Method and Evaluation Thereof of Yeast Extract Separation on the Growth of Microalgae

by

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Abstract

Microalgae grow heterotrophically and produce high value pigments, nutraceuticals, pharmaceuticals, feeds, and biofuels. The growth rate, nutrient medium, growth density, and oil content strongly affect the economics of heterotrophic microalgae fermentation systems. This thesis explores the growth promoting properties of yeast extract, a complex media component, on the growth of *Auxenochlorella protothecoides* by solvent separation of yeast extract.

Complete yeast extract was found to increase the growth rate of *A. protothecoides*, but the effect saturates above initial media concentrations of 4 g/L. Yeast extract partitions in several solvents. In methanol, the yeast extract partially dissolves. The solid portion boosts the initial growth rate of *A. protothecoides* above that of complete yeast extract by 149.4% in cell count and 24.7% in dry weight. The isopropanol/water separation is a liquid/liquid separation and produces a denser extract fraction (IT) that promotes the initial cell count of *A. protothecoides* by 55.6% above the maximal effect of yeast extract.

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Table of Contents

List of Tables

List of Figures

1 Introduction

Microalgae can use light energy, chemical energy, or a mix of the two and store a portion of this energy as usable and valuable products. The biodiversity of the microalgae cannot be understated—the genetic difference covered in the spectrum of algae is vast. Microalgae growth can be quantified using a number of direct and indirect methods. The key parameters that can be used to compare the growth properties between microalgae are respiration, carbon dioxide evolution rate, oxygen uptake rate, specific growth rate, doubling time, biomass yield, and product yield (where the algae are being used to create a specific compound, such as starch). The growth rate, nutrient medium, growth density, and oil content have been shown to strongly affect the algal fermenter economics[1].

This thesis will explore the growth properties of a complex medium component, yeast extract, and its subfractions on microalgae. More specifically, the initial growth promotion of the yeast extract and its subfractions will be evaluated by comparing the dry weights, cell counts, and/or optical densities of the microalgae *Auxenochlorella protothecoides.* Yeast extract is a complex mixture of compounds in a yeast cell. The components in yeast extract will be partitioned with various solvents and the differences between the partitions will be confirmed with measuring physical characteristics, such as differential scanning calorimetry. The growth properties of microalgae will also be tested with supplementation with the partitions or fractions of yeast extract. The solvent properties and other thermodynamic considerations will govern solvent selection. Finally, the very initial growth effects of the refined yeast extract on the growth metrics of microalgae will be evaluated.

2 Background

2.1 Microalgae

Fossil fuels represented 81.3 % of the 514 ZJ world total primary energy supply in 2008[2], with energy demand expected to increase to 742 ZJ by 2030[3]. The energy supplied from non-renewable resources must eventually be substituted with energy derived from renewable sources. Microalgae have the potential to completely replace conventional petroleum-based liquid fuels[4, 5] (from diesel to jet fuel) with an algal-based, fungible fuel. Microalgae have much higher growth rates and oil productivity compared to agricultural crops, and they do not require arable land. Most algae can grow almost anywhere autotrophically and only require sunlight and simple nutrients in photo bioreactors[6, 7]. Photo-bioreactors can be open or closed to the environment, with open reactors requiring ambient conditions favourable to growth[8]. Photobioreactors are less favourable in Alberta due to the ambient winter temperatures; however heterotrophic growth of algae in closed bioreactors for oil production remains a viable year-round option. Many studies have shown the technical feasibility of heterotrophic[1, 9-23] and autotrophic[4, 6, 8, 24-30] algal growth for oil production. These studies have focused on the growth rate, nutrient medium, growth density, and oil content which have been shown to strongly affect the economics of algal oil production in fermenters[1]. Low yields of algae biomass obtained in large-scale reactors, coupled with the high capital cost necessary have resulted in a formidable cost of production for algae, regardless of their end use[7]. Slight changes in feeding strategies have been shown to significantly affect the lipid content of algae during heterotrophic growth[31].

Algal oil can be converted into a variety of liquid fuels. Basha et al. completed a review of 130 scientists up to 2008 for the conversion of agricultural crop oil to biodiesel[32]. Algal oil can be converted into ASTM 7566 compliant jet fuel with product, mass yields between 77% and 94%[33-37]. A detailed review of the diversity, applications, and means of culture of algal species is given by de la Hoz Siegler[38]. This thesis will focus on the microalgae species *Auxenochlorella protothecoides*

which has been shown to have achieved high oil content (up to 50 % lipid), high biomass concentrations (up to 120 g/L), and maximum growth rates (up to 0.04 1/h) [38].

2.1.1 *Auxenochlorella protothecoides*

Chlorella sp. are spherical or ellipsoidal cells with a simple life cycle and nutritional requirements. They reproduce asexually, with each mature cell dividing to produce 2, 4, 8, or 16 autospores. The composition of Chlorella is highly variable, with lipid content reported from 4.5 to 85.6 % of the dry-weight[7]. The subgenus *Auxenochlorella protothecoides* was proposed by Krauss and Shihira on the basis of its requirement for thiamine[39]. A picture of *Auxenochlorella protothecoides* under x400 light microscope magnification is shown in Figure 1.

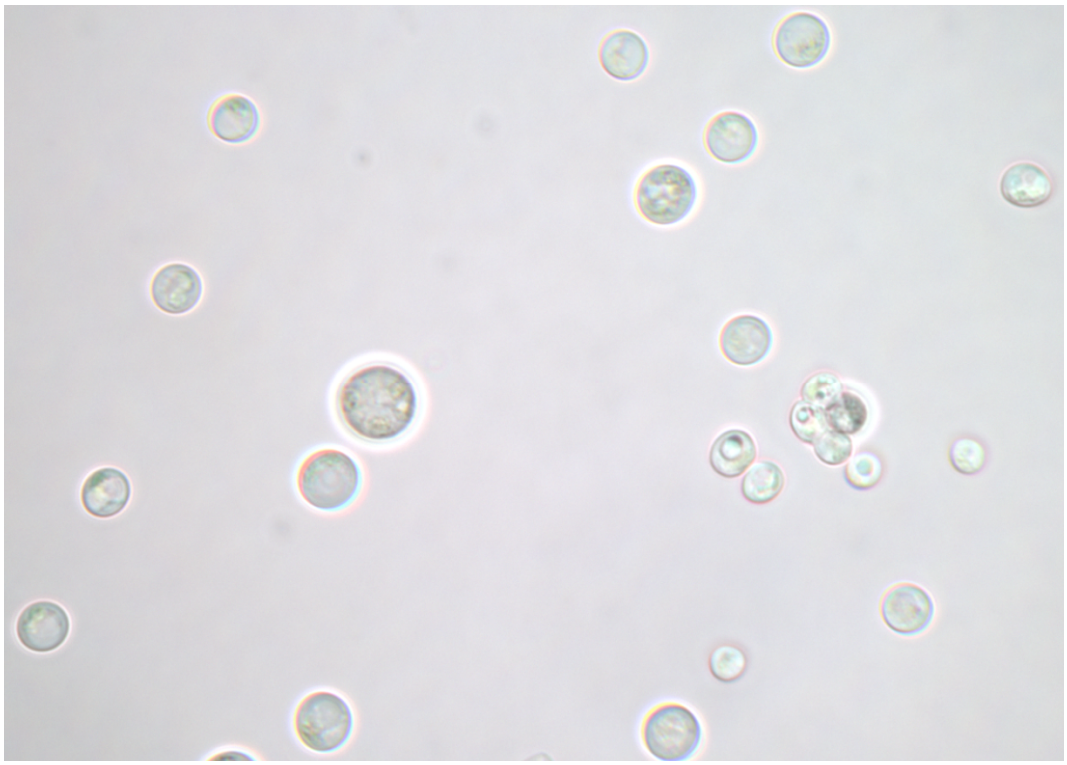


Figure 1. *Auxenochlorella protothecoides* observed under light microscope, x400 magnification.

2.2 Heterotrophic algal growth

Microbial growth media can be defined or complex. Defined media is prepared from purified compounds such that all constituents' identity and concentration are known. Complex media is often made using a mixture of purified compounds and biological mixtures, such as soil, yeast extract, or digests of organic material. Microalgae generally require a variety of macronutrients, micronutrients, and vitamins.

2.2.1 Carbon source

Energy for heterotrophic microbial growth is typically derived from the carbon source. Samejima *et al.* (1958) found that glucose, galactose, and acetate supported *Chlorella pyrenoidosa* in heterotrophic conditions whereas arabinose, xylose, cellobiose, glycerol, mannitol, i-inositol, mannose, fructose, maltose, sucrose, and lactose did not[40]. However, Gao *et al.* (2010) reported that heterotrophic growth of *A. protothecoides* on fructose resulted in a higher biomass than growth on the same concentration of glucose, with similar lipid contents in the cells[41], which demonstrates the change in biomass yield for different substrates. Heredia-Arroyo *et al.* found that *A. protothecoides* can grow on glycerol, acetate, and glucose[42]. Algae can utilize both a chemical and a light source of energy simultaneously. Illumination accelerated the growth of *Chlorella vulgaris* significantly when grown with glucose as an energy/carbon source[40]. Many algae utilize a preferred substrate rather than utilize more than one form of chemical energy at a time. Addition of a second sugar did not result in faster growth[40]. Retovsky *et al.* found magnesium deficiency caused enlargement of *Chlorella vulgaris* cells[43] which suggests that the regulation systems for *Chlorella sp.* can be manipulated with extracellular media components. Richmond summarized that the lack of versatility in the use of organic substrates for *Chlorella sp.* result from restrictions on permeability of the organic substrate. This thesis will use glucose as the sole carbon source to limit the scope of the project. The biological efficacy of the produced compounds of yeast extract

may be different for mixotrophic, phototrophic, or heterotrophic growth using other carbon sources.

2.2.2 Nitrogen source

Nitrogen is an essential nutrient required for growth of algae[44]. Nitrogen-limiting growth is used to regulate algal cell growth and division by host species in symbiotic algae-invertebrate relationships[45]. High levels of certain nitrogen-containing compounds, such as ammonia, are toxic and inhibit cell growth[44]. A nitrogen-limited environment is required to achieve high lipid content in algal cells. Thomas *et al.* studied the nitrogen metabolism of *Scenedesmus obliquus*. He found that nitrogen deficiency (cellular nitrogen content of 20 mgN/gDW) results in drastically reduced growth rate[46]. The growth rate of cultures could be restored by addition of KNO_3 until the nitrogen level in the cells was 80 mgN/gDW[46]. It was found that in 12 hours, 10 % of cell nitrogen is lost when placed in nitrogen-deficient media, probably due to the shift from protein production to lipid production, with the nitrogen released into the media. Protein nitrogen represents 90 to 95 % of the total nitrogen in an algal cell[46] and nitrogen analysis is sometimes multiplied by an empirical factor to estimate protein content. Yung *et al.* found that illumination stimulates both nitrogen reduction of the media and lipid production in *Chlorella pyrenoidosa*. He also found that a media pH of 7.5 promotes maximum conversion of glucose to lipids because of the availability of bicarbonate ions[47]. Richardson *et al.* found that cellular nitrogen content must drop to 3 % of dry weight (30 mgN/g DW) before appreciable increase in lipid synthesis occurs in *Chlorella sorokiniana*. They found that no significant change in liqids content/amount in continuously cultured algae with changing nitrogen content. Richardson identified that potassium starvation may also increase lipid content[48]. Richmond concluded that dehydration of dewatered algal mass accounts for about 15 % of the overall production cost[7].

2.2.2.1 Nitrogen regulation

A. protothecoides has been shown to store nitrogen intracellularly. A sufficiently high nitrogen level inhibits the growth of algae. Thus in a single stage process, the algae must metabolize their intercellular nitrogen to a certain level before lipid synthesis is stimulated. While model-based control strategies that control the extracellular nitrogen concentration in a single-stage batch fed process have been used to increase the maximum oil content and oil productivity[13], the authors propose that extracellular mitotic stimulation will further enhance the oil productivity of heterotrophic algal growth processes. Spoehr and Milner found that when cell division stops, the energy content (lipid content) rises sharply[49]. While Spoehr and Milner used cell age and/or nitrogen depletion to stop cell division, a mitotic depressant could potentially perform the same function. In the hypothetical two stage process, mitotic stimulation is used in the first reactor to induce rapid cell division. The rapid cell division and cell growth utilizes the intercellular nitrogen and nitrogen present in the media. A mitotic depressant is added to the second reactor to induce lipid production by directing energy into lipid synthesis rather than cell division.

2.3 Yeast extract

Yeast (*Saccharomyces cerevisiae*) is used in ethanol fermentation, bread dough leavening, and as a source of yeast extract. Yeast extract is the water soluble portion of lysed yeast cells and is a critical component of many biological media formulations, including those used for microalgae growth.

Sherwood *et al.* found that several yeast extracts varied in their toxicity to two strains of soil bacteria, *Rhizobium trifolii*. The toxic factor was removed from Difco yeast extract powder by repeated extractions with methanol or aqueous ethanol (80 % v/v), but was insoluble in absolute ethanol, ether, benzene, or chloroform. A 20 % (w/v) YE was eluted in a Sephadex G-15 column and subsequent growth found two types of inhibitors: glycine and sodium. The glycine was the primary cause of

YE toxicity to *Rhizobium trifolii*; the toxicity is increased by monovalent cations and decreased by Calcium ions[50].

Davis *et al*. found that the source of yeast and method of autolysis did not influence the growth-promotion of *Streptococcus* and *Lactobacillus*[51]. A previously unidentified yeast extract factor that stimulates growth of *Streptococcus faecalis* was concentrated 1000 fold by extraction with aqueous acetone and subsequent treatment with charcoal and superfilrol by Hoffmann *et al*[52]. Hoffmann *et al.* found that supplementation of synthetic medium with extracts of yeast significantly shortened the lag phase and incubation time required for maximum growth of lactic acid bacteria[52], with 50 to 60 % of the growth promotion effect caused by uridine. Beinert *et al.* studied the coenzyme A content of different lots of yeast extract, which was found to vary largely[53]. Krauss *et al.* found sulfanilamide as an ineffective selective bacteriostatic agent for boosting xenic culture of *Scenedesmus sp.* because it also inhibited the microalgae[54]. Ikawa found that yeast extract was supplying growth stimulatory substances rather than additional absolute growth requirements, with the favorable effect of yeast extract addition being a pronounced reduction in the lag phase of algae. They found that more purified yeast extract preparations showed that the growth stimulation factors fell into two well-defined categories: nucleic acids and peptides. The growth promotion of nucleic acids is higher after hydrolysis with sodium hydroxide (which degrades the nucleic acids into nucleotides). They found that uridine, cystidine, uridylic acid, uridine-5-phosphate, and cytidylic acid exhibited activities of the order of 50 to 200 times that of yeast extract, but this high order of activity could not be consistently duplicated. Uridylic acid was the most active component tested, which gave half-maximal growth at 20 micrograms per mL, whereas YE required 300 to 500 micrograms per mL. Other unidentified stimulatory substances, among which some are most likely peptides, are undoubtedly present, which must contribute to the effects of yeast extract to explain its high activity relative to that of known compounds[55]. Grant *et al.* found the ash of yeast extract to be required for growth of the nematode-trapping fungus, *Arthrobotrys conoides*. The samples were

analysed for Al, Ba, Cu, Fe, Mg, Mn, Sr, Cd, Co, Cr, Ga, Mo, Ni, Pb, Sn, Ti, V, and Zn, and are shown in Table 1.

Table 1. Minor element composition of yeast extracts reproduced Grant *et al.*

Element	Minimum µg /g dry wt.	Maximum µg/g dry wt.	Average µg/g dry wt.
Al	2.1	3.8	3.1
Ba	1.0	1.7	1.3
Cd	1.2	2.0	1.5
Co	1.0	6.1	3.5
Cr	9.4	17.4	12.0
Fe	121	185	150
Ga	0.01	0.20	0.09
Mg	980	1580	1270
Mn	1.4	3.2	2.3
Mo	2.6	9.1	5.9
Ni	6.3	32.9	18.2
Pb	2.6	12	6.8
Sn	0.03	0.18	0.09
Sr	0.84	1.4	1.1
Ti	1.4	4.8	3.0
V	31.2	66.1	43.7
Zn	46.2	104.0	74.0

Smith *et al.* separated yeast extract into seven fractions on a Sephadex G-25 HPLC column. The fraction most stimulatory to *Streptococcus lactis* contained over 70 % of the free amino acids with purine, pyrimidine bases, and inorganic constituents also contributing to the stimulation. Complete yeast extract resulted in higher growth rates than any of the fractions. The yeast extract fraction could be simulated with a mixture of four nucleotide bases (adenine, guanine, uracil, and xanthine) and tryptone[56].

2.4 Yeast extract separation

Yeast extract is a common additive in microbiological media formations because it stimulates cell growth. Coming from an undefined source (autolyzed cells of *Saccharomyces cerevisiae*), yeast extract may also contain compounds that are toxic to microalgae. The component(s) which stimulate growth have not been identified. Previous studies on yeast extract's growth promotion have isolated and partially characterized fractions of yeast extract on the acidophilic thermophilic mycoplasma *Thermoplasma acidophilum*[57], though no previous work has been conducted using the methods within this thesis. Among several hundred compounds tested, only glutathione plus iron elicited growth response in *Thermoplasma acidophilum*[57].

Previous experience demonstrated qualitatively that sterile filtered yeast extract exhibits different growth stimulation than the same yeast extract when thermally sterilized with an autoclave. It was hypothesized that the difference in growth stimulation of yeast extract was due to either a stimulatory compound (YESC) or an inhibitory compound (YEID) that was destroyed upon autoclaving, or remained in the filter cake of the sterile filter. A process which isolates the active component of yeast extract could be protected by patent law whereas the addition of unaltered yeast extract cannot be protected. This subchapter details the separation and verification of the YESC and YEID fractions, with the methodology shown in Figure 2.

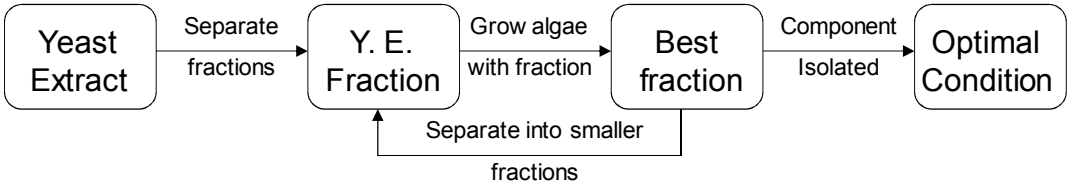


Figure 2. Overview of stimulant isolation process

Several methods exist to separate biological materials based on size, charge, reactivity, and solubility among other methods. This chapter deals with separation of yeast extract into two or more fractions by differential solubility in solvents.

2.4.1 Thermodynamic considerations solvent-based separation

In order for a separation method to be commercially viable, the solvent must be recovered for reuse. An established process of separating two miscible solvents with different volatilities is distillation. During distillation, the more volatile components of a mixture are evaporated from the bulk liquid and condensed in a concentrated form. Many solvent properties, including volatility, depend directly on the molecular weight. If there were no thermodynamic interactions between solvent molecules, the evaporation rate of the solvent would depend on the molecular weight of the solvent[58]. Thus, a low molecular weight solvent would require less energy to recover from a solution than a higher molecular weight solvent. The solvents were selected if they were miscible with water, had a lower boiling point than water, and were relatively non-toxic. Alcohols were chosen due to the relative ease in separation from water via distillation despite azeotrope formation occurring for some alcohols. A summary of the solvents as well as their properties is given in Table 2.

Table 2. Solvent selection: Solvent properties[59].

	Boiling Point b.p. (°C)	Heat of Vaporization H_v (kJ/mol)	Solubility* x_i
water	100	40.7	-
methanol	65	35.2	miscible
ethanol	43	38.6	miscible
1-propanol	97	41.4	miscible
2-propanol	83	39.9	miscible
1-butanol	118	43.3	.0184
2-butanol	99	40.8	.0295
2-methyl-2-propanol	82	39.1	miscible
acetone	56	29.1	miscible
***solubility refers to maximum mass fraction of solvent in water at 25 °C**			

First, a suitable single solvent was identified by testing the solubility of yeast extract. It was postulated that the single solvent method could be improved by using a water/solvent mixture using a cheaper, less-toxic, or more-effective solvent. To select the appropriate solvent/water mixtures, the Hansen Solubility Parameter was used. Following the selection of the solvent, the kinetics of dissolution is also important. In this study, the Noyes-Whitney theory of dissolution will be used. Differences in each of the final fractions were investigated using differential scanning calorimetry.

