Developing a Sustainable Bioprocess for Chitin Extraction from Atlantic Lobster Shell Waste

by

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Abstract

The goal of this research was to develop a fermentative chitin extraction method from Atlantic lobster shell waste. Results showed initial inoculum ratio was the most statistically significant variable in chitin extraction efficiency at a 5% shell ratio. Novel pre-treatments (MAE and UAE) were not found to have a significant influence on extraction efficiency under the tested conditions regardless of the presence of lactose in the pre-treated slurry. As determined by degree of decalcification and deproteinization as well as further measurement of fermentation dynamics, the most efficient of the tested formulations was 5% shell: 5% lactose: 5% inoculum (w/v) without a pre-treatment. The fermentation achieved 95.0% decalcification and 72.7% deproteinization when maintained at 37℃ for 5 days with constant stirring. This fermentative approach using lactose and lactobacilli inoculum successfully yielded high levels of mineral and protein removal however further studies are required to optimize the process.

List of Abbreviations and Symbols Used

α	Alpha
β	Beta
γ	Gamma
℃	Degrees Celsius
<	Less than
%	Percent
µL	Microlitres
µm	Micrometers
ANOVA	Analysis of Variance
cfu	Colony forming units
DES	Deep eutectic solvent
DM	Demineralization
DP	Deproteinization
g	Grams
HCl	Hydrochloric Acid
HNO_3	Nitric Acid
H_2SO_4	Sulfuric Acid
Ins. frac	Insoluble fraction
kHz	Kilohertz
L	Litre
M	Molar

MAE	Microwave-assisted extraction
mg	Milligram
mm	Millimeter
mM	Millimolar
mL	Millilitre
N_C	Chitinous nitrogen
N_T	Total nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_2	Amino
nm	Nanometers
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
rpm	Rotations per minute
UAE	Ultrasound-assisted extraction
W	Watts
w/v	Weight per volume

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Chapter 1 : Introduction

Chitin is a versatile biopolymer found in crustacean shells, insects, fungi and yeasts. Along with its derivatives, chitin has applications in a wide variety of industries from agriculture to biomedicine. Lobster shells are a rich source of chitin as well as protein, pigments and minerals however they are vastly underutilized as a bioresource. The majority of lobster shells are landfilled, composted or disposed back into the ocean disrupting both marine and land environments due to their slow degradation process (Ilangumaran et al., 2017).

Conventional chitin extraction from crustacean shells employs harsh acid and alkali treatments for demineralization and deproteinization processes, respectively. These chitin extraction methods are costly, produce harmful effluents, can result in an inconsistent chitin product and are unable to produce co-products. In the last 5 years, alternative solvents such as ionic liquids and deep eutectic solvents have been investigated as possible replacements to the strong acids and bases used in conventional chemical extraction. In addition, over the past fifteen years, many biological chitin extraction methods such as enzymatic protein hydrolysis and microbial fermentation have gained significance in the literature. Indirect heat treatments such as microwave and ultrasound have also been used in conjunction with chemical and biological methods in an attempt to make chitin extraction processes more environmentally sustainable and cost-effective.

Microbial fermentation is a favourable alternative chitin extraction method due to the low impact and low costs associated as well as the potential for value added co-product recovery. Fermentations employing lactic acid and non-lactic producing inoculum have been studied both individually and in combinations to achieve efficient demineralization

and deproteinization of crustacean shells. The fermentation medium results in a soluble fraction containing minerals, proteins and pigments and an insoluble fraction containing chitin. Lactic acid fermentation has been studied significantly more than other alternative biological methods; however, optimization is still required to achieve complete mineral and protein removal from crustacean shells.

Most research regarding alternative chitin extraction methods has been performed using shrimp shell waste (Arbia, Arbia, Adour, & Amrane, 2013; Kaur & Dhillon, 2015). Although lobster shells are a rich source of chitin and large volumes of lobster shell waste are produced annually, there is a lack of research pertaining to their use in alternative chitin extraction methods. Although the principles applied to other types of crustacean shells should be similar, they may not always translate directly to a comparable process for lobster shells. Lobster shells tend to be more difficult to degrade than shrimp shells due to their thicker and more rigid structure. A few more recent studies have examined alternative chitin extraction methods using lobster shell waste (Ilangumaran et al., 2017; Nguyen, Barber, Corbin, & Zhang, 2017; P. Zhu, Gu, Hong, & Lian, 2017) and to date only one study has been found regarding the lactic acid fermentation of lobster shells (Chakravarty, Yang, Palmer, & Brigham, 2018). Further research regarding chitin extraction from lobster shells is required prior to the development of a sustainable process on an industrial scale.

Chapter 2 : Objectives and Hypotheses

This research project focuses on the development of an environmentally and economically sustainable fermentative chitin extraction process from Atlantic lobster shells using a commercial blend of lactic acid producing inoculum and lactose as a fermentative carbon source. The objectives of the study were to:

1. Determine the impact of key variables of the fermentative process including initial shell, lactose and inoculum ratios on the responses of demineralization and deproteinization of lobster shells.
2. Study the impact of pre-treating the shell slurry (microwave and ultrasound) on the responses of demineralization and deproteinization with and without lactose in the pre-treated shell slurry.
3. Fully define and characterize the dynamics of the most efficient combination of fermentation conditions of those tested and thereby characterize the resultant chitin product.

The following hypotheses are proposed:

1. A lactic acid fermentation process can be developed to achieve degrees of demineralization and deproteinization of lobster shells comparable to values found in the literature.
2. The use of novel pre-treatments can increase extraction efficiency due to increased degradation of the shell prior to the addition of inoculum.

