factors such as carbon to nitrogen ratio, oxidative stress and inhibitory compounds can impact the MVA pathway and can therefore be used to manipulate its products (Xie et al., 2017). For example, a higher carbon to nitrogen ratio has a positive impact on the accumulation of terpenoids, as well as fatty acids such as DHA (Aki et al., 2003). It has been suggested that depriving thraustochytrid cells of nitrogen can lead to the accumulation of citrate

in the mitochondria. This accumulation of citrate is a result of adenosine monophosphate (AMP) consumption to produce inosine monophosphate (IMP), also referred to as AMP deaminase. It is possible that the citrate is then exported into the cytosol and converted into acetyl-CoA by ATP citrate lyase which could lead to greater activity of the MVA pathway (Lee Chang et al., 2013, 2014; Xie et al., 2017). Additionally, the type of carbon source would also have an impact on the synthesis and accumulation of squalene and carotenoids through this pathway (Aki et al., 2003).

Oxidative stress has also been shown to increase the production of DHA through the polyketide synthase pathway (PKS) and terpenoids in the MVA pathway by reducing respiratory strength (Jain et al., 2007; Xie et al., 2017). The PKS and MVA pathway share the same initiating building block (acetyl-CoA) and therefore strategies used to manipulate one can often be applied to the other. Inhibitory compounds such as the antifungal terbinafine hydrochloride ((E)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthylmethylamine hydrochloride) and methyl jasmonate have been studied for their ability to inhibit squalene monooxygenase, which is vital in the conversion of squalene to other sterol products (Fan et al., 2010; Yue & Jiang, 2009). The concept behind using these compounds is that inhibiting metabolic products which occur after squalene production would therefore promote the accumulation of squalene in the cell. Additionally, squalene has been shown to accumulate in exponential phase of thraustochytrid growth cycles and then begins to decrease after this growth stage. This is likely due to the conversion of squalene into other MEP or MVA metabolites (Chen et al., 2010; Li et al., 2009a). Figure 2.4 shows the point (exponential growth phase) within the accumulation of biomass and depletion of glucose in which squalene typically reaches peak productivity.

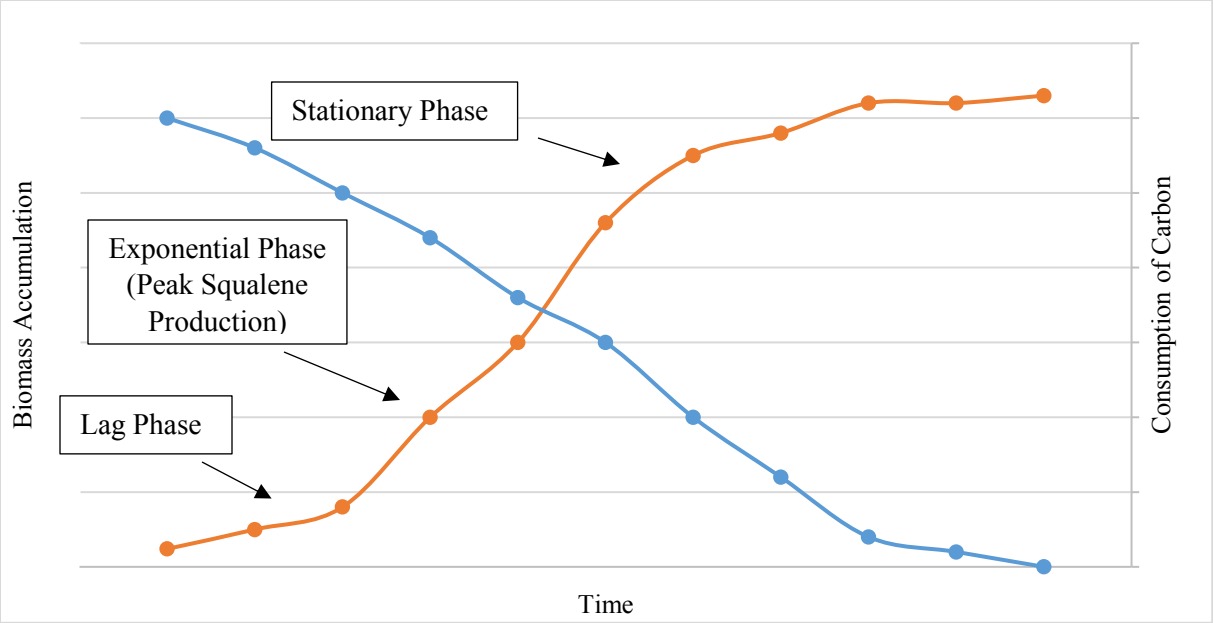

Figure 2.4. Typical growth curve for thraustochytrids including the accumulation of biomass (orange line) and depletion of a carbon source (blue line).

2.2.3 Microbial sources of squalene

As previously discussed, the major source of squalene for industrial use is from the liver of deep-sea sharks. Plant sources have been studied as potential alternatives for squalene production, but due to low productivity and inconsistencies in crop characteristics, plant alternatives are not suitable long term solutions. Microbial sources of squalene have been successfully implemented for industrial DHA production and are therefore microbial sources should be a consideration for a potential solution for sustainable squalene production (Burja et al., 2006). In recent decades, microorganisms, including bacteria, yeast, fungi and algae, have been used in a variety of industries for the production of proteins, bioinsecticides, biofertilizers, nutritional oils and biofuels (Thangadurai & Sangeetha, 2014). Typical squalene contents for yeasts and thraustochytrids have been reported as 0.43-340 mg L^{-1} dry cell weight (DCW) and 0.72 mg L^{-1} DCW respectively (Song et al., 2015). These values exclude strains which produce

exceptional amounts of squalene that show commercial potential. Table 2.1 shows a list of strains including both yeasts and thraustochytrids that have been studied for their ability to produce squalene. These values are not productive enough for commercialization, however, there is potential for the discovery of new strains that are inherently capable of producing sufficient amounts of squalene for this purpose. For example, in a recent study by Song et al. (2015), a yeast strain identified as *Pseudozyma* sp. SD301 was found to produce 2.445 g L^{-1} of squalene, which is close to double the previously highest recorded squalene yield by *Aurantiochytrium* sp. 18W-13a (Kaya *et al*., 2011; Song *et al*., 2015). The yield for strain Pseudozyma sp. SD301 was obtain in a fermentation study by which higher amounts of glucose were supplied to the culture (60 g L^{-1} compared to 20 g L^{-1}).

It is important to note that the SD301 yeast strain obtained its yield via a bioreactor fermentation, whereas the 18W-13a strain was cultured in a flask. These different scales in fermentation could lead to discrepancies in the values and are therefore not easily compared. However, the high squalene yields from 18W-13a and SD301 support the notion that microorganisms, especially thraustochytrids and thraustochytrid-like strains, should be researched as potential industrial squalene producers under fermentative conditions. In addition, thraustochytrids have a demonstrated a propensity to produce dense biomass containing high lipid content when grown in various types of liquid cultures (Burja et al., 2006; Gupta et al., 2013).

Table 2.1 Squalene yield and productivity from different microbial sources.

Strain	Squalene yield (g L^{-1})	Squalene productivity (g L^{-1} h^{-1})	Growth Vessel	Carbon source	Reference
Aurantiochytrium sp. 18W-13a	1.29	0.014	Flask	Glucose (20g L^{-1})	Kaya *et al*., 2011
Aurantiochytrium sp. 18W-13a	0.9	0.0125	Flask	Glucose (20g L^{-1})	Nakazawa *et al*., 2012
Aurantiochytrium sp. 18W-13a	0.86	-	Flask	Glucose (20g L^{-1})	Nakazawa *et al*., 2014
Aurantiochytrium sp. AR-4a	0.77	-	Flask	Glucose (20g L^{-1})	Nakazawa *et al*., 2014
Aurantiochytrium sp. Yonez5-1	1.079	0.011	Flask	Glucose (20g L^{-1})	Nakazawa *et al*., 2014
Schizochytrium mangrovei PQ6	1.019	0.011	Batch Fermentation	Glucose (60g L^{-1})	Hoang *et al*., 2014
Pseudozyma sp. JCC207	0.341	0.004	Flask	-	Chang *et al*., 2008
Pseudozyma sp. SD301	0.82	-	Flask	Glucose (30g L^{-1})	Song *et al*., 2015
Pseudozyma sp. SD301	1.65	0.039	Batch Fermentation	Glucose (60g L^{-1})	Song *et al*., 2015
Pseudozyma sp. SD301	2.445	0.031	Fed-Batch Fermentation	Glucose (140 g L^{-1})	Song *et al*., 2015

2.2.4 Squalene Extraction Methods

The process of extracting squalene from biomass involves disruptions of the cell wall to release cellular contents which includes the squalene-containing lipid fraction. Extraction of the lipid phase from thraustochytrids has been performed in many studies, including those conducted by Jiang et al. (2004), Burja et al. (2006), Fan et al. (2007), Aasen et al. (2016) and Lowrey et al. (2016). Commonly the Bligh and Dyer or the Folch method are used for total lipid extraction and require water, chloroform and methanol as their primary solvents (Kumar et al., 2015). The extraction of squalene from dried thraustochytrid cells has been performed successfully using

methods like these. For example, Nakazawa et al. (2014) used chloroform and methanol in a modified Folch protocol, whereas Jiang et al. (2014) used the original Bligh and Dyer method to extract squalene. Additionally, a combination of KOH and ethanol have also been used in the extraction of squalene from thraustochytrids cells (Li et al., 2009a).

2.3 Approach for Isolation of Commercially Viable Thraustochytrid Strains

Bioprospecting to discover new thraustochytrid strains has been successfully conducted by several research groups (Bowles, 1999; Burja et al., 2006; Gupta, 2013; Song et al., 2015). When seeking new strains, a suitable location must first be selected. Then, isolation techniques are used to collect microorganisms from the environment and the strains are evaluated for commercial viability. In this section, the characteristics of locations from which thraustochytrids have been isolated will be described. Following this, common isolation techniques used for thraustochytrids are outlined: baiting, direct plating and filtration. Finally, the bio-rational approach that is used for isolating commercially viable strains is discussed.

2.3.1 Location

Thraustochytrids occur ubiquitously throughout marine environments, where conditions can vary with respect to salinity, pH and temperature. They have been found in marine estuary and mangrove environments, where there is plentiful organic material necessary for heterotrophic growth. Thraustochytrids tend to concentrate on decaying (but not fully decomposed) plant matter, marine invertebrates, aggregated phytoplankton and water columns (Li et al., 2009; Raghukumar et al., 2000). Although fully decomposed organic matter, such as fallen trees and leaves, are not ideal for thraustochytrid isolation due to competition from yeast and mold species (Porter, 1990), this competition could reap positive benefits when looking for a

viable strain for industrial fermentation. Indeed, Barclay (1994) suggests that microorganisms that are capable of out-competing other species in their natural environment are more robust strains, which makes them more suitable for commercial scale up.

2.3.2 Common Isolation Techniques

2.3.2.1 Pollen Baiting

Baiting techniques for the isolation of thraustochytrids use chemoattractants from baiting substrates to attract thraustochytrid zoospores (Garrity, 1996). Baiting, as a means of isolating thraustochytrids, was first described by Gaertner (1968) and is one of the most common methods used in the field. Pollen from the genus *Pinus* (pine pollen grains) was Gaertner's preferred bait of choice and remains the most commonly used bait amongst researchers (Burja et al., 2006; Fan et al, 2002; Gupta et al, 2013; Li et al., 2009). Examples of other kinds of bait include onion skin, apple skin, bleached grass leaves, corn leaves, cellophane, shrimp and insect exoskeleton, snake skin and *Sorghum* sp. seed (Bremer, 2000a; Gupta et al., 2013; Porter, 1990). As pine pollen is the most widely used baiting material, further discussion of this technique will focus on pine pollen grains.

The first step in the baiting technique is to sterilize the bait. Pollen grains are sterilized before being used to inoculate either vegetative (plant materials) or water samples, to reduce the introduction of non-thraustochytrid species. Sterilization using dry heat, at temperatures between 90^{o}C an d 100 ^{o}C, was suggested by Raghukumar et al. (2000), whereas Bowles (1999) used γ-irradiation at a 246 kGy dosage as originally outlined by Gaerter (1968). A lower dosage of 25 kGy γ-irradiation has also been suggested by Bremer (2000b) as well as fumigation with propylene dioxide.

Once sterilized, pine pollen grains are dusted into the prepared sample suspension, at a recommended concentration of 25-30 mg of pollen grain per 20 mL of sample suspension media (Gupta et al., 2016). Where the vegetative samples would be washed with sterile sea water before being placed in a baiting vial, and the water samples would be added directly to the vial (Bremer, 2000a; Porter, 1990.). The sample may also be treated with antibiotics such as penicillin, streptomycin sulfate, ampicillin, rifampicin, and/or nystatin to discourage bacterial growth (Gupta *et al*., 2013; Taoka *et al*., 2010; Yang *et al*., 2010). Amphotericin B can also act as both an antifungal and antibiotic agent in this application (Taoka et al., 2010). Ideally, baited samples are incubated at the original isolation temperature (18 ^{o}C-25 ^{o}C), however, incubation generally occurs at room temperature.

After 48 hours of incubation, the pollen grains should be examined for the presence of thraustochytrid thalli (Bremer, 2000); however, other researchers suggest a longer incubation period of 8 – 10 days to allow for colony formation (Gupta et al., 2016) and prevent bacteria, yeast and fungi from taking over the culture. During incubation, the spherical thraustochytrid zoospores, will attach between two large air sacs on the pollen grain. Pollen grains, with visually identifiable thraustochytrid zoospores attached, are plated onto agar plates containing similar selective agents as the isolation incubation media (Bremer, 2000). After growth for 7-10 days, the colonies formed on the plates should be observed for thraustochytrid-like properties and picked for further purification on selective media plates (Gupta et al., 2016; Gupta et al., 2013).

2.3.2.2 Direct Plating

The direct plating technique can be used in combination with pollen baiting or as a stand-alone method. This technique involves directly spreading sediment, solid organic matter or sea water samples onto a selective media plate containing antibiotic and antifungal agents (Fan *et al*.,

2002; Gupta *et al*., 2013; Liu, 2014; Yang *et al*., 2010). Although this method can be used with several types of samples, solid samples from plant and animal tissue are preferred. Solid tissue, commonly from leaf samples, is cut into small discs and washed with sterilized sea water to reduce bacteria, mold and fungi colonies. The cut size of samples has not been consistent between various studies. For example, sizes of 1.5 cm^2, 5 mm^2, 1.0 cm^2 and 0.5 cm^2 have been reported by Fan *et al.,* (2002), Raghukumar (1995), Porter (1990) and Bremer (2002), respectively. In addition, each of the mentioned studies offers its own method of sample plating. For example, Fan *et al* (2002) performed three washing steps before using the leaf sample to inoculate the selective media plate. Bremer (2000) washed once using a flask at 200 RPM for two to three hours and also suggested an additional washing step and further reduction of the 0.5 cm^2 disk. Porter (1990) omitted the washing step and instead used a smearing technique, where the sample was directly spread across the agar plate. Although washing steps will greatly reduce the presence of non-thraustochytrid species on the samples, there is also the increased risk of removing thraustochytrids by washing. Water samples can be challenging for direct plating, since the concentration of thraustochytrids in water samples is much lower than for solid samples. Sediment samples can be directly plated using the same smearing technique described above by Porter (1990).

After plating, disk samples from plant or animal tissue are overlaid with sterile seawater to encourage zoospore release. This will increase the amount of thraustochytrid thalli on the plate, thereby increasing the probability of isolation (Bremer, 1995). During incubation, the same considerations regarding temperature that were outlined in the baiting technique should be used (Bowles et al., 1999; Bremer, 2000b) and the plates should be observed under a microscope every 24 hours for the formation of thalli. Identified thraustochytrid strains would then undergo

several sub-culturing steps to purify the sample, until axenic cultures can be stored on agar slants (Fan *et al*., 2002).

2.3.2.3 Filtration

Filtration is a useful technique for isolating thraustochytrids from water samples. Through filtration, thraustochytrids in a sample are concentrated, thus increasing the chance of isolating thraustochytrids from originally dilute samples. Filtration methods have been described by Barclay et al. (1994), Bremer (2000) and Honda et al. (1998). The filtration process described by Bremer (2000) involves a one-step filtration through a 0.4 µm membrane filter. This allows for a decrease in the number of bacteria present in the water sample while leaving the larger microflora, including thraustochytrids, to remain. However, the technique can also concentrate competitive microflora within the same size range as the thraustochytrids. Thus, there is an increased chance of contamination from yeast, mold and protozoa.

Barclay et al. (1994) describes a rapid sandwich method for filtration, involving the use of two different filter sizes to address the contamination issues that can arise from a one-step filtration approach. The first filter is a 20-25 µm filter which removes particulates greater than 25 µm, therefore reducing the incidence of molds but allowing thraustochytrids and other unicellular organisms to pass through. This is beneficial since molds are concentrated in marine coastal environments and can dominate a plate if not removed or suppressed. The filtered sample is then passed through the second 1.0 µm polycarbonate filter, which allows many bacteria to continue through with the filtrate. The organisms corresponding to the size range appropriate for thraustochytrids are retained on the 1.0 µm filter and can be plated on selective media containing similar antibiotics and antifungal agents listed in section 2.3.2.1. This two-step filtration method allows the concentration of microflora within the 1.0 µm- 20 µm cell size range.

2.3.3 Bio-rational approach for industrial strain selection

The methods and techniques that are used for bioprospecting and isolating wild thraustochytrids have been outlined in this section. However, in order to be successful in isolating a strain with commercial viability, a bio-rational approach should be used. A bio-rational approach involves identifying the desirable characteristics that a strain should ideally possess in order to produce high levels of target compounds (e.g. DHA, EPA, carotenoids, squalene), as well as being able to thrive under typical industrial operating conditions (Barclay et al., 2010). This knowledge aids the bioprospecting strategy, as it can influence the selection of the location chosen for collecting samples from which to isolate thraustochytrids. For example, thraustochytrids are saprotrophic organisms that obtain their nutrients by absorbing dissolved organic matter and are commonly found in areas with detritus, decomposing plant material and in sediment, so locations with estuaries, marine tidal pools, inland saline lakes, playas and springs should be sampled (Barclay *et al*., 2010; Burja *et al*., 2006). Organisms found in these areas are subject to changing tidal temperatures, salinity and a variety of carbon sources, and would be likely to possess desirable traits for industrial production, as outlined in Table 2.2. Once an isolate that possesses these desirable traits is found, it would ideally be capable of producing the target product at a commercially viable level.

The bio-rational approach can also be used to determine which strains can grow under process-relevant conditions and to eliminate any strains that are not suitable. As shown in Table 2.2, certain fermentation process parameters can influence the selection of preferred traits for an ideal industrial strain, using a bio-rational approach. As heterotrophic fermentation is typically performed in conventional stainless steel bioreactors, it is important to select organisms that can

thrive in conditions that are operationally advantageous, allow the process to run at a lower cost, and can remain biologically stable.

Table 2.2. Targeted process parameters and corresponding rationale for isolating an industrially suitable thraustochytrid strain using a bio-rational approach

Targeted process parameter	**Bio-rationale for selection of industrially suitable strain**
Temperature	Organism should tolerate elevated temperatures, as greater productivity will generate more heat from exothermic reaction, therefore reducing the need for cooling (Barclay *et al.*, 2010).
pH	Organism should grow at a pH of less than 7 to reduce the risk of bacterial contamination (Montville & Matthews, 2007)
Salinity	Organism should thrive at low salt concentrations to reduce corrosion to the fermenter
Cell type	Organism should be unicellular and ≤ 25 nm to reduce energy needed for mixing with an impeller (Barclay *et al*., 2010)
Carbon Source	Organism should be able to maintain a high productivity while consuming an inexpensive carbon source (Barclay *et al*., 2010)

Chapter 3 : Methods and Materials

3.1 Schematic of methods development

Figure 3.1 represents the project schematic which includes a comprehensive view of the flow of experiments as well as method and process developments. The project flow includes stages in which method development took place in terms of squalene extraction and media selection for secondary and tertiary screening. Also included are the selection criteria evaluated after primary, secondary and tertiary strain screening steps.

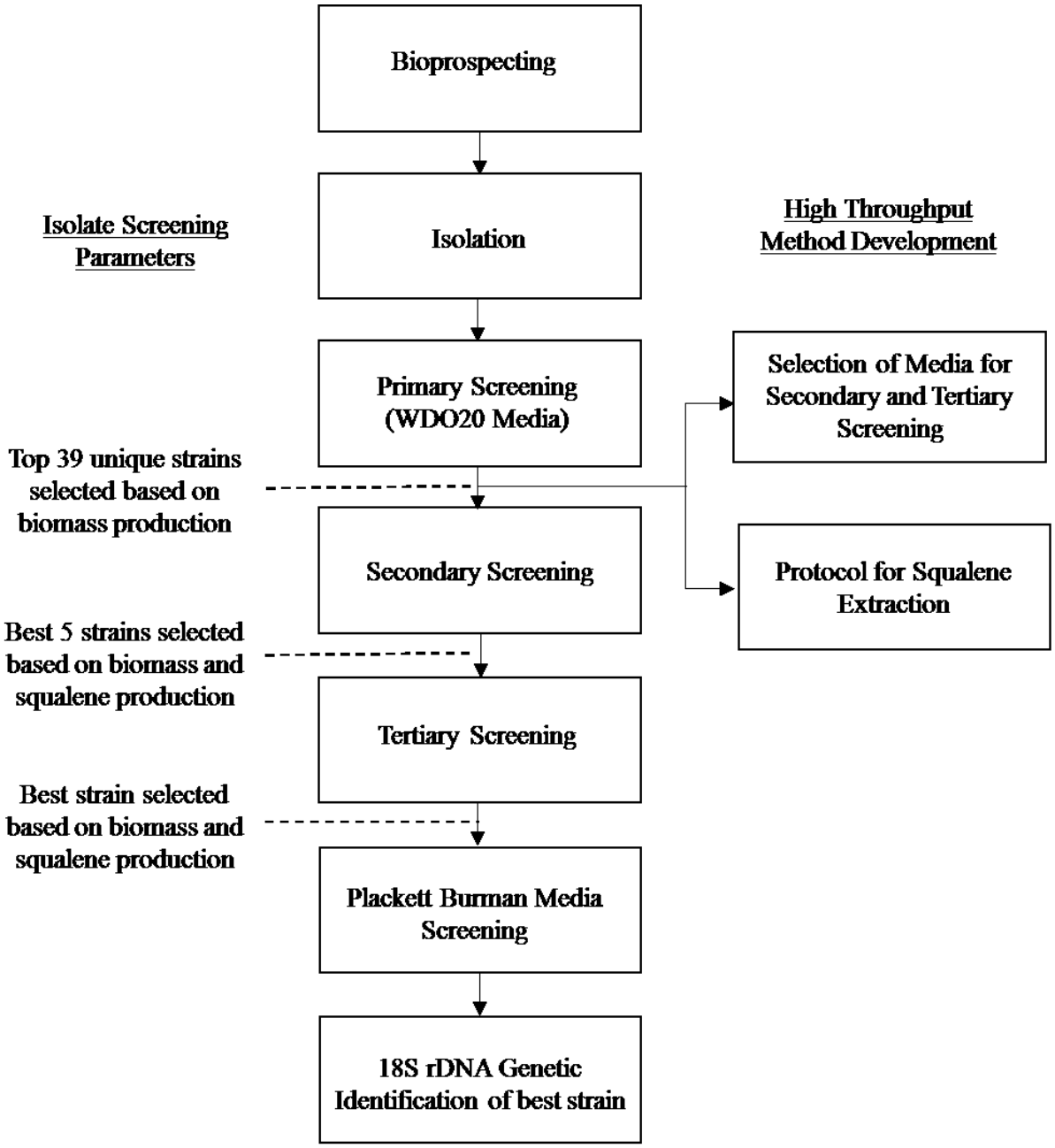

Figure 3.1.Flow Diagram of Experimental Work for Screening and Isolate Selection.