2.4.1.1 Hansen Solubility Parameter

The Hansen Solubility Parameter (HSP) is taken from Hansen and is based on the total cohesion energy[60]. The cohesion energy is partitioned into three forces that govern dissolution: non-polar atomic (dispersion) interactions, D; permanent dipole-permanent dipole interactions, P; and the hydrogen bonding molecular interaction, referred to as an electron exchange parameter, H[60].

$$E = E_D + E_P + E_H \quad (1)$$

The D, P, and H components of total cohesion energy, E, divided by the molar volume, V, give the Hansen Solubility Paramter:

$$E/_V = {E_D}/_V + {E_P}/_V + {E_H}/_V \quad (2)$$

$$\delta = \delta_D + \delta_P + \delta_H \quad (3)$$

The solubility parameter distance, R_a, relates likeness of a solvent (S) to dissolve a particular compound (X) as follows:

$$R_a = \sqrt{4\left(\delta_{D,S} - \delta_{D,\mathrm{X}}\right)^2 + \left(\delta_{P,\mathrm{S}} - \delta_{P,\mathrm{X}}\right)^2 + \left(\delta_{H,\mathrm{S}} - \delta_{H,\mathrm{X}}\right)^2} \quad (4)$$

Solubility parameters of solvent mixtures are weighted by volume fraction ($\theta_{V,i}$) of solvent(s):

$$\delta_{D,\mathrm{Mixture}} = \sum \theta_{V,i} \times \delta_{D,i} \quad (5)$$

$$\delta_{P,\mathrm{Mixture}} = \sum \theta_{V,i} \times \delta_{P,i} \quad (6)$$

$$\delta_{H,\mathrm{Mixture}} = \sum \theta_{V,i} \times \delta_{H,i} \quad (7)$$

2.4.1.2 Dissolution kinetics

Solid particle dissolution of a uniform particle size follows the Noyes-Whitney differential equation[61-63]. The Noyes-Whitney theory of dissolution implies that the dissolution behaviour for a single particle with a certain size can be extended to the dissolution of other particles with known sizes as well. The dissolution rate ($\frac{\mathrm{d}m}{\mathrm{d}t}$) is related to the surface area (S), diffusion coefficient (D), height of the boundary layer (h), mass concentration of yeast extract in the bulk phase (C_t), and the mass concentration of yeast extract on the surface (C_s):

$$-\frac{\mathrm{d}m}{\mathrm{d}t} = \frac{DS}{h}(C_s - C_t) \quad (8)$$

2.4.1.3 Differential scanning calorimeter

Previous work has examined whole organisms and parts of their cells using differential scanning calorimetry[64, 65]. A Differential Scanning Calorimeter (DSC) measures the heat flow of a sample required to reach a set temperature profile relative to a reference cell. Comparison of the yeast extract fractions with literature values for cell components using differential scanning calorimetry will provide insight into the mechanism of the yeast extract separation or reaction.

3 Standard Materials and Methods

3.1 Chemicals, solvent handling

All chemicals were obtained from Fisher Scientific Canada, were anhydrous, reagent grade or higher and were used as received. Deionized water was produced by Milli-Q Academic purification system with a water resistivity of 18.2 MΩ·cm. Weights were measured by an analytical balance with a maximum weight of 210 g and a 0.0001 g accuracy, Sartorius BL210S, certified to ISO9001. The solvent removal was accomplished under vacuum in a Büchi Rotavapor R-200 equipped with a Büchi Heating Bath B-490. The vacuum was produced from a 30 L KNF Neuberger vacuum pump. The residual solvent was removed under high vacuum using a Fisher Scientific Maxima C Plus vacuum pump with a maximum pressure of 0.0133 Pa (0.0001 torr). Solids were dried at 45 °C in a vessel, evacuated with the 30 L KNF Neuberger vacuum pump. The yeast extract factions were sent to the mass spectrometry laboratory in the Department of Earth and Atmospheric Sciences at the University of Alberta for analysis of the boron, phosphorous, manganese, iron, cobalt, copper, zinc, and molybdenum content.

3.2 Glassware cleaning

All glassware was initially washed with soap and hot water, and then placed in a self-cleaning Kenmore oven (550 °C) for four hours on self-clean to oxidize any trace organics bound to the glass. The glassware was then scrubbed with Sparkleen from Fisher Scientific and rinsed three times with deionized water.

3.3 Development of Growth Media

3.3.1 Thermal and size sterilization effect on yeast extract

The fraction identifiers for growth media prepared with thermal and size sterilization are given in Table 3. To further test if the yeast extract has a protein portion that is responsible for the growth promotion of yeast extract, a method for

determination of ruminal protein degradation of alfalfa using commercial protease was adapted for yeast extract[88].

Table 3. Growth media prepared with thermal and size sterilization.

Fraction Identifier	Description
YE+Protease 48h	Sterile filtered yeast extract, as received with sterile filtered protease incubated for 48 hours before sterilization to denature the protease.
Protease 48h	Sterile filtered protease incubated for 48 hours before sterilization to denature the protease. Added to HGM with yeast extract, as received added to the media before autoclave sterilization.
Protease 0h	Sterile filtered protease incubated for 0 hours before sterilization to denature the protease. Added to HGM with yeast extract, as received added to the media before autoclave sterilization.
YE	Sterile filtered yeast extract, as received.

3.3.2 Methanol Separation

For each methanol separation, a weighed amount of yeast extract was added to a 500 mL beaker with a stir bar. Methanol was measured with a graduated cylinder and was added to the beaker containing yeast extract. The heterogeneous mixture was stirred at 300 rpm. The total contact time of the solvent was measured from when the methanol was added to the yeast extract until the liquid level disappeared under the level of the filter cake. The mixture was vacuum filtered through Whatman No. 5 filter paper. The filter cake was dried in a vacuum oven and the methanol was removed with a rotary evaporator at 50 °C under vacuum in a tarred round bottom flask. The residual solvent was removed under high vacuum. The weight of the dried filter cake was measured. Table 4 contains a summary of the growth media prepared using the methanol separation method.

Table 4. Growth media prepared with methanol separated yeast extract fractions.

Fraction Identifier	Description
MIS16.6	Methanol insoluble fraction of yeast extract, extracted at 16.6 g/L concentration of yeast extract in methanol.
MIS20	Methanol insoluble fraction of yeast extract, extracted at 20 g/L concentration of yeast extract in methanol.
MIS20.4	Methanol insoluble fraction of yeast extract, extracted at 20.4 g/L concentration of yeast extract in methanol.
MIS25.5	Methanol insoluble fraction of yeast extract, extracted at 25.5 g/L concentration of yeast extract in methanol.
MIS40	Methanol insoluble fraction of yeast extract, extracted at 40 g/L concentration of yeast extract in methanol.
MIS80	Methanol insoluble fraction of yeast extract, extracted at 80 g/L concentration of yeast extract in methanol.
MIS120	Methanol insoluble fraction of yeast extract, extracted at 120 g/L concentration of yeast extract in methanol.
MIS160	Methanol insoluble fraction of yeast extract, extracted at 160 g/L concentration of yeast extract in methanol.
MS6.6	Methanol soluble fraction of yeast extract extracted at 6.6 g/L concentration of yeast extract in methanol.
MS20	Methanol soluble fraction of yeast extract, extracted at 20 g/L concentration of yeast extract in methanol.
MYE	Yeast extract that was mixed with methanol at 20 g/L. The solvent was removed in the rotary evaporator under vacuum. The yeast extract was not separated.
YE	Yeast extract, as received

3.3.3 Isopropanol/Water Separation

In the two solvent system-based separation of yeast extract, an amount of yeast extract was mixed with the isopropanol in one of two ways. In the first method, the isopropanol was first mixed with water to 70%, and the yeast extract dissolved in this mixture. Alternatively, the yeast extract was dissolved fully in water and then the corresponding volume of isopropanol added. The yeast extract dissolves more readily in the water before isopropanol addition making the second method the method of choice. Regardless of the mixing technique above, the resulting two immiscible liquid layers were separated in a 1 litre separatory funnel. The extract and raffinate layers were dried under vacuum in a rotary evaporator at 50 °C under vacuum. Residual solvent was removed under high vacuum. Table 5 contains a summary of the growth media prepared using the isopropanol separation method. Parts of this table will be repeated in the sequential separation section for comparison.

Table 5. Growth media prepared with isopropanol/water separated yeast extract fractions.

Fraction Identifier	Description
IB	Yeast extract isopropanol extract, the portion of yeast extract dissolved in the more dense liquid phase formed from yeast extract's addition to isopropanol/water mixtures.
IB70	Sterile filtered isopropanol extract portion of yeast extract, produced from 70% vol. isopropanol/water mixture at 70 g/L concentration of yeast extract in isopropanol/water solvent.
IB90	Sterile filtered isopropanol extract portion of yeast extract, produced from 70% vol. isopropanol/water mixture at 90 g/L concentration of yeast extract in isopropanol/water solvent.
IT	Yeast extract isopropanol raffinate, the portion of yeast extract dissolved in the denser liquid phase formed from yeast extract's addition to isopropanol/water mixtures.
IT70	Sterile filtered isopropanol raffinate portion of yeast extract, produced from 70% vol. isopropanol/water mixture at 70 g/L concentration of yeast extract in isopropanol/water solvent.
IT90	Sterile filtered isopropanol raffinate portion of yeast extract, produced from 70% vol. isopropanol/water mixture at 90 g/L concentration of yeast extract in isopropanol/water solvent.
YE	Yeast extract, as received

3.3.4 Sequential separation

The methanol and isopropanol/water extractions were completed in series, as demonstrated in Figure 3. Initially, 72 g of yeast extract was added to 4 litres of methanol in a 5 litre beaker with a 2 inch stir bar. The mixture was mixed for 15 seconds, and then filtered through Watman No. 5 filter paper under vacuum. The total contact time (from first adding the methanol until the filter cake had no liquid on top of it) was two minutes. The filter cake was dried under high vacuum and stored under nitrogen at room temperature in the dark. The methanol was removed under vacuum in a rotary evaporator and the resulting solids dried under high vacuum. 7 g of dry filtrate or filter cake was added to 30 mL of deionized water in a 50 mL beaker with a 2.54 cm stir bar and placed on a magnetic stirrer until dissolved. The solution was transferred to a 100 mL separatory funnel. The residual solution in the beaker was washed into the separatory funnel with 70 mL of anhydrous isopropanol. The mixture was mixed vigorously, and allowed to settle for 5 to 30 minutes. The bottom fraction (Extract) was isolated from the top fraction (Raffinate) into two tarred round bottom flasks. The Extract and Raffinate were dried using the rotary evaporator and the resulting solids dried under high vacuum.

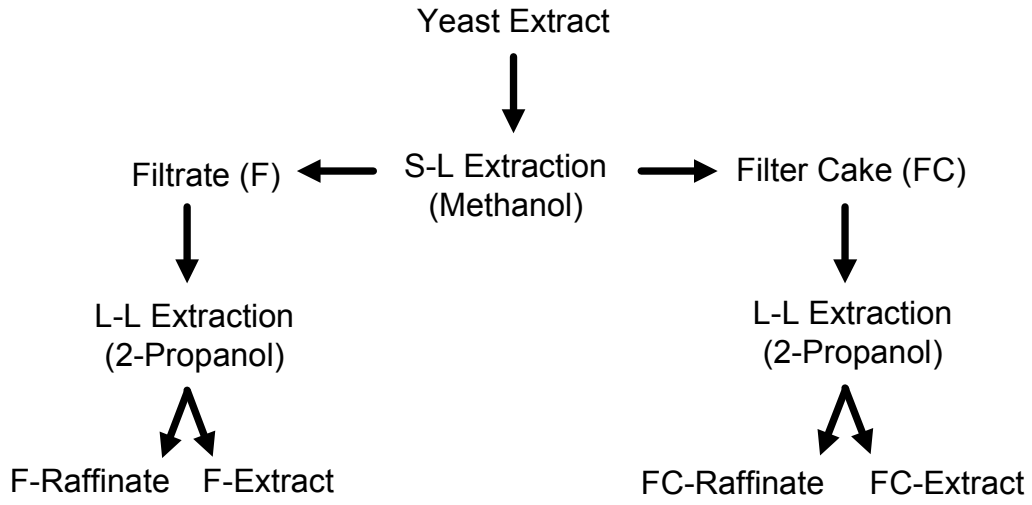


Figure 3. Sequential separation fractions and methodology.

Table 6 contains a summary of the growth media prepared using a combination of methanol and isopropanol separation methods.

Table 6. Growth media prepared with sequential methanol then isopropanol/water separated yeast extract fractions.

Fraction Identifier	Description
IB70MIS15	Sterile filtered FC-Extract fraction from Figure 3. The yeast extract is first partially dissolved in methanol at 15 g/L concentration. The filter cake is removed via vacuum filtration and dried under high vacuum. This portion is then extracted with 70% vol. isopropanol/water at 70 g/L. The yeast extract fraction dissolved in the denser phase is separated then dried under high vacuum.
IB70MS15	Sterile filtered F-Extract fraction from Figure 3. The yeast extract is first partially dissolved in methanol at 15 g/L concentration. The filter cake is removed via vacuum filtration and dried under high vacuum. This portion is then extracted with 70% vol. isopropanol/water at 70 g/L. The yeast extract fraction dissolved in the denser phase is separated then dried under high vacuum.
IT70MIS15	Sterile filtered FC-Raffinate fraction from Figure 3. The yeast extract is first partially dissolved in methanol at 15 g/L concentration. The filter cake is removed via vacuum filtration and dried under high vacuum. This portion is then extracted with 70% vol. isopropanol/water at 70 g/L. The yeast extract fraction dissolved in the denser phase is separated then dried under high vacuum.
IT70MS15	Sterile filtered F-Raffinate fraction from Figure 3. The yeast extract is first partially dissolved in methanol at 15 g/L concentration. The filter cake is removed via vacuum filtration and dried under high vacuum. This portion is then extracted with 70% vol. isopropanol/water at 70 g/L. The yeast extract fraction dissolved in the denser phase is separated then dried under high vacuum.
YE	Yeast extract, as received

3.3.5 Thermogravametric analysis

A TA Instruments Q1000 Differential Scanning Calorimeter was used to collect the thermal analysis data. The instrument was calibrated previously by qualified technicians using indium. All measurements were made at a linear scanning rate of 10 °C/min, with samples hermetically sealed in aluminum pans. An empty, sealed aluminum pan was used as a reference. One DSC measurement was made for each sample. Sample masses ranged from 6.0 to 8.0 mg. Nitrogen purge gas was used at a flow rate of 50 mL/min.

3.4 Microalgal Growth

3.4.1 Culture Species

Auxenochlorella protothecoides (UTEX 25) was received from the University of Texas at Austin's UTEX the Culture Collection of Algae. The algae culture is no longer available and was replaced by the xenic microalgal culture *Auxenochlorella protothecoides* (UTEX B25). The axenic cultures were transferred from the received agar into HGM-agar and into liquid heterotrophic growth media (HGM). Agar cultures were stored at 4 °C in the event that the liquid stock cultures were contaminated. Liquid cultures were transferred into new HGM liquid media as required.

3.4.2 Growth Media

Table 7 shows the stock solution concentration, amounts used in media, and final concentration in growth media. The efficacy of the refined yeast extract fractions was tested by the initial growth (less than 72 hours) in algae growth media (AGM), glycerol growth media (GGM), and/or heterotrophic growth media (HGM). The concentration of the refined yeast extract fractions was 4 g/L unless otherwise stated. The experiments are labeled with descriptive identifiers of the growth media, fraction, and how the fraction was compared. For example, AGM+YE means that yeast extract (YE) was sterile filtered into autotrophic growth medium (AGM).

Table 7. Stock solutions and final media concentrations compared to B4-Fe medium.

Component	Stock (g/L)	AGM* (mg/L)	GGM* (mg/L)	HGM* (mg/L)	B4-Fe[38] (mg/L)
Glucose	-	20000	-	20000	varied
Glycerol	-	-	15000	-	varied
Yeast Extract	40.0 g/L[+]	-	-	4000	-
KH_2PO_4	17.5 g/L	525	525	525	280
K_2HPO_4	7.5 g/L	225	225	225	120
$MgSO_4 \cdot 7\ H_2O$	6.0 g/L	180	180	180	120
NaCl	6.0 g/L	180	180	180	-
$CaCl_2 \cdot 2\ H_2O$	2.5 g/L	75	75	75	10
Heavy Metals					
$Na_2EDTA \cdot 2\ H_2O$	0.75 g/L	12	12	12	64
$FeCl_3 \cdot 6\ H_2O$	0.097 g/L	1.55	1.55	1.55	48[a]
$MnCl_2 \cdot 4\ H_2O$	0.041 g/L	0.656	0.656	0.656	7.2
H_3BO_3	0.011 g/L	0.176	0.176	0.176	11.6
$ZnCl_2$	0.005 g/L	0.080	0.080	0.080	0.88[b]
$CoCl_2 \cdot 6\ H_2O$	0.002 g/L	0.032	0.032	0.032	-
$CuSO_4 \cdot 5\ H_2O$	0.002 g/L	0.032	0.032	0.032	0.32
$Na_2MoO_4 \cdot 5\ H_2O$	-	-	-	-	0.12
Thiamine HCl	-	-	-	-	0.040

*** indicates final concentration.**
[+] Yeast extract cannot be stored in a stock solution.
[a] as $FeSO_4 \cdot 7\ H_2O$
[b] as $ZnSO_4 \cdot 7\ H_2O$

For growth experiments, 30 mL of base stock solutions, and 16 mL of the stock heavy metal solution were used per litre of liquid growth medium. The fractions will be compared as required throughout this thesis. Aliquots of 100 or 125 mL of liquid growth medium was added to a 250 mL Erlenmeyer flask with side arm flask. The flasks were topped with custom made stoppers and tinfoil. The flasks were autoclaved with a 20 minute sterilization time, allowed to cool to room temperature, and inoculated with 1 mL of inoculum[38].

3.4.3 Cell growth metrics

3.4.3.1 Dry weight determination

A 5 mL sample of well-mixed algae was vacuum filtered through tarred, dry Whatman # 5 filter paper. The filter paper was pre-dried in an oven at 80 °C for at least 48 hours. The filter cake of algae was washed with deionized water, then allowed to dry in an oven at 80 °C for at least 48 hours before the dry weight was measured. Weights were measured on a calibrated analytical balance with a maximum weight of 210 g and 0.0001 g accuracy, Sartorius BL210S.

3.4.3.2 Optical density

The optical density measurements were taken using a Spectronic Spec 20D Spectrophotometer in specialized Erlenmeyer flasks with 15 mm diameter test tubes as side arms. The specialized glassware allowed optical density measurements to be taken without disturbing the sterile cotton seal on the top of the Erlenmeyer flasks. An extra flask for each media tested was kept sterile and used as the machine's blank. The spectrophotometer was allowed to warm up for at least two hours prior to use. With nothing in the chamber, the transmittance was adjusted to 0%. The wavelength used was 630 nm. The optical density is an indirect measure of dry weight and is dependent on the cellular constituency. The method assumes that two cells have the same size (dry weight) if they adsorb the same amount of light at the wavelength tested. The dry weight/optical density relationship for *Auxenochlorella protothecoides* is shown in Figure 4.

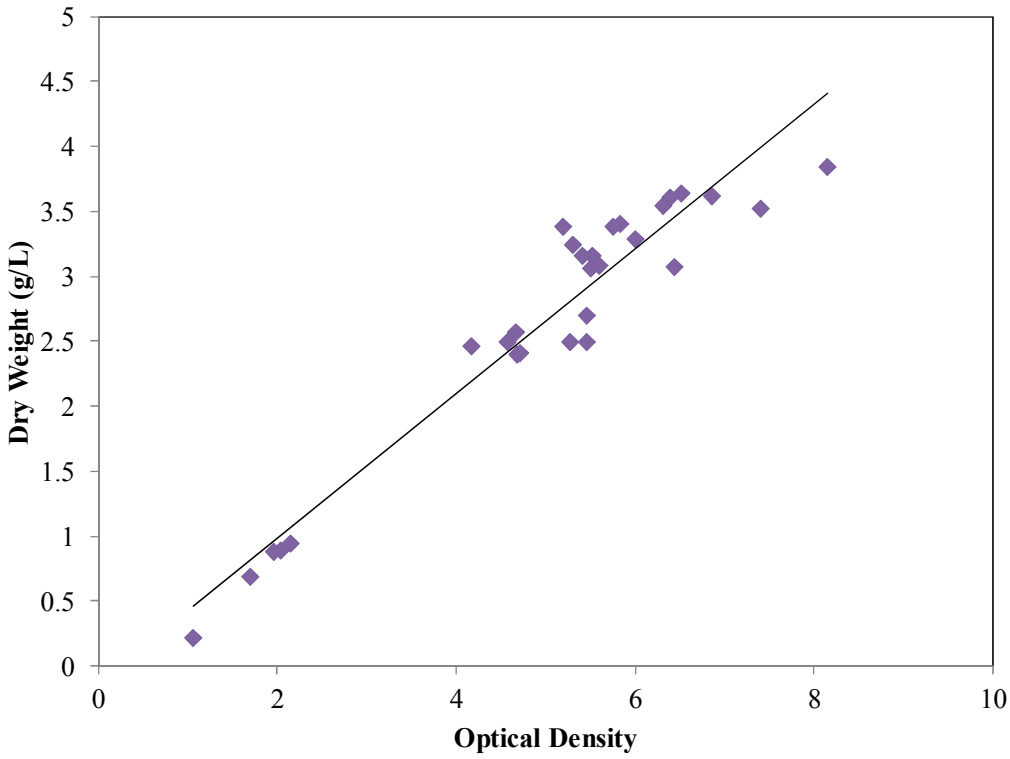


Figure 4. Dryweight as a function of optical density for *Auxenochlorella protothecoides*.

3.4.3.3 Cell count and cell size

A Beckman Coulter Z2 Coulter Counter particle counter and size analyzer was used to obtain simultaneous particle size and distributions. The Coulter counter measures the changes in electrical resistance through an aperture as algal cells pass through it. The Coulter Counter was equipped with a 70 µm aperture. *A. protothecoides* cells range in diameter from 1.5 to 20 µm, with an average cell size between 4 and 6 µm. The gain and current of the instrument are adjusted to measure a range of cell sizes, with the maximum value three times the minimum value. Two measurements were taken on the same Isoton™ diluted algal samples between 1.2 µm to 4.0 µm by adjusting the gain and current to 256 and 2000 mA and from 3.5 µm to 12.73 µm by adjusting the gain and current to 32 and 500 mA change numbers. Each measurement was stored on a hard drive disk in a separate file with extension .z2.

The files were combined and the data summarized using an excel macro, given in Appendix A: Supplementary Figures.Appendix B: Computer Code.

3.4.4 Inoculum and inoculation

Inoculum was grown in HGM for 48 to 72 hours before being added to the test medium. The inoculum flask was shaken to ensure homogeneity, and 1 mL was transferred from the inoculum flask to the test medium with a Fisherbrand Finnpipette II Single-Channel 100-1000 µL Pipetter in a laminar flow, biosafety cabinet. The optical density, dry weight, and cell count of the test medium were taken after inoculum addition.

3.4.5 Growth conditions

Algae were grown in modified 250 mL Erlenmeyer flasks equipped with a 25 mm side arm test tube under axenic conditions with 100 mL working volume. The side arm flasks allowed spectrophotometric analysis without the removal of sample, reducing the chances of contamination. Custom stoppers were made from cheesecloth and sterile cotton. The custom stoppers overlapped the Erlenmeyer flask lid, preventing settled spores from being inadvertently transferred to the medium. Algae were grown heterotrophically by placing the custom Erlenmeyer flasks in an incubated shaker (New Brunswick Scientific Innova 4330) at 100 rpm and 28 °C. Mixotrophic or phototrophic growth was conducted on a New Brunswick Scientific Innova 2300 platform shaker at 100 rpm and room temperature (between 25 and 27 °C), shown in Figure 5. The photosynthetically active radiation (PAR) of the growth lights fastened above the platform shaker was 81.89 $\mu mol/s \cdot m^2$. The PAR was measured by a LI-COR Biosciences LI-250A Light meter.

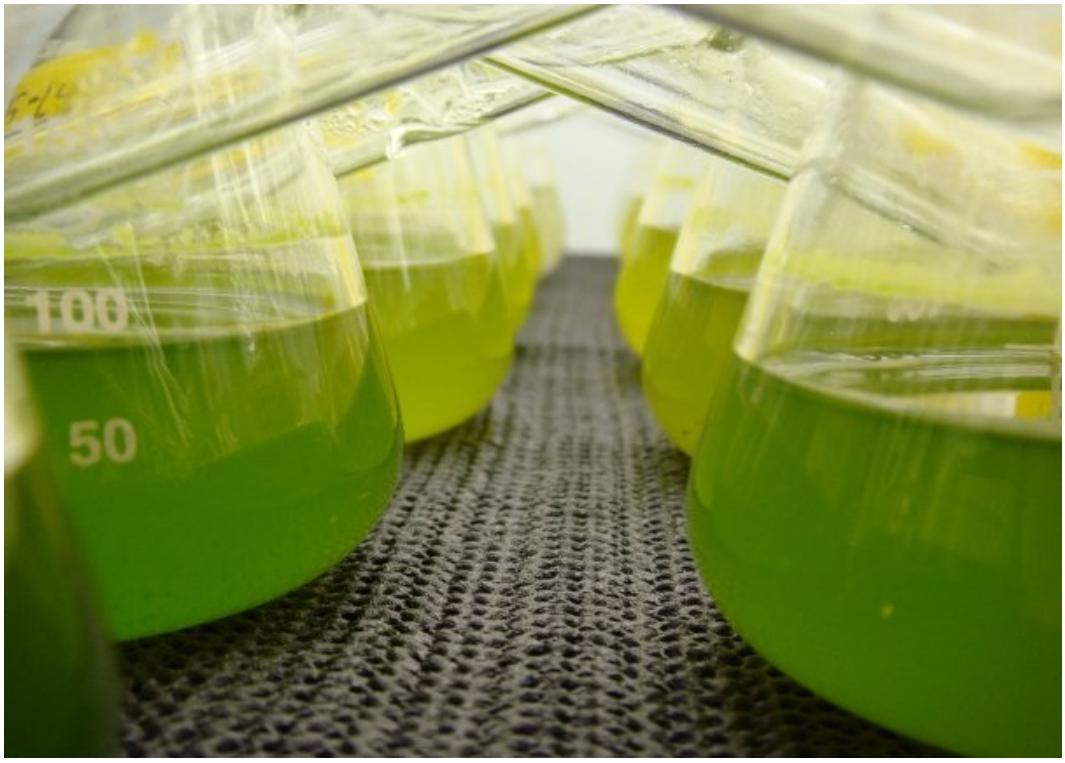


Figure 5. Phototrophic growth of *Auxenochlorella protothecoides* demonstrating side arm flask.

3.5 Analysis of Variance

ANOVA analysis was used, where appropriate, to determine if two data sets were statistically the same (null hypothesis). Calculations were completed using an online calculator developed by the College of Saint Benedict Saint John's University[66].

4 Results and Discussion: Physical properties of Yeast Extract

4.1 Methanol Separation of Yeast Extract

Yeast extract was found to be partially soluble in methanol. The kinetics of dissolution followed Noyes-Whitney kinetics, depicted in **Figure 6**. The rate of dissolution decreased as a function of time as the amount of dissolved yeast extract in methanol approached a maximum value.

The solid components of yeast extract could be isolated via filtration and subsequent drying. The filter cake yield is shown in Figure 6 for various concentrations of yeast extract in methanol and contact times. A two dimensional representation is available in Appendix A: Supplementary Figures. The contact time is recorded from the moment the methanol touches the yeast extract until the liquid level passes below the filter cake. The filter cake yield is calculated as the ratio of dry filter cake weight (the portion of the yeast extract that is insoluble in yeast extract) to original yeast extract weight is proportional to the concentration gradient present between the particles of yeast extract and the concentration of yeast extract in the bulk phase. The mass yield of methanol-insoluble yeast extract is characterized by a sharp drop upon addition of methanol until about 30 seconds, when the decrease in yield slows until the yield approaches a constant value (i.e. the solubility limit) (see Figure 6 and Appendix A: Supplementary Figures. The experiment did not reach equilibrium after 5 minutes. The yeast extract dissolution in methanol follows the first order dissolution rate process described by the Noyes-Whitney theory of dissolution. The concentration gradient is the driving force for dissolution. The maximal rate of dissolution is achieved at the initial time, at t=0 because the largest concentration gradient is present. Lower concentrations of yeast extract in methanol result in higher sustained concentration gradients for a given time period than higher concentrations of yeast extract. The higher sustained driving force allows a greater portion of the yeast extract to dissolve, which results in a lower filter cake yield for lower concentrations of yeast extract in methanol.

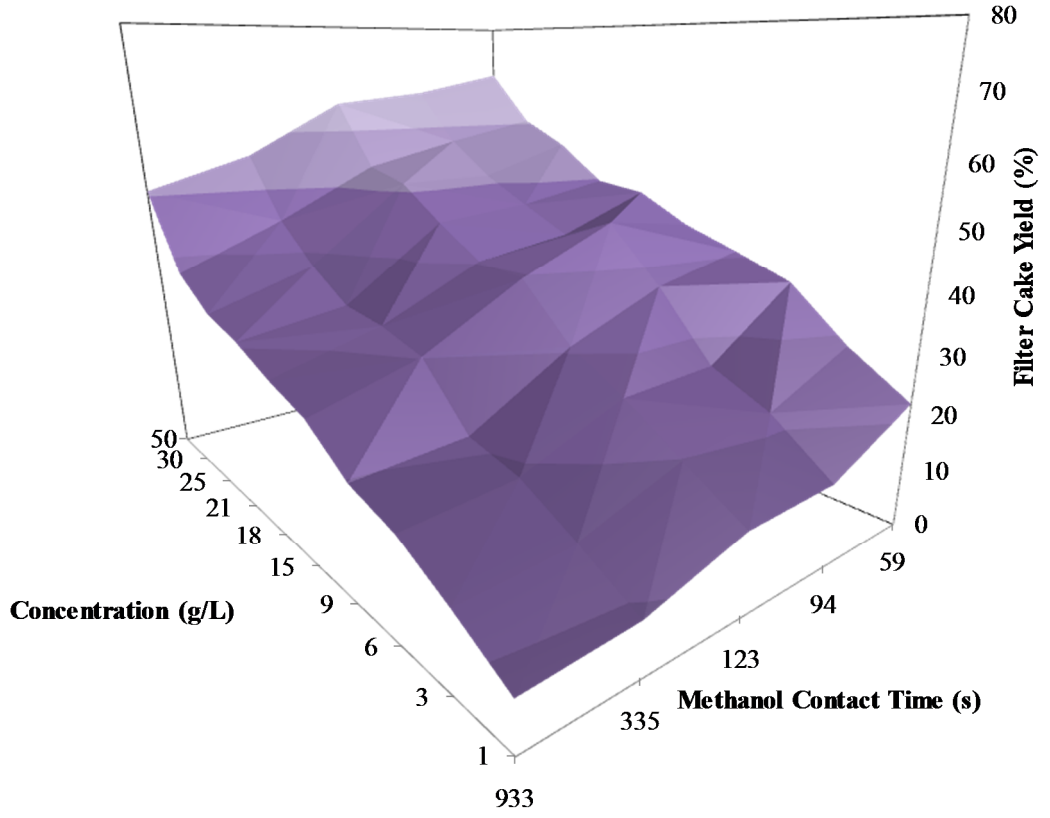


Figure 6. Effect of methanol contact time on filter cake yield for the partially soluble yeast extract in methanol-separation.

4.2 Isopropanol/Water Separation of Yeast Extract

The Hansen Solubility Parameter (HSP) of water/alcohol mixtures was matched to methanol by varying the mixture-solvent volume fraction. The resulting mixture HSP and pure solvent HSP are compared in Table 8. The HSPs were calculated using the method of Hansen[67-71]. The Hansen Solubility Parameter (HSP) radius of dissolution (R_a) of an isopropanol/water mixture was minimized with respect to methanol by varying the volume fraction of water. The resulting 72 vol % isopropanol had a R_a of 4.2 which is assumed to be below an arbitrary threshold for good interactions. The isopropanol/water method produces two immiscible liquids when mixed with yeast extract, a phenomenon referred to as "salting-out"[72] when salt is used to separate isopropanol from aqueous mixtures.

Table 8. Hansen Solubility Parameters (δ_D, δ_P, and δ_H) of various water/solvent mixtures.

Solvent	δ_D	δ_P	δ_H	Ra
Methanol	14.7	12.3	22.3	0.0
Water	15.5	16.0	42.3	20.40
2-propanol	15.8	6.1	16.4	8.84
Ethanol	15.8	8.8	19.4	5.05
Acetone	15.5	10.4	7.0	15.55
72% 2-propanol	15.7	8.9	23.7	4.21
84% Ethanol	15.8	10.0	23.1	3.24
57% Acetone	15.5	12.8	22.2	1.68

The 57% acetone fully dissolved the yeast extract. The 84% ethanol separated into two phases, a small amount of more dense viscous dark brown liquid and a cloudy light-yellow liquid. The dense brown liquid adhered to glass and could be isolated by decanting the light-yellow liquid and subsequent dissolution with water. The ethanol separation method was not studied further.

When added to 70% volume isopropanol/water mixtures, the yeast extract 'salts-out' the isopropanol. The components of yeast extract split disproportionately into the extract phase (dense, water-rich, bottom layer), and raffinate phase (less dense, isopropanol-rich, top layer) according to Figure 7. The amount of yeast extract in each phase depends on the ratio of yeast extract to solvent. At higher concentrations of yeast extract, a greater proportion of yeast extract enters the isopropanol-rich extract fraction than the water-rich raffinate fraction. The clear extract and raffinate phases were collected in such a way that the other phase was not present, leaving a residual amount in the separatory funnel. These samples were used for growth tests on *A. protothecoides* with residual unsettled material at the interface left in the separatory funnel. If the mass fraction of raffinate and extract is of interest, future tests should apply greater separation forces in a centrifuge to result in complete fractionation of the two layers prior to separation. The error in measurement is

caused mostly by incomplete separation of the phases in a separatory funnel and residual yeast extract lost in the separatory funnel.

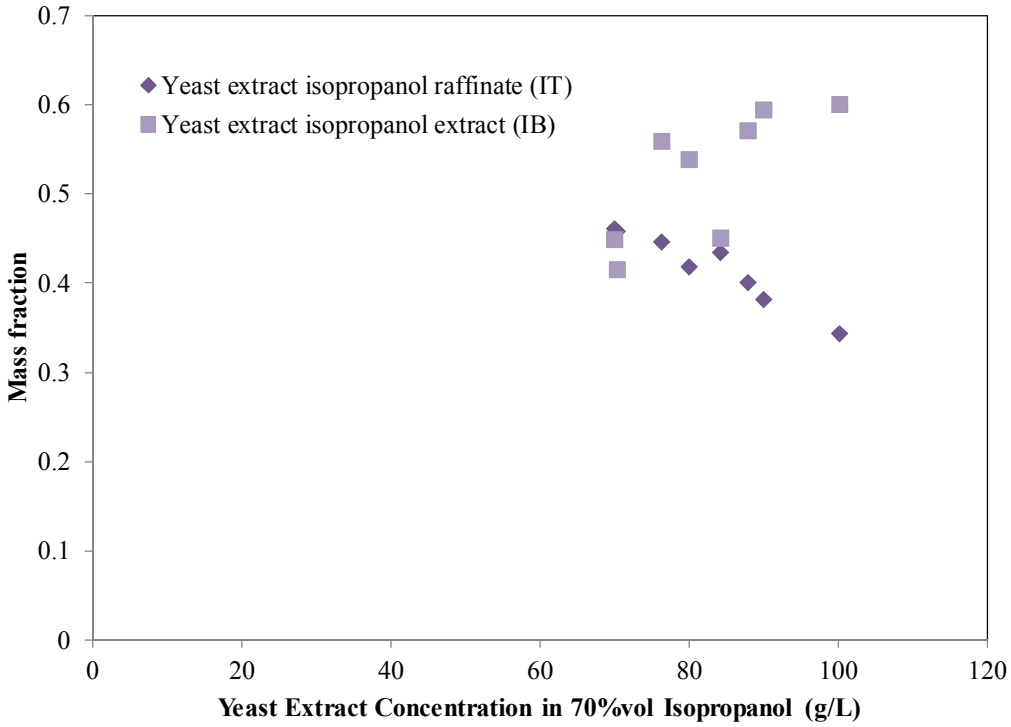


Figure 7. Mass fractions of yeast extract distributed among the less dense isopropanol-rich layer (extract fraction) and the more dense water-rich layer (raffinate fraction).

4.3 TGA/DSC Solvent effects

To verify the physical uniqueness of subfractions from complete yeast extract, the differential scanning calorimetry (DSC) thermal behaviours of the produced extracts are analyzed. At a high level, DSC will confirm that the extracts are in fact distinct moieties. Figure 8 and shows the thermal events for the complete yeast extract, the methanol insoluble fraction, and the methanol soluble fraction. Figure 9 shows the thermal events for complete yeast extract, the isopropanol extract fraction, and the isopropanol raffinate fraction. Part of this chapter was submitted as part of course work for Ch E 694: Advanced Topics in Chemical Engineering at the University of Alberta in the winter 2011 term.

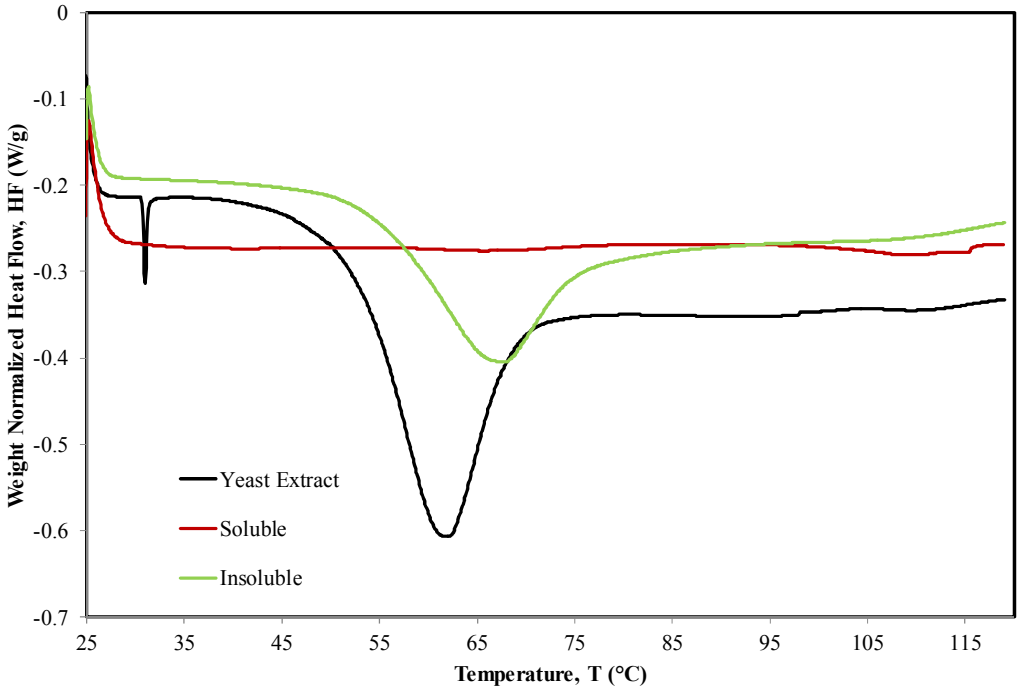


Figure 8. DSC thermal behaviour of yeast extract, methanol soluble fraction (filtrate), and methanol insoluble fraction (filter cake). DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

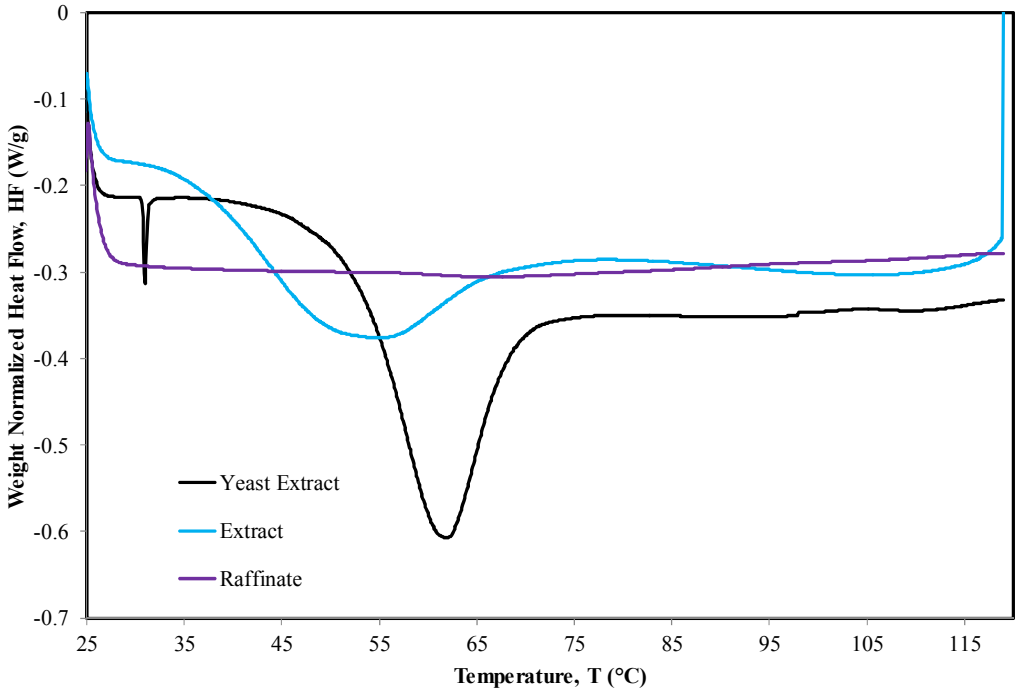


Figure 9. DSC thermal behaviour of: yeast extract, isopropanol/water extract fraction, and isopropanol/water raffinate fraction. DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

The complete yeast extract displays two endothermic peaks, an initial endothermic peak at approx. 28 °C and a second endothermic peak at 61.8 °C. The raffinate produced from extraction of yeast extract with 70 g/L of 70% isopropanol and the methanol soluble portion of yeast extract do not exhibit endothermic peaks. The extract produced from extraction of yeast extract with 70 g/L of 70% isopropanol and the methanol insoluble portion of yeast extract exhibit endorthermic peaks the onset and; peak temperatures are summarized in Table 9.