3.2 Strain collection locations

All strain collection, isolation and primary growth procedures outlined in sections 3.2 – 3.5 were performed along side the strain discovery team a Mara Renewables Corp. who included Dr. Jeremy Benjamin and Kaitlyn Tanner. A total of four sampling collections were completed

for this project. The first collection designated as the MA collection included 3 unique sampling sites from Lawrencetown Beach, Nova Scotia (*Figure 3.2*) in July 2018. The second collection trip, the MB collection (*Figure 3.3*), spanned across the eastern region of Canada, beginning in Quebec and ending in Nova Scotia from July 21–23, 2018. A total of 26 unique sampling sites were sampled from coastal regions over approximately 1000 km distance. The third collection, MC collection, was on the eastern shore of Nova Scotia from an inlet in East Petpeswick (*Figure 3.4*) on July 31, 2018. A total of 36 unique sampling sites were visited over an 8 km distance. The final collection labeled the MD collection included 48 sampling sites from the coastline of Prince Edward Island (*Figure 3.5*) covering approximately 1000 km and samples taken every 20 km. The MD collection was completed between September 19 – 21, 2018.

Figure 3.2. MA collection sites (each black dot represents one sample site) located in Lawrencetown Beach, Nova Scotia, Canada

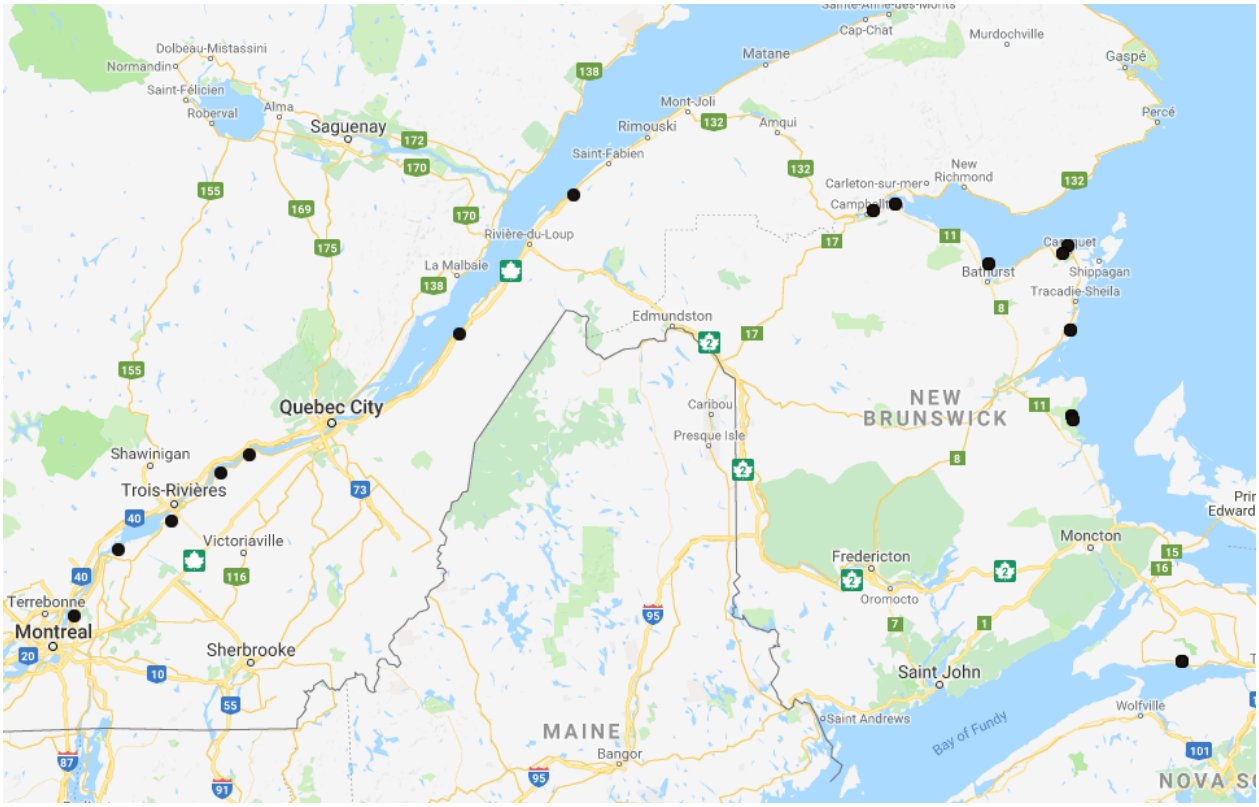

Figure 3.3. MB collection sites spanning eastern Canada (Quebec, New Brunswick and Nova Scotia).

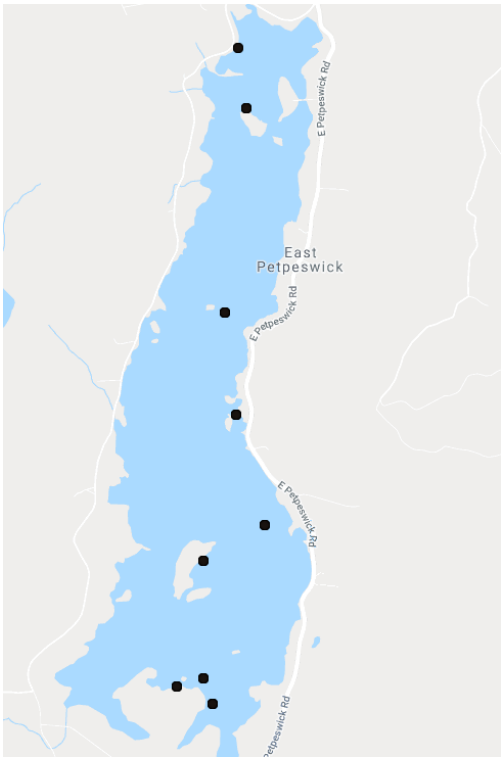

Figure 3.4. MC collection sites (four unique samples where obtained from each site) located in East Petpeswick, Nova Scotia, Canada.

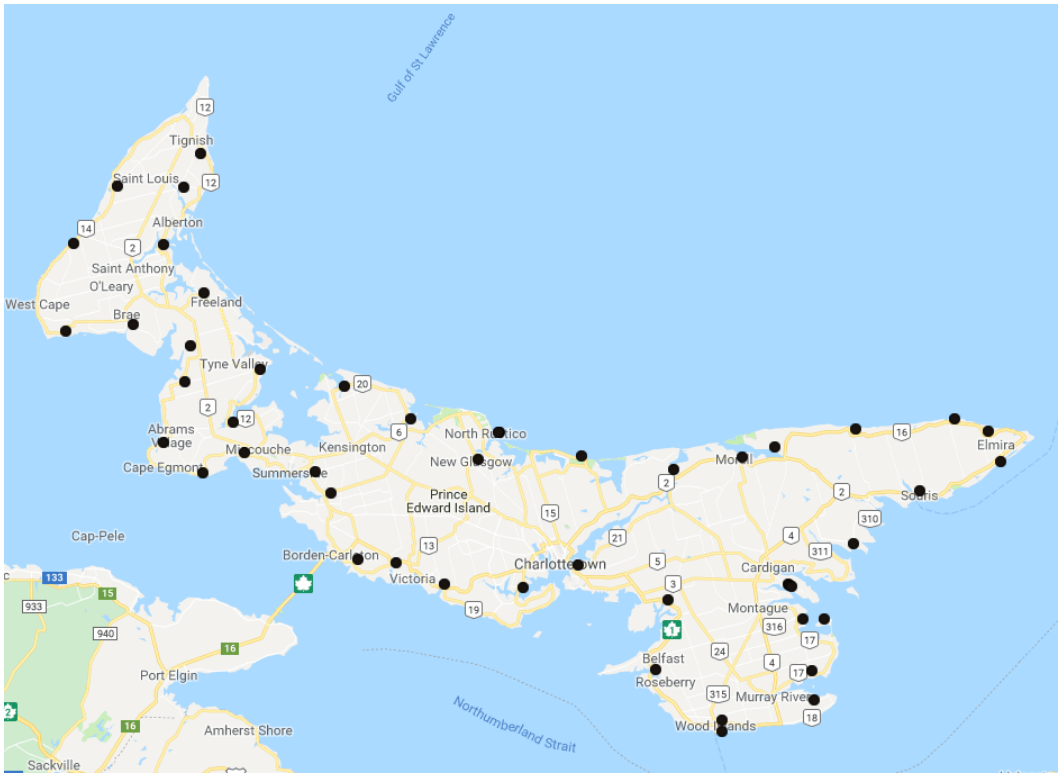

Figure 3.5. MD collection sites covering the coast line of Prince Edward Island, Canada.

3.3 Sample collection procedure

At each sampling site, two samples of *Spartina alterniflora* (a type of cordgrass) were identified, where the first sample would be fresh with little to no decay and the second sample would have partial decay. Both the fresh and the decayed samples were collected into clean plastic sealable bags with a small amount of water from the sample area added to the bag to keep the sample moist. The sample bags were immediately stored on ice and transported to the laboratory within 24 - 72 h. Once in the laboratory the samples were refrigerated at 4°C until isolation procedures commenced.

3.4 Strain Isolation Procedure – Pollen Baiting

3.4.1 Sample preparation

Prior to isolation the samples were retrieved from 4°C and were washed using a solution of 30 g L^{-1} artificial seawater salts (ASW, InstantOcean®, Spectrum Brands Inc., Blacksburg, Virgina, United States) containing 1% (v/v) penicillin G and streptomycin solution (PS) which was made up of 10,000 units penicillin G and 10 mg streptomycin per mL in 0.9% (w/v) (Sigma-Aldrich, Oakville, Ontario, Canada). The ASW was sterilized at 121°C for 20 minutes in an autoclave. Individual samples were then cut using sterilized scissors to roughly 0.5 cm^2 pieces and transferred into baiting (scintillation) vials.

3.4.2 Baiting samples

Samples were placed into 50 mL scintillation vials containing 10 mL of 30 g L^{-1} ASW and left to set for a minute to allow the sample to sink to the bottom of the vial. Next pre-sterilized pollen grains were dusted over the surface of the ASW, so they were just visible to the naked eye. The vials were then capped and stored in darkness for 24 – 72 hours at room temperature without agitation. The contents of the vials were observed daily using a Nikon Eclipse E600 microscope at a 10x and 40x magnification (Nikon, New York, U.S.A) set to brightfield illumination to check for thraustochytrid colonization on the pollen grains.

3.4.3 Plating and strain microscopic isolate identification

Once colonization was observed via microscopy, a sterile loop was used to dilution streak onto WDO plates to allow for picking of individual colonies. The WDO plates contained 2 g L^{-1} yeast extract (Leiber Yeast, Monrovia, California, USA), 2 g L^{-1} soy peptone (HiMedia, West Chester, Pennsylvania, USA), 20 g L^{-1} ASW salts and 15 g L^{-1} agar (Sigma-Aldrich, Oakville, Ontario, Canada) and brought to volume using distilled water. The plates were left covered on the bench top for 24 – 96 h until thraustochytrid colonies formed. Plates were observed daily for fungal contamination and aseptically cut from the WDO plate when necessary. Colonies were observed using an AmScope LED dissection light microscope (AmScope Inc., Irvine, California, USA) and positive identification of thraustochytrid colonies was based on the appearance of typical morphologies observed with Thraustochytrid T18 (available from the strain library at Mara Renewables, Canada, Dartmouth, Nova Scotia), which was used as a control strain for comparison. Individual colonies were picked from the plates using sterile flat-edged tooth picks.

The tooth picks were then used to streak the colonies onto new WDO plates. This colony picking and streaking procedure was carried 3 – 4 times until axenic colonies could be confirmed.

3.4.4 Cryopreservation of axenic isolates

Axenic isolates were patched on WDO plates and maintained on these types of plates via weekly re-patching until they could be grown in liquid media and cryopreserved. These patched plates were used to inoculate 50 mL flat bottom flasks (Fisher Scientific, Canada, Ottawa, Ontario) containing 10 mL of WDO5 media which was made up of 5 g L^{-1} glucose 2 g L^{-1} yeast extract, 2 g L^{-1} soy peptone, 20 g L^{-1} ASW. Cultures were grown at 25°C and 200 RPM in a New Brunswick Scientific Innova 44 incubator (Eppendorf, Mississauga, Ontario, Canada) for 72 h before harvesting. An aliquot of harvested cells was then mixed with 200 µL of 50 % reagent grade glycerol (Sigma-Aldrich, Oakville, Ontario, Canada) to 800 µL of homogeneous culture. A total of 9 cryopreserved glycerol stocks per isolate was mixed in these proportions and frozen at -80°C in Nalgene® cryogenic vials in 1 mL volumes (Sigma-Aldrich, Oakville, Ontario, Canada).

3.5 Primary flask screening of isolates

Cryopreserved strains were retrieved from -80°C and placed on the bench top to reach room temperature. The entire contents of each tube were inoculated into 250 mL flat bottom Pyrex Erlenmeyer flasks (Fisher Scientific, Canada, Ottawa, Ontario) containing 60 mL of WDO5. Cultures were grown at 25 °C and 200 RPM in a shake flask incubator for 96 h. Each flask was checked using 100x magnification to ensure the culture remained axenic and if so, 50 mL of culture was transferred into a pre-weighed 50 mL centrifugation tube. Cultures were centrifuged at 3750 x g at 4 °C for 20 min (Sorvall Legend RT+, Thermo Scientific, Asheville, North Carolina, USA). The supernatant was discarded, and 10 mL of distilled water was added to

each tube to wash the pellets. The tubes were then vortexed for 10 – 15 sec to allow a homogenous solution to form. Tubes were re-centrifuged at 3750 x g at 4°C for 20 min. The supernatant was discarded, and the tubes were capped with foil and placed at -80°C for a minimum of 4 h. Frozen samples were removed from the freezer and lyophilized (FreeZone18, Labconco, Kansas City, Missouri, USA) for 48 – 72 h. The washed and lyophilized samples were then weighed to determine biomass concentration.

To determine the strains that would be most robust at higher glucose concentrations, selected isolates that had shown high biomass concentrations when grown in WDO5 media were then grown under the same conditions using WDO20. This media is identical to WDO5 except for its glucose concentration of 20 g L^{-1}. The results from the WDO20 media were used to determine the strains that would be evaluated further. The selection was based on biomass concentration, sampling site as well as colony morphology on WDO plates.

3.6 Development of squalene production media for high throughput screening

A known squalene producing strain, *Aurantiochytrium tsukuba-3,* was grown in four different thraustochytrid culture media to determine the most appropriate medium to culture the isolated strains in for secondary screening. This strain was acquired from the International Patent Organism Depositary in Tokyo, Japan to act as a control strain for all subsequent experiments. The four media for this experiment were chosen because thraustochytrids are known to thrive under these media conditions (Burja et al., 2006; Kaya et al., 2011; Nakazawa et al., 2014). *Aurantiochytrium tsukuba-3* was grown in WDL20 media (20 g L^{-1} glucose (Sigma-Aldrich), 5 g L^{-1} yeast extract , 9 g L^{-1} sodium chloride, 0.03 mg L^{-1} B_{12} (Sigma-Aldrich), 6 g L^{-1} thiamine hydrochloride (Sigma-Aldrich), 0.03 mg L^{-1} ferric chloride (Sigma-Aldrich), 0.25 g L^{-1} calcium chloride (Sigma-Aldrich), 3.0 mg mL^{-1} copper (II) sulfate pentahydrate (Sigma-Aldrich), 1.5 mg

mL^{-1} sodium molybdate (Sigma-Aldrich), 3.0 mg mL^{-1} zinc sulfate (Sigma-Aldrich), 1.5 mg mL^{-1} cobalt (II) chloride (Sigma-Aldrich), 3.0 mg mL^{-1} manganese chloride (Sigma Aldrich) and 1.5 mg mL^{-1} nickel sulfate (Sigma-Aldrich), 6.81 g L^{-1} ammonium sulfate (Fisher Scientific, Ottawa, Ontario, Canada), 1.59 g L^{-1} potassium sulphate monobasic (Fisher Scientific), 1.74 g L^{-1} potassium sulphate dibasic (Fisher Scientific)),WDL60 media (20 g L^{-1} glucose, 5 g L^{-1} yeast extract, 9 g L^{-1} sodium chloride, 0.03 mg L^{-1} B_{12}, 6 g L^{-1} thiamine hydrochloride, 0.03 mg L^{-1} ferric chloride, 0.25 g L^{-1} calcium chloride, 3.0 mg mL^{-1} copper (II) sulfate pentahydrate, 1.5 mg mL^{-1} sodium molybdate, 3.0 mg mL^{-1} zinc sulfate , 1.5 mg mL^{-1} cobalt (II) chloride, 3.0 mg mL^{-1} manganese chloride and 1.5 mg mL^{-1} nickel sulfate, 6.81 g L^{-1} ammonium sulfate, 1.59 g L^{-1} potassium sulphate monobasic, 1.74 g L^{-1} potassium sulphate dibasic), SQM20 media (20 g L^{-1} glucose, 10 g L^{-1} soy peptone, 5 g L^{-1} yeast extract, 15 g L^{-1} ASW) and SQM60 (60 g L^{-1} glucose, 10 g L^{-1} soy peptone, 5 g L^{-1} yeast extract, 15 g L^{-1} ASW). The media in which the control strain performed best in terms of squalene content and biomass was chosen as the media used for secondary flask screening.

3.7 Development of protocol to determine squalene content for high throughput screening

A method for squalene determination was developed for high throughput screening of thraustochytrid biomass by: (1) determining the best solvent combination for extraction and (2) determining the most suitable storage conditions for biomass prior to squalene extraction.

3.7.1 Squalene extraction methods

Three treatments with different solvent combinations were assessed for extraction of squalene and are described as follows:

For **Treatment 1,** freeze dried biomass (20 - 80 mg) was weighed into 2 mL screw capped microcentrifuge tubes containing approximately 0.25 g of acid washed, 425 - 600 µm glass beads

(Sigma-Aldrich). Then 1 mL of hexane (Caledon, Georgetown, Ontario, Canada) was added to the microcentrifuge tubes containing the biomass and glass beads as a first solvent wash. The tubes were then placed in a BioSpec minibeadbeater™ (Cole-Parmer, Montreal, Quebec, Canada) for 3 minutes. The tubes were then vortexed for 10 – 15 seconds before being sonicated (Branson 3210) for 15 minutes. Following sonication, the tubes were centrifuged at 14 x g for 5 minutes. The supernatant was then collected into a glass tube and dried under nitrogen gas. In the second extraction step, 750 µL of hexane and 750 µL of chloroform (Caledon) was then added to the tube and the biomass was subjected to 2 minutes of bead beading, 10 – 15 sec of vortexing and 5 minutes centrifugation. The supernatant was collected in placed into the same glass tube corresponding to the biomass sample and dried under nitrogen gas. A third and final extraction step was performed using 750 µL of hexane and 750 µL of chloroform, 1 minute of bead beating, 10 – 15 seconds of vortexing and again centrifuged for 5 minutes. The supernatant was removed from the tube and placed into the glass tube where it was dried under nitrogen gas. Once dried the residual lipid layer in the glass tube was suspended in 1 mL toluene and vortexed to ensure homogeneity. The entire volume of the tube was then pipetted into a 1.5 mL centrifugation tube and centrifuged to remove and impurities (biomass or glass), then the toluene layer with the dissolved lipid fraction was transferred into a glass vial for squalene analysis.

Treatments 2 and **3** followed the same bead beating, sonication, vortexing and drying protocol as **Treatment 1** and only differed in the solvents used to extract the squalene. **Treatment 2** used hexane as the sole solvent for each of the three extractions in a 1 mL volume. **Treatment 3** used chloroform and methanol (Caledon, Georgetown, Ontario, Canada) in a 1:1 ratio for a 1 mL total volume for each extraction step.

For squalene analysis of samples from all treatments, samples were loaded on to an Agilent Technology 1200 series HPLC using a poroshell 120 EC-C18, 4.6x100mm, 2.7µm column (Agilent, Santa Clara, California, USA) with a column temperature of 30°C and UV/Vis wavelength of 198nm. The mobile phase was 100 % acetonitrile (Caledon, Georgetown, Ontario, Canada) using isocratic flow at 1 mL min^{-1} for 23 min, with a run time per injection of 23 minutes and injection volume of 1 µL.

3.7.2 Biomass storage conditions

The stability of the squalene in lyophilized biomass samples was examined under three different storage conditions to identify the most appropriate way to preserve the samples for further screening stages of this project. Three flasks of *A. Tsukuba-3* biomass which was grown for 96 hours in WDL20 media at 25 °C was used in this set of experiments. The lyophilized biomass from each flask was subject to the following three conditions: 1) room temperature of 20-22 °C in capped falcon tube, 2) room temperature of 20-22 °C, nitrogen purged capped falcon tube and 3) -20 °C, nitrogen purged capped falcon tube. The squalene was extracted and quantified using the protocols described in section 3.7.1 each week from each sample for around 4 weeks.

3.8 Secondary flask screening of isolates

The top 39 isolates were chosen based on their biomass concentrations from the initial flask screening in WDO20 media. These strains were grown in WDL20 determined by the results from the experiments performed in section 3.5. Each isolate was grown in 500 mL flat bottom Erlenmeyer flasks in a 120 mL volume at 25 °C for 4 days. For high through-put screening, each isolate was grown in single flasks without replicates. The biomass was washed

and dried as per the descriptions in section 3.5, and then analyzed for squalene using treatment 1 described in section 3.7.

3.9 Tertiary flask screening of isolates

Based on the results from section 3.8.1., the five best performing strains determined by their biomass and squalene content were evaluated further in triplicate flask experiments on WDL20 culture media using *Aurantiochytrium tsukuba-3* as a control strain. Each isolate was grown in 500 mL flat bottom Erlenmeyer flasks in a 120 mL volume at 25°C for 5 days. Each flask was sampled daily to monitor biomass accumulation, squalene content as well as glucose. Glucose was monitored using a YSI 2900D Biochemistry Analyzer (YSI, St. Petersburg, Florida, USA). The results of this more detailed strain analysis were used to determine the best thraustochytrid strain to use in Plackett-Burman experiments as well as 18s rRNA genetic identification.