Table 9. Onset and peak temperature of selected yeast extract fractions.

Fraction Analysed	Onset Temperature	Peak Temperature
Complete Yeast Extract	53 °C	61.8 °C
Insoluble Fraction	54 °C	67.7 °C
Extract Fraction	36 °C	54.9 °C

The liquid-liquid isopropanol separation following the solid-liquid methanol extracts (according to Figure 3) results in four fractions. The isopropanol extract and raffinate fractions of the soluble fraction are shown in Figure 13. Figure 11 demonstrates the isopropanol extract and raffinate fractions of the insoluble fraction. The isopropanol extracts from each source are summarized in Figure 12. Similarly, Figure 13 summarizes the isopropanol raffinate fractions from various sources.

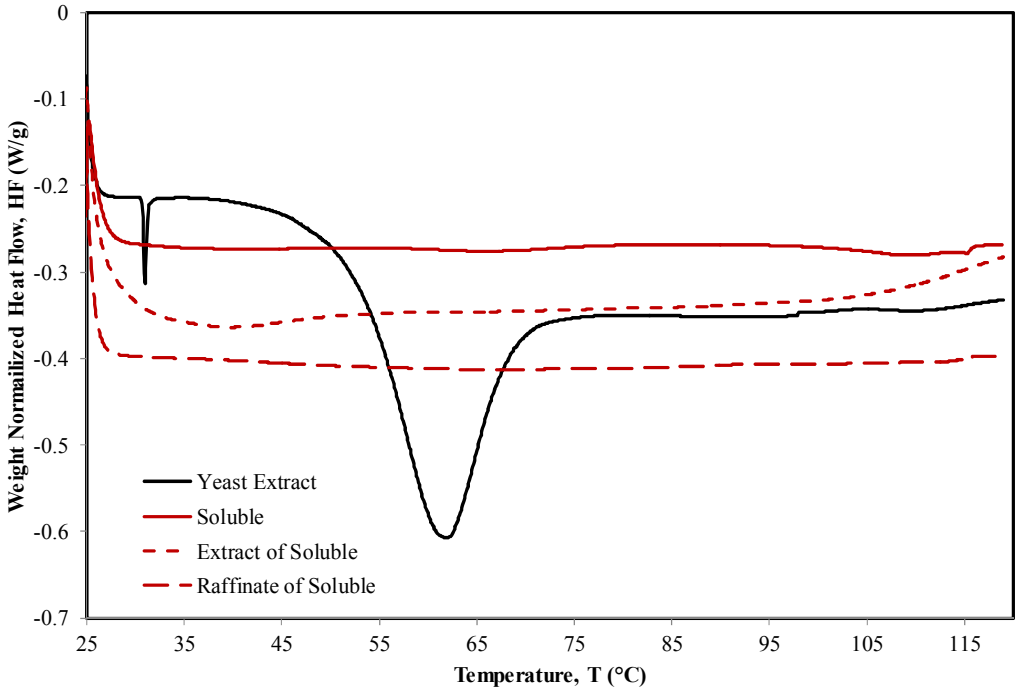


Figure 10. DSC thermal behaviour of: yeast extract, the methanol soluble fraction, the isopropanol extract of the methanol soluble fraction, and the isopropanol raffinate of the methanol soluble fraction. DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

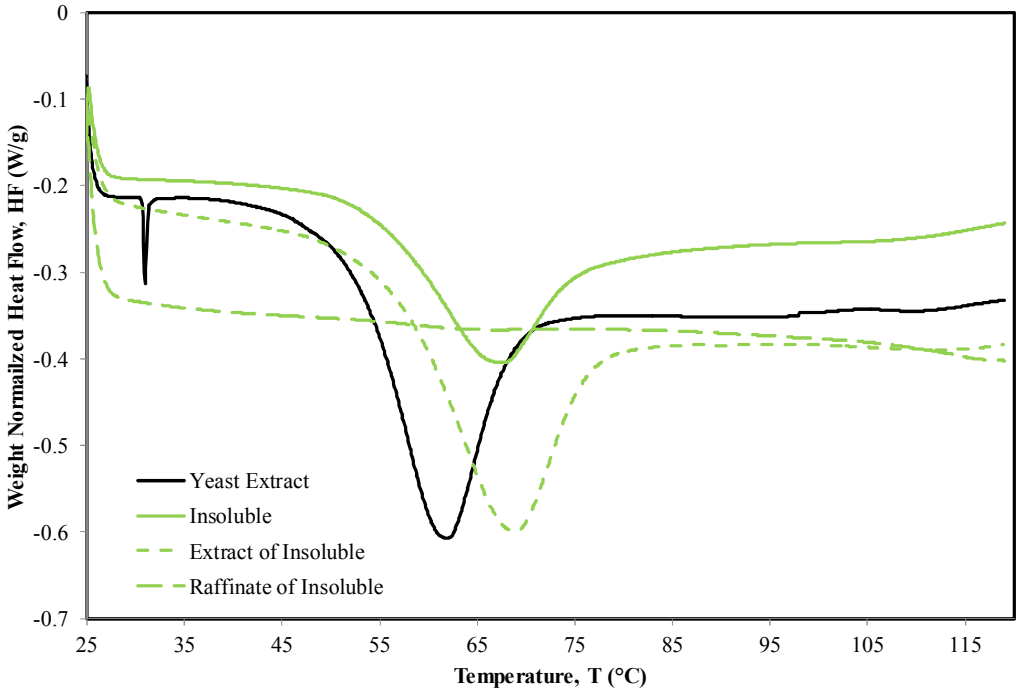


Figure 11. DSC thermal behaviour of yeast extract, the methanol insoluble fraction, the isopropanol extract of methanol insoluble fraction, and the isopropanol raffinate of methanol insoluble fraction. DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

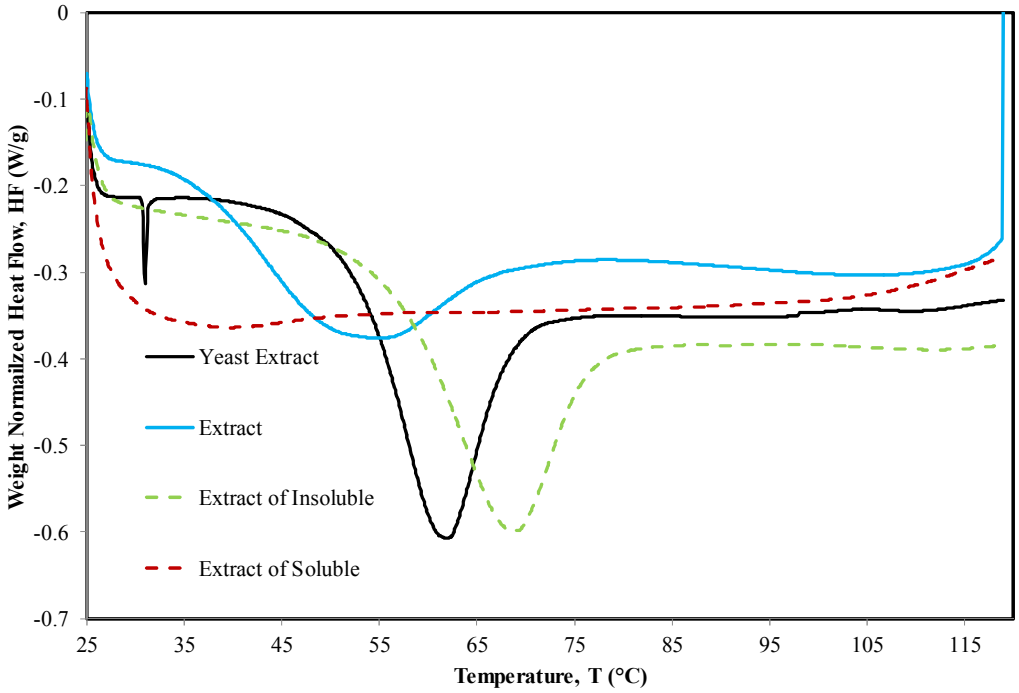


Figure 12. DSC thermal behaviour of yeast extract, the isopropanol extract fraction, the isopropanol extract of methanol insoluble fraction, and isopropanol extract of methanol soluble fraction. DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

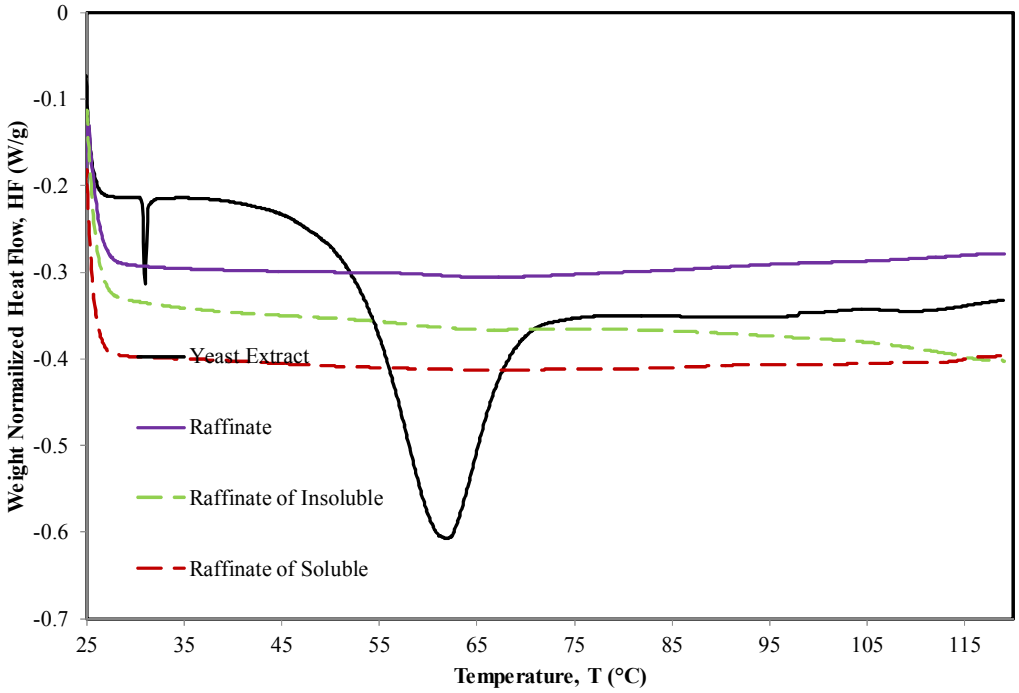


Figure 13. DSC thermal behaviour of yeast extract, the isopropanol raffinate fraction, the isopropanol raffinate of methanol insoluble fraction, and the isopropanol raffinate of methanol soluble fraction. DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

To confirm if the solvent role in the techniques was solely separation, the fractions were recombined in the same mass fraction as they were created. Figure 14 shows the DSC thermal analysis for the recombined S-L and L-L extracts.

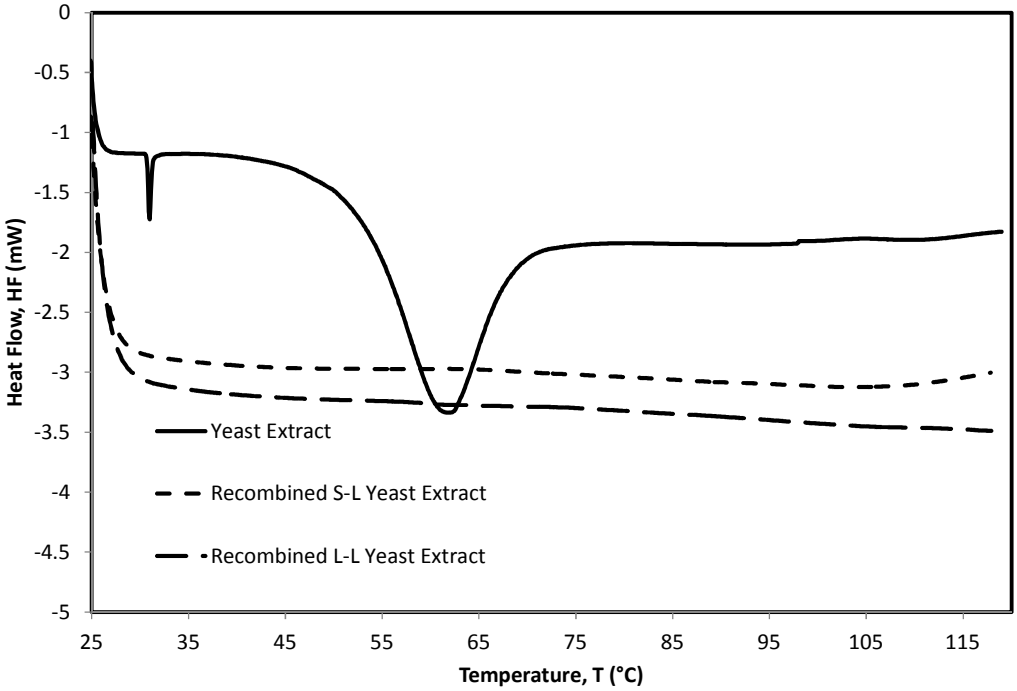


Figure 14. DSC thermal behaviour of yeast extract, recombined soluble fraction and insoluble fraction (Recombined S-L Yeast Extract), and recombined extract fraction and raffinate fraction (Recombined L-L Yeast Extract). DSC was taken at 10 °C/min with a nitrogen purge gas at 50 mL/min in hermetically sealed aluminum pans.

Neither recombined yeast extract produces the same DSC signal as the original yeast extract. This suggests that the solvent for both system reacts with the yeast extract irreversibly. To ensure homogeneity in the samples, the fractions were dissolved in water and the water was subsequently removed. The higher temperature required to dry the samples may have denatured the proteins.

5 Results and Discussion: Initial Growth Properties of Yeast Extract

Each flask was inoculated with 1 mL of inoculum in the log phase of growth. Due to the variability between inocula, direct comparison of values between experiments is difficult as each inoculum was slightly different. To standardize the results for comparison, a flask of heterotrophic growth media (HGM) was grown as a living control. The relative difference between a test experiment's growth and that of HGM can be compared across all experiments.

5.1 Yeast extract: concentration

The optical density of *A. protothecoides* grown heterotrophically on AGM supplemented with various concentrations of yeast extract is shown in Figure 16. Each test was performed in triplicate and the standard deviation is presented as vertical error bars. The dryweights after 48 hours are presented as a function of initial concentration of yeast extract in Figure 15.

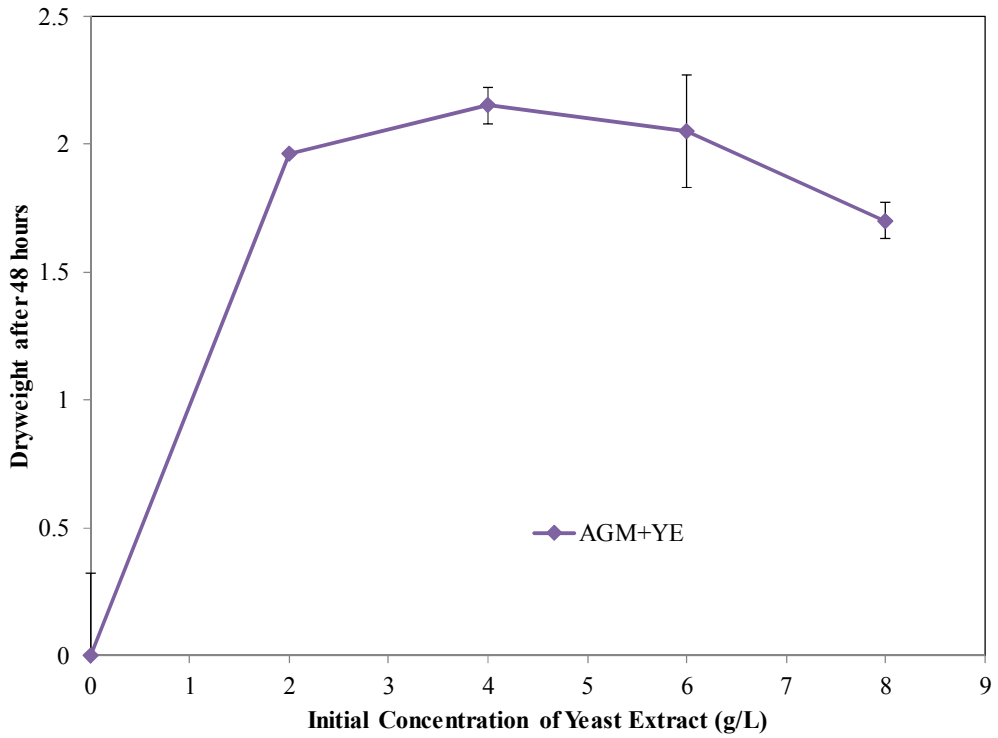


Figure 15. Final dry weight and its variance of *A. protothecoides* grown heterotrophically with various initial yeast extract concentrations.

A 4 g/L concentration of yeast extract resulted in the highest optical density and dry weight in the shortest time. The optical density of AGM+YE (4 g/L) is better than the AGM+YE (2 g/L) and AGM+YE (6 g/L) of yeast extract with 95.5% confidence. The dry weight of AGM+YE (4 g/L) is statistically significant at 95% confidence compared to AGM+YE (2 g/L), but not to the AGM+YE (6 g/L). The AGM and AGM+YE (8 g/L) were found to be distinct from the other tests with 99.99% confidence. The 95% confidence interval for the final optical density of AGM+YE (4 g/L) was 0.9063 thru 0.9704. A statistically significant increase of 5.8% in optical density between the AGM+YE (4 g/L) and AGM+YE (2 g/L) may not justify using twice the yeast extract substrate for industrial applications, but biomass yield is not the only factor to consider. However for the laboratory data, the use of 4 g/L is statistically justified on the basis of dry weight and optical density and 4 g/L was assumed to be the optimal concentration for the refined yeast extract fractions, unless otherwise stated.

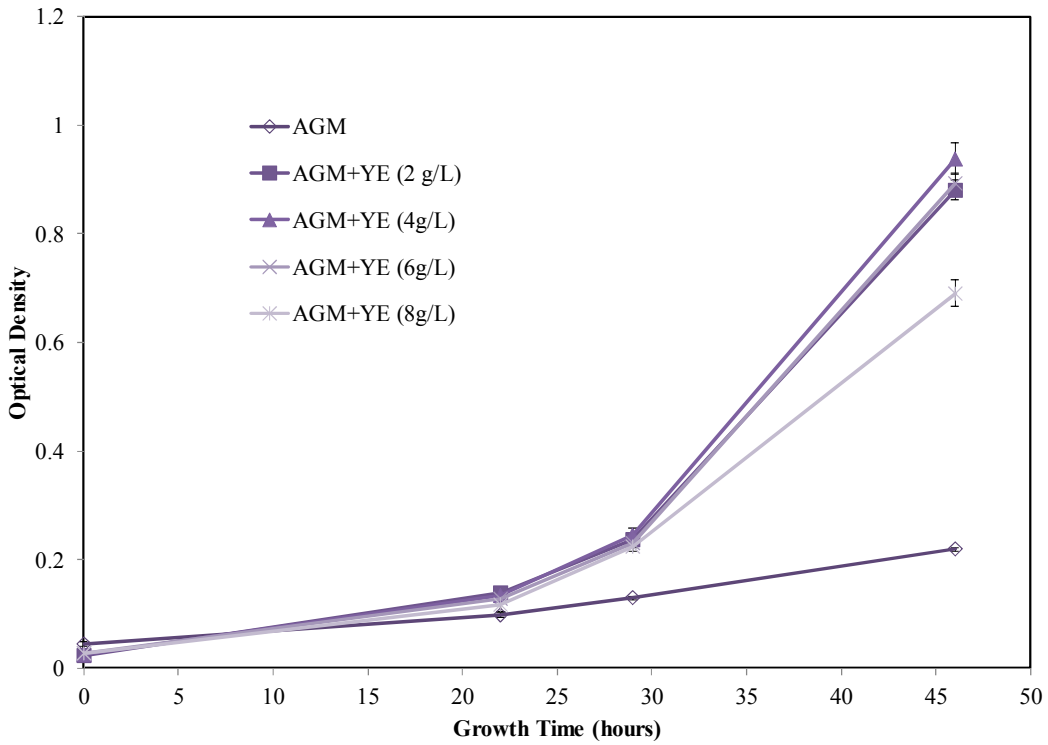


Figure 16. Optical density of *A. protothecoides* grown heterotrophically on AGM supplemented with various concentrations of yeast extract.