3.10 Plackett-Burman Media Screening

3.10.1 Factors and responses

Plackett-Burman (PB) experimental design was used to determine important media factors that affect biological responses. Factors were chosen based on published work by Fan et al. 2010, Martins et al. 2018, and Chen et al. 2010, for improving squalene yields in thraustochytrids via media optimization. The eight factors chosen for the PB experiments were temperature, sodium chloride, carbon source (glucose and glycerol), soy peptone, yeast extract, monosodium glutamate (MSG), tryptone and terbinafine. The variables that were analyzed as responses were biomass concentration and squalene concentration. Each media factor had a corresponding high (+) and low (-) designation (Table 3.1). *Table 3.1. Plackett-Burman media design factors high and low designations.*

Table 3.1. Plackett-Burman media design factors high and low designations.

Factor	High (+)	Low (-)
Temperature (°C)	28	25
NaCl (g L^{-1})	15	10
Carbon (g L^{-1})	30 (glycerol)	30 (glucose)
Soy Peptone (g L^{-1})	7	3
Yeast Extract (g L^{-1})	7	3
MSG (g L^{-1})	3	1
Tryptone (g L^{-1})	7	3
Terbinafine (g L^{-1})	0.02	0.01

These factors were combined in different combinations to create the full PB experimental design. Minitab18 statistical software was used as a platform to build the Plackett-Burman design for media factor screening. The design was set up to test 12 unique media combinations for a total of 36 runs when performed in triplicate (Table 3.2). Each run was grown in 500 mL Erlenmeyer flasks with an initial volume of 120 mL and a 10% seed by volume grown from the same set of cryopreserved stocks. These flasks were sampled daily and analyzed for biomass content, carbon source consumption, pH and squalene content over 120 hours. Final time points were analyzed in Minitab18 to determine which media factors were significant for squalene production and *biomass* content.

Table 3.2). The vitamins, trace elements, ammonium sulfate, and phosphates remained in the same concentration as the WDL20 media.

3.10.2 Plackett-Burman Experimental design

Minitab18 statistical software was used as a platform to build the Plackett-Burman design for media factor screening. The design was set up to test 12 unique media combinations for a total of 36 runs when performed in triplicate (Table 3.2). Each run was grown in 500 mL

Erlenmeyer flasks with an initial volume of 120 mL and a 10% seed by volume grown from the same set of cryopreserved stocks. These flasks were sampled daily and analyzed for biomass content, carbon source consumption, pH and squalene content over 120 hours. Final time points were analyzed in Minitab18 to determine which media factors were significant for squalene production and biomass content.

Table 3.2. Plackett-Burman experimental matrix for 36 runs where '+' represents the high and '-' represents the low value. The carbon source '+' value represents the glycerol factor and '-' the glucose factor.

Run	Temperature	NaCl	Carbon Source	Soy Peptone	Yeast Extract	MSG	Tryptone	Terbinafine
1	-	+	+	+	-	+	+	-
2	-	+	+	-	+	-	-	-
3	-	-	-	-	-	-	-	-
4	+	-	-	-	+	+	+	-
5	-	-	-	+	+	+	-	+
6	-	-	+	+	+	-	+	+
7	-	+	-	-	-	+	+	+
8	+	+	+	-	+	+	-	+
9	+	+	-	+	-	-	-	+
10	+	-	+	+	-	+	-	-
11	+	+	-	+	+	-	+	-
12	+	-	+	-	-	-	+	+
13	-	-	-	-	-	-	-	-
14	-	-	+	+	+	-	+	+
15	+	+	-	+	-	-	-	+
16	+	-	-	-	+	+	+	-
17	-	+	+	-	+	-	-	-
18	+	+	+	-	+	+	-	+
19	-	+	+	+	-	+	+	-
20	+	-	+	+	-	+	-	-
21	-	-	-	+	+	+	-	+
22	+	-	+	-	-	-	+	+
23	-	+	-	-	-	+	+	+
24	+	+	-	+	+	-	+	-
25	+	-	-	-	+	+	+	-
26	-	+	+	-	+	-	-	-
27	-	-	+	+	+	-	+	+

28	+	+	-	+	-	-	-	+
29	-	+	+	+	-	+	+	-
30	+	+	-	+	+	-	+	-
31	+	-	+	-	-	-	+	+
32	-	-	-	-	-	-	-	-
33	+	-	+	+	-	+	-	-
34	+	+	+	-	+	+	-	+
35	-	-	-	+	+	+	-	+
36	-	+	-	-	-	+	+	+

3.11 Genetic identification of best isolate

The 18S rDNA sequence was amplified from the top performing strain's (MD-24A-8) DNA. Taq DNA polymerase and primers JBo119 (5'-CAACCTGGTTGATCCTGCCAGTA-3') and JBo120 (5'-TCACTACGGAAACCTTGTTACGAC-3') were used to extract the 18s sequence from the selected strain (Burja et al., 2006). The 50 µl PCR reaction contained 1.25 units Taq DNA polymerase (New England Biolabs, Massachusetts, USA), 1x standard reaction buffer, 200 µM of each dNTP (A,G,C,T) (Bioline, London, UK) 0.2 µM of each primer (JBo119 and JBo120), 3% DMSO and diluted using PCR reagent water (Thermo Fisher Scientific). This PCR reaction was incubated at 94°C for 4 minutes, then subjected to 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 68°C for 1 minute and 45 seconds, followed by a final incubation at 68°C for 30 minutes, before being held at 4°C. The ~1.7 kb amplicon was gel purified using a Necleo spin DNA purification kit (Tankara Bio., Mountain View, California, USA).

The pool of amplified fragments was cloned into pCR2.1 vector by TA cloning using a TA Cloning®Kit containing One Shot™ TOP10 competent *E.coli* cells (Invitrogen, San Francisco, California, USA) to produce plasmid LP1-17 and was sent to Genewiz (Boston, Massachusetts,

USA) for sequencing. Each clone was sequenced with eight primers including four forward primers and four reverse primers. Two of the sequencing primers, M13R and T7, are universal primers that bind vector sequences flanking the TA cloning site and were provided by Genewiz. The other 6 primers, including JBo119 and JBo120 used to amplify the 18S rDNA, bind within the 18S rDNA sequence and were designed based on previously reported primer sequences (Burja et al., 2006; Mo et al., 2002). The 8 primer sequences are outlined in Table 3.3.

Table 3.3. Primers and corresponding sequences used for the 18s rRNA identification of the best thraustochytrid strain.

Primer	Sequence	Reference
M13R	5'-CAG GAA ACA GCT ATG AC-3'	(universal primer)
T7	5'-TAA TAC GAC TCA CTA TAG GG-3'	(universal primer)
JBo119	5'-CAACCTGGTTGATCCTGCCAGTA-3'	(Burja et al., 2006)
JBo120	5'-TCACTACGGAAACCTTGTTACGAC-3'	(Burja et al., 2006)
JBo121	5'-GTCTGGTGCCAGCAGCCGCG-3'	(Mo et al., 2002)
JBo122	5'-CTTAAAGGAATTGACGGAAG-3'	(Mo et al., 2002)
JBo123	5'-AGCTTTTTAACTGCAACAAC-3'	(Mo et al. 2002)
JBo124	5'-GGCCATGCACCACCACCC -3'	(Mo et al. 2002)

The 8 sequencing reactions for the pLP1-17 clone were trimmed by deleting the 5' and 3' sequences containing ambiguous nucleotides. These were assembled into a single contig using ChromasPro software which contained 18S rDNA sequences as well as flanking vector sequences. The vector sequences were trimmed from the contig leaving only the 18S rDNA sequence amplified by JBo119 and JBo120. Any ambiguous nucleotides indicated by ChromasPro were manually corrected based on the chromatograph peaks in overlapping areas. There were very few ambiguities in the sequencing data. 18S rDNA sequence was covered by at least 2 sequencing reads over its entire length but had at least 3 reads coverage for the vast majority of its length.

The phylogenetic analysis on the sequence obtained for the strain of interest was performed using Molecular Evolutionary Genetics Analysis (MEGA10) software. Multiple sequence alignments were generated in order to infer the evolutionary relationships between the sequences. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The bootstrap consensus tree created was inferred from 1000 replicates and was taken to represent the evolutionary history of the taxa analyzed. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. This analysis involved 41 nucleotide sequences in which positions with less than 75% site coverage were eliminated. This means that fewer than 25% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Chapter 4 : Results and Discussion

4.1 Strain Isolation

A total of 406 strains were isolated from the MA, MB, MC and MD collections (Figures 3.1 - 3.4) using the pollen baiting method described in section 2.3 (Figure 4.1). These results pertaining to strain isolation and initial flask screening in WDO5 and WDO20 were obtain by working along side the strain discovery team at Mara. Although the goal was to target thraustochytrid species for collection, yeast isolates were also collected during this process. The MA collection yielded 9 thraustochytrid strains, the MB collection yielded 43 thraustochytrid strains and 3 yeast strains, the MC collection yielded 113 thraustochytrid strains and 1 yeast strain, and the MD collection yielded 105 thraustochytrids and 121 yeast and 11 unidentified isolates. The strains were identified based on colony morphology on WDO plates and later confirmed by examination under a microscope during the secondary flask screening. The isolates were assigned names in the following format: "**collection name – sample site # – sample A or B – isolate number from sample**".

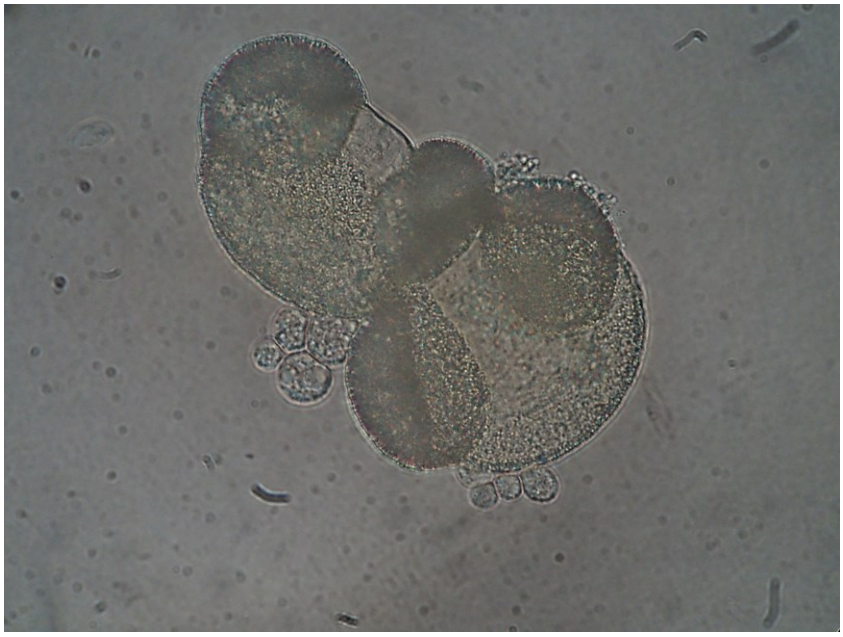

Figure 4.1. Pollen grains showing successful colonization (100x magnification).

4.2 Primary flask screening of isolates – 5 g L^{-1} glucose

Each of the 406 isolates were grown in 250 mL flasks that were inoculated from a liquid culture. The intent of this primary flask screening using WDO5 media was to determine the strains that could viably grow on glucose (5 g L^{-1}) and to rank them based on their biomass concentration. These strains were grown using T18 as a reference thraustochytrid strain for what is considered a high biomass producer. Collections MA-MC were compared with a T18 biomass concentration of 1.52 g L^{-1} and the MD collection with T18 biomass concentration of 2.98 g L^{-1}. It is uncertain why the control strain performed differently in terms of biomass between the MA-MC and MD collection, however each strain was still compared to its respective T18 control. It may be due to variability in media ingredients (e.g. differences between lot numbers) or biological variability. Table 4.1, Table 4.2,Table 4.3 and Table 4.4 list the biomass concentrations achieved for each isolate from the primary screening experiments. This flask

screening was used to check the viability of the cryopreserved stocks as well as to identify cell types as either thraustochytrids or yeasts.

Table 4.1 MA collection isolate names, cell morphology type and biomass concentration. T = Thraustochytrid, Y = Yeast.

Isolate Name	Cell Type	Biomass (g L^{-1})
MA-1-B-1	T	0.274
MA-1-B-2	T	0.384
MA-1-B-3	T	0.342
MA-1-B-4	T	0.248
MA-1-B-5	T	0.246
MA-1-B-6	T	0.378
MA-1-B-7	T	0.452
MA-1-B-8	T	0.372
MA-1-B-9	T	0.332

Table 4.2. MB collection isolate names, cell morphology type and biomass concentration. T=Thraustochytrid, Y=Yeast and ?=unable to identify.

Isolate Name	Cell Type	Biomass (g L^{-1})	Isolate Name	Cell Type	Biomass (g L^{-1})
MB 10A-1	?	0.278	MB 16B-4	T	0.412
MB 10A-2	?	0.092	MB 17A-1	?	0.3
MB 11B-1	Y	0.284	MB 17A-2	T	0.288
MB 11B-2	T	0.192	MB 17A-3	?	0.344
MB 11B-3	Y	0.75	MB 17B-1	T	1.082
MB 12B-1	Y	3.152	MB 19B-1	T	0.678
MB 12B-2	Y	3.092	MB 19B-3	T	0.946
MB 12B-3	T	0.454	MB 19B-5	T	0.658
MB 12B-4	T	0.732	MB 19B-6	T	0.912
MB 13A-3	Y	1.338	MB 19B-7	?	0.198
MB 13B-1	T	1.934	MB 19B-8	?	1.338
MB 13B-2	T	1.968	MB 20B-1	T	0.59
MB 13B-3	T	3.286	MB 20B-2	T	0.676
MB 15B-1	T	0.418	MB 21A-3	T	0.312
MB 15B-2	T	1.346	MB 21A-4	T	0.22
MB 15B-3	T	0.956	MB 22A-1	T	1.012
MB 15B-4	T	0.818	MB 22A-2	T	0.764
MB 15B-5	T	0.738	MB 23A-1	T	0.26

MB 15B-6	T	1.086	MB 23A-3	T	0.214
MB 16A-2	T	0.536	MB 25A-1	T	0.36
MB 16B-1	T	0.232	MB 25A-2	T	0.278
MB 16B-2	T	0.248	MB 25B-1	T	0.34
MB 16B-3	T	1.274	MB 25B-2	T	0.468

Table 4.3. MC collection isolate names, cell morphology type and biomass concentration. T=Thraustochytrid, Y=Yeast and ?=unable to identify.

Isolate Name	Cell Type	Biomass ($g\ L^{-1}$)	Isolate Name	Cell Type	Biomass ($g\ L^{-1}$)	Isolate Name	Cell Type	Biomass ($g\ L^{-1}$)
MC 10A-2	T	0.518	MC 21A-1	T	0.396	MC 35A-2	T	0.504
MC 10A-3	T	0.533	MC 21A-2	T	0.410	MC 35A-3	T	0.362
MC 10B-1	T	0.002	MC 21A-3	T	0.300	MC 35A-4	T	0.638
MC 10B-2	T	2.276	MC 24A-1	T	0.378	MC 35B-1	?	0.518
MC 10B-4	T	0.491	MC 24A-4	T	0.444	MC 35B-2	?	0.400
MC 11A-1	T	0.629	MC 25A-1	T	0.420	MC 35B-4	T	0.346
MC 11A-2	T	0.718	MC 25A-4	T	0.422	MC 35B-5	T	0.372
MC 12A-2	T	0.478	MC 25B-1	T	0.364	MC 35B-6	Y	2.150
MC 12A-4	T	0.371	MC 25B-4	T	0.254	MC 35B-7	T	0.314
MC 12A-6	T	0.409	MC 26A-1	T	0.368	MC 36B-1	T	0.374
MC 12A-7	T	0.411	MC 26A-2	T	0.942	MC 36B-3	Y	0.318
MC 12B-1	T	3.544	MC 26A-3	T	0.324	MC 03B-1	T	0.593
MC 12B-2	T	1.248	MC 26A-4	T	0.298	MC 03B-3	T	0.047
MC 12B-3	?	1.084	MC 27A-1	T	0.306	MC 04A-1	T	0.102
MC 12B-4	T	0.452	MC 27A-2	T	0.276	MC 04A-3	T	0.531
MC 12B-6	T	0.356	MC 27A-3	T	0.306	MC 04B-2	T	0.231
MC 13A-1	T	0.594	MC 27A-4	T	0.278	MC 04B-4	T	0.536
MC 13A-3	?	0.594	MC 28A-1	T	0.530	MC 05A-1	T	0.411
MC 13B-2	T	0.276	MC 28A-2	T	1.042	MC 05A-2	Y	-
MC 13B-3	T	0.350	MC 28B-3	T	0.320	MC 05B-1	T	1.078
MC 13B-5	T	1.066	MC 28B-4	T	0.302	MC 05B-2	T	0.469
MC 13B-7	T	0.410	MC 29B-3	T	0.348	MC 05B-3	T	1.636
MC 14B-2	T	0.420	MC 29B-4	T	0.292	MC 05B-4	T	0.551
MC 14B-4	T	0.412	MC 30A-1	T	0.334	MC 06A-2	T	0.642
MC 15B-1	T	2.028	MC 30A-2	T	0.660	MC 06A-4	T	0.449
MC 15B-4	T	0.400	MC 30B-1	T	0.470	MC 07A-1	T	0.376
MC 17A-1	T	0.264	MC 30B-2	?	1.084	MC 07A-3	T	0.480
MC 17A-2	T	0.338	MC 31A-1	?	1.070	MC 07B-1	T	0.804
MC 17B-1	T	0.250	MC 31A-2	T	0.310	MC 07B-2	T	0.624
MC 17B-3	T	0.302	MC 31B-2	T	0.328	MC 07B-3	T	0.551
MC 19A-1	T	1.364	MC 31B-4	T	0.400	MC 07B-4	T	0.384

MC 19A-2	T	0.984	MC 32B-1	T	0.362	MC 08A-2	T	0.491
MC 01A-1	T	0.233	MC 32B-3	T	0.448	MC 08A-4	T	0.418
MC 01A-2	T	0.493	MC 33A-1	T	0.374	MC 08B-1	T	0.069
MC 01B-1	T	0.413	MC 33A-3	T	2.662	MC 08B-3	T	1.098
MC 01B-2	T	0.529	MC 34A-1	T	1.232	MC 08B-4	T	0.431
MC 01B-3	T	0.538	MC 34A-2	T	0.684	MC 09A-3	T	0.507
MC 01B-4	T	0.538	MC 35A-1	T	0.578	MC 09A-4	T	0.493

Table 4.4. MD collection isolate names, cell morphology type and biomass concentration. T=Thraustochytrid, Y=Yeast and ?=unable to identify

Isolate Name	Cell Type	Biomass (g L^{-1})	Isolate Name	Cell Type	Biomass (g L^{-1})	Isolate Name	Cell Type	Biomass (g L^{-1})
MD 10A-1	?	1.002	MD 28A-7	Y	4.534	MD 42B-2	Y	3.112
MD 10A-2	T	0	MD 28B-1	Y	4.246	MD 43A-1	Y	1.138
MD 10A-5	T	0.584	MD 28B-2	Y	4.512	MD 43A-2	Y	3.244
MD 10A-6	T	0.564	MD 28B-3	T	1.522	MD 43A-7	T	2.842
MD 10B-1	Y	3.422	MD 28B-4	T	1.564	MD 43A-8	T	2.59
MD 10B-2	T	1.642	MD 29A-1	?	0.872	MD 43B-1	T	2.56
MD 10B-5	Y	3.992	MD 29A-10	Y	3.866	MD 43B-2	T	2.712
MD 10B-6	Y	4.45	MD 29A-2	?	1.15	MD 44A-1	Y	4.406
MD 10B-7	Y	4.33	MD 29A-5	T	0.548	MD 44A-2	Y	4.434
MD 10B-8	Y	3.478	MD 29A-6	T	0.698	MD 44A-5	T	2.852
MD 11A-1	T	2.312	MD 29A-9	Y	3.032	MD 44A-6	Y	4.818
MD 11A-2	T	2.576	MD 29B-1	Y	4.422	MD 44A-7	T	1.37
MD 11B-1	T	1.37	MD 29B-2	Y	4.082	MD 44A-8	T	2.792
MD 11B-2	T	1.336	MD 29B-3	Y	3.728	MD 44B-1	Y	4.112
MD 11B-3	Y	3.424	MD 02A-1	T	2.454	MD 44B-2	T	1.606
MD 11B-4	Y	3.972	MD 02A-2	T	0.648	MD 44B-3	T	1.692
MD 13A-1	Y	3.512	MD 02A-3	T	1.372	MD 44B-5	Y	4.38
MD 13A-2	Y	3.722	MD 02A-4	T	0.618	MD 44B-6	Y	3.936
MD 13A-5	Y	5.964	MD 30B-1	Y	2.854	MD 44B-7	Y	3.776
MD 13A-6	Y	5.652	MD 30B-2	Y	3.118	MD 45A-1	Y	4.33
MD 13B-1	T	0.574	MD 31A-1	Y	4.63	MD 45A-2	Y	3.56
MD 13B-2	T	0.704	MD 31A-2	Y	4.67	MD 45A-3	Y	3.424
MD 14A-1	?	1.242	MD 31A-3	Y	5.018	MD 45A-4	Y	3.71
MD 14A-2	T	1.322	MD 31A-4	Y	4.83	MD 45A-5	Y	2.516
MD 14A-3	T	1.488	MD 32B-1	Y	4.588	MD 45A-6	Y	3.296
MD 14B-1	T	2.23	MD 32B-2	Y	4.188	MD 45A-8	Y	2.942
MD 14B-2	T	2.43	MD 32B-5	Y	4.79	MD 45B-2	Y	3.426
MD 17A-1	T	0.69	MD 32B-6	Y	4.024	MD 45B-3	Y	3.58
MD 17A-4	T	0.76	MD 33A-1	Y	3.562	MD 46A-1	Y	4.17