5.2 Yeast Extract: thermal and filter sterilization

The cell count as a function of growth time is shown in Figure 17 for autoclaved (HGM) and 0.22 µm sterile filtered (AGM+YE) yeast extract. In addition, the HGM and AGM+YE media had 1.03 g of protease sterile filtered into the media and was autoclaved after a 0 or 48 hour incubation period.

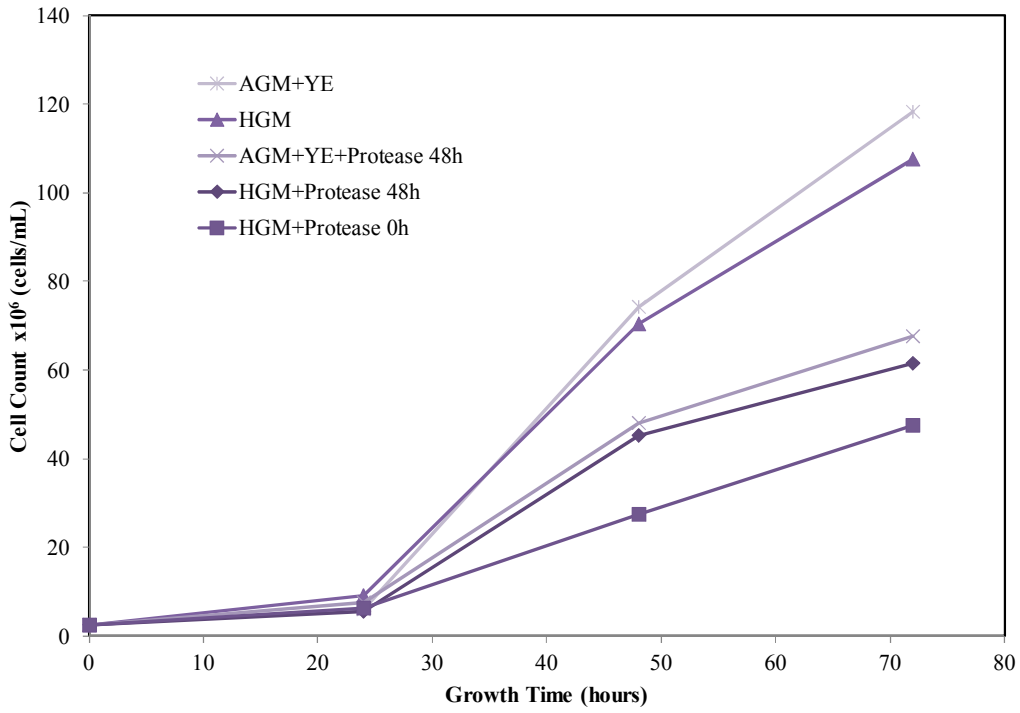


Figure 17. Cell count as a function of growth time for *A. protothecoides* grown with autoclaved and sterile filtered yeast extract subjected to protease for a varied incubation time.

The yeast extract is produced from autolysis of whole yeast cells and is a mixture of intact vitamin B complexes[73], amino acids, peptides, water soluble vitamins and water soluble carbohydrates. *Auxenochlorella protothecoides* requires thiamine (Vitamin B1), but not cobalamin (Vitamin B12), nor biotin (also known as Vitamin H or Vitamin B7)[74]. Growth curves fit into two groupings of specific growth rates based on cell counts displayed in Table 10. The region of fastest average growth rate occurs for HGM and AGM+YE that were not subjected to protease treatment. The yeast extract in the AGM+YE fraction was not subject to thermal degradation and had the highest growth rate. The yeast extract in the HGM media was autoclaved and therefore most likely thermally degraded; however it had a slightly lower maximum growth rate to HGM+Protease. The region of moderate average growth rate occurs for HGM and AGM+YE with 1.03 g of protease, incubated for 48 hours. The AGM+YE have a slightly higher final cell count than the HGM

regardless of protease treatment. The yeast extract-containing media had the same order of magnitude of maximum specific growth rate for autoclaved yeast extract media (between 0.77 and 0.88 h^{-1}) which was less than the maximum specific growth rate for sterile filtered yeast extract media (0.104 h^{-1}).

Table 10. Specific growth rates for autoclaved (HGM), and sterile filtered (AGM+YE) growth media with or without addition of protease.

Growth Media	Max. Growth Rate (h^{-1})*	Final Dry Weight (g/L)
AGM+YE	0.104	4.06
HGM	0.086	3.68
AGM+YE+Protease 48h	0.077	3.02
HGM+Protease 48h	0.088	2.84
HGM+Protease 0h	0.061	2.58
*** Growth rate is based on cell counts**		

The thermal degradation products of autoclaved yeast extract are concluded to be less efficacious than sterile filtered yeast extract. The decreased efficacy of yeast extract when subjected to protease treatment is unexpected due to the increased protein (and therefore nitrogen) content of the media. The presence of protease negatively affected the growth of algae, HGM+Protease 0h was less effective than the HGM+Protease 48 h. This test should be confirmed with a negative control in which denatured protease is added to HGM. The protease may bind an important component in the media, as addition of any protease reduces the growth rates. Had the cause of the increased algal growth rate been a protein degraded by protease, the 0h hour incubation time treatment would have outperformed the 48 hour incubation time treatment.

5.3 Yeast Extract Fraction Growth: Methanol Separation

The optical density as a function of growth time for *A. protothecoides* grown on AGM supplemented with methanol insoluble fraction (MIS) and methanol soluble

fraction (MS) produced with a methanol contact time of 90 seconds and 20 g yeast extract per litre of methanol in the yeast extract refining step is shown in Figure 18.

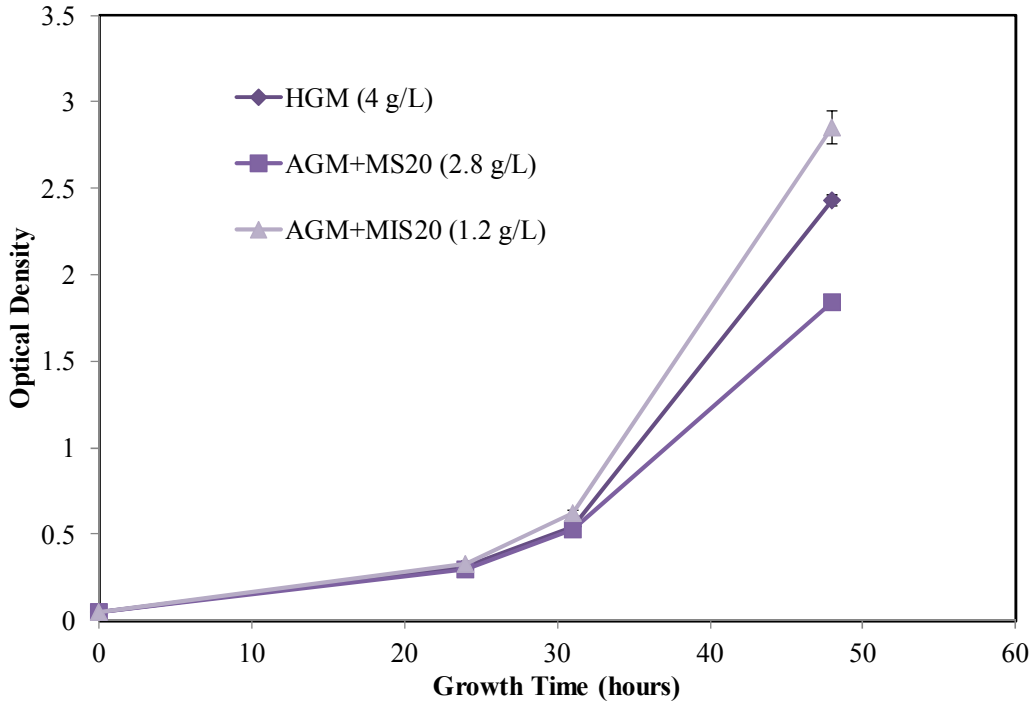


Figure 18. Optical density as a function of time for growth of *A. protothecoides* growth on AGM supplemented with methanol insoluble fraction (MIS20) and the methanol soluble fraction (MS20) produced from 20 g YE per litre of methanol. Methanol contact time was 90 seconds and approximately 100 mL of methanol was used.

Four grams of yeast extract was partially dissolved in methanol to yield 1.2 g of insoluble fraction (MIS20) and 2.8 g of soluble fraction (MS20). The MIS20 resulted in an increase of 17% in optical density after 48 hours for *A. protothecoides* and the MS20 resulted in a decrease of 24% in optical density at the very high statistical significance. The final dry weight is given in Table 11 confirm the optical density findings.

Table 11. Final dry weight of *A. protothecoides* after 48 hours growth on AGM supplemented with methanol insoluble fraction (MIS40) and methanol soluble fraction (MS40) produced from 40 g YE per litre of methanol. Methanol contact time was 90 seconds and 100 mL of methanol was used.

Growth Media	Dry Weight (g/L)
AGM+MS40	1.32±0.07
AGM+MIS40	2.05±0.08
HGM	1.84±0.04

The optical density as a function of growth time for *A. protothecoides* grown on AGM supplemented with methanol insoluble fraction (MIS) produced with a methanol contact time of 60 seconds for various concentrations of yeast extract per litre of methanol in the yeast extract refining step is shown in Figure 19.

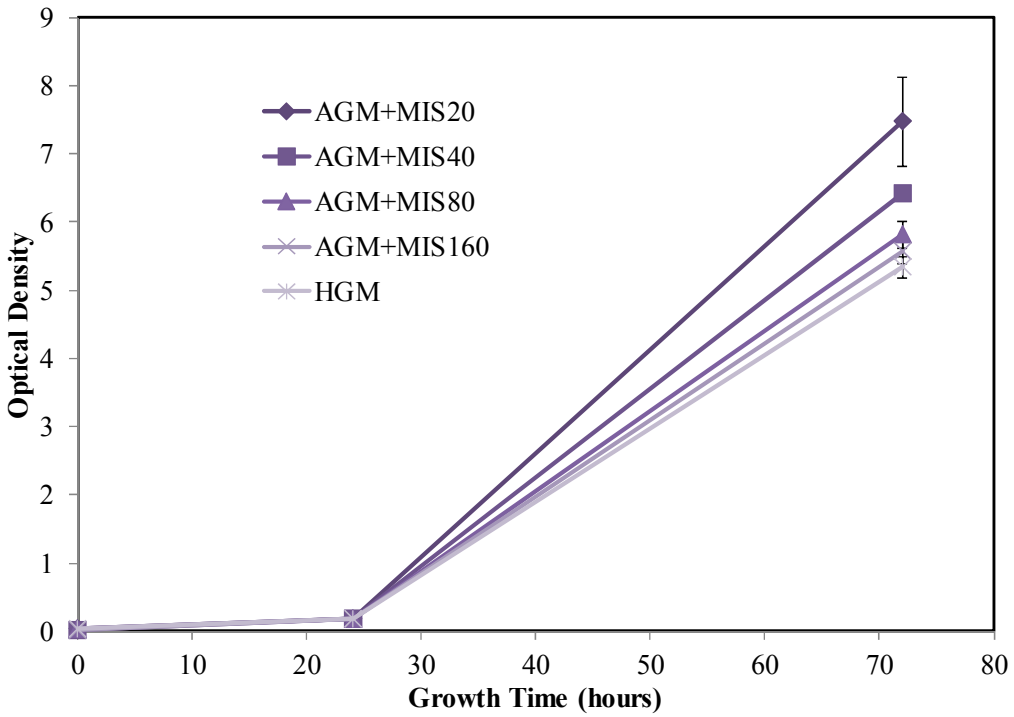


Figure 19. Optical density as a function of time for growth of *A. protothecoides* growth on AGM supplemented with methanol insoluble fraction (MIS) produced from 20 g YE per litre of methanol (MIS20), 40 g YE per litre of methanol (MIS40), 80 g YE per litre of methanol (MIS80), and 160 g YE per litre of methanol (MIS160). All media contained 4 g/L of MIS fraction. Methanol contact time was 60 seconds and 500 mL of methanol was used.

The highest final optical density was for the most dilute methanol extraction (MIS20), at 20 g YE per litre of methanol and was found to be statistically different 99.97% confidence from the other treatments. The MIS40 was found to be distinct from the MIS80, MIS160, and HGM treatments at 99.98% confidence. The MIS80 was different to 95% confidence. The same trend of increased growth at more dilute YE in methanol is displayed in Table 12 for the final dry weight, cell counts, and average cell sizes. The MIS20 results in a 65% increase in cell count over HGM. The rapid induced cell division results in a decrease in the average cell size.

Table 12. Final dry weight of *A. protothecoides* after 72 hours growth on AGM supplemented with methanol insoluble fraction (MIS) produced from 20 g YE per litre of methanol (MIS20), 40 g YE per litre of methanol (MIS40), 80 g YE per litre of methanol (MIS80), and 160 g YE per litre of methanol (MIS160). All media contained 4 g/L of MIS fraction. Methanol contact time was 60 seconds and 500 mL of methanol was used.

Growth Media	Dry Weight (g/L)	Cell Count (10^6 cell/mL)	Avg. Cell Size (µm)
AGM+MIS20	3.66±0.16	91.1±1.2	5.23±0.04
AGM+MIS40	3.59±0.05	68.2±2.6	5.79±0.04
AGM+MIS80	3.25±0.16	59.7±4.5	6.05±0.14
AGM+MIS160	3.25±0.13	56.4±1.1	6.36±0.06
HGM	3.23±0.16	55.0*	6.36*
*** Standard deviation unavailable**			

The optical density as a function of growth time for *A. protothecoides* grown on AGM supplemented with methanol washed yeast extract (i.e. not separated) and methanol insoluble fraction (MIS) produced with a methanol contact time of 60 seconds for various concentrations of yeast extract per litre of methanol in the yeast extract refining step is shown in Figure 20.

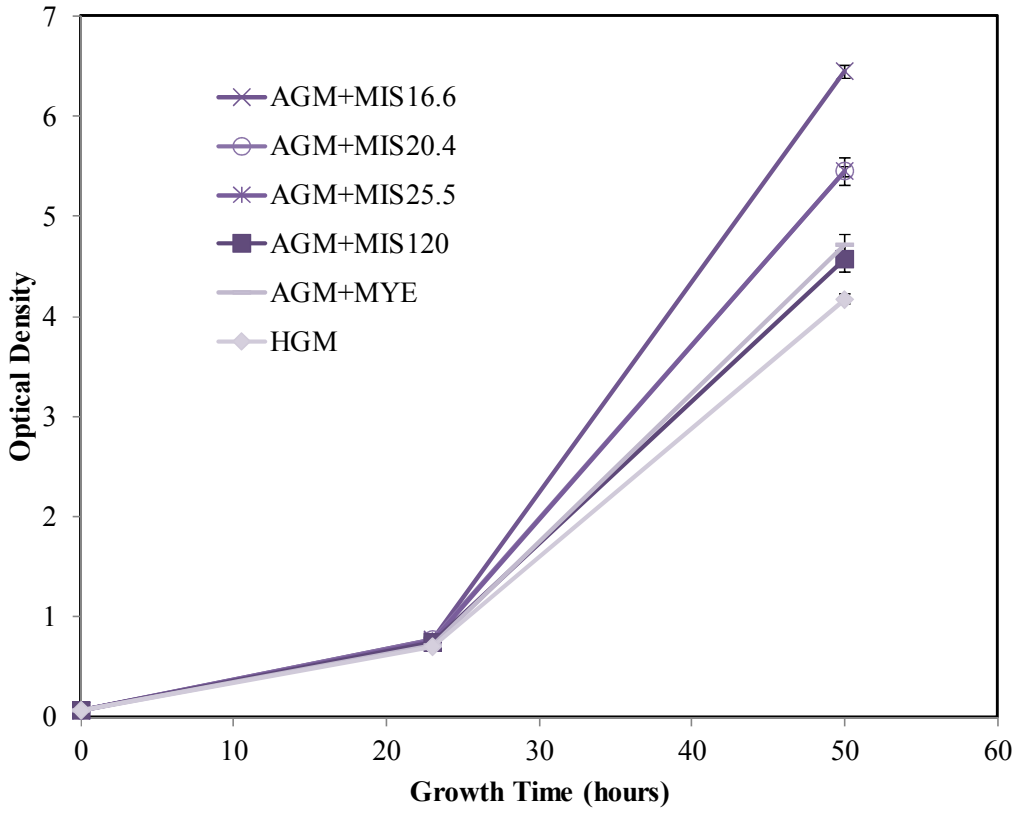


Figure 20. Optical density as a function of time for growth of *A. protothecoides* growth on AGM supplemented with methanol insoluble fraction (MIS) produced from 16.6 g YE per litre of methanol (MIS16.6), 20.4 g YE per litre of methanol (MIS20.4), 25.5 g YE per litre of methanol (MIS25.5), 120 g YE per litre of methanol (MIS120), and 20 g YE per litre of methanol wherein the methanol was removed and the washed yeast extract was used (MYE). All media contained 4 g/L of MIS or MYE fraction. Methanol contact time was 90 seconds and 500 mL of methanol was used.

The final dry weight, cell count, and average cell size for the fractions in Figure 20 is shown in Table 13. The optical density, dry weight, cell count, and cell size AGM+MIS20.4 and AGM+MIS25.5 were not statistically distinct from each other at 95% confidence. The AGM+MIS16.6 had a highly significant increase over the other fractions tested increase of 24.7% and 149.4% for dry weight and cell count, respectively compared to HGM. The lower the concentrations of yeast extract in methanol during the extraction, the more effective the resulting insoluble fraction.

Table 13. Final dry weight of *A. protothecoides* after 72 hours growth on AGM supplemented with methanol insoluble fraction (MIS) produced from 16.6 g YE per litre of methanol (MIS16.6), 20.4 g YE per litre of methanol (MIS20.4), 25.5 g YE per litre of methanol (MIS25.5), and 120 g YE per litre of methanol (MIS120). All media contained 4 g/L of MIS fraction. Methanol contact time was 90 seconds and 500 mL of methanol was used.

Growth Media	Dry Weight (g/L)	Cell Count (10^6 cell/mL)	Avg. Cell Size (µm)
AGM+MIS16.6	3.07±0.10	76.1±2.3	5.04±0.03
AGM+MIS20.4	2.49±0.08	49.7±1.9	5.63±0.07
AGM+MIS25.5	2.70±0.16	48.4±5.4	5.78±0.13
AGM+MIS120	2.49±0.12	28.3±2.0	6.68±0.05
MYE	2.55±0.09	28.3±2.0	6.36±0.01
HGM	2.46±0.08	30.5±2.7	6.57±0.11

The optical density as a function of growth time for *A. protothecoides* grown on 20 g/L glucose (AGM) and 15 g/L glycerol (GGM) supplemented with methanol insoluble fraction (MIS) produced with a methanol contact time of 60 seconds for various concentrations of yeast extract per litre of methanol in the yeast extract refining step is shown in Figure 21.

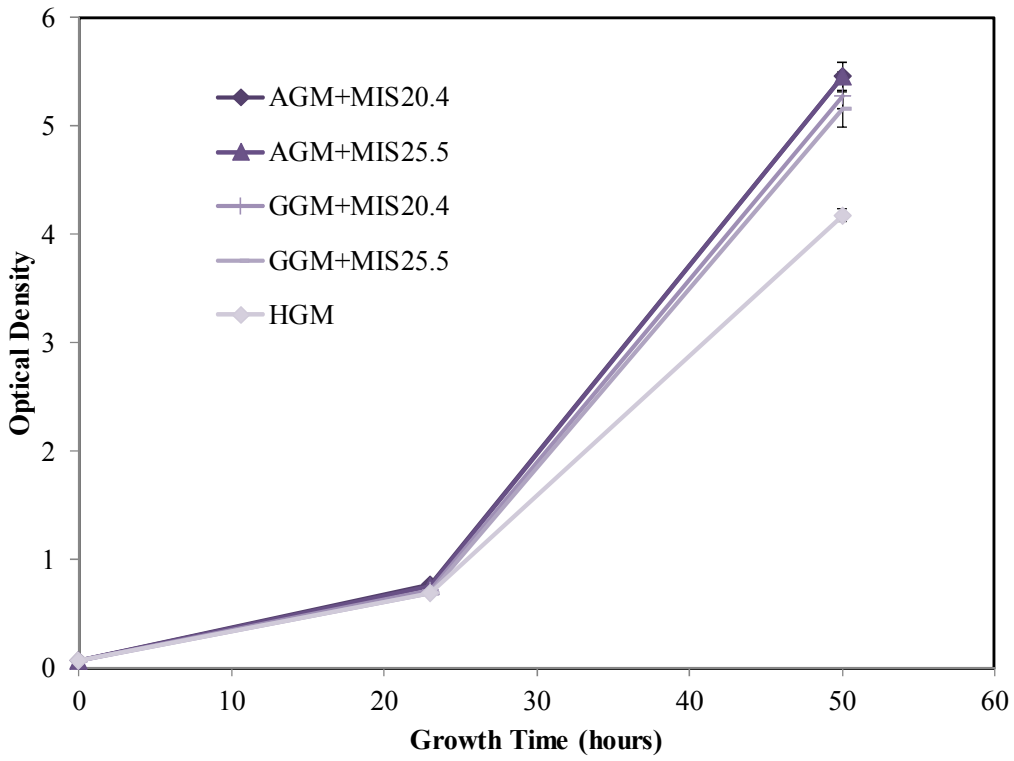


Figure 21. Optical density as a function of time for growth of *A. protothecoides* growth on glucose (AGM) and glycerol (GGM) supplemented with methanol insoluble fraction (MIS) produced from 20.4 g YE per litre of methanol (MIS20.4) and 25.5 g YE per litre of methanol (MIS25.5). Methanol contact time was 90 seconds and 500 mL of methanol was used.

The final dry weight, cell count, and cell sizes are shown in Table 14. The optical density and dry weight of AGM+MIS20.4, AGM+MIS25.5, GGM+MIS20.4, and GGM+MIS25.5 were not statistically distinct at 95% confidence. Extraction of yeast extract at 20.5 and 25.5 g of yeast extract per litre of methanol resulted in the same increase in optical density (average 27.8%) for a 20 g/L glucose (AGM) and 15 g/L glycerol (GGM) carbon source compared to HGM. The dry weight of the fractions on glucose or glycerol was not distinct from HGM. The cell count of the glycerol grown *A. protothecoides* was significantly higher than the glucose grown *A. protothecoides* (an average increase of 123.4% over HGM). The cells are smaller when grown in glycerol than glucose due to any number of reasons, such as osmotic stresses induced from the glycerol, or rapid cell division, etc.

Table 14. Final dry weight, cell count, and average cell size of *A. protothecoides* after 72 hours growth on glucose (AGM) and glycerol (GGM) supplemented with methanol insoluble fraction (MIS) produced from 20.4 g YE per litre of methanol (MIS20.4) and 25.5 g YE per litre of methanol (MIS25.5). All media contained 4 g/L of MIS fraction.

Growth Media	Dry Weight (g/L)	Cell Count (10^6 cell/mL)	Avg. Cell Size (µm)
AGM+MIS20.4	2.49±0.08	49.7±1.9	5.63±0.07
AGM+MIS25.5	2.70±0.16	48.4±5.4	5.78±0.13
GGM+MIS20.4	2.41±0.07	70.5±3.0	4.85±0.07
GGM+MIS25.5	2.49±0.08	65.9±4.6	4.86±0.00
HGM	2.46±0.08	30.5±2.7	6.57±0.11

The optical density as a function of growth time for *A. protothecoides* grown on 20 g/L glucose (AGM) supplemented with methanol insoluble fraction (MIS) produced with a methanol contact time of 120 seconds and a 16.6 g yeast extract per litre of methanol for various concentrations of yeast extract fraction in the growth media is shown in Figure 22. The final cell counts and average cell size of *A. protothecoides* grown heterotrophically on AGM supplemented with yeast extract fraction MIS16.6 is shown in Table 15.

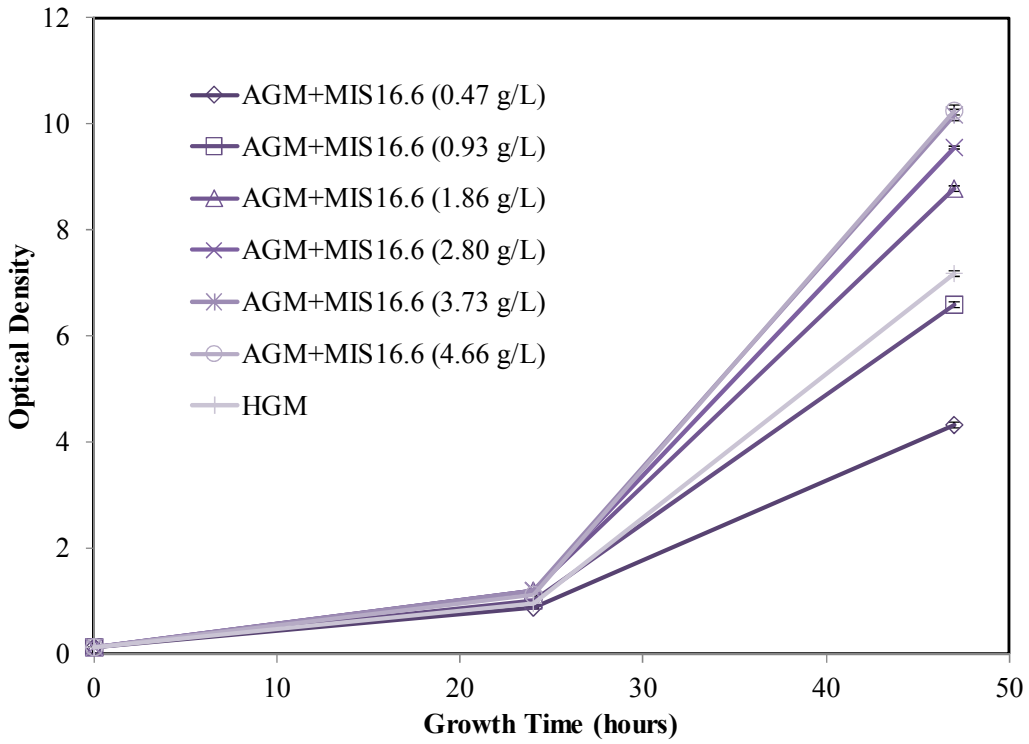


Figure 22. Optical density as a function of time for growth of *A. protothecoides* growth on glucose (AGM) supplemented with methanol insoluble fraction (MIS) produced from 16.6 g YE per litre of methanol (MIS16.6). Methanol contact time was 120 seconds and 3.6 L of methanol was used.

Table 15. Final cell count and average cell size of *A. protothecoides* after 72 hours growth on glucose (AGM) supplemented with methanol insoluble fraction (MIS) produced from 16.6 g YE per litre of methanol (MIS16.6). Methanol contact time was 120 seconds and 3.6 L of methanol was used.

Growth Media	Cell Count (10^6 cell/mL)	Avg. Cell Size (µm)
AGM+MIS16.6 (0.47 g/L)	10.3±0.5	4.68±0.04
AGM+MIS16.6 (0.93 g/L)	13.5±0.6	4.96±0.03
AGM+MIS16.6 (1.86 g/L)	17.8±0.6	5.45±0.02
AGM+MIS16.6 (2.80 g/L)	18.0±1.0	5.69±0.06
AGM+MIS16.6 (3.73 g/L)	17.0±0.9	6.07±0.05
AGM+MIS16.6 (4.66 g/L)	16.0±0.9	6.29±0.05
HGM	14.0±1.3	6.73±0.18

The optical density of 4.66 g/L and 3.73 g/L were not statistically significant from each other after 48 hours with each other test being statistically significant at the 95% confidence interval. In general, the incremental increase in concentration of MIS16.6 had diminishing returns on the increase of optical density that could be obtained after 48 hours, shown in Figure 23.

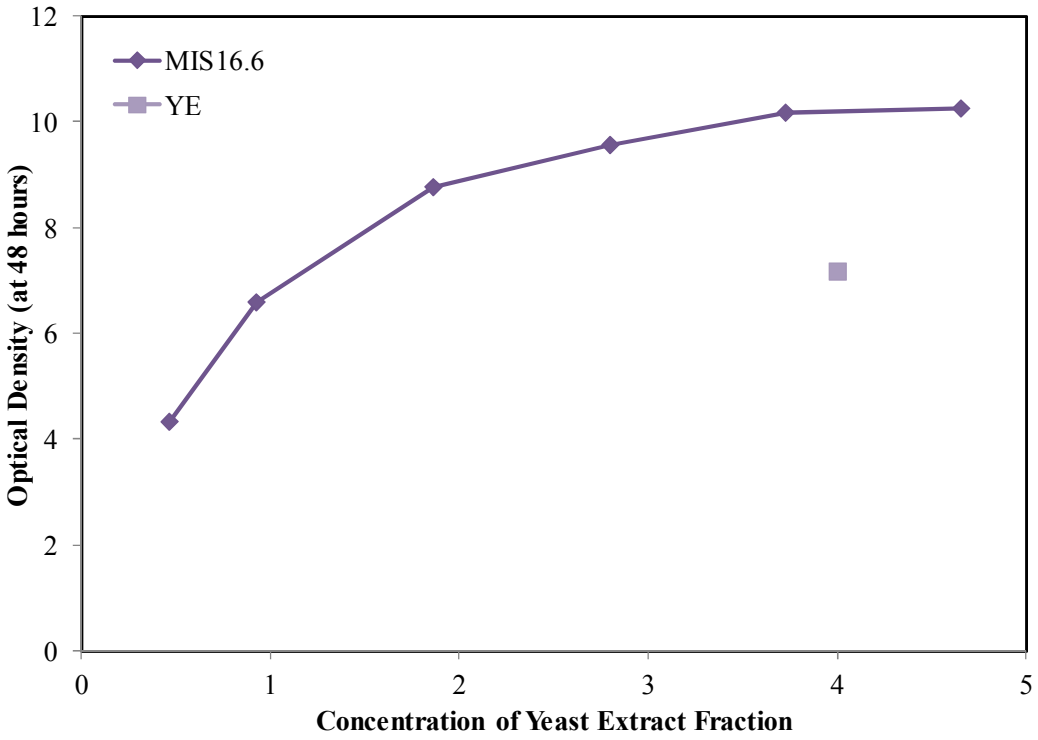


Figure 23. Optical density after 48 hours as a function of yeast extract fraction concentration of *A. protothecoides* growth on glucose (AGM) supplemented with methanol insoluble fraction (MIS) produced from 16.6 g YE per litre of methanol (MIS16.6). Methanol contact time was 120 seconds and 3.6 L of methanol was used.

The optical density as a function of time for *A. protothecoides* growth on glucose medium (AGM) supplemented with various concentrations of the methanol soluble portion of yeast extract (MS6.6) separated at 6.6 g of yeast extract per litre of methanol is shown in Figure 24. The optical density of *A. protothecoides* after 48 hours was statistically lower when grown with the tested concentrations of the methanol soluble portion of yeast extract than with 4 g/L of yeast extract. The optical density of *A. protothecoides* grown on the methanol soluble portion of yeast extract can approach value when grown on complete yeast extract at very high concentrations (above 6 g/L), but is less efficacious at lower concentrations (lower than 6 g/L).

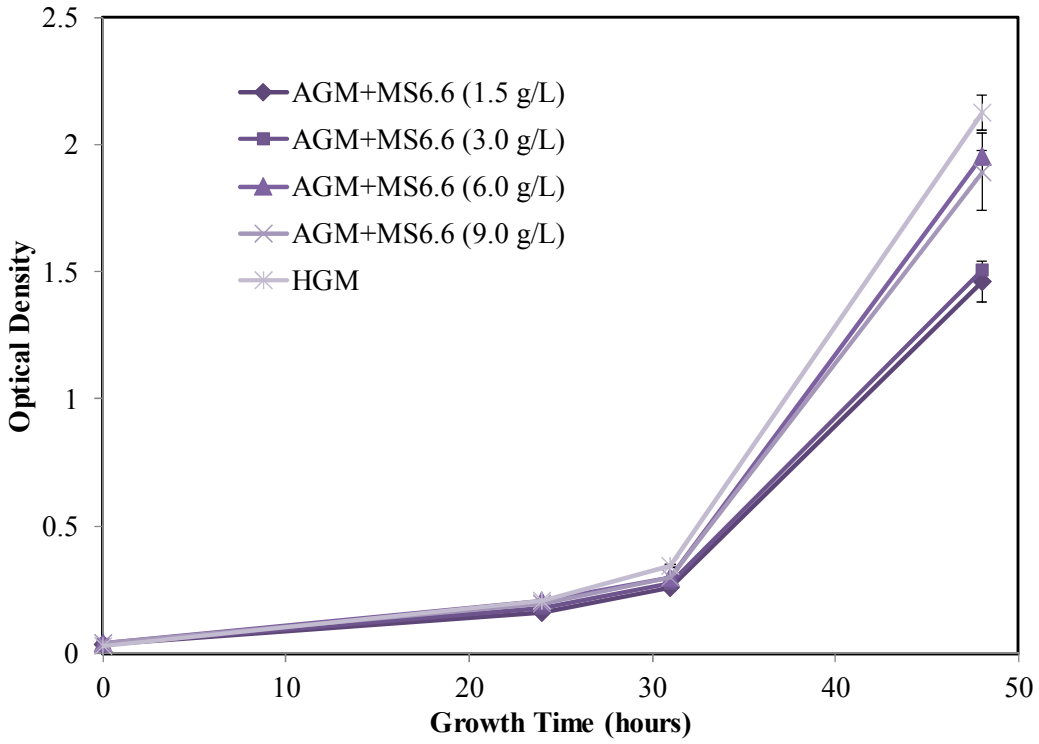


Figure 24. Optical density as a function of time for A. protothecoides growth on glucose medium (AGM) supplemented with various concentrations of the methanol soluble portion of yeast extract (MS6.6) separated at 6.6 g of yeast extract per litre of methanol.

The methanol soluble and methanol insoluble portion of yeast extract were added back together in both a 50/50 mixture, and by the proportions in which they are found in yeast extract. The optical density as a function of time for *A. protothecoides* grown on glucose medium supplemented with reconstituted yeast extracts (the sub fractions of yeast extract added back together) is shown in Figure 25. The natural proportion of yeast extract sub fractions do not recreate the same growth as complete yeast extract. However, the biological efficacy of yeast extract can be restored by separating the yeast extract, then recombining the resulting fractions in a slightly different proportion. The 50/50 mixture of methanol insoluble and methanol soluble yeast extract fractions are statistically the same at 95% confidence.

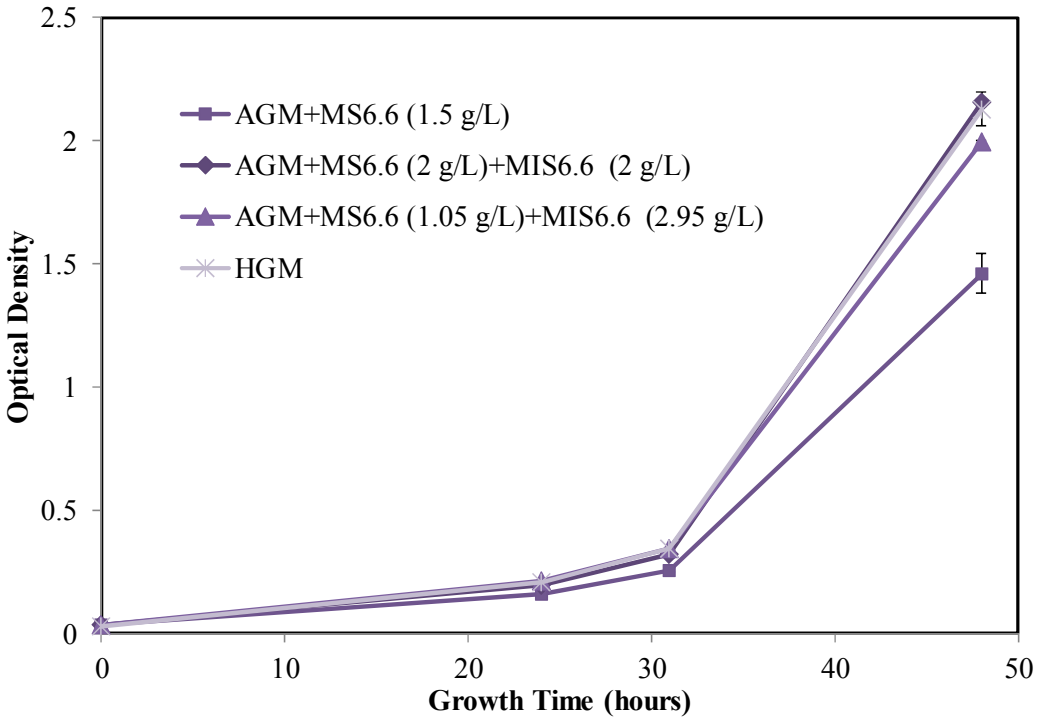


Figure 25. Optical density as a function of time for *A. protothecoides* grown in glucose medium (AGM) supplemented with the methanol soluble portion of yeast extract that was separated using 6.6 g of yeast extract in methanol (MS6.6), and the methanol insoluble portion of yeast extract that was separated using 6.6 g of yeast extract in methanol (MIS6.6).

5.4 Yeast Extract Fraction Growth: Isopropanol/Water Separation

The cell count as a function of time for *A. protothecoides* growth on glucose media (AGM) supplemented with isopropanol extract (IT) and isopropanol raffinate (IB) produced from 70 g YE per litre of isopropanol is shown in Figure 26. The concentration of the yeast extract (fractions) in the medium was 4 g/L. The components of yeast extract in the less dense raffinate (IT70) result in 51.0% decrease in the cell count of *A. protothecoides* after 72 hours compared to complete yeast extract (HGM). The growth promoting compounds of yeast extract appear to be concentrated into the more dense extract (IB70) with an increase of 55.6% in the cell count of *A. protothecoides* after 72 hours.

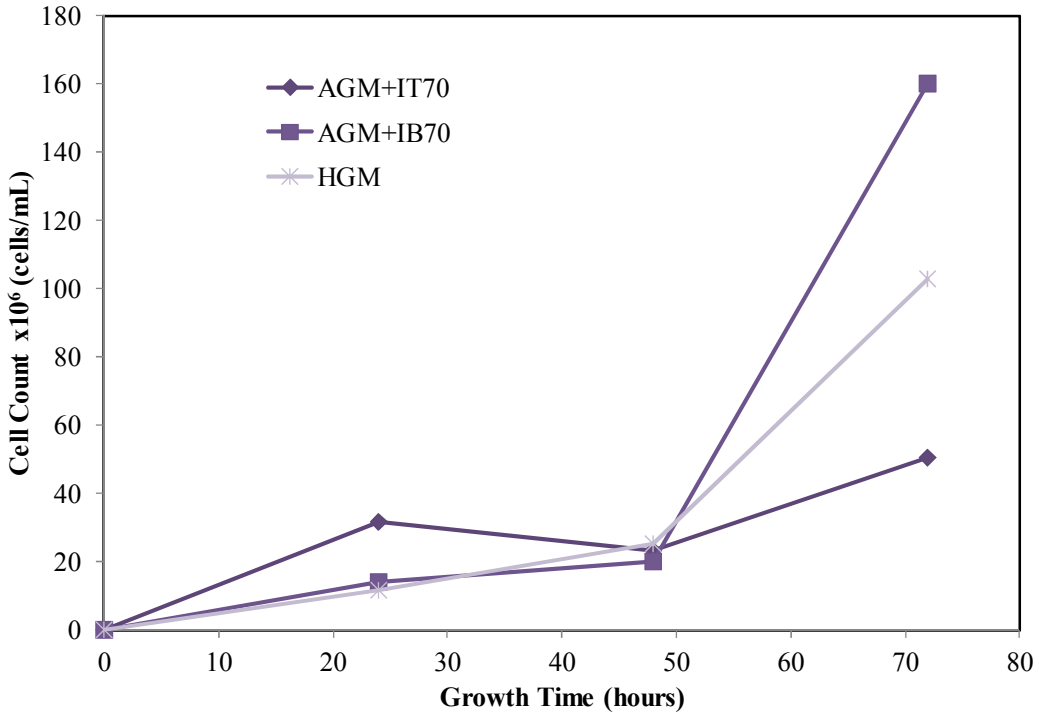


Figure 26. Cell count as a function of time for *A. protothecoides* growth on glucose (AGM) supplemented with isopropanol extract (IT) and isopropanol raffinate (IB) produced from 70 g YE per litre of isopropanol.