MD 17A-4	T	0.76	MD 33A-3	Y	3.306	MD 46A-2	Y	3.656
MD 17B-1	Y	3.122	MD 33A-5	Y	3.498	MD 46A-3	Y	4.212
MD 17B-2	Y	3.132	MD 33A-6	Y	4.124	MD 46B-1	Y	3.688
MD 18A-1	T	2.038	MD 33B-2	Y	3.646	MD 46B-2	Y	3.18
MD 18A-3	T	2.952	MD 33B-3	Y	3.86	MD 46B-6	T	1.6
MD 18B-1	Y	3.342	MD 34A-1	?	1.41	MD 46B-8	T	0
MD 18B-2	Y	3.628	MD 34A-4	T	3.402	MD 47B-1	T	2.09
MD 01A-1	T	1.004	MD 34A-5	T	1.832	MD 47B-2	T	2.316
MD 01A-2	?	0.764	MD 34A-6	T	2.538	MD 47B-3	T	0.614
MD 01A-3	T	0.856	MD 34B-3	T	0.706	MD 47B-4	T	0.546
MD 01A-4	?	0.88	MD 34B-4	T	0.8	MD 48A-1	Y	3.178
MD 01A-5	?	0.624	MD 34B-5	T	2.664	MD 48A-2	Y	4.054
MD 20B-1	Y	3.0	MD 34B-6	T	3.006	MD 48A-5	T	1.452
MD 20B-2	Y	4.57	MD 36A-2	Y	3.622	MD 48A-6	T	0
MD 20B-4	Y	4.094	MD 36A-3	Y	2.816	MD 48A-7	T	2.006
MD 22A-2	Y	3.848	MD 36B-1	Y	4.072	MD 48A-8	T	1.464
MD 22A-4	Y	3.606	MD 36B-2	Y	3.24	MD 48B-1	Y	4.458
MD 22B-1	T	0.64	MD 37A-1	Y	2.572	MD 48B-10	T	2.78
MD 22B-2	Y	3.738	MD 37A-2	Y	4.158	MD 48B-11	T	0.476
MD 22B-5	?	1.062	MD 37A-3	T	3.768	MD 48B-2	Y	2.874
MD 22B-6	T	1.59	MD 37A-4	T	3.642	MD 48B-4	T	2.742
MD 23A-1	Y	3.458	MD 37B-1	T	3.658	MD 48B-5	T	2.744
MD 23A-2	Y	3.504	MD 37B-2	Y	4.452	MD 48B-6	Y	2.376
MD 23A-3	T	2.726	MD 37B-3	Y	4.292	MD 48B-7	T	2.534
MD 23A-4	Y	2.932	MD 38B-1	Y	3.078	MD 48B-8	Y	2.452
MD 23A-5	Y	4.764	MD 38B-2	Y	3.182	MD 48B-9	T	2.492
MD 23A-6	Y	4.588	MD 39A-1	Y	2.95	MD 49A-1	T	0.864
MD 23B-2	Y	4.566	MD 39A-2	Y	3.34	MD 49A-2	T	0.434
MD 23B-4	Y	4.328	MD 39A-3	Y	2.018	MD 49A-3	T	0.682
MD 24A-1	T	1.318	MD 39A-4	Y	3.504	MD 49A-4	T	0.656
MD 24A-2	?	1.434	MD 39B-1	Y	4.216	MD 04A-1	Y	3.32
MD 24A-5	T	2.576	MD 39B-2	Y	6.492	MD 04A-2	Y	3.71
MD 24A-7	Y	4.952	MD 39B-3	Y	4.264	MD 04B-1	Y	4.118
MD 24A-8	T	3.048	MD 39B-4	Y	2.972	MD 04B-4	Y	3.912
MD 24B-1	Y	2.732	MD 40B-1	Y	3.462	MD 05A-1	T	2.644
MD 24B-2	T	0.798	MD 40B-2	Y	2.982	MD 05A-2	T	0.722
MD 24B-3	T	4.174	MD 40B-3	T	1.38	MD 05A-3	T	2.158
MD 26A-1	T	3.006	MD 41A-1	Y	4.638	MD 06A-1	T	2.724
MD 26A-2	T	2.734	MD 41A-2	Y	4.52	MD 06A-2	T	2.808
MD 26A-3	T	3.052	MD 41B-3	Y	4.53	MD 06B-1	Y	3.872
MD 26A-4	Y	0.1	MD 41B-4	Y	4.938	MD 06B-2	Y	3.242
MD 26B-2	T	1.296	MD 42A-1	T	2.852	MD 06B-3	Y	4.656
MD 26B-3	?	0.178	MD 42A-2	T	1.258	MD 07A-1	T	0.444

MD 26B-5	T	2.392	MD 42A-3	T	3.296	MD 07A-2	T	1.73
MD 26B-6	T	2.928	MD 42A-4	Y	4.044	MD 08A-1	T	0.536
MD 26B-7	Y	2.922	MD 42A-5	T	2.292	MD 08A-4	T	0.462
MD 26B-8	Y	3.27	MD 42A-6	T	0.718	MD 08A-5	T	1.018
MD 28A-1	T	2.23	MD 42A-7	T	2.868	MD 08A-6	T	1.46
MD 28A-2	T	1.808	MD 42A-8	T	2.928	MD 08B-1	T	2.658
MD 28A-6	Y	3.488	MD 42B-1	Y	2.634	MD 08B-2	T	2.924

4.3 Primary flask screening of Thraustochytrid isolates – 20 g L^{-1} glucose

The purpose of this stage of primary flask screening was to select the strains that produced the highest biomass concentration when grown in WDO20 media, which provided a higher level of glucose than the WDO5 used previously. A total of 255 thraustochytrids were grown under these conditions and 69 strains grew to a biomass concentration of greater than 1.5 g L^{-1} in WDO5 media (Table 4.1,

Table 4.2, Table *4.3* and Table *4.4*), where this level of growth is a good indicator of potentially suitable strains for development at the industrial scale (A.Windust, Personal communication, June 2017).

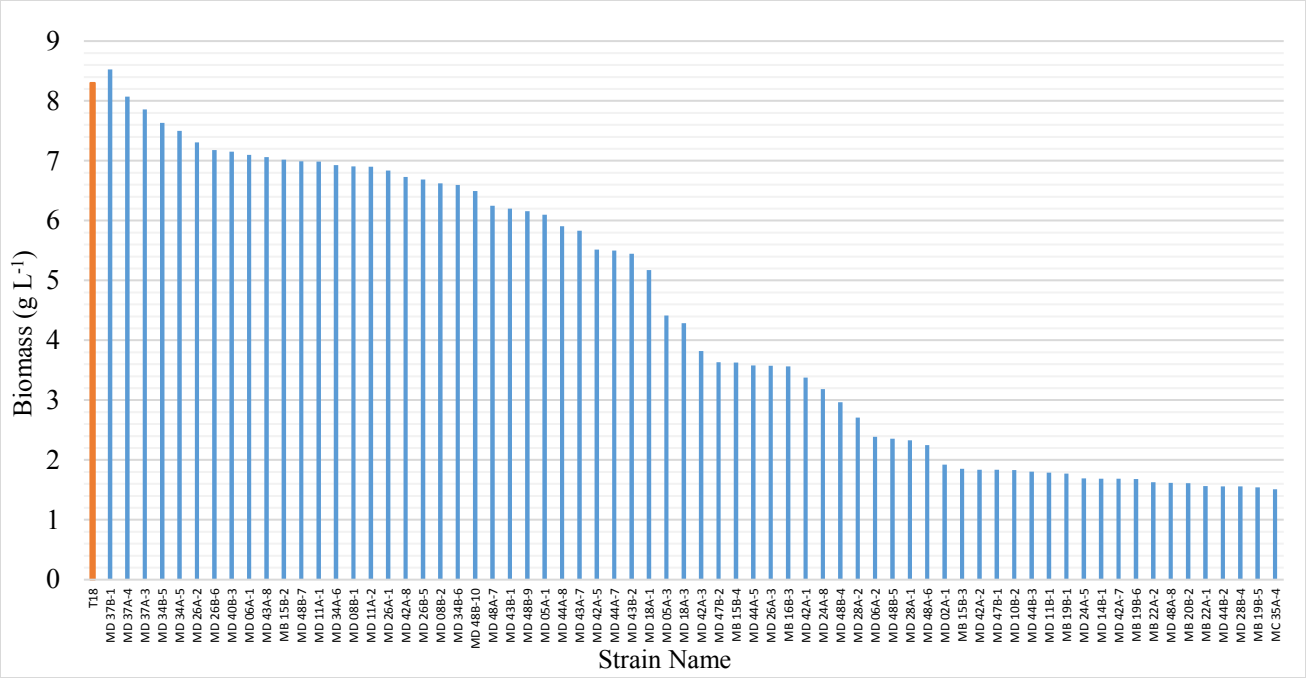

Figure 4.2. Thraustochytrid strains with biomass concentrations > 1.5 g L^{-1} that were grown in 20 g L^{-1} media. Orange bar represents the control strain for biomass production (T18).

Of the 69 strains shown in Figure 4.2, 39 strains were chosen for secondary flask screening. The selection was based on biomass concentration, sampling site, as well as colony morphology on WDO plates. The strain names were abbreviated to simple number designations at this point to simplify strain recognition. The formal strain identification name and abbreviated strain number can be found in Table A 3.

One of the aims of this research is to find a thraustochytrid strain that could potentially be used in industrial production. It is important that these strains can produce high amounts of biomass as a primary selection criterion. Additionally, demonstrating the ability of a strain to grow in higher concentrations of glucose is an indicator of a more robust strain. A strain which is tolerant of high substrate concentrations is also desirable for large scale fermentations, especially with respect to glucose (Le et al., 2017). A tolerance to high glucose concentration has also been associated with higher DHA contents in some organisms and the ability to grow at

concentrations < 100 g/L (Wong et al., 2008). As previously mentioned, both the PKS pathway, which gives rise to DHA, and the MVA pathway, which gives rise to squalene, both have the same initiating metabolite (Acetyl Co-A), therefore it can be speculated that what is beneficial for DHA production could also be beneficial for squalene production. Additionally, carbon tolerance is important as large- scale fermentations operated in batch mode rely on media with high initial levels of glucose to obtain the greatest amount of biomass per batch. Fed-batch fermentations also benefit from using strains with high glucose tolerance, as this can be translated to a high level of biomass per volume fed into the bioreactor. Although this work does not include fermentation studies in vessels larger than shake flasks, its important to consider the future development of any strain that may eventually be used for fermentation studies to further the strain development.

The evaluation of carbon tolerance and biomass production are relatively simple results to obtain but hold a lot of significance when reviewing a large library of strains. These outcomes can therefore determine a favourable starting point for investigation when beginning to identify strains of interest to analyze specifically for squalene production. It is a more economical approach in terms of time and materials to perform these simpler experiments before more in-depth investigations are conducted in subsequent secondary and tertiary screening experiments.

4.4 Development of squalene production media for high throughput screening

To determine the most appropriate media to be used in squalene screening for the chosen isolates, three media combinations described in section 3.6 were tested using the squalene control strain *Aurantiochytrium tsukuba-3*. Both the WDL20 and the WDL60 media out-performed the SQM20 media. The WDL20, WDL60 and SQM20 media resulted in average squalene contents (mg g^{-1}) of 50.72 ± 7.01, 48.09 ± 17.08 and 8.28 ± 1.49, respectively. The

WDL20 media was chosen as the base-line squalene media to be used in subsequent experiments, based on its highest average squalene content as well as its consistent results in comparison to the WDL60 media.

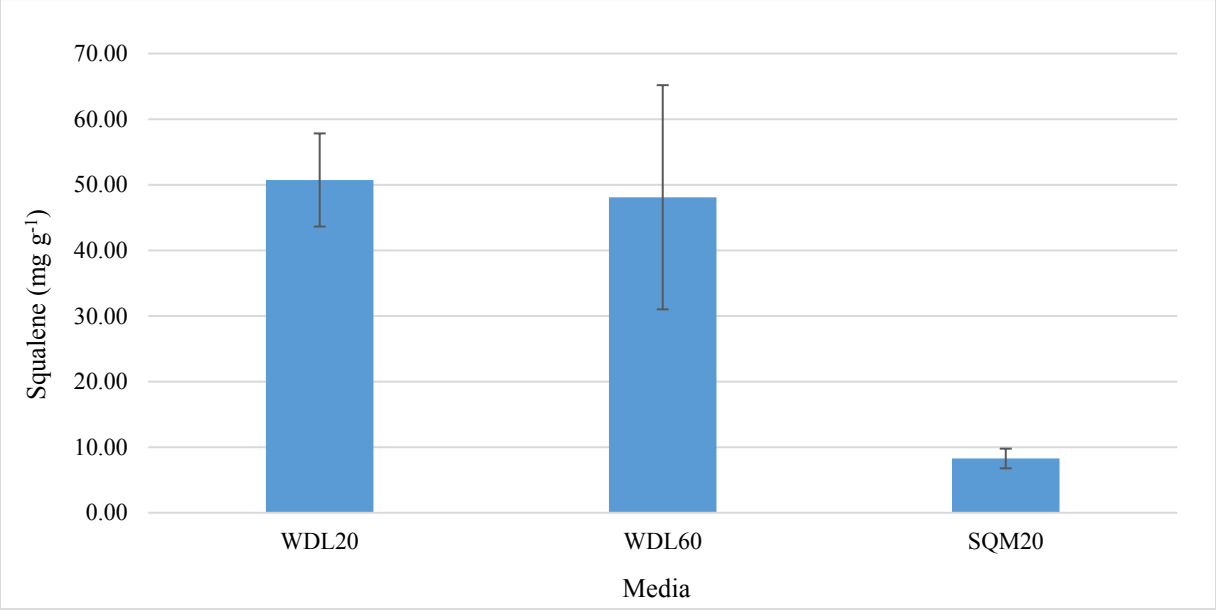

Figure 4.3. Squalene content in mg g^{-1} of *Aurantiochytrium tsukuba-3* grown on WDL20, WDL60 and SQM20 media with standard deviations (n=8).

4.5 Development of protocol to determine squalene content for high throughput screening

4.5.1 Extraction of Squalene

Due to the high numbers of isolate samples in this project, an efficient squalene extraction method needed to be developed. The aim was to create a protocol that would work using < 80 mg of dried thraustochytrid biomass (control strain *Aurantiochytrium tsukuba-3*) and require minimal amounts of solvents and time. To determine the best solvent combination to use for squalene extraction, three thraustochytrid biomass samples labeled as A, B and C were subject to Treatments 1, 2 and 3 as outlined in section 3.7. Treatment 1 used hexane as solvent, Treatment 2 used chloroform and hexane, and Treatment 3 used chloroform and methanol.

Figure 4.4 shows the average squalene content from each biomass sample under each extraction condition.

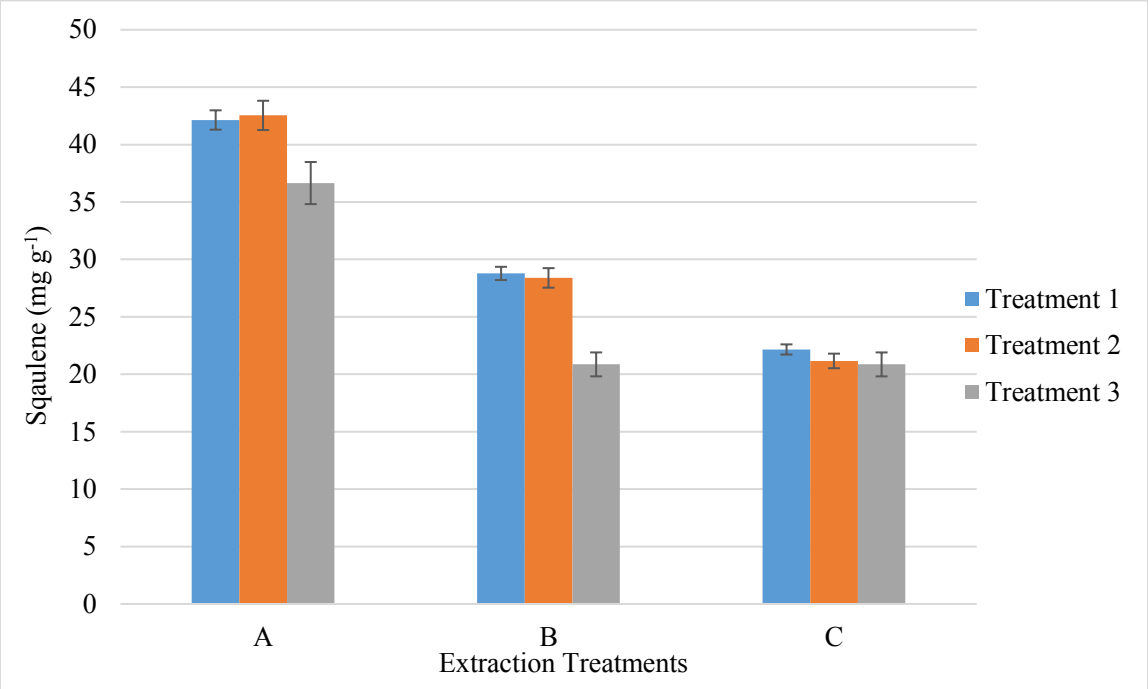

Figure 4.4. Squalene in mg g^{-1} and standard deviations from biomass samples A, B and C that have been extracted using Treatments 1 (hexane & chloroform), 2 (hexane) and 3 (chloroform and methanol) (n=3).

A one-way ANOVA analysis was performed on each biomass sample to compare the extraction treatments at a 95% confidence level (α=0.05) followed by a Tukey test to compare the treatments (Figure A 1, Figure A 2, Figure A 3). Biomass sample A had a P-value of 0.004 indicating that there was a significant difference in the data set. To determine if the results from the treatments were significantly different, a Tukey Pairwise comparison was performed, which determined that Treatment 3 was significantly different than Treatments 1 and 2, whereas there was no significant difference between Treatments 1 and 2. Biomass sample B had a P-value of 0.001, again indicating a significant difference between treatments. The Tukey test revealed that Treatment 3 was again significantly different than 1 and 2, and that 1 and 2 showed no

significant difference. Biomass sample C had a P-value of 0.052, indicating no significant difference between treatments.

The studies by Jiang et al. (2004); Lee et al. (2010) and Nakazawa et al. (2012, 2014) were used to develop the protocols for squalene extraction investigated here. Both the Jiang and Nakazawa studies used modified Bligh and Dyer methods accompanied with lipid fractionation to obtain a more concentrated squalene stream. The study by Lee et al. (2010) also describes a modified Bligh and Dyer method with bead beating as physical disruption method. In this study, all extraction treatments involved bead beating and sonication, and different solvent combinations were investigated.

While Treatment 1 used hexane and chloroform (1:1 v/v), Treatment 2 used hexane, and Treatment 3 used chloroform and methanol (1:1 v/v) being the most similar to the Bligh and Dyer method. Of the three extractions tested, Treatment 3 was the least successful and most variable, and although it used the main solvents also involved in the Bligh and Dyer method, under these extraction conditions it did not perform well. Typically, these solvents would be used in a ternary solution including water. The water allows for the separation of a top organic chloroform phase that is rich in oil and a bottom water-rich phase where the methanol also resides. This partitioning, based on hydrophobicity, is what drives the Bligh and Dyer method (Breil et al., 2017). All the methods tested used nitrogen drying to evaporate the solvents after bead beating and sonication. The methanol and chloroform combination in Treatment 3 took the longest time to evaporate and yielded lower squalene than the other methods. The chloroform performed better when paired with hexane rather than methanol under these conditions. Methanol in combination with chloroform may be better suited to and extraction that involves a ternary solvent combination with water to allow efficient phase separation.

Figure 4.5 shows many common solvents and their relative polarity to each other. Squalene is a non-polar hydrocarbon and therefore will dissolve in non-polar solvents (Bhattacharjee & Singhal, 2003). Hexane was chosen based on its low polarity as an extraction solvent both on its own and in combination with chloroform. As previously mentioned, there was no significant difference in the amount of squalene extracted from Treatment 1 (hexane and chloroform) and Treatment 2 (hexane). The decision was made to use the chloroform and hexane-based protocol for all squalene extractions rather than the hexane-only protocol. Performing an extraction with only one solvent would be a simpler process, however, extraction efficiency can change based on the biomass being processed (Lee et al., 2010). It is possible that by having the two different solvents (chloroform and hexane), the protocol could be more inclusive when performed on different strains.

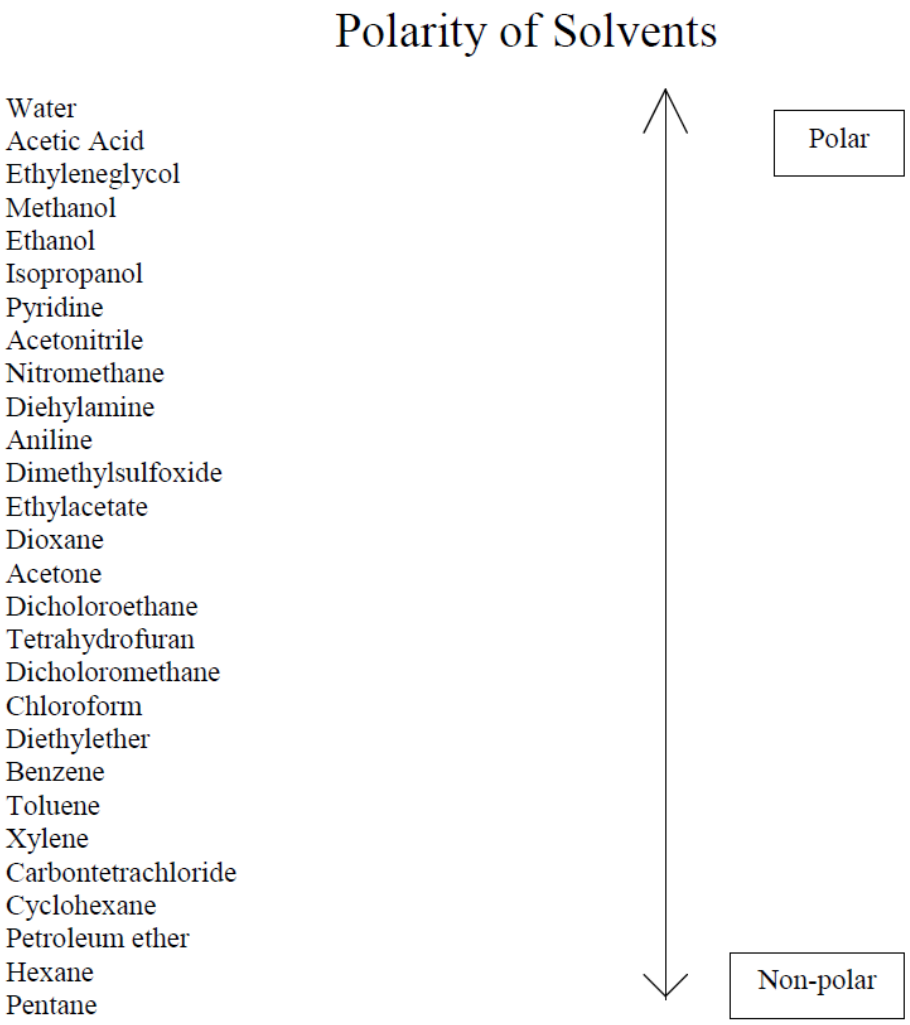

Figure 4.5. Diagram depicting highest to lowest polarity of common solvents (University of Ohio, Biochemistry Department)

4.5.2 Biomass storage conditions

The stability of squalene was evaluated under different storage conditions for lyophilized biomass, to determine the most suitable conditions to use for screening experiments prior to squalene extraction. The parameters that were evaluated included temperature (room temperature vs -20 °C), exposure to air, and light vs dark conditions. Biomass from three flasks of *A. Tsukuba-3* grown under the same conditions (flasks A, B and C) was subjected to three different

storage conditions and the squalene content was monitored over 27 days. Condition 1 was at room temperature, with light exposure and air exposure in a capped tube, Condition 2 was at room temperature, with no light exposure and purged with nitrogen in capped tube, and Condition 3 was at - 20 °C, no light exposure and purged with nitrogen in capped tube.