The cell count as a function of time for *A. protothecoides* growth on glucose media (AGM) supplemented with isopropanol extract (IT) and isopropanol raffinate (IB) produced from 90 g YE per litre of isopropanol is shown in Figure 27. The concentration of the IT90, IB90, and complete yeast extract (HGM) was 4 g/L. The IB90 resulted in an increase in cell count of 29.0% and the IT90 resulted in a decrease in cell count of 49.0% over complete yeast extract (HGM).

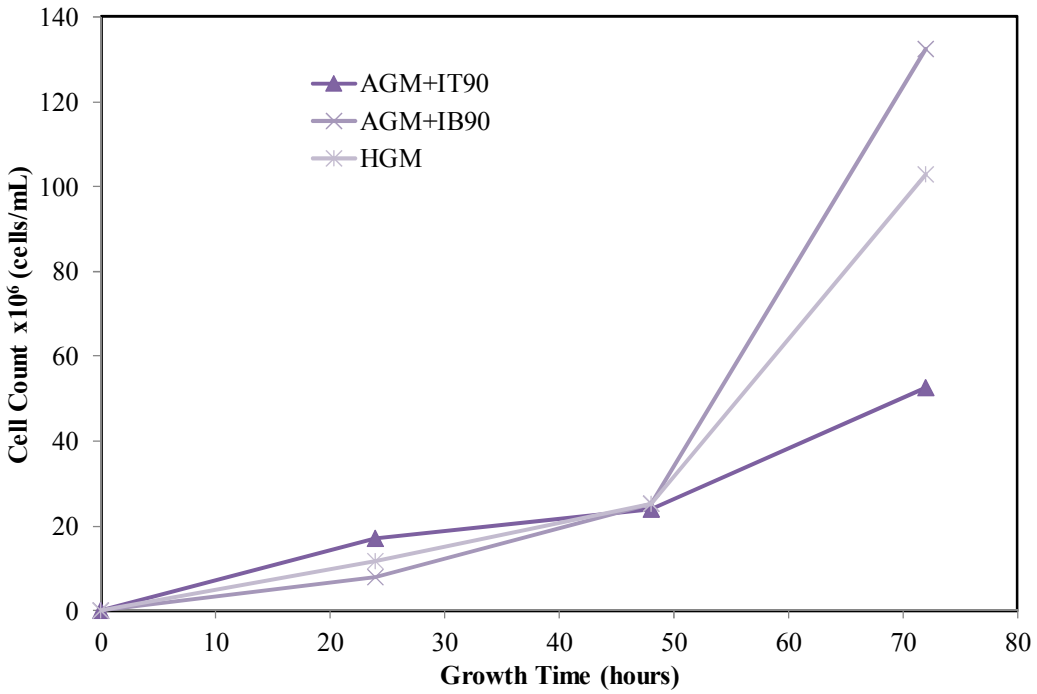


Figure 27. Cell count as a function of time for *A. protothecoides* growth on glucose (AGM) supplemented with isopropanol extract (IT) and isopropanol raffinate (IB) produced from 90 g YE per litre of isopropanol.

The isopropanol extraction of yeast extract results in two sub fractions of yeast extract: a raffinate (IT) and an extract (IB). The yeast extract was dissolved in 70%vol isopropanol/water at 70 g/L and 90 g/L. The lower the concentration of yeast extract in 70%vol isopropanol/water, the more efficacious the resulting more-dense extract fraction (IB) was at increasing the cell count of algae.

5.5 Sequential separation

The 70%vol isopropanol/water and methanol separations are predicted to separate the same components given their similar Hansen Solubility Parameters. However, the isopropanol separation does not result in a solid precipitate like the methanol separation. The different behaviour of isopropanol to produce two liquid phases may also result in the isopropanol extraction separating out different components of yeast extract in the isopropanol extract (IB) than the methanol insoluble fraction (MIS).

The cell count as a function of time is shown in Figure 28 for heterotrophic growth of *A. protothecoides* on glucose media (AGM) supplemented with the methanol soluble (MS) portion of yeast extract. The MS fraction was produced from 15 g/L of yeast extract in methanol was fully dried and then extracted with 70%vol isopropanol at 70 g/L to yield an extract fraction (IB70MS15) and a raffinate fraction (IT70MS15). The methanol soluble portion of yeast extract (MS15) decreases growth by 24% in optical density (see Figure 18) but the growth promotion of yeast extract can be partially restored to that of complete yeast extract via separation with isopropanol. The isopropanol raffinate of the methanol soluble portion should contain the least growth promoting compounds, or inhibitory compounds. However, the growth promotion does not decrease further when the methanol soluble portion of yeast extract is further separated with isopropanol. Therefore, it is proposed that the mechanism of action of yeast extract is solely growth promotion with inhibitory substances not being present at sufficient quantities to affect algal growth.

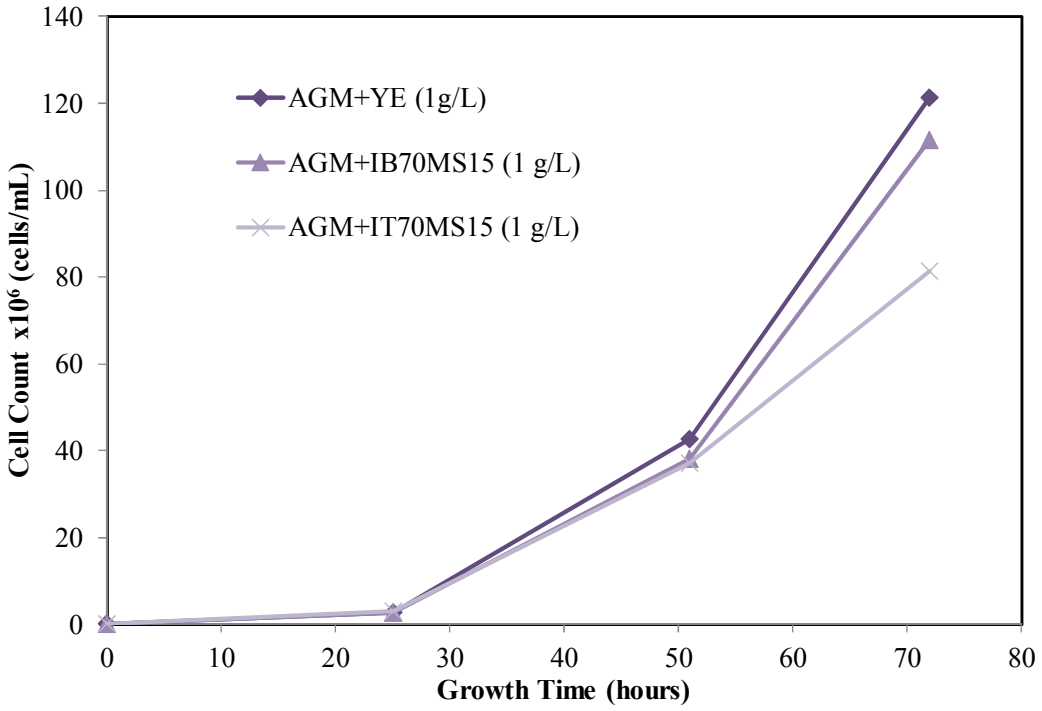
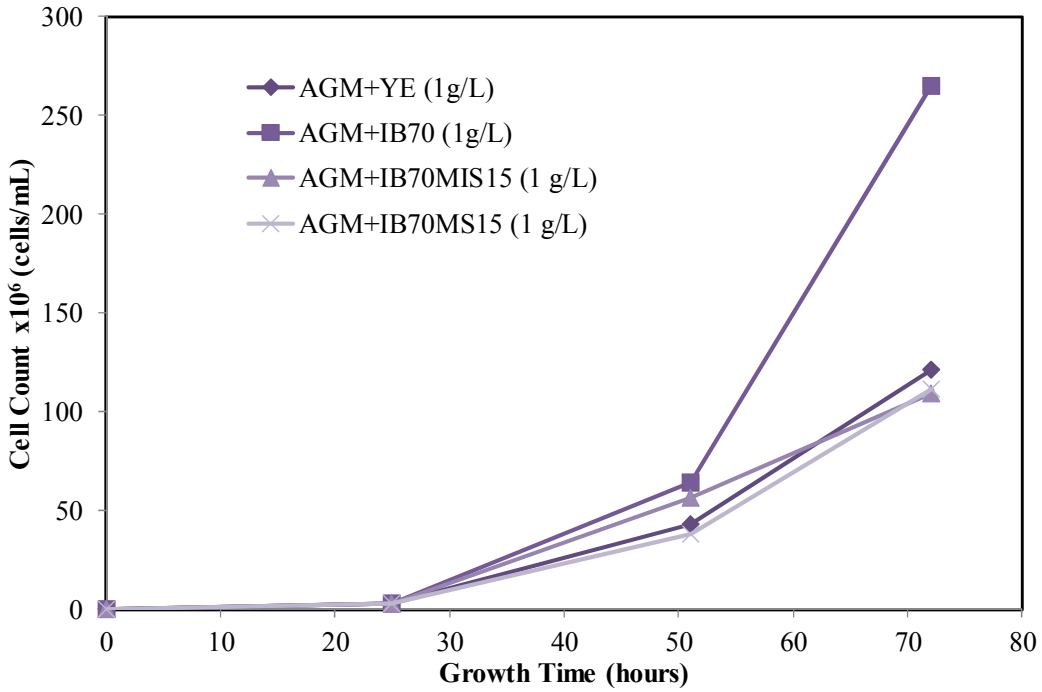


Figure 28. Cell count as a function of time for *A. protothecoides* grown in glucose medium (AGM) supplemented with the dried methanol soluble yeast extract that was extracted with 70%vol isopropanol at 70 g/L to yield an extract (IB70MS15) and a raffinate (IT70MS15). The methanol soluble portion of yeast extract was prepared at 15 g/L and a contact time of 200 seconds.

The cell count as a function of time is shown in Figure 29 for heterotrophic growth of *A. protothecoides* on glucose media (AGM) supplemented with sequentially solvent-separated yeast extract. To produce the fractions, the methanol soluble portion of yeast extract, produced from 15 g/L of yeast extract in methanol (MS15) was fully dried and then extracted with 70%vol isopropanol at 70 g/L to yield an extract fraction (IB70MS15) and a raffinate fraction (IT70MS15). The methanol soluble portion of yeast extract (MS15) decreases growth by 24% in optical density (see Figure 18) but the growth promotion of yeast extract can be partially restored to that of complete yeast extract via separation with isopropanol.



18

Figure 29. Cell count as a function of time for *A. protothecoides* grown in glucose medium (AGM) supplemented with the dried methanol insoluble yeast extract that was extracted with 70%vol isopropanol at 70 g/L to yield an extract (IB70MIS15) and a raffinate (IT70MIS15). The methanol insoluble portion of yeast extract was prepared at 15 g/L and a contact time of 200 seconds.

6 Discussion

The nutritional requirements of *Auxenochlorella protothecoides* has been studied extensively and a variety of complex and defined media are used for its culture[75]. Algae cultured on yeast extract containing complex media generally grows faster than defined media. Yeast extract is a complex mixture of biological materials composition is source dependant. The relative amounts of the biological components are critical to growth promotion of microorganisms.

Yeast extract was chosen for fractionation to explore which moieties of yeast extract promote growth and in what way (i.e. cell count, dry weight, oil content, etc.). Previous studies on yeast extract's growth promotion have isolated and partially characterized fractions of yeast extract on the acidophilic thermophilic mycoplasma *Thermoplasma acidophilum*[57], though no previous work has been conducted using the methods within this thesis. Among several hundred compounds tested, only glutathione plus iron elicited growth response in *Thermoplasma acidophilum*[57].

Yeast extract has a maximum grow stimulating effect for *A. protothecoides* at 4 g/L yeast extract, as is presented in Figure 15 and Figure 16 which shows that adding more or less initial concentrations of yeast extract results in a lower specific growth rate. This suggests that the stimulatory effect of yeast extract becomes saturated and even inhibitory at high concentrations. The effect of yeast extract follows the same pattern as that of nitrogen and carbon feeds where at low concentrations, adding more nitrogen or carbon increases the growth of microalgae[44, 45, 48, 76]. The growth stimulating effect of whole yeast extract cannot be improved by simply increasing its concentration. The stimulating effect of yeast extract is however thermally sensitive. Figure 17 shows that when yeast extract is filter sterilized, the dry weight after 72 hours is 10% higher than when it is sterilized. This difference shows a moiety of yeast extract is thermally sensitive.

Given the complex nature of the composition of yeast extract, it is possible that yeast extract could be improved by separation. There are a variety of methods used

to separate natural materials, such as separation based on size, solubility, charge, volatility, etc. Gaudreau *et al.* found that ultrafiltration with 1, 3, and 10 kD pore size did not yield fractions of yeast extract that significantly better than the whole parent mixture[77]. Yeast extract has also been separated using adsorbance via liquid chromatography. Smith *et al.* fractionated yeast extract on a Sephadex G-25 column and found 70% of the amino N present in one fraction as well as a component which decomposed H2O2, an inhibitory metabolite which accumulates in *Streptococcus lactis* growth[78].

This study investigates solvent separation as a means to concentrate the growth promoting or growth inhibiting moieties present in yeast extract. Yeast extract partitions in several solvents. As will be discussed later, the fractions from solvent separation of yeast extract have different stimulating effects than whole yeast extract.

Yeast extract is proposed to be separated by the alcohol solvents in the presence of water. Methanol, ethanol and isopropanol precipitate nucleic acids and DNA fragments. DNA precipitates at 35% in isopropanol and 0.5M salt solution where ethanol requires 75% with 0.5M salt[79]. The alcohol-based solvents will most probably separate bioavailable growth factors which could include DNA, nucleic acids, proteins, and cofactors.

In methanol, the yeast extract partially dissolves. The residual solid can be separated from the methanol solution. The proportion of dissolved yeast extract is dependent on the solvent-yeast extract contact time and concentration (as shown in Figure 7). The mass yield of methanol-insoluble yeast extract is characterized by a sharp drop upon contact with methanol until about 30 seconds. This behaviour is described by the Noyes-Whitney equation.

When the methanol is first added to solution (t=0), the concentration in the bulk of the solution is also zero ($c_t(0) = 0$). After approximately thirty seconds, the bulk phase concentration is appreciable. The surface concentration (c_s) of methanol soluble compounds is the same for all concentrations initially, but decreases with

time more rapidly at low concentrations as it becomes depleted $\left[(c_s - c_t)_{low} > (c_s - c_t)_{high}\right]_{initial}$ and $(c_t \rightarrow c_s)_{low}$ faster than $(c_t \rightarrow c_s)_{high}$. For all concentrations of yeast extract, the bulk concentration approaches a limit, such that the bulk concentration approaches the surface concentration ($c_t \rightarrow c_s$).

The Hansen Solubility Parameter is a method of predicting if a given material will dissolve in similar solvent systems using three parameters: dispersion, polarity, and hydrogen bonding (δ_D, δ_P, and δ_H). The dispersion portion of the HSP accounts for the Van Der Waals forces (induced dipoles), the polarity term accounts for permanent dipole-dipole interactions, and a third term for hydrogen bonding. The HSP is a means of quantifying that like dissolves like. Methanol has the solubility parameters of (14.7, 12.3, and 22.3) whereas isopropanol has the solubility parameters of (15.8, 6.1, and 16.4). From the HSP, we expect that isopropanol will dissolve more non-polar substances than methanol. An 84% ethanol mixture HSP (15.8, 10.0, and 23.1) is closer to methanol than a 72% isopropanol HSP (15.7, 8.9, 23.7). The Hansen Solubility Parameter of several mixtures of low molecular weight solvents (ethanol, isopropanol, acetone, among others) and water was matched to that of methanol by adjusting the proportion of water in Table 8. Of the solvents tested, isopropanol/water was the most promising (it had two distinct easily separated phases). An isopropanol/water mixture of 72% volume was calculated to be the most close to methanol in the isopropanol. However, Hansen Solubility Parameters are approximate and a 70% isopropanol mixture was selected and formed two separable phases when added to yeast extract at 70 and 90 g/L of initial yeast extract in solvent.

The isopropanol/water separation is time-independent (see Figure 7). The isopropanol/water/yeast extract system separates into two phases: a denser phase (extract) and a less dense phase (raffinate). In general, a higher concentration of yeast extract results in a larger mass fraction of extract and a lower mass fraction of raffinate.

The complex nature of yeast extract makes characterizing it and its subebsequent fractions difficult. Compositional analysis by thermogravimetric analysis (TGA) has been used previously for purity determinations, and characterization of active ingredients[80]. DSC has the ability to give valuable physical property information such as melting points, specific heat capacities, glass transition points, and vapour pressures. Differential scanning calorimetry measures the difference in heat required between a reference cell and the sample. DSC is very sensitive to minute changes in composition, phase, morphology, and organic degradation (such as protein denaturation) and can be used to evaluate complex biological sample's purity[64]. Previous work has used specific heat capacities from thermal analysis to find minute differences in complex mixtures[80]. As the identity of the growth promoting compounds present in yeast extract are yet unknown, the thermoanalytical tool, DSC, was chosen to confirm the physical differences between yeast extract fractions and whole yeast extract. A change in measured physical properties will be due to chemical changes or differential compositional partitioning in the solvent systems.

Yeast extract partitions in methanol into two fractions (a dissolved fraction and a solid). These two fractions of yeast extract were found to be physically different using differential scanning calorimetry. The solid portion retains an endothermic peak also present in complete yeast extract. However, the soluble portion of yeast extract does not exhibit defined peaks in Figure 8.

The endothermic peaks in Figure 8 at 28 °C and 61.8 °C could be caused by any number of compound types, from sugars such as trehalose[65], starch[81], glycol-glycerophospholipids[82], organic acid assemblies[83], and protein[84]. Neither the soluble nor the raffinate fraction's DSC analysis contained an endothermic peak in Figure 8 and Figure 9.

The insoluble fraction and extract fraction exhibit an endothermic peak similar to that of the complete yeast extract in Figure 8 and Figure 9. The onset and peak temperatures of the insoluble and extract are summarized with those of complete

yeast extract in Table 9. The onset temperature for the complete yeast extract and insoluble fraction are similar, however the insoluble fraction has a higher peak temperature. The similar packing, mass, and sample collection suggests that this is due to a composition change. A different composition in the extract fraction is thought to cause a lower onset and peak temperature than complete yeast extract. The extract fraction and the soluble fraction both promote the heterotrophic growth of algae. The DSC thermal behaviour of the fractions suggests that the S-L and L-L is primarily separation, with minor reactions that change the peak temperatures.

The methanol soluble fraction of yeast extract does not exhibit an endothermic peak in Figure 8 and when it is further separated with isopropanol, the resulting fractions also do not contain an endothermic peak in Figure 11. Likewise, the raffinate fractions do not exhibit an endothermic peak, regardless of the source before separation. The soluble fraction, its derivative L-L extract fractions, and any raffinate fractions (see Figure 13 and Figure 12). The extract fraction of the insoluble fraction (see Figure 10 has the same onset and peak temperatures, but at a more endothermic heat flow. This suggests that the L-L extraction has further purified the compound which produces the endothermic peak. Contradictorily, the extract fraction grew faster than the extract of the insoluble fraction. Therefore the S-L technique's solvent (i.e. methanol) reacts with the yeast extract as well as separates its constituents. The growth was not repeated, so the observation is not reliable. The extract of the insoluble fraction did not shift the endothermic peak to a lower temperature (see Figure 11) as expected from the extract's effect on yeast extract (see Figure 10). Subsequent L-L separation with isopropanol solely separated the mixture because the methanol had already reacted with the functional groups in the yeast extract. This suggests that the isopropanol also reacts with the yeast extract, but cannot displace methanol in the yeast extract.

The isopropanol/water extract fraction produced has a greater stimulatory response in *A. protothecoides* than the methanol fraction (see Figure 23, Figure 26, and Figure 27). The isopropanol/water separation may be better at separating the growth promoting factor(s) from yeast extract, but probably separates a distinct mixture of

compounds than the methanol separation. The DSC behaviours of the methanol and isopropanol/water fractions are quite different from yeast extract (see Figure 8 and Figure 9). The insoluble fraction of methanol shifts the peak to a higher temperature, to 67.6 °C where the isopropanol raffinate fraction shifts the peak to a lower temperature of 54.9 °C from the complete yeast extract (67.7 °C). The DSC behaviour suggests that different compound(s) are separated from the complete yeast extract depending on the solvent selected because the peak temperature shifts substantially.

The methanol insoluble portion of yeast extract increases the biological efficacy of the yeast extract by one third (at 4 g/L of insoluble fraction) over 4 g/L of complete yeast extract (see Figure 15 and Figure 23). Increasing the amount of methanol insoluble fraction in the initial growth media increases the growth rate of *A. protothecoides* to a maximum.

The methanol soluble portion of yeast extract is less efficacious than the insoluble portion, but at sufficiently high concentrations (6 g/L), it approaches the same values as complete yeast extract (at 4 g/L) as shown in Figure 24. The methanol soluble fraction exhibits much different DSC behaviour than yeast extract as well. Whereas the complete yeast extract had an endothermic peak at 61.8 °C, the methanol soluble fraction is devoid of any well-defined peaks in Figure 8 and Figure 10.

The kinetics of yeast extract dissolution in methanol solvent strongly affected the biological efficacy of the methanol yeast extract fraction. Yeast extract was found to be partially soluble in methanol. The kinetics of dissolution followed Noyes-Whitney kinetics, depicted in **Figure 6**. The rate of dissolution decreased as a function of time as the amount of dissolved yeast extract in methanol approached a maximum value. The initial amount of yeast extract in methanol affected the maximal amount of yeast extract that dissolved in methanol and the time required to reach the maximal concentration. The growth of *Auxenochlorella protothecoides* on the insoluble portion of yeast extract that was produced using initial yeast extract

in methanol concentrations from 16.6 g/L to 120 g/L is shown in Figure 20. The AGM+MIS16.6 (with an initial yeast extract in methanol concentration of 16.6 g/L) had a highly significant increase (99% confidence) over the other fractions tested. The growth factor in yeast extract could be a number of compounds, such as carbohydrates, proteins, RNA, DNA, nucleic acids, amino acids, cofactors, etc. Andreu *et al.* used yeast extract as a source of RNA for production of disodium 5'-inosinate and 5'-guanylate (flavour enhancers and food additives). They found that the RNA could be purified from yeast extract using 0.5 v/v ethanol with 1M NaCl with 61% yield and 45% purity[85]. Kollar *et al.* describe in detail the method for producing yeast extract in which yeast cells are autolysate, centrifuged, then spray dried after ultrafiltration to yield yeast extract[86]. If the growth promotion was due to RNA or precipitating proteins, one would expect that a longer solvent contact time would precipitate more of the growth factor and increase the efficacy of the compound. The initial yeast extract in solvent concentration should also be independent from the growth factor efficacy, however using a short solvent contact time and low yeast extract in solvent concentration results in the highest growth rates (an increase of 24.7% and 149.4% for dry weight and cell count, respectively compared to HGM, which contains complete yeast extract).

Protease was added to complete yeast extract and was incubated for 0 or 48 hours before being inactivated via thermal sterilization in an autoclave and the resultant growth curves are in Figure 17. If the growth promoting moiety of yeast extract had a protein component, and that component was degradable by protease, one would expect that the addition of protease would decrease the amount of available protein and thus there would be a decrease in the growth promotion of the yeast extract. The addition of protease and incubation for 48 hours resulted in a decrease in final dry weight of 23% compared to adding water. However, the addition of protease and incubation for 0 hours acted as a control for the effect of protease addition to the growth of *A. protothecoides* and resulted in a decrease of 28% in final dry weight. Had the growth promotion been due to a protein in yeast extract, the yeast extract would have been just as effective with 0 hours of incubation, as the protease

would have been deactivated immediately. Rather, there may be two effects at work: the protease inhibits the growth of *A. protothecoides* (seen by the decrease upon addition of protease regardless of incubation period) and the protease degrades a portion of the insoluble yeast extract to produce more bioavailable moieties that increase the growth of *A. protothecoides* (as seen by the relative increase in dry weight of 5% for protease addition with 48 hours of incubation to 0 hours of incubation).

Surprisingly, sequential separation using methanol separation twice, the isopropanol/water separation twice, or using a combination of the two separation techniques does not result in a better yeast extract fraction (see Figure 28 and Figure 29). Yeast extract is a heat-sensitive media component. During the drying of the yeast extract subfractions following treatment with solvent, temperatures of up to 60 °C are applied under vacuum. The DSC behaviours of the fractions show there is an irreversible chemical change to the fractions at this approximate temperature (see Figure 8). The actual process of drying the fractions may be degrading them, which is supported by Figure 10, Figure 11, Figure 12, Figure 13, and Figure 14, in which the endothermic peak disappears from the twice extracted yeast extract fractions.

To test the effects of producing the fractions (heat, air-contact, solvent-adsorption, solvent-desorption, etc.), complete yeast extract was subjected to the conditions of the methanol separation without being separated in Figure 20. The yeast extract was then dried without being separated and the resulting fraction (MYE) was grown against complete yeast extract. The optical density of MYE was slightly better than complete yeast extract. The growth of the algae was completed in triplicate and the result was found to be statistically significant at 95% confidence from complete yeast extract which could be caused by the methanol reacting irreversibly with the yeast extract. The resulting increase in optical density of approximately 10% does not translate into the dry weight, cell count, or average cell size suggesting that the methanol separation is only a separation. The optical density of a population is highly dependent on the cellular makeup, which is highly variable and the dry

weight, cell count, and average cell size measurements are more accurate and precise.

The discovery of these novel growth factors may prove useful for increasing the specific growth rates of microalgae cultures—a key economic parameter in feasibility studies. Deeper study of the specific compounds in the subfractions was deemed to be too big of a scope for this thesis and should be undertaken in future work. DSC should be used initially as the tool to evaluate if the sub separations were successful by the presence of an endothermic peak at approximately 60±10 °C. The presence of an endothermic peak would not quantify the growth promoting properties, however.

7 Conclusion

Microalgae have much higher growth rates and oil productivity compared to agricultural crops, and they do not require arable land. Microalgae can grow heterotrophically using chemical energy and phototrophically using light energy and store a portion of this energy as valuable products—pigments, nutraceuticals, pharmaceuticals, feeds, and biofuels. Heterotrophic growth of microalgae has much higher growth rates and densities than phototrophic growth of microalgae, but requires large amount of chemical feedstock.

The economic feasibility of heterotrophic algal growth depends on the growth parameters of microalgae: the specific growth rate, biomass yield, and cell densities[87, 88]. The maximum specific growth rate achievable under batch flask growth is comparable to that achievable under fed batch, open loop, and adaptive predictive, non-linear control strategies[38]. The maximum specific growth rate of microalgae in batch flasks can be used to predict the maximum specific growth rates at a larger scale[38]. The specific growth rate achievable in a hypothetical microalgae facility relates inversely to the capital cost of that facility[89].

The scope of this project was to examine the growth promotion of yeast extract on the microalgae, *Auxenochlorella protothecoides* by separating the yeast extract into smaller subfractions using solvent separation. Several factors including the key parameters of growth (e.g. specific growth rate) have been shown to affect the economics of microalgae facilities. Microalgae can produce a variety of valuable products—from food to fuel. In this study, yeast extract was fractionated using solvents and the subfractions were grown in glucose media. It was found that:

- yeast extract promotes growth of *A. protothecoides* to a maximum amount
- the maximum growth promotion can be increased by purifying the growth promotion compounds using methanol
- the methanol separation depends on the solvent contact time and the yeast extract in solvent concentration

- the Hansen Solubility Parameter predicted the isopropanol/water ratio that would separate the yeast extract in a similar function to methanol
- the maximum growth promotion of the isopropanol/water fractions were greater than the methanol fraction
- the isopropanol/water, methanol, and yeast extract growth promotion fractions shared a similar DSC peak at approximately 60 °C
- sequential separation of yeast extract did not improve the growth promotion properties and removed the endothermic DSC peak
- future work should identify the active compound(s) in yeast extract by subfractionation using DSC as tool to see if the subfractions contain a growth promoting compound

8 References

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Appendix A: Supplementary Figures.

This appendix contains supplementary figures. All data here is contained in the thesis and presented in a different way.

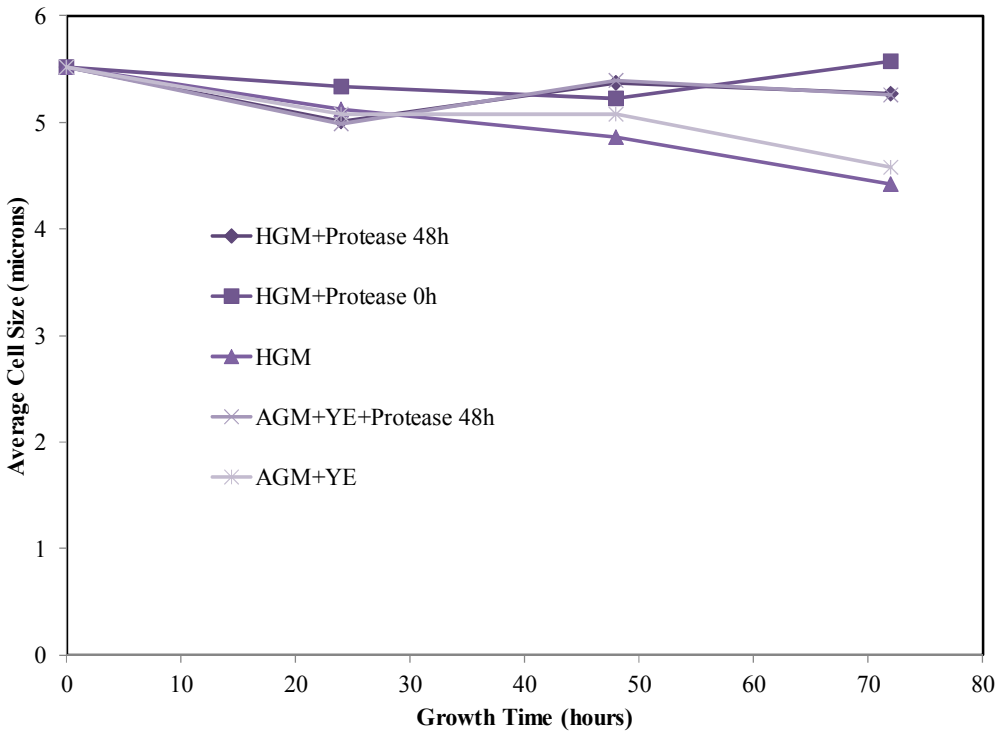

Figure Appendix A1: Average cell size of *Auxenochlorella protothecoides* grown on glucose media as a function of growth time for yeast extract with protease for 0 and 48 hour incubating time.

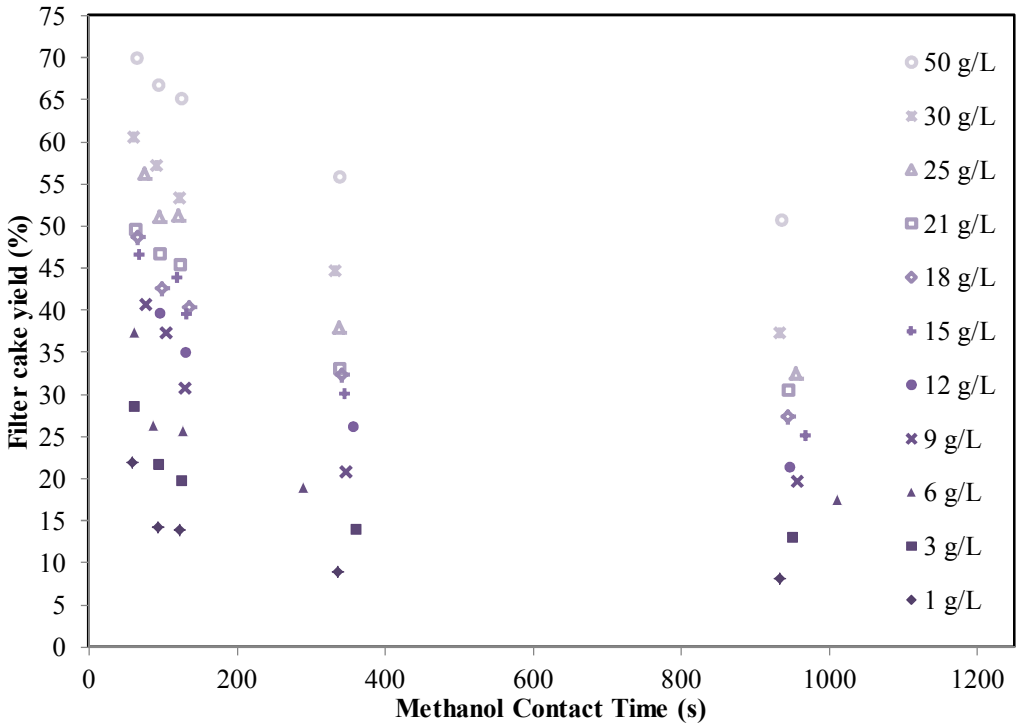

Figure Appendix A2: Effect of methanol contact time on filter cake yield for the partially soluble yeast extract in methanol-separation.

Appendix B: Computer Code

This appendix contains the excel macro (visual basic) code used to summarize and consolidate the cell count files. It was modified from code available for free from Ron de Burin's Excel automation website.

(de Bruin, Ron. Excel Automation Website, www.rondebruin.nl/win/s3/win022.htm)

```
Sub ConsolidateData()
'For Excel 2000 and higher
    Dim Fnum As Long
    Dim mysheet As Worksheet
    Dim basebook As Workbook
    Dim TxtFileNames As Variant
    Dim QTable As QueryTable
    Dim SaveDriveDir As String
    Dim ExistFolder As Boolean

    'Save the current dir
    SaveDriveDir = CurDir

    'You can change the start folder if you want for
    'GetOpenFilename,you can use a network or local folder.
    'For example ChDirNet("C:\Users\Ron\test")
    'It now use Excel's Default File Path

    ExistFolder = ChDirNet(Application.DefaultFilePath)
    If ExistFolder = False Then
        MsgBox "Error changing folder"
        Exit Sub
    End If

    TxtFileNames = Application.GetOpenFilename _
    (filefilter:="=#Z2 Files (*.=#Z2), *.=#Z2", MultiSelect:=True)

    If IsArray(TxtFileNames) Then

        On Error GoTo CleanUp

        With Application
            .ScreenUpdating = False
            .EnableEvents = False
        End With
```

```
'Add workbook with one sheet
Set basebook = Workbooks.Add(xlWBATWorksheet)

'Loop through the array with txt files
For Fnum = LBound(TxtFileNames) To UBound(TxtFileNames)

    'Add a new worksheet for the name of the txt file
    Set mysheet = Worksheets.Add(After:=basebook. _
                  Sheets(basebook.Sheets.Count))
    On Error Resume Next
    mysheet.Name = Right(TxtFileNames(Fnum), Len(TxtFileNames(Fnum)) - _
                   InStrRev(TxtFileNames(Fnum), "\", , 1))
    On Error GoTo 0

    With ActiveSheet.QueryTables.Add(Connection:= _
            "TEXT;" & TxtFileNames(Fnum), Destination:=Range("A1"))
        .TextFilePlatform = xlWindows
        .TextFileStartRow = 1

        'This example use xlDelimited
        'See a example for xlFixedWidth below the macro
        .TextFileParseType = xlDelimited

        'Set your Delimiter to true
        .TextFileTabDelimiter = True
        .TextFileSemicolonDelimiter = False
        .TextFileCommaDelimiter = False
        .TextFileSpaceDelimiter = False

        'Set the format for each column if you want (Default = General)
        'For example Array(1, 9, 1) to skip the second column
        .TextFileColumnDataTypes = Array(1, 9, 1)

        'xlGeneralFormat  General         1
        'xlTextFormat     Text            2
        'xlMDYFormat      Month-Day-Year  3
        'xlDMYFormat      Day-Month-Year  4
        'xlYMDFormat      Year-Month-Day  5
        'xlMYDFormat      Month-Year-Day  6
        'xlDYMFormat      Day-Year-Month  7
        'xlYDMFormat      Year-Day-Month  8
        'xlSkipColumn     Skip            9

        ' Get the data from the txt file
        .Refresh BackgroundQuery:=False
```

```
        End With
        ActiveSheet.QueryTables(1).Delete
        Next Fnum

        'Delete the first sheet of basebook
        On Error Resume Next
        Application.DisplayAlerts = False
        basebook.Worksheets(1).Delete
        Application.DisplayAlerts = True
        On Error GoTo 0

CleanUp:

        ChDirNet SaveDriveDir

        With Application
            .ScreenUpdating = True
            .EnableEvents = True
        End With
    End If


'Summarize the data

    Dim Sh As Worksheet
    Dim Newsh As Worksheet
    Dim myCell As Range
    Dim ColNum As Integer
    Dim RwNum As Long
    Dim TtlRw As Long

    With Application
        .Calculation = xlCalculationManual
        .ScreenUpdating = False
    End With

    'Delete the sheet "Summary-Sheet" if it exist
    Application.DisplayAlerts = False
    On Error Resume Next
    basebook.Worksheets("Summary-Sheet").Delete
    On Error GoTo 0
    Application.DisplayAlerts = True

    'Add a worksheet with the name "Summary-Sheet"

    Set Newsh = basebook.Worksheets.Add
```

```
    Newsh.Name = "Summary-Sheet"

    'The links to the first sheet will start in row 2
    RwNum = 1

    Range("A1").Value = "Sample Name"
    Range("B1").Value = "Cell Count"
    Range("C1").Value = "Cell Size"
    Range("D1").Value = "Weighting"
    Range("E1").Value = "STD"
    Range("F1").Value = "Dilution Factor"
    Range("G1").Value = "Lower Value"
    Range("H1").Value = "Count LV <"
    Range("I1").Value = "Upper Value"
    Range("J1").Value = "Count GV <"
    Range("K1").Value = "Dilution Factor"

    For Each Sh In basebook.Worksheets
       If Sh.Name <> Newsh.Name And Sh.Visible Then
          ColNum = 1
          RwNum = RwNum + 1
          'Copy the sheet name in the A column
          Newsh.Cells(RwNum, 1).Value = Sh.Name
            Sh.Range("A682").Formula                                                    =
"=Round(Sumproduct(A164:A420,A424:A680)/Sum(A424:A680),3)"
            Sh.Range("A683").Formula = "=Sum(A424:A680)"
            Sh.Range("A684").Formula                                                    =
"=Round(((SUMPRODUCT((A164:A420)*(A164:A420),A424:A680)/A683)-
(A682^2))^(0.5),2)"
            Sh.Range("A685").Formula = "=A73"
          For Each myCell In Sh.Range("A151,A682,A683,A684,A685")    '<--Change the range
             ColNum = ColNum + 1
             Newsh.Cells(RwNum, ColNum).Formula = _
             "='" & Sh.Name & "'!" & myCell.Address(False, False)
          Next myCell
          TtlRw = RwNum
       End If
    Next Sh

    Newsh.UsedRange.Columns.AutoFit

    With Application
       .Calculation = xlCalculationAutomatic
       .ScreenUpdating = True
    End With
```

```
' Text to Columns
Dim Panda As Worksheet

'Add a worksheet with the name "CCTemp"

    Set Panda = basebook.Worksheets.Add
    Panda.Name = "CCTemp"

'Copy data to temporary file

Sheets("Summary-Sheet").Select
Range("B2:B" & TtlRw).Select
Selection.Copy

'Paste into CCTemp

Sheets("CCTemp").Select
Range("A2:A" & TtlRw).Select
Selection.PasteSpecial Paste:=xlValues

'Split text

Range("A2:A" & TtlRw).TextToColumns _
     Destination:=Range("B2"), _
     DataType:=xlDelimited, _
     TextQualifier:=xlDoubleQuote, _
     ConsecutiveDelimiter:=False, _
     Tab:=False, _
     Semicolon:=False, _
     Comma:=True, _
     Space:=False, _
     Other:=True, _
     OtherChar:="="

'Copy CCTemp Slit Cells
Range("C2:F" & TtlRw).Select
Selection.Copy

'Paste back into Summary=Sheet
Sheets("Summary-Sheet").Select
Range("G2:J" & TtlRw).Select
Selection.PasteSpecial Paste:=xlValues

' Text to Columns
Dim Panda1 As Worksheet
```

```
'Add a worksheet with the name "CCTemp1"

    Set Panda1 = basebook.Worksheets.Add
    Panda1.Name = "CCTemp1"

'Copy data to temporary file

Sheets("Summary-Sheet").Select
Range("F2:F" & TtlRw).Select
Selection.Copy

'Paste into CCTemp

Sheets("CCTemp1").Select
Range("A2:A" & TtlRw).Select
Selection.PasteSpecial Paste:=xlValues

'Split text

Range("A2:A" & TtlRw).TextToColumns _
      Destination:=Range("B2"), _
      DataType:=xlDelimited, _
      TextQualifier:=xlDoubleQuote, _
      ConsecutiveDelimiter:=False, _
      Tab:=False, _
      Semicolon:=False, _
      Comma:=True, _
      Space:=False, _
      Other:=True, _
      OtherChar:="="

'Copy CCTemp Slit Cells
Range("C2:C" & TtlRw).Select
Selection.Copy

'Paste back into Summary=Sheet
Sheets("Summary-Sheet").Select
Range("K2:K" & TtlRw).Select
Selection.PasteSpecial Paste:=xlValues

'Delete CCTemp Sheet
Application.DisplayAlerts = False
basebook.Worksheets("CCTemp1").Delete
Application.DisplayAlerts = True
End Sub
```