Figure 4.6 shows the plot of squalene (mg g^{-1}) over 27 days for Condition 1 (room temperature, light exposure and air exposure in a capped tube). The initial squalene content in flasks A, B and C was 35.32, 29.65 and 39.77 mg g^{-1} respectively. By day 6 there was already a steep drop in squalene content for all three flasks and by day 27 the content in flasks A, B and C was 5.54, 1.32 and 4.67 mg g^{-1} respectively.

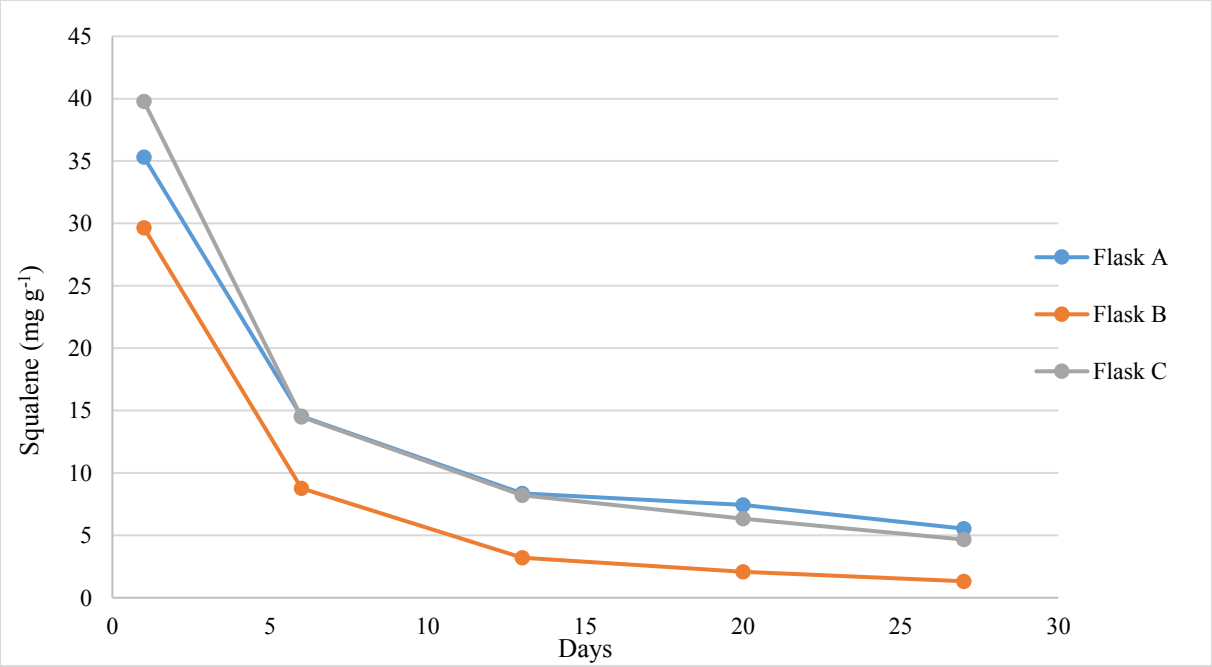

Figure 4.6. Condition 1 (room temperature, light exposure and air exposure in capped tube) showing squalene content over 27 days in biomass from flasks A, B and C (n=1).

The results from storage under Condition 2 (room temperature, no light exposure and nitrogen purged in capped tube) are shown in Figure 4.7. Here, a similar trend was observed as for Condition 1, where a large portion of the squalene content was lost in the first 6 days of

storage. The initial squalene content in flasks A, B and C was 35.72, 31.11 and 40.40 mg g^{-1} respectively. By day 27 the content in flasks A, B and C was 5.94, 2.95 and 5.31 mg g^{-1} respectively.

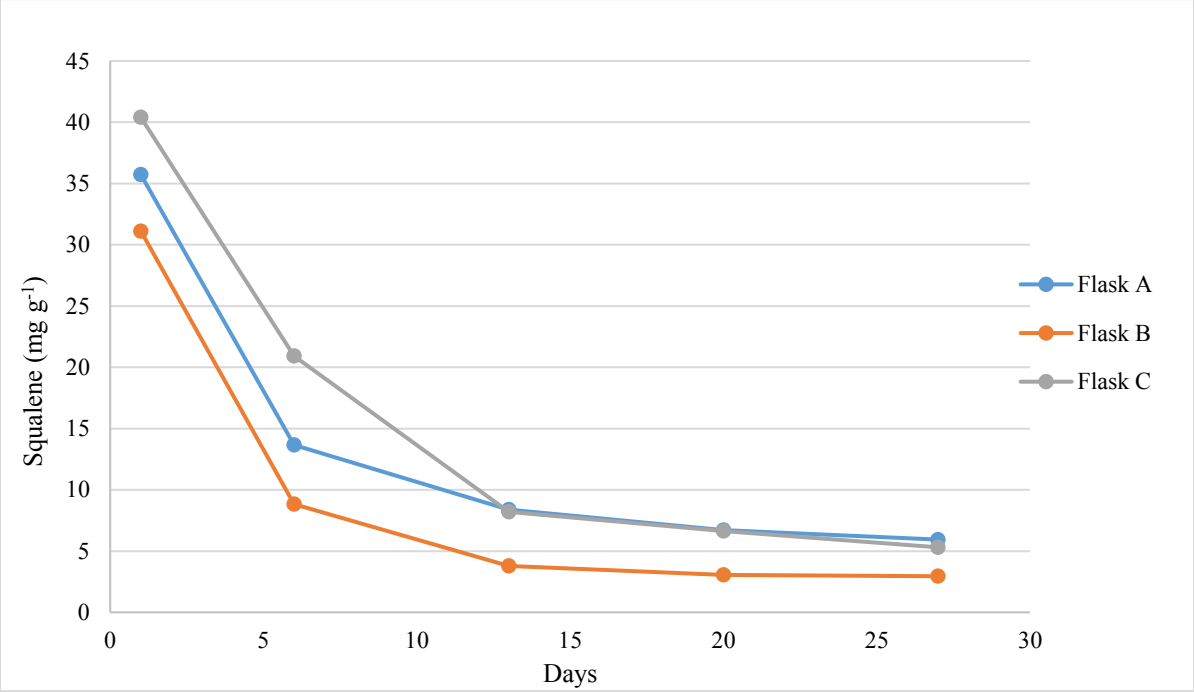

Figure 4.7. Condition 2 (room temperature, no light exposure and nitrogen purged in capped tube) showing squalene content over 27 days in biomass from flask A, B and C (n=1)

Results from Condition 3, shown in Figure 4.8 (- 20 °C, no light exposure and nitrogen purged in capped tube), did not have the same steep drop in squalene content as the biomass stored under Conditions 1 and 2. Rather, the biomass appeared to be unaffected under these conditions. The initial squalene content in flasks A, B and C was 35.38, 25.89 and 37.56 mg g^{-1} respectively. By day 27 the content in flasks A, B and C was 26.07, 27.83 and 33.43 mg g^{-1}, respectively. The discrepancy between the initial and final time point (the final being slightly higher than the initial) in flask B is likely due to experimental variance; however, flask A showed a decrease in squalene content on day 27. From these results, the best storage conditions for freeze-dried biomass were determined for future screening experiments to ensure squalene

stability, where samples would be maintained for no longer than 20 days at -20°C, with no light and purged with nitrogen before analysis.

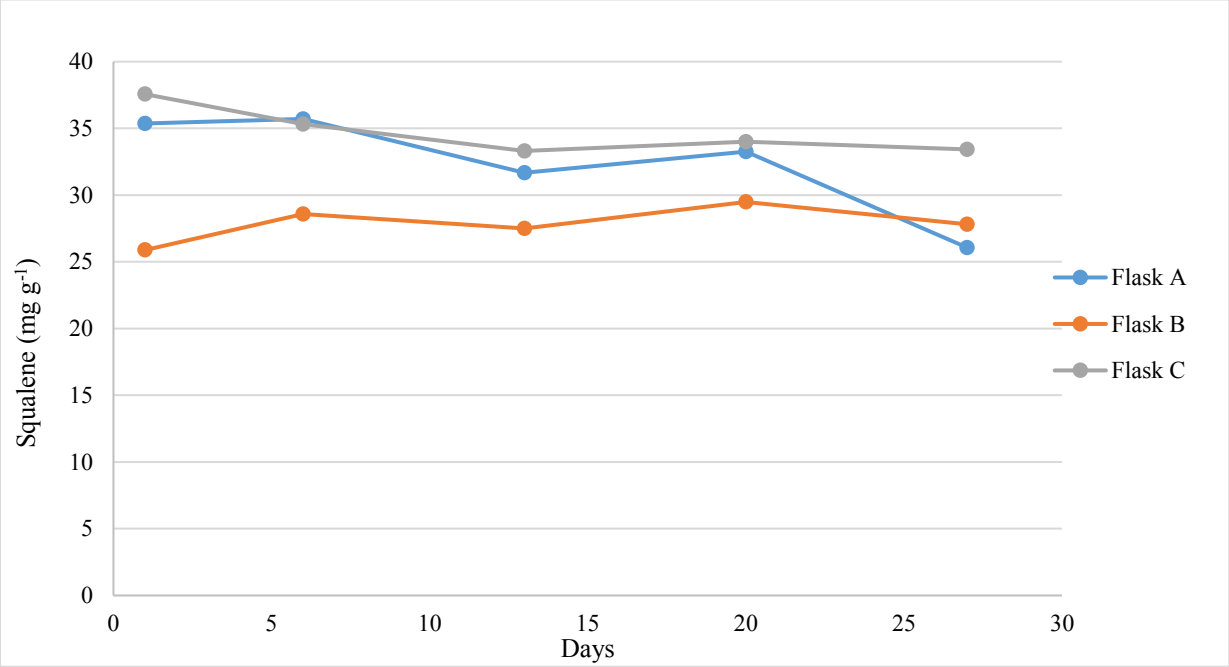

Figure 4.8. Condition 3 (-20 °C, no light exposure and nitrogen purged in capped tube) squalene content over 27 days in biomass from flask A, B and C (n=1)

As squalene is a highly unsaturated compound, it is prone to oxidative reactions which can be impacted by the selected storage parameters (Naziri et al., 2014). Conditions 1 and 2 showed a steep decrease in squalene within the first 6 days. Flasks A, B and C stored under Condition 1 lost 58.83%, 70.35 % and 63.50% of squalene respectively within the first 6 days. Similarly, for Condition 2 flasks A, B and C lost 61.73 %, 71.62 % and 48.24 %. The storage temperature for these two conditions were identical (room temperature) but differed in light exposure (condition 1 = light exposed and condition 2 = no light) and oxygen exposure (condition 1 = air exposure and condition 2 = nitrogen purge). The removal of light and oxygen under this temperature did not improve the squalene retention in the sample, which was surprising as biologically active compounds such as squalene and carotenoids are extremely

sensitive towards heat and light as well as acting as efficient oxygen scavengers (Bonnie & Choo, 1999). The findings of the current study supports work by Chandrasekaram et al. (2009) in which squalene, carotenes, vitamin E, and sterols were evaluated in crude palm oil for their storage stability over a 3 month period at 30 °C and -16 °C. Squalene degradation at 30 °C after 1 and 2 months was 59 % and 72 % respectively and at -16 °C was 1% and 1.3% respectively. When held under storage conditions of -20 °C in the current study, squalene in the freeze-dried thraustochytrid biomass showed little to no degradation in the first 6 days of storage. After 20 days, flasks A and C began showing signs of deterioration (5.9 % and 9.5 % respectively). The decrease in squalene at day 27 for flask A and C was 26.2 % and 10.5 % respectively where flask B did not experience any degradation. Using a one-way ANOVA tukey comparison in Minitab18 at a confidence level of 95%, treatments 3 was deemed statistically different than treatments 1 and 2 in flasks A and B. In flask 3, the data did not show a significant difference between treatments. When comparing the analysis of variance of the data sets for flasks A, B and C for treatments 1, 2 and 3, the p-values were 0.024, 0.017 and 0.039 at a 95% confidence interval, therefore the null hypothesis that all means are equal can be rejected (Figure A 4). These results indicate that temperature seems to play a key role in the stability of squalene. This is an important concept to consider for future work and harvesting squalene from thraustochytrid biomass, as it can be rapidly lost from samples if not stored properly. This sensitivity to storage temperature could also be a challenge for large scale production.

4.6 Secondary flask screening of isolates

The 39 isolates selected from primary flask screening in WDO20 (section 3.8) were chosen based on their biomass concentrations and their thraustochytrid-like morphology and

subjected to for secondary flask screening. Each isolate was grown in WDL20 media in a 500 mL flat bottom flask and grown for 96 hours. *A. Tsukuba-3* was used as a benchmark for squalene production in these growth experiment. Figure 4.9 shows the results of this flask study ranked in order from highest to lowest based on the amount of squalene in mg L^{-1}. The top five strains with respect to both biomass and squalene content included strains 91, 90, 94, 171 and 170. Due to similarities in the fatty acid methyl ester (FAME) profiles of strains 91 and 90 performed by another group within the Mara laboratory (data not shown), it was determined that strains 91 and 90 were likely duplicate strains; therefore, strain 91 was excluded from further screening and strain 286 was included. The control strain, *A. Tsukuba-3,* grew to a biomass concentration of 2.6 g L^{-1} which is equivalent to a squalene content per biomass dry weight of 10.44 mg g^{-1} and a squalene concentration of 27.19 mg L^{-1}. The biomass concentration for strains 90, 94, 171, 170 and 286 were 7.30 g L^{-1}, 6.56 g L^{-1}, 5.94 g L^{-1}, 3.42 g L^{-1} and 3.78 g L^{-1}, respectively; the squalene concentration per biomass dry weight were 2003.01 mg L^{-1}, 1980.52 mg L^{-1}, 1629.63 mg L^{-1}, 2040.82 mg L^{-1} and 874.52 mg L^{-1} respectively; and the squalene content in the biomass was 14.61 mg L^{-1}, 12.99 mg L^{-1}, 9.61 mg L^{-1}, 6.97 mg L^{-1}, 3.31 mg L^{-1} respectively.

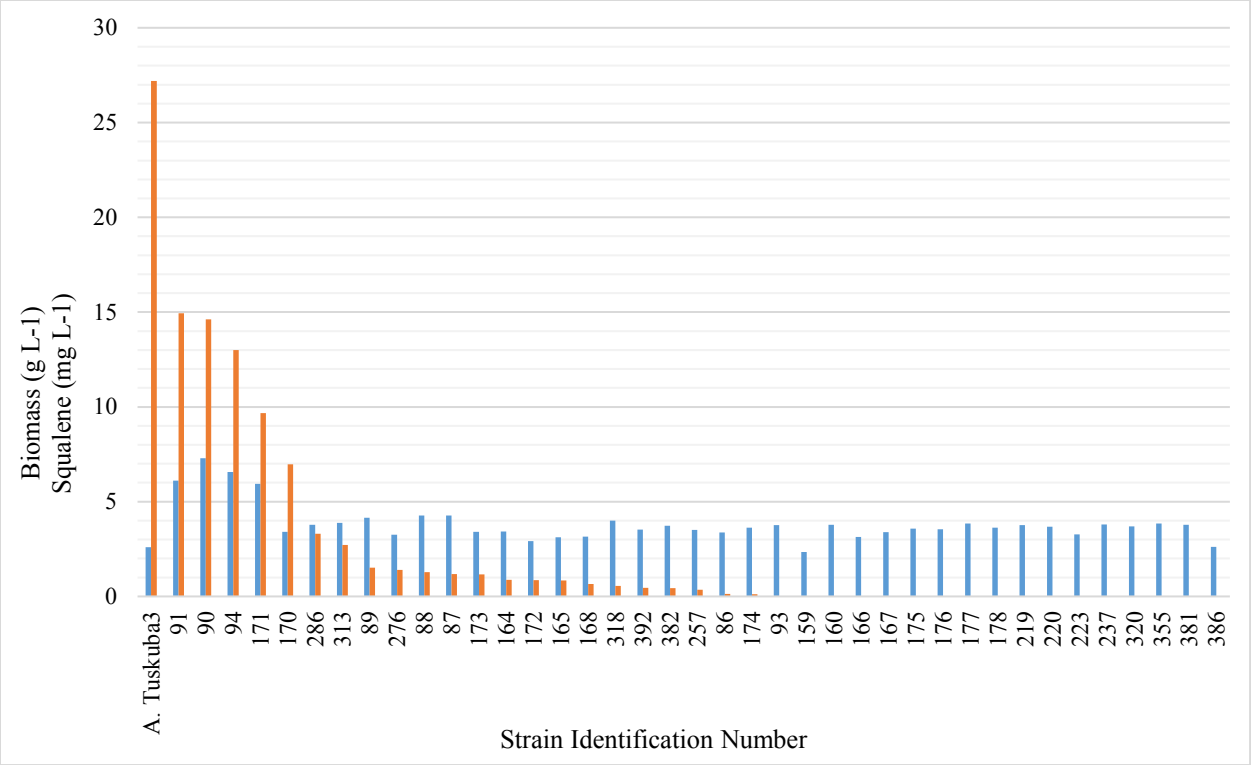

Figure 4.9. Biomass (g L^{-1}) (blue bars) and squalene (mg L^{-1}) (orange bars) for the 39 highest biomass producers chosen from the primary flask screening experiment in Section 4.3 (n=1).

These 39 strains were evaluated for both their squalene and biomass production using WDL20 media. This media had been chosen for high through-put screening (Section 4.4) as it was determined to be the best readily available media for squalene production using the squalene control strain, *A. Tsukuba-3*. The control strain out-performed all 39 strains in terms of squalene content, however, the top 5 strains all out-performed the control in terms of biomass produced (Figure 4.9). All 39 strains were ranked according to squalene content as the primary determinant for selecting the strains to go for subsequent tertiary screening. The biomass production was used as a secondary screening parameter for evaluation, which resulted in selection of the top 5 strains.

4.7 Tertiary flask screening of isolates

The five best performing strains from secondary flask screening were grown in triplicate flask experiments and sampled each day for 5 days for tertiary screening. The purpose of the tertiary screening was to evaluate in a more detailed fashion, the way these strains grow and accumulate squalene. Until this level of screening occurred, only end points from flask fermentations had been sampled for biomass and squalene content. Each strain was grown in WDL20 and sampled at 46, 66, 96, 114 and 138 hours. Results for biomass accumulation and squalene content are shown in Figure 4.109 and Figure 4.10, respectively.

Strain 90 had the highest rate of biomass concentration (7.51 g L^{-1}). Additionally, strain 90 depleted the glucose in the media 48 hours earlier than the other isolates (data not shown). Strains 94 and 171 depleted their glucose in 114 hours ending with biomass concentrations of 6.52 g L^{-1} and 5.48 g L^{-1}. Strains 286, 170 and *A. tsukuba-3* reached biomass concentrations of 6.33 g L^{-1} 6.98 g L^{-1} and 4.48 g L^{-1}, respectively, after 141 hours of incubation. All strains, apart from strain 286 and *A. tsukuba-3,* depleted all the available glucose in the media, with 3.69 g L^{-1} of glucose remaining for strain 286 and 3.53 g L^{-1} remaining for *A. tsukuba-3*.

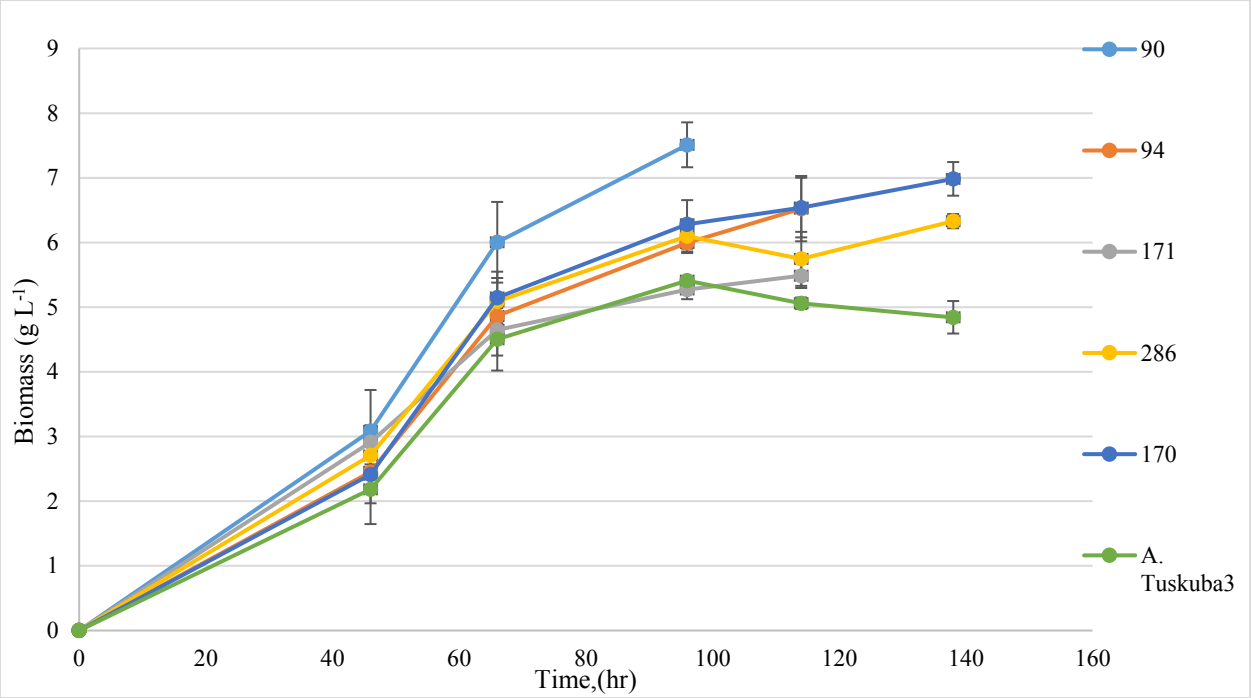

Figure 4.10. Biomass accumulation curve with standard deviations for strain 90 (light blue), 94 (orange), 171 (grey), 286 (yellow) and 170 (dark blue) (n=3).

Squalene content (mg g^{-1}) was also monitored in each flask at every time point after inoculation. There was a trend among each flask in which the squalene content peaked somewhere between 46 and 66 hours and then began to decrease as biomass accumulated (Figure 4.11) and glucose depleted. The highest squalene content for strains 90, 94, 171, 286, 170 and *A. tsukuba-3* were 44.29 mg L^{-1}, 26.23 mg L^{-1}, 49.20 mg L^{-1} 48.05 mg L^{-1}, 23.80 mg L^{-1} and 50.57 mg L^{-1}. Strains 90, 286 and 171 were the best performers based on their peak squalene concentration. When considering squalene content in mg g^{-1}, strains 90, 286 and 171 reached values of 14.85 ± 3.65,17.12 ± 4.67 and 17.68 ± 1.14. These peak values were subject to a one-way ANOVA analysis of variance at the 95% confidence level to yield a p-value of 0.603 which means the null hypothesis must be accepted indicated that all the means are not significantly different.

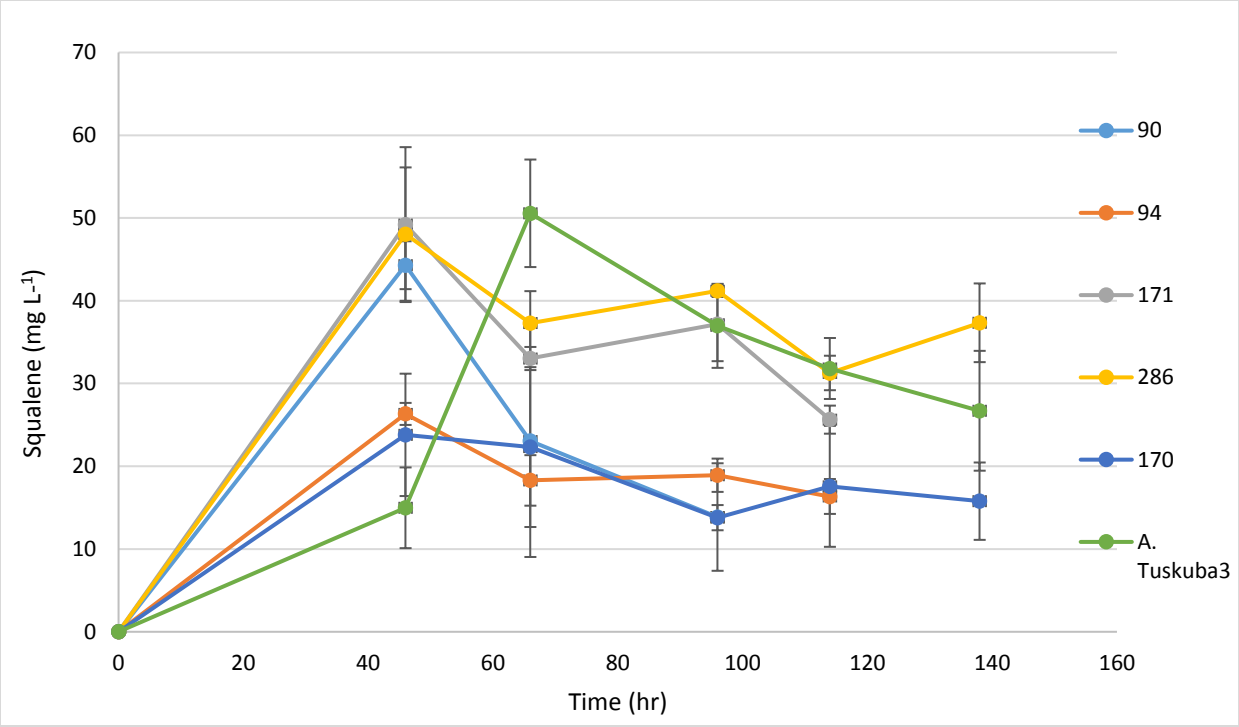

Figure 4.11. Squalene accumulation curve for strain 90 (light blue), 94 (orange), 171 (grey), 286 (yellow) and 170 (dark blue).

Squalene accumulation and peak production in the exponential growth phase has been observed in other thraustochytrid strains. For example, Fan et al. (2010) studied the effect of terbinafine on *Auratiochytrium* sp. *mangrovei FB3* over a 5 day flask level fermentation. In this study the highest squalene content and concentrations, 0.37 mg g^{-1} and 2.21 mg L^{-1}, were achieved in exponential growth (initial 24 hours) and then decreased as the cells entered initial stationary phase. The squalene content and concentration, respectively, decreased ten and four-fold from day 1 to 5. The same trend was previously noted with this strain by Jiang et al. (2004). In work by Li et al. (2009), the squalene content of *Aurantiochytrium* sp. BR-MP4-A1 was also detrimentally impacted by increased culture age. The highest squalene content was achieved at 36 hours of culture time (0.567 mg g^{-1}) but declined to less than 0.1 mg g^{-1} after 60 hours.

Figure 2.3 describes the pathway by which squalene is produced in thraustochytrids. It is a key intermediate in sterol biosynthesis and it is possible that by the end of exponential growth, squalene is converted into the downstream products of this pathway (Bhattacharjee et al., 2001). Additionally, squalene is catalyzed by squalene epoxidase to produce (3S)-2,3-oxidosqualene. This conversion requires molecular oxygen in sufficient concentrations to enable the activity of this step (Wentzinger et al., 2002). In a study by Lewis et al. (2001), the impact of dissolved oxygen on squalene and sterol biosynthesis was examined. Lewis postulated that under these types of growth conditions in flask fermentations, cellular dissolved oxygen may increase and reach high enough concentrations that could promote squalene epoxidase activity leading to a reduction in squalene content, as the culture exits exponential growth. They also speculated that dissolved oxygen present in the culture has a greater impact on cellular squalene and other individual sterols than culture age or temperature.

Strains 90, 171 and 286 all performed better than the other strains and accumulated similar concentrations of squalene as the control strain. The control strain grew slower than strains 90, 171 and 286 and therefore reached its peak squalene content roughly 24 hours later. When determining which of the three top isolates to be carried forward in Placket Burman experiments, strain 90 was chosen based in its glucose depletion rate and biomass concentration. Strain 90 depleted its glucose and reached the highest biomass concentration (7.56 g L^{-1}) of all the strains in the shortest amount of time (96 hours). Because high biomass and productive growth is important when choosing an industrially suitable strain (Barclay et al., 2010), these parameters were used to select the top performing strain.

4.8 Plackett-Burman media screening

Twelve unique media conditions were evaluated by cultivating strain 90 in each, using a Plackett-Burman design for experiments that were conducted in triplicate, and described in detail in section 3.10. As previously mentioned, the Plackett-Burman experiments have both factors and responses. Different combinations of the 8 factors chosen in high and low values makes up the 12 unique media conditions. Table 4.5 summarizes the 12 media used for the PB design as well as the final response values for biomass accumulation in g L^{-1}, the squalene concentration in mg L^{-1}, and the squalene content in mg g^{-1}; Figure 4.14, 4.13 and 4.14 represent how the responses for each media factor combination change over culture time.

Table 4.5 Plackett-Burman media compositions (factors) and responses (biomass, squalene content and squalene concentration).

Factors									Responses		
Media #	**Temp (°C)**	**NaCl (g L^{-1})**	**Carbon source (30 g L^{-1})**	**SP (g L^{-1})**	**YE (g L^{-1})**	**MSG (g L^{-1})**	**Tryp (g L^{-1})**	**Terb (g L^{-1})**	**Biomass (g L^{-1})**	**Squalene (mg g^{-1})**	**Squalene (mg L^{-1})**
1	30	10	Gly	3	3	2	7	0.01	8.57	30.09	257.29
2	30	15	Glu	7	3	2	3	0.02	11.17	10.15	111.17
3	25	15	Gly	3	7	2	3	0.01	11.48	58.37	665.96
4	30	10	Gly	7	3	4	3	0.02	10.77	28.59	309.91
5	30	15	Glu	7	7	2	7	0.01	12.83	10.73	137.08
6	30	15	Gly	3	7	4	3	0.02	10.47	34.01	357.81
7	25	15	Gly	7	3	4	7	0.01	8.90	63.94	567.23
8	25	10	Gly	7	7	2	7	0.02	5.90	50.12	293.07
9	25	10	Glu	7	7	4	3	0.02	1.33	0.00	-
10	30	10	Glu	3	7	4	7	0.01	12.23	3.29	63.79
11	25	15	Glu	3	3	4	7	0.02	11.93	10.02	118.70
12	25	10	Glu	3	3	2	3	0.01	11.73	5.41	40.27

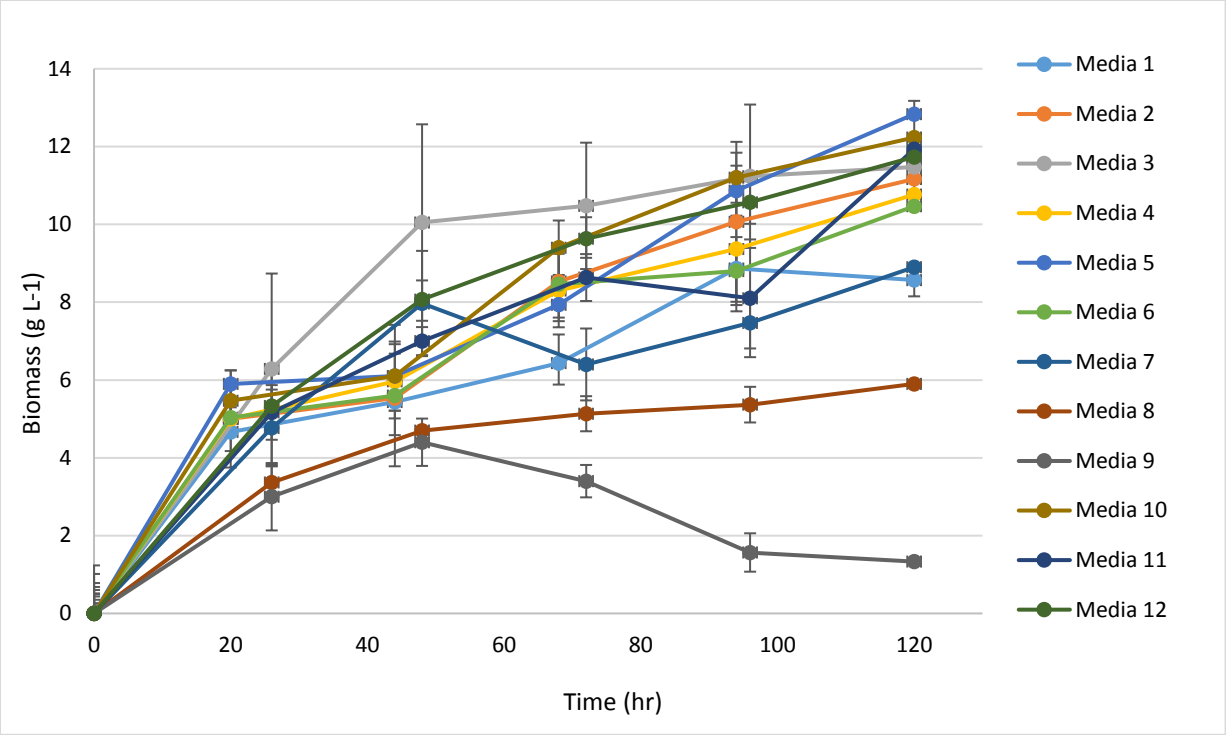

Figure 4.12. Biomass accumulation curves (g L^{-1}) and standard deviations for the 12 media used for Plackett-Burman experiments inoculated with strain 90 (n=3).

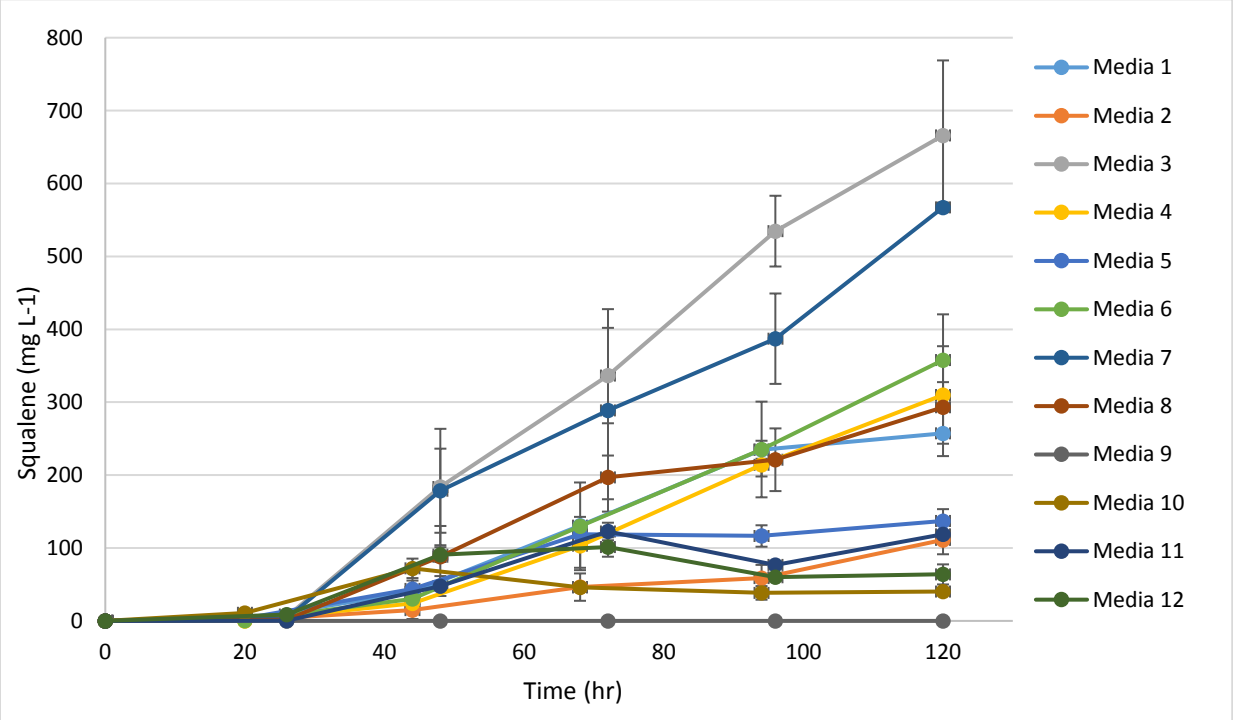

Figure 4.13. Squalene accumulation curves (mg L^{-1}) and standard deviations for the 12 media used for Plackett-Burman experiments inoculated with strain 90 (n=3).

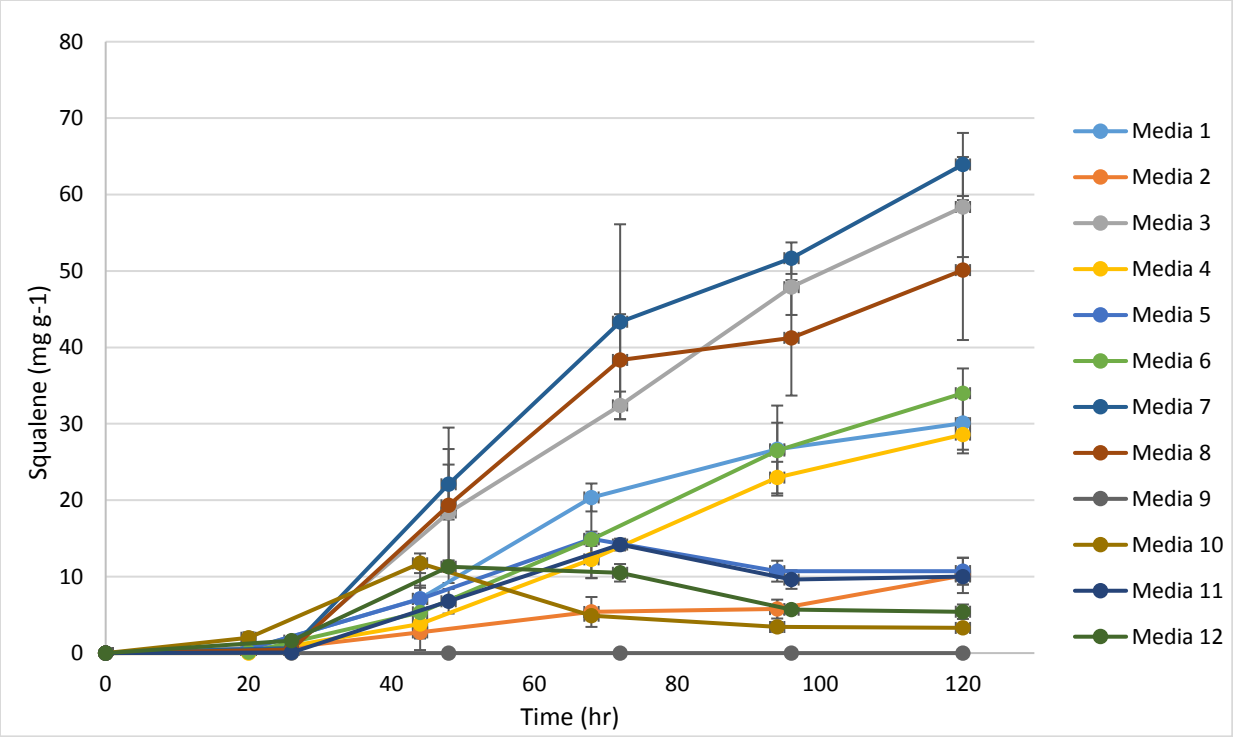

Figure 4.14. Squalene accumulation curve (mg g^{-1}) for the 12 medias used for Plackett-Burman experiments inoculated with strain 90 (n=3).

Most of the media combinations, with exceptions to 8 and 9 had final biomass concentrations at 120 hours of > 8 g L^{-1} with the highest biomass concentrations belonging to 5 at a value of 12.83 g L^{-1} (Figure 4.12). In terms of squalene accumulation, the content within the dry biomass (mg g^{-1}) as well as the concentration with respect to cell concentration (mg L^{-1}), were recorded and plotted. Because the biomass concentration was so low in media 9, the analysis for squalene qualification was unable to be performed. There are three apparent groupings for squalene content: higher, middle and lower groupings. In terms of squalene concentration (mg L^{-1}) (Figure 4.13), media 3 and 7 outperformed the rest while 1, 4, 6 and 8 fell in the mid area of the data and 5, 9, 10, 11 and 12 were the lower valued grouping. Media 3 and 7 produced squalene concentrations of 665.96 mg L^{-1} and 567.23 mg L^{-1}. The same trend can be seen for the squalene accumulation in terms of squalene content (mg g^{-1}) (Figure 4.14), media 3, 7 and 8 outperformed the rest while 1, 4 and 6 fell within the middle range of the run values and

2, 5, 7, 9, 11 and 12 were at the bottom. Media 3, 7 and 8 had squalene contents of 63.94 mg L^{-1}, 58.73 mg L^{-1} and 50.12 mg L^{-1}.

Using the final time points of each run, values for the three responses biomass (g L^{-1}), squalene content (mg g^{-1}) and squalene concentration (mg L^{-1})) were input into Minitab 18 software. The Plackett-Burman design was analyzed at a 95 % confident interval (α=0.05) to determine the factors which contribute significantly, either positively or negatively, to the responses. The Pareto charts for each of the response variables are shown in Figures 4.15, 4.16 and 4.17, where the relative magnitude and the statistical significance of both the main and interaction effects can be compared. The effects are shown in decreasing order of their absolute values (from the most to least significant). The reference line on the chart indicates which effects are significant, meaning that any factor with a standardized effect greater than the reference line is considered significant. The factors are designated in Figures 4.15, 4.16 and 4.17 are as follows: A = Temperature, B = NaCl, C = Carbon Source, D = Soy Peptone, E = Yeast Extract, F = Monosodium Glutamate, G = Tryptone and H = Terbinafine.

Figure 4.15 represents the Pareto chart for the biomass response with respect to the listed 8 factors labeled A-H. In these results, the effects that are statistically significant (α = 0.05) include terbinafine, NaCl, soy peptone, temperature and yeast extract. From ANOVA, these factors have p-values of 2.0 x 10^{-5}, 1.1 x 10^{-4} , 1.9 x 10^{-4}, 3.4 x 10^{-4} and 2.0 x 10^{-2} respectively (Table 4.6). This implies that these five factors have either a positive or negative significant impact on the biomass produced by strain 90. According to the F-values in Table 4.6, terbinafine has the most significant impact on biomass production. The model summary for this analysis has a R^2 of 79.52 % representing how well the model fits the data.

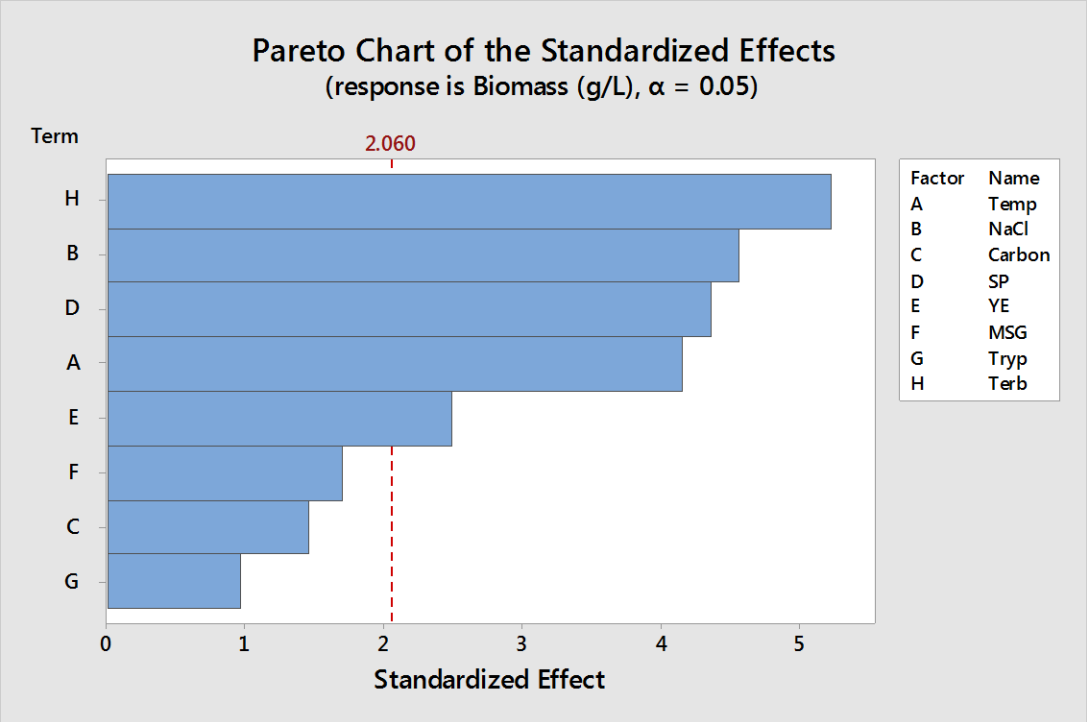

Figure 4.15. Pareto chart for biomass response from the 8 factors analyzed in Minitab 18.

Table 4.6. Analysis of variance (ANOVA) of the regression model from the Plackett-Burman design for media factor contribution to biomass concentration in un-coded units. Model Summary; S = 1.79, R^2 = 79.52 %, R^2 (adjusted) = 71.32 %, R^2 (predicted) = 57.52 %, P<0.05 significance level.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	306.876	30.6	9.70	0.000
Blocks	2	1.499	0.74	0.24	0.791
Linear	8	305.377	38.1	12.07	0.000
Temp	1	54.514	54.5	17.24	0.000
NaCl	1	66.043	66.0	20.88	0.000
Carbon	1	6.622	6.62	2.09	0.160
SP	1	60.218	60.21	19.04	0.000
YE	1	19.507	19.50	6.17	0.020
MSG	1	9.161	9.16	2.90	0.101
Tryp	1	2.947	2.94	0.93	0.344
Terb	1	86.366	86.36	27.31	0.000
Error	25	79.057	3.1623		
Total	35	385.933			

Figure 4.16 represents the Pareto chart for the squalene content response for the 8 factors of interest. In these results, the effects that are statistically significant ($\alpha = 0.05$) include carbon source, temperature, NaCl, terbinafine, tryptone and monosodium glutamate. From ANOVA, these factors have p-values of 1.0×10^{-16}, 2.1×10^{-6}, 2.5×10^{-6}, 2.5×10^{-5}, 4.7×10^{-3}, 1.1×10^{-2}, 3.9×10^{-2} respectively (

Table 4.7). According to the F-values in Table 4.7, the carbon source plays the most significant role in squalene content in strain 90. The model summary for this analysis has a R^2 of 95.05 % representing how well the model fits the data.

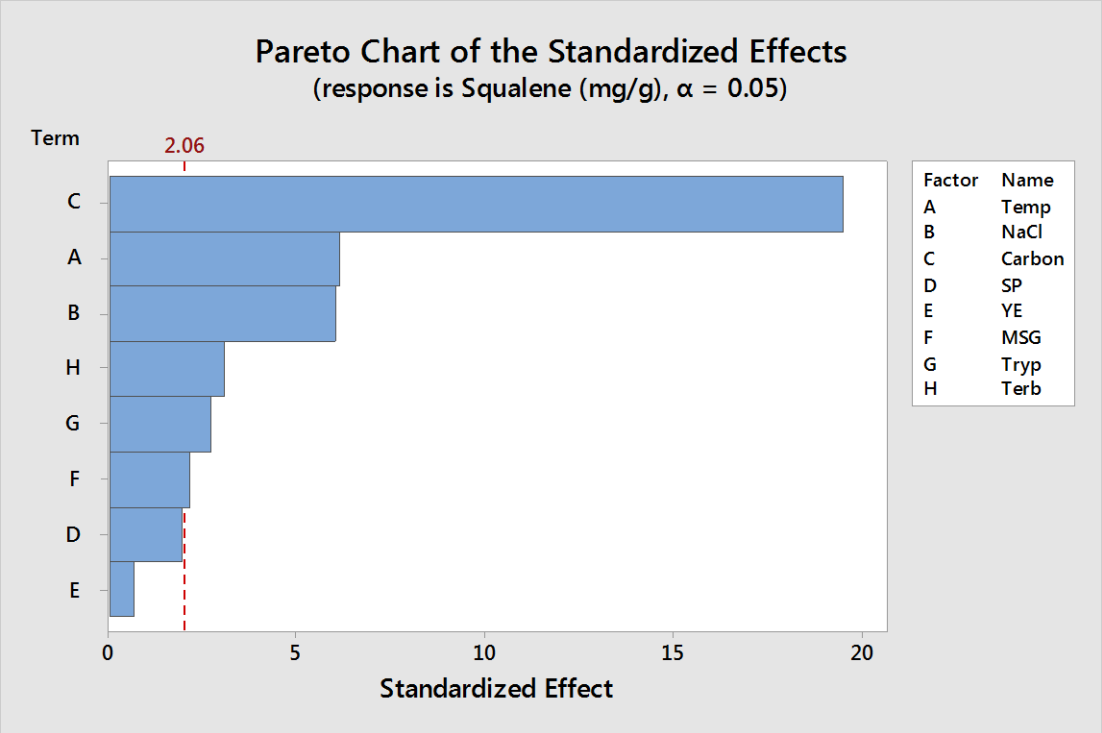

Figure 4.16. Pareto chart for squalene content from 8 factors analyzed in minitab18.

Table 4.7. Analysis of variance (ANOVA) of the regression model from the Plackett-Burman design for media factor contribution to squalene content in un-coded units. Model Summary; S = 5.78, R^2= 95.05 %, R^2(adjusted) = 93.06 %, R^2(predicted) = 89.73 %, P<0.05 significance level.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	16051	1605	48	0.000
Blocks	2	1	0	0	0.988
Linear	8	16050	2006	60	0.000
Temp	1	1256	1256	38	0.000
NaCl	1	1224	1224	37	0.000
Carbon	1	12699	12699	379	0.000
SP	1	127	127	4	0.062
YE	1	16	16	0	0.491
MSG	1	158	158	5	0.039
Tryp	1	249	249	7	0.012
Terb	1	321	321	10	0.005
Error	25	837	33		
Total	35	16887			

Figure 4.17 represents the Pareto chart for the squalene concentration response for the 8 factors of interest. In these results, the effects that are statistically significant ($\alpha = 0.05$) include carbon source, NaCl, terbinafine and temperature. From ANOVA, these factors have p-values of 2.8×10^{-15}, 6.4×10^{-9}, 8.9×10^{-6} and 2.5×10^{-4} respectively (Table 4.8). According to the F-values in Figure 4.8, the carbon source and NaCl have the greatest impact on squalene concentration in strain 90. The model summary for this analysis has a R^2 of 94.33 % representing how well the model fits the data.

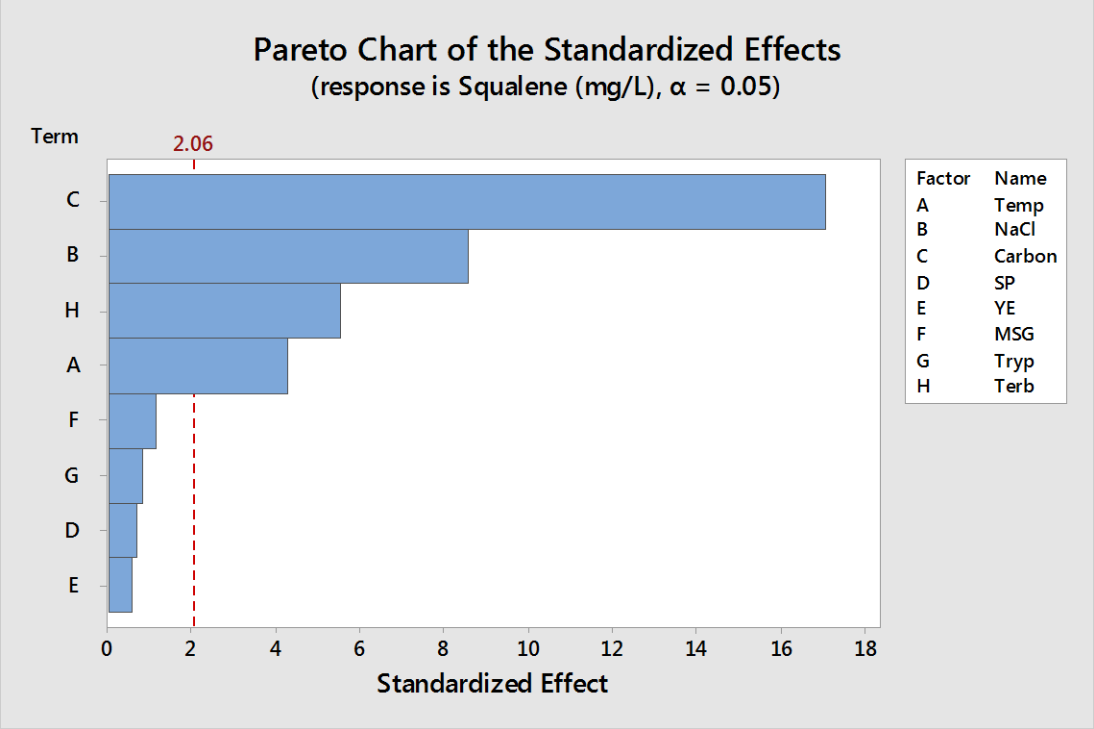

Figure 4.17. Pareto chart for squalene concentration from 8 factors analyzed in minitab18.

Table 4.8. Analysis of variance (ANOVA) of the regression model from the Plackett-Burman design for media factor contribution to squalene concentration in un-coded units. Model Summary; S = 58.04, R^2 = 94.33 %, R^2(adjusted) = 92.07 %, R^2 (predicted) = 88.25 %, P<0.05 significance level.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	1401930	140193	41.62	0.000
Blocks	2	202	101	0.03	0.971
Linear	8	1401728	175216	52.01	0.000
Temp	1	60978	60978	18.10	0.000
NaCl	1	248080	248080	73.64	0.000
Carbon	1	979044	979044	290.64	0.000
SP	1	1716	1716	0.51	0.482
YE	1	1012	1012	0.30	0.589
MSG	1	4613	4613	1.37	0.253
Tryp	1	2323	2323	0.69	0.414
Terb	1	103962	103962	30.86	0.000
Error	25	84215	3369		
Total	35	1486145			

The design matrix of the Plackett-Burman design for the effects of 8 growth/media factors on biomass (g L^{-1}), squalene content (mg g^{-1}) and squalene concentration (mg L^{-1}) and their responses are shown in Table 4.5. Results showed the highest biomass concentration of 12.83 g L^{-1} in media 5, highest squalene content of 63.94 mg g^{-1} in media 7 and the highest squalene concentration of 665.96 mg L^{-1} in media 3. These results of the factor modeling experiment by Plackett-Burman design revealed that 5, 6 and 4 out of the 8 media-related factors significantly influenced the biomass production, squalene content and squalene concentration, respectively. As previously indicated Plackett-Burman design is a small, two-level factorial experimental design which aims to identify critical physicochemical parameters and is able to able detect the large main effects (Ekpenyong et al., 2017). Although main effects (negative and positive) can be identified in this experimental design, this still only contributes to screening media rather than true optimization. Further work in the form of a full factorial design, which can be based on the results of a Plackett-Burman design, can identify interactions between factors as well as focus on determining more precise media concentrations that will yield the best result.

The main effects plot for biomass concentration (Figure 4.18) shows all the factors that were examined, with the significant ones being temperature, NaCl, soy peptone, yeast extract and terbinafine. Temperature and NaCl contributed to higher biomass, where NaCl made a larger contribution than temperature. Thraustochytrids require sea water (or artificial substitutes) to grow, therefore it is not surprising that NaCl had a positive influence on the biomass accumulation of this strain (Raghukumar, 2008). The tolerance and optimal NaCl concentration varies strain by strain (Burja et al., 2006), but with regards to strain 90, biomass production is positively influenced at 15 g L^{-1} in culture media.

Optimal temperature conditions also vary depending on the strain. Biomass production was positively influenced by the increased temperature of 28 °C. Temperature, with respect to lipid accumulation, can change the lipid profile of the strain, especially the DHA content. Lower temperatures can negatively impact biomass productivity but increase DHA content (Gupta et al., 2012; Qiu, 2003). The concept behind this resides in membrane fluidity. In microalgae, saturated fatty acids at lower temperature tend to lose physical properties associated with fluidity and therefore genes encoding for acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases will be overexpressed in order to produce PUFAs (Sánchez et al., 2017). This is relevant to the current study because it can be hypothesized that squalene could also play a role in membrane fluidity given its highly unsaturated structure. Additionally, as mentioned above, in microalgae, decreased temperature leads to overexpression of acetyl-CoA desaturase which is a key enzyme in the biosynthesis on squalene as well as DHA (Honda et al., 1998; Spanova & Daum, 2011; Xie et al., 2017).

The higher concentrations of terbinafine, soy peptone and yeast extract did not have positive impacts on the biomass concentration. Terbinafine had the largest negative impact on biomass according to Figure 4.18. It was included in this study for its reported ability to inhibit squalene monooxygenase by binding to the regulatory site and by disrupting a specific lipid domain of the enzyme (Fan et al., 2010; Favre & Ryder, 1996). Inhibition of squalene monooxygenase will promote the accumulation of squalene by inhibiting the steps which lead to cholesterol formation. Work performed by Fan et al. (2010) showed that on strain *A. mangrovei* FB3 treated with terbinafine concentrations ranging from 0.1 to 100 mg L^{-1}, it was shown that the addition of terbinafine resulted in a decreased biomass concentration. The current study used

a high terbinafine concentration of 20 mg L^{-1} and low concentration of 10 mg L^{-1} and noted the same impact on biomass as the Fan study.

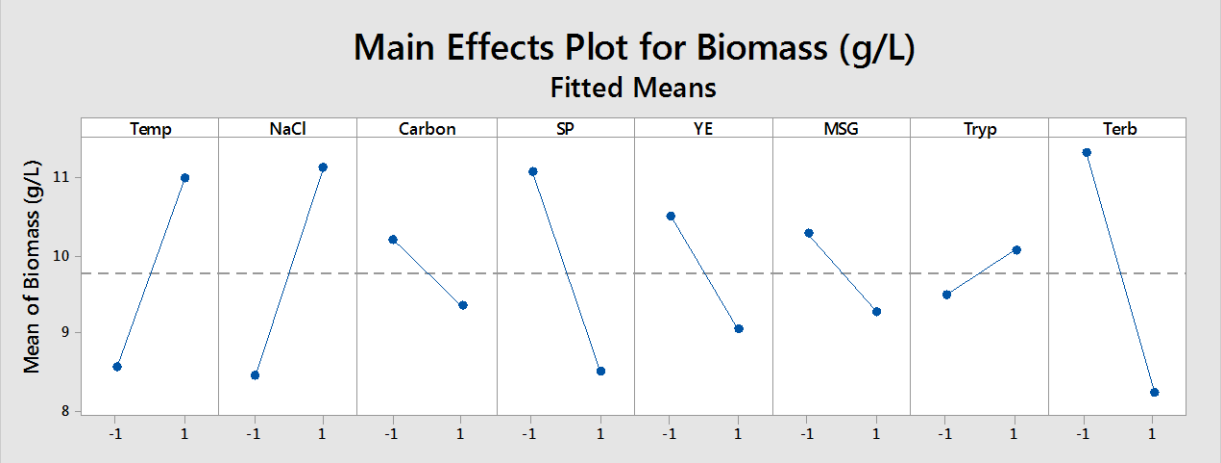

Figure 4.18. Main effects plot for biomass for no significant and non-significant media factors for biomass response.

The main effects plot for squalene concentration and squalene content are represented in Figure 4.19 and Figure 4.20 respectively. The major significant positive effects are carbon source and NaCl. As previously discussed, NaCl is an obligate growth component for marine thraustochytrids. The effect of NaCl is greater with respect to the concentration than content, however, the concentration is a term which connects both the biomass production and squalene content. The impact of carbon source on squalene was the most significant of all the components in the medias. The 'low' carbon source referred to glucose and the 'high' carbon source referred to glycerol. Both sources were added to each flask in the same concentration (30 g L^{-1}) so it could be determined whether a difference between the two exists. Glycerol has been used as major carbon source in a number of studies where the focus was to improve the yields for both terpenoids and PUFA production (Chang et al., 2013; Gupta et al., 2013). Glycerol has been used successfully as a primary carbon source in work by Scott et al. (2011), in which strain T18 (the same biomass control strain used in the current study), produced high levels of biomass, oil and

PUFA using glycerol as a sole carbon source. Additionally, a study on *Aurantiochytrium limacinum* SR21 by Li et al. (2015), showed that glycerol is able to promote the accumulation of DHA better than glucose during nitrogen limited growth. Gupta et el. (2013) was able to increase carotenoid content in several newly isolated thraustochytrid strains by using glycerol to replace of glucose.

There have been preliminary results that support the fact that glycerol may be able to enhance the production of secondary metabolites leading to increased terpenoids (such as squalene), carotenoids and DHA (Chen et al., 2016). The results of this study, performed on *Schizochytrium sp*. S056, showed that glycerol promoted the biosynthesis of secondary metabolites in thraustochytrids primarily through enhancing the glycolytic activity and the production of NADH. This study suggests that there was up-regulation of transcription level genes which occurs in both the glycolysis pathway and the acyl-CoA pathway. The genes are as follows; bkdA1 which codes for 2-oxoisovalerate dehydrogenase, pyk gene which codes for pyruvate kinase and finally the gapA gene coding for glyceraldehyde-3-phosphate dehydrogenase. The pyk and gapA are genes within glycolytic biosynthetic pathway which catalyze two irreversible reactions which lead to the synthesis of phosphoenol pyruvate and glycerate-3-phosphate. The increase in these secondary metabolites when cultured with glycerol increases the glycolysis metabolism and associated genes are significantly up-regulated which results in increased acetyl-CoA production. Additional to this, increased acetyl-CoA was made available by the downregulation in genes involved in glycogenesis and the citric acid cycle. The impact of glycerol on squalene content can also be seen when ranking the both the content and concentration of each media. The 6 types of media which contain glycerol as the sole carbon source outperformed every media which contained glucose as the sole carbon source.

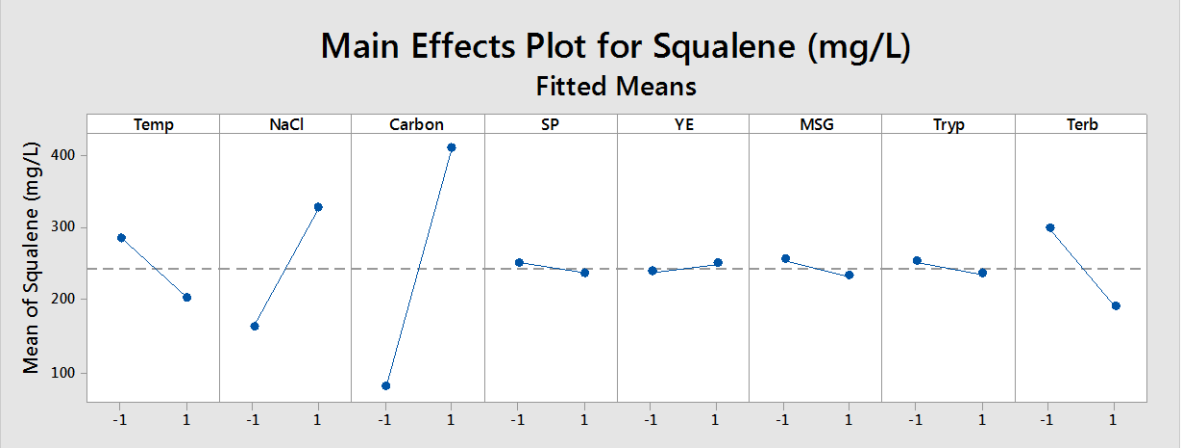

Figure 4.19. Main effects plot for biomass for no significant and non-significant media factors for squalene concentration response.

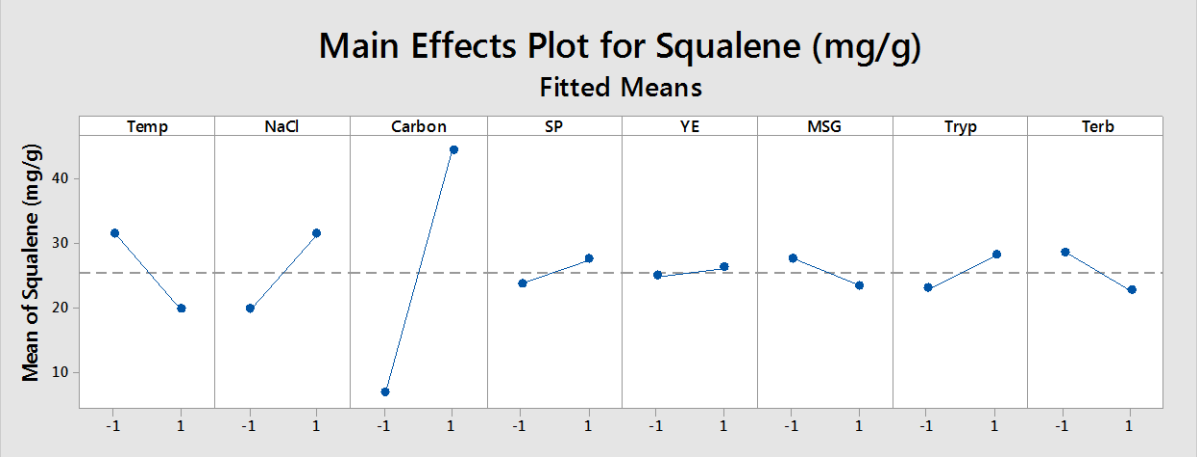

Figure 4.20. Main effects plot for biomass for no significant and non-significant media factors for squalene content response.

Strain 90 reached a maximum squalene content of 63.94 mg g^{-1} (media 7) and a maximum squalene concentration of 665.96 mg L^{-1} (media 3). Both values are greater than what was produced by the squalene control strain (*A. Tsukuba-3)* throughout secondary and tertiary screening. The highest reported squalene content and concentration achieved by control strain in the tertiary screening was 11.23 mg g^{-1} and 50.57 mg L^{-1}. However, the control strain had performed better in the prior media screening experiments reaching a peak squalene content of 50.72 mg g^{-1} in WDL20 media (Figure 4.3). This value is lower, but more comparable to strain 90 when cultured in medias 3 and 7. Table 4.9 lists known squalene producing thraustochytrids

and their corresponding squalene values. The most notable are *Aurantiochytrium* Yonex5-1, *Schiziochytrium mangrovei* PQ6 *and Aurantiochytrium sp*. 18W -13a, which produced 1073.8 mg L^{-1}, 1019.0 mg L^{-1} and 863.31 mg $^{L-1}$ respectively (Hoang et al., 2014; Nakazawa et al., 2014). Strain 18W-13a is the same squalene strain used in this study just under a different identification (*A. Tsukuba-3*). In the current work, squalene values that were reported in the Nakazawa et al. (2014) studies were not able to be replicated. The *Schiziochytrium mangrovei* PQ6 strain which produced a squalene concentration of 1019.0 mg L^{-1}, cannot be compared directly to the results obtained for strain 90, as that work was done in a 30 L bioreactor and was not a flask level study. Given the preliminary media screening, it is clear that strain 90 is a natural high producer of squalene and with further optimization of its culture conditions may be able to increase values in terms of squalene content and biomass. It is important to additionally note that the strains identified in Table 4.9 are all novel strains that have not undergone genetic modification. Genetic modification of a yeast strains by different biotechnology groups such as Amco Corporations and the Korean Research Institute of Bioscience and Biotechnology have been able to improve the production of squalene to 159.9 mg g^{-1} and 143 mg g^{-1} (DCW) respectively. Squalene contents of this magnitude have never been reported in novel thraustochytrid strains (Alejandro & Laurentano, 2009; Grünig et al., 1992)

Table 4.9. Published thraustochytrid squalene producing strains and their highest recorded squalene content or concentration.

Strain Name	**Squalene**	**Reference**
Schiziochytrium mangrovei	0.162 mg g^{-1}	(Jiang et al., 2004)
Aurantiochytrium sp. BR-MP4-A1	5.9 mg L^{-1}	(Chen et al., 2010)
Aurantiochytrium sp. 18W -13a	863.31 mg L^{-1}	(Nakazawa et al., 2014)
Aurantiochytrium sp. Yonez5-1	1073.8 mg L^{-1}	(Nakazawa et al., 2014)
Schiziochytrium mangrovei PQ6	1019.0 mg L^{-1}	(Hoang et al., 2014)

It is also interesting to note that in addition to the promising squalene concentrations achieved through the Plackett-Burman screening, there was a gradual accumulation of squalene throughout the entire culture time in most of the different media. In sections 3.8, the trend of increasing and peaking squalene during exponential phase followed by a steady decline as the culture aged, was noted in all strains. The phenomena has also been noted in work by Jiang et al. (2004), Kaya et al. (2011), Fan et al. (2010) and Li et al. (2009). It is possible that the terbinafine which was added to the media aided in this successful accumulation over a culture time of 120 hour. However, Fan et al. (2010) also utilized terbinafine and still noted the same decreasing trend after exponential growth. It is possible that there is an interaction in the media combinations which is unable to be detected by Plackett-Burman design which is contributing to this result. The increasing trend of squalene accumulation is noted most predominately in the flask where glycerol is the carbon source. This may have been due to a combination of the previously discussed positive impact of glycerol and terbinafine on terpenoid biosynthesis that could have resulted in this outcome.

4.9 Genetic identification and phylogeny

MEGAX software was used to phylogenetically reconstruct the aligned thraustochytrid-derived 18s rRNA sequences and placed strain 90 (referred to as LP90 in Figure 4.21) in the *Aurantiochytrium* clade. Bootstrap values of 100% supported the placement of strain 90 in the *Aurantiochytrium* clade. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1668 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.

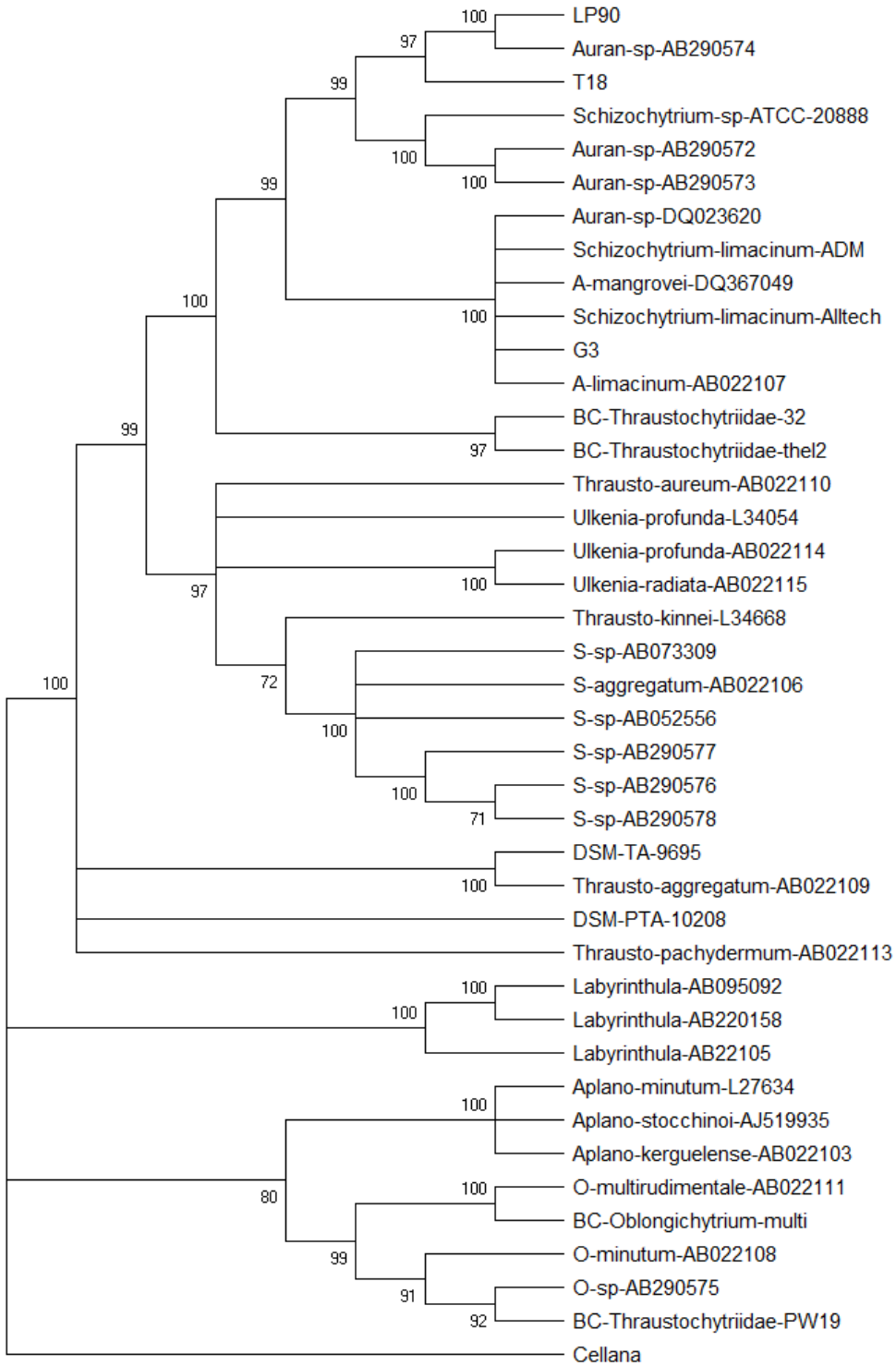

Figure 4.21. Rooted phylogenetic tree established from 18s rRNA sequences retrieved from NCBI's GenBank as well as the 18s rRNA sequence from strain 90. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible mode to create the bootstrap consensus tree.

Chapter 5 : Conclusions and Future Work

The overall goal of this study was to explore squalene production from marine microorganisms by isolating and screening suitable strains for media development and potential large-scale industrial processing. Through this work, the following outcomes relating to the specific objectives, have been achieved:

1. A total of 406 strains (the majority identified as thraustochytrids) were isolated from Canadian marine environments, where three strains (90, 171 and 286) showed the potential to produce squalene in meaningful amounts.
2. A high throughput method for screening strains was established, which involved selecting a screening media for secondary/tertiary screening and developing a protocol for extracting squalene from thraustochytrids.
3. The best strain for squalene production was identified (*Aurantiochytrium* strain 90); screening experiments indicated that it performed comparably to other high squalene producers in the literature.
4. A culture media was developed using Plackett-Burman design to promote squalene production in the best strain (*Aurantiochytrium* strain 90). Important media and culturing factors which promote squalene production were found to be glycerol, NaCl and temperature. Glycerol had the greatest positive impact on squalene production and may interact with other media components to promote the sustain accumulation of squalene past the exponential growth of biomass.

There is potential for future work on strain 90 to improve culture conditions to promote higher biomass and squalene production. Now that important media and growth factors have

been identified by means of Plackett-Burman design, a smaller factorial design of experiments could be performed in order to optimize the media conditions and identify significant interactions between factors. This information would result in a base media that could be used and further developed in bioreactor studies to test the feasibility and fitness of strain 90 in fermentations conducted at a larger scale. Using a bioreactor would also allow a greater degree of process control, which could lead to higher levels of squalene production. It is possible that operating a bioreactor in semi-continuous mode could be beneficial for producing squalene from this strain. In a semi-continuous fermentation, culture broth is harvested and replenished with balanced media when the target compound reaches a maximum level of production. Since squalene has demonstrated a tendency to decrease after a certain point of biomass growth, a semi-continuous operating strategy could be used to extend the period that squalene is produced at maximum levels.

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Appendix

Table A 1. Squalene values used to determine the best media for screening experiments.

Media	SQ ($\mu g\ g^{-1}$)	Avg ($mg\ g^{-1}$)
WDL20	46384.84	50.72
WDL20	44379.36	
WDL20	51345.18	
WDL20	53724.97	
WDL20	44668.47	
WDL20	44287.55	
WDL20	63212.03	
WDL20	57795.62	
WDL60	22010.8	48.09
WDL60	25190.06	
WDL60	38662.58	
WDL60	53536.23	
WDL60	60086.58	
WDL60	58484.85	
WDL60	62987.99	
WDL60	63781.39	
SQM20	6540.36	8.28
SQM20	6401.99	
SQM20	9686.47	
SQM20	8820.83	
SQM20	9059.7	
SQM20	10500	
SQM20	8098.5	
SQM20	7123.89	

Table A 2. Raw HPLC data from squalene extraction experiments (n=3).

Sample name	Sample Mass (g)	[HPLC] (ug mL^{-1})	Squalene (ug g^{-1})	Squalene (mg g^{-1})
A-1-1	0.0641	2683.0	41856.5	41.86
A-1-2	0.0526	2190.0	41635.0	41.63
A-1-3	0.0550	2361.0	42927.3	42.93
A-2-1	0.0552	2321.0	42047.1	42.05
A-2-2	0.0578	2463.0	42612.5	42.61
A-2-3	0.0562	2414.0	42953.7	42.95
A-3-1	0.0533	2096.0	39324.6	39.32
A-3-2	0.0543	1917.0	35303.9	35.30
A-3-3	0.0551	1946.0	35317.6	35.32
B-1-1	0.0516	1503.0	29127.9	29.13
B-1-2	0.0569	1614.0	28365.6	28.37
B-1-3	0.0601	1734.0	28851.9	28.85
B-2-1	0.0533	1636.0	30694.2	30.69
B-2-2	0.0522	1379.0	26417.6	26.42
B-2-3	0.0479	1344.0	28058.5	28.06
B-3-1	0.0490	1027.0	20959.2	20.96
B-3-2	0.0569	1140.0	20035.1	20.04
B-3-3	0.0501	1081.0	21576.8	21.58
C-1-1	0.0677	1504.0	22215.7	22.22
C-1-2	0.0655	1443.0	22030.5	22.03
C-1-3	0.0608	1351.0	22220.4	22.22
C-2-1	0.0479	1013.0	21148.2	21.15
C-2-2	0.0525	1087.0	20704.8	20.70
C-2-3	0.0610	1319.0	21623.0	21.62
C-3-1	0.0490	1027.0	20959.2	20.96
C-3-2	0.0569	1140.0	20035.1	20.04
C-3-3	0.0501	1081.0	21576.8	21.58

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	64.99	32.495	16.09	0.004
Error	6	12.12	2.019		
Total	8	77.11			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.42099	84.29%	79.05%	64.65%

Means

Factor	N	Mean	StDev	95% CI
Treatment 1A	3	42.140	0.691	(40.132, 44.147)
Treatment 2A	3	42.538	0.458	(40.530, 44.545)
Treatment 3A	3	36.65	2.32	(34.64, 38.66)

Pooled StDev = 1.42099

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Treatment 2A	3	42.538	A
Treatment 1A	3	42.140	A
Treatment 3A	3	36.65	B

Means that do not share a letter are significantly different.

Figure A 1. ANOVA and Tukey test obtained from Minitab18, analyzing Biomass A against treatments 1, 2, and 3.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	119701314	59850657	33.22	0.001
Error	6	10811476	1801913		
Total	8	130512789			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1342.35	91.72%	88.95%	81.36%

Means

Factor	N	Mean	StDev	95% CI
Treatment 1B	3	28782	386	(26885, 30678)
Treatment 2B	3	28390	2157	(26494, 30286)
Treatment 3B	3	20857	776	(18961, 22753)

Pooled StDev = 1342.35

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Treatment 1B	3	28782	A
Treatment 2B	3	28390	A
Treatment 3B	3	20857	B

Means that do not share a letter are significantly different.

Figure A 2. ANOVA and Tukey test obtained from Minitab18, analyzing Biomass B against treatments 1, 2, and 3.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	2770751	1385376	5.04	0.052
Error	6	1649204	274867		
Total	8	4419955			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
524.278	62.69%	50.25%	16.05%

Means

Factor	N	Mean	StDev	95% CI
Treatment 1C	3	22155.5	108.3	(21414.9, 22896.2)
Treatment 2C	3	21159	459	(20418, 21899)
Treatment 3C	3	20857	776	(20116, 21598)

Pooled StDev = 524.278

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Treatment 1C	3	22155.5	A
Treatment 2C	3	21159	A
Treatment 3C	3	20857	A

Means that do not share a letter are significantly different.

Figure A 3. ANOVA and Tukey test obtained from Minitab18, analyzing Biomass C against treatments 1, 2, and 3.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	1110	555.0	5.18	0.024
Error	12	1285	107.0		
Total	14	2395			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
10.3465	46.36%	37.42%	16.18%

Means

Factor	N	Mean	StDev	95% CI
Treatment 1-A	5	14.23	12.26	(4.15, 24.31)
Treatment 2-A	5	14.08	12.47	(4.00, 24.16)
Treatmeat 3-A	5	32.41	3.94	(22.32, 42.49)

Pooled StDev = 10.3465

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Treatmeat 3-A	5	32.41	A
Treatment 1-A	5	14.23	B
Treatment 2-A	5	14.08	B

Figure A 4. ANOVA and Tukey test obtained from Minitab18, analyzing Biomass A against treatments 1, 2, and *3*.

Table A 3. Formal strain identification and abbreviated strain identification number for top 39 strains.

Strain ID	Strain Name
86	**MD-26A-3**
87	**MD-37A-3**
88	**MD-37A-4**
89	**MD-37B-1**
90	**MD-24A-8**
91	**MD-24A-5**
93	**MD-34B-6**
94	**MD-42A-3**
159	**MB-13A-3**
160	**MB-15B-2**
164	**MD-5A-1**
165	**MD-6A-2**
166	**MD-8B-1**
167	**MD-8B-2**
168	**MD-11A-2**

170	**MD-26A-2**
171	**MD-43A-7**
172	**MD-43A-8**
173	**MD-43B-2**
174	**MD-44A-5**
175	**MD-44A-8**
176	**MD-48B-10**
177	**MD-42A-8**
178	**MD-34B-5**
219	**MD 5A-3**
220	**MD-6A-1**
223	**MD-11A-1**
237	**MD-18A-1**
257	**MD-26A-1**
276	**MD-43B-1**
286	**MD-44A-7**
313	**MD-48A-7**
318	**MD-48B-7**
320	**MD-48B-9**
355	**MD-26B-5**
381	**MD-34A-5**
382	**MD-34A-6**
386	**MD-40B-3**
392	**MD-42A-5**

Table A 4. Averaged data from tertiary screening of strains 90, 94, 171, 286, 170 and squalene control strain.

90

Time (h)	Avg Glu (g/L)	Avg Biomass (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	18.40								
46	11.05	3.087	14.85	44.29	0.43	1.63	0.63288	3.65131	2.891
66	5.13	6.003	4.04	23.04	0.45	1.45	0.62389	2.90996	14.005
96	0.67	7.510	1.87	13.87	0.42	1.10	0.34699	0.96205	6.497

94

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	18.40								
46	12.73	2.453	10.76	26.33	0.43	0.15	0.1159	1.0645	1.3304
66	8.07	4.863	3.76	18.29	0.47	0.42	0.25403	0.5393	3.0517
96	4.71	5.993	3.16	18.92	0.44	0.73	0.13317	0.4095	2.011
114	1.20	6.523	2.53	16.35	0.38	0.79	0.50501	0.4808	2.1068

171

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	18.40								
46	11.83	2.913	17.12	49.20	0.46	1.34	0.25106	4.6662	9.3591
66	8.73	4.650	7.15	33.02	0.49	1.23	0.3985	0.8530	1.3970
96	6.09	5.273	7.06	37.16	0.43	0.97	0.15044	0.9817	4.4571
114	0.78	5.487	4.68	25.63	0.31	0.91	0.19035	0.4693	1.6938

286

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	18.40								
46	11.23	2.707	17.68	48.05	0.38	0.81	0.29687	1.1408	8.0590
66	7.78	5.090	7.38	37.31	0.48	0.48	0.36097	1.1624	3.8548
96	5.39	6.097	6.77	41.21	0.47	0.53	0.26006	0.2602	0.7513

114	4.43	5.747	5.47	31.27	0.41	0.74	0.41741	0.7047	2.0838
138	3.69	6.330	5.90	37.34	0.43	0.72	0.11	0.7243	4.7549

170									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	18.40								
46	12.97	2.410	9.89	23.80	0.44	0.40	0.76374	0.3886	7.3930
66	7.72	5.150	4.42	22.32	0.48	1.20	0.3985	2.1254	9.6580
96	3.68	6.280	2.21	13.79	0.43	0.96	0.3747	0.3565	1.5219
114	3.77	6.540	2.69	17.58	0.45	0.79	0.4613	1.0816	7.3075
138	0.00	6.983	2.25	15.78	0.38	0.00	0.26006	0.6293	4.6740

A. *Tsukuba-3*									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	18.40								
46	12.93	2.183	6.77	14.98	0.40	0.46	0.21595	1.5216	4.8767
66	7.66	4.503	11.23	50.57	0.42	0.36	0.48387	0.7401	6.4969
96	5.00	5.410	6.83	36.97	0.40	0.15	0.06083	0.9122	5.0761
114	4.01	5.060	6.30	31.82	0.35	0.25	0.08888	0.8177	3.6843
138	3.53	4.843	5.48	26.71	0.33	0.34	0.25146	1.19287	7.2413

Table A 5. Plackett-Burman raw averaged data from medias 10-12 grown using strain 90 (n=3).

Media 1									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	31.81	0.00				0.70			
20	27.66	4.67	0.49	2.40	1.30	2.54	0.49	0.45	2.31
44	26.88	5.43	7.11	39.55	1.15	0.50	0.85	1.73	15.75
68	22.02	6.43	20.36	130.67	0.66	1.94	0.55	1.83	12.01
94	18.26	8.87	26.67	234.75	0.66	1.50	0.74	3.48	12.36
120	9.82	8.57	30.09	257.29	0.39	0.83	0.42	3.95	31.24

Media 2									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	27.43	0.00				0.27			

20	24.63	5.00	0.14	0.73	1.86	0.70	1.01	0.24	1.27
44	18.67	5.53	2.70	14.81	0.56	1.62	0.35	2.31	12.26
68	15.83	8.53	5.38	46.33	0.68	0.57	0.32	1.95	18.75
94	10.56	10.07	5.76	58.71	0.56	0.84	0.93	1.24	18.14
120	5.58	11.17	10.15	111.17	0.48	1.34	2.06	2.30	19.76
Media 3									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	33.63	0.00				0.70			
26	27.93	6.29	0.89	5.65	1.18	0.73	0.52	0.32	2.15
48	21.37	10.05	18.40	183.65	0.82	2.29	2.45	6.27	79.84
72	17.50	10.48	32.41	336.53	0.64	1.41	2.52	1.81	65.44
96	13.01	11.24	47.88	534.66	0.54	1.21	1.62	3.64	48.53
120	11.55	11.48	58.37	665.96	0.52	1.61	1.84	6.55	102.88
Media 4									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	30.60	0.00				0.59			
20	28.07	5.00	0.00	0.00	2.37	0.96	0.44	0.00	0.00
44	28.15	5.97	3.83	24.21	4.14	1.90	1.25	1.92	14.62
68	18.07	8.30	12.26	103.17	0.77	6.02	0.95	2.47	30.32
94	15.64	9.37	22.96	214.13	0.63	1.74	0.95	2.07	16.02
120	6.27	10.77	28.59	309.91	0.44	3.83	1.60	1.98	66.88
Media 5									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	27.05	0.00				0.17			
20	26.00	5.90	0.55	3.29	5.79	0.35	0.26	0.32	1.97
44	19.13	6.10	7.08	43.89	0.77	0.42	0.35	4.10	27.10
68	14.80	7.93	14.95	118.77	0.65	0.44	0.58	0.94	13.51
94	10.80	10.87	10.72	116.47	0.67	0.62	0.06	1.37	14.70
120	7.07	12.83	10.73	137.08	0.64	1.06	0.64	1.75	16.21
Media 6									
Time (h)	**Avg Glu (g/L)**	**Avg X (g/L)**	**Avg SQ (mg/g)**	**Avg SQ Yield (mg/L)**	**Avg X Yield (g/g)**	**Glu St Dev**	**Biomass StDev**	**SQ St. Dev**	**SQ Yield St. Dev**
0	31.69	0.00				2.42			
20	29.80	5.03	0.10	0.52	2.83	1.97	0.21	0.05	0.25
44	28.52	5.60	5.33	30.13	3.71	4.66	0.26	3.21	18.31
68	24.53	8.47	14.88	129.74	1.25	4.98	1.82	5.05	60.13

94	18.74	8.80	26.50	235.14	0.69	5.02	0.95	5.89	65.65
120	8.80	10.47	34.01	357.81	0.46	4.37	0.87	3.24	62.72

Media 7

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	31.07	0.00				1.23			
26	31.20	4.77	0.65	3.21	-11.12	0.28	0.68	0.29	1.65
48	21.31	7.97	22.09	178.57	0.82	0.98	0.99	4.61	57.69
72	18.87	6.40	43.36	288.77	0.52	1.37	1.35	12.76	138.88
96	13.30	7.47	51.68	387.12	0.42	2.63	0.92	2.06	62.01
120	11.93	8.90	63.94	567.23	0.47	3.12	0.66	4.13	6.62

Media 8

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	31.99					0.58			
26	30.83	3.37	0.47	1.57	15.85	0.62	1.23	0.14	0.87
48	22.68	4.70	19.34	88.03	0.51	1.44	0.44	10.17	42.20
72	21.48	5.13	38.36	196.84	0.50	2.63	0.06	5.99	29.97
96	18.61	5.37	41.25	221.07	0.42	2.90	0.45	7.55	43.01
120	23.40	5.90	50.12	293.07	0.73	2.25	0.46	9.15	34.46

Media 9

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	30.34	0.00				0.34			
26	30.63	3.00	0.00	0.00		0.42	0.78	0.00	0.00
48	26.47	4.40	0.00	0.00	1.13	0.25	0.87	0.00	0.00
72	27.53	3.40	0.00	0.00	1.24	0.40	0.61	0.00	0.00
96	27.23	1.57	0.00	0.00	0.50	0.76	0.42	0.00	0.00
120	26.63	1.33	0.00	0.00	0.35	1.06	0.49	0.00	0.00

Media 10

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	27.66	#DIV/0!	#DIV/0!	#DIV/0!		0.28	#DIV/0!	#DIV/0!	#DIV/0!
20	26.23	5.47	2.01	11.10	5.50	0.95	0.35	0.71	4.25

44	18.80	6.10	11.75	72.08	0.69	0.62	0.52	1.29	13.36
68	14.07	9.40	4.89	45.97	0.67	0.59	0.89	0.37	5.83
94	10.40	11.20	3.41	38.39	0.66	0.80	0.70	0.66	9.33
120	7.38	12.23	3.29	40.27	0.60	0.72	0.64	0.19	2.75

Media 11

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	30.30	0.00				0.40			
26	28.47	5.17	0.09	0.43	3.45	0.68	0.70	0.16	0.74
48	23.67	7.00	6.78	47.82	1.17	2.72	0.36	1.61	13.66
72	18.77	8.63	14.20	122.65	0.75	0.06	0.60	0.77	12.02
96	12.53	8.10	9.62	76.77	0.46	1.19	1.51	1.22	5.29
120	6.77	11.93	10.02	118.70	0.51	1.40	1.24	1.06	0.72

Media 12

Time (h)	Avg Glu (g/L)	Avg X (g/L)	Avg SQ (mg/g)	Avg SQ Yield (mg/L)	Avg X Yield (g/g)	Glu St Dev	Biomass StDev	SQ St. Dev	SQ Yield St. Dev
0	29.47	0.00				1.10			
26	27.70	5.33	1.63	8.68	9.37	0.46	0.60	0.10	0.76
48	23.33	8.07	11.29	90.92	1.36	0.91	0.97	0.69	10.13
72	16.43	9.63	10.51	101.33	0.75	0.93	0.49	1.14	13.29
96	10.87	10.57	5.67	59.84	0.57	0.80	0.55	0.47	3.77
120	6.15	11.73	5.41	63.79	0.51	0.91	0.55	0.95	13.75