Chapter 3 : Literature Review

3.1 Canadian Lobster Industry

Lobsters are one of Atlantic Canada's most valuable marine resources with Canadian lobster exports of 85,440 metric tonnes valued over 2.2 billion CAD in 2018 (Fisheries and Oceans Canada, 2019). The global demand for lobster has continued to have an upward trend since 2009 as shown in Figure 3.1 (Fisheries and Oceans Canada, 2018). Provinces that contribute to the Canadian lobster landings include Nova Scotia, New Brunswick, Prince Edward Island, Quebec and Newfoundland and Labrador with over half of total landings contributed by Nova Scotia each year (Fisheries and Oceans Canada, 2017). The lobster fishing season is short in Atlantic Canada and peaks twice a year, once from April to June and again in November to December (Lobster Council of Canada, 2011).

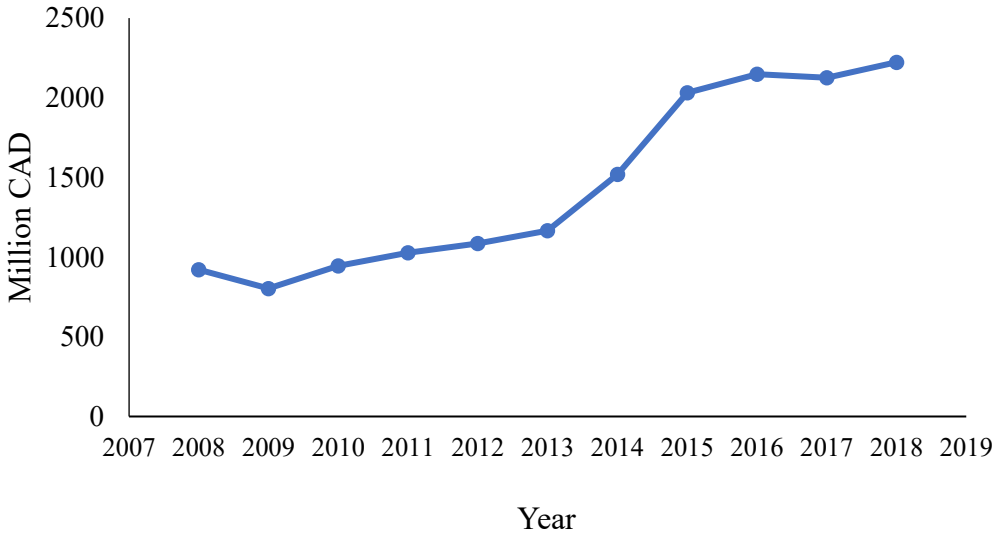


Figure 3.1: Total annual Canadian lobster export value by year (2008-2018) (Based on data from Fisheries and Oceans Canada)

Canadian lobsters are exported to over 50 countries (Fisheries and Oceans Canada, 2017) and 50-55% of the total landed lobsters in Canada are processed each year while remaining lobsters are typically sold live (Lobster Council of Canada, 2011). Lobster products include canned or frozen meat and require up to 75% (w/w) of the lobster to be discarded during processing as it is inedible (Nguyen et al., 2017; Zhu et al., 2017). In 2017, lobster landings totaled 97,452 tonnes therefore it is evident that the processing of Canadian lobsters generates large amounts of waste. Since lobster fishing has a short season and the marine resource is not commercially farmed like other crustaceans such as shrimp, the production of shell waste fluctuates throughout the year.

Canada, the United States and Australia are the three major lobster producing countries globally. It is estimated that over 45,000 tonnes of lobster processing waste is produced each year between these three countries (Nguyen et al., 2017). The majority of this waste is either landfilled, composted or directly discarded into the ocean. These current disposal methods pose a threat to both marine and land environments (Ilangumaran et al., 2017). A small fraction of the shell waste is used in low-value products such as fertilizer or animal feed, but the shells are largely underutilized (Kaur & Dhillon, 2015). Lobster shells have potential to produce multiple value-added products as they are a rich source of minerals, proteins, pigments and chitin.

3.2 Chitin

Chitin is the second most naturally abundant biopolymer following cellulose and is composed of β-(1,4)-N-acetyl-D-glucosamine units. The biopolymer is a structural

component found in the exoskeleton of shellfish and insects as well as the cell walls of fungi and yeast.

There are three polymorphic forms of chitin: α-chitin, β-chitin and γ-chitin. The most stable and naturally abundant form is α-chitin, which is found in the exoskeleton of crustaceans such as shrimp, crab and lobster. A less stable form of chitin is isolated from squid pen known as β-chitin while γ-chitin is the least common form and has been found in the cell walls of fungi and yeast. The three forms have slight differences in their structure as α-chitin has an antiparallel chain arrangement, β-chitin has a parallel chain arrangement and γ-chitin is a mixture of the antiparallel and parallel arrangements (Arbia et al., 2013).

Chitin is water insoluble due to strong hydrogen bonds but can be converted into its derivative chitosan, which is soluble in dilute acids, through partial chitin deacetylation (Arbia et al., 2013). Concentrated sodium hydroxide (NaOH), typically at concentrations of 40-50% by weight, is used for the deacetylation process (Hamed et al., 2016). The deacetylation causes N-acetyl glucosamine to transform into D-glucosamine units with free NH_2 groups as shown in Figure 3.2. The degree of acetylation refers to the ratio of glucosamine to N-acetyl glucosamine units within the structure. When the structure has more than 50% N-acetyl glucosamine units, the product is considered chitin and when the structure contains more than 50% D-glucosamine units, the product is considered chitosan (Hamed et al., 2016; Romano, Fabritius, & Raabe, 2007). The degree of acetylation is an important factor as it has an effect on molecular weight as well as the functionality of the product (Jung & Zhao, 2011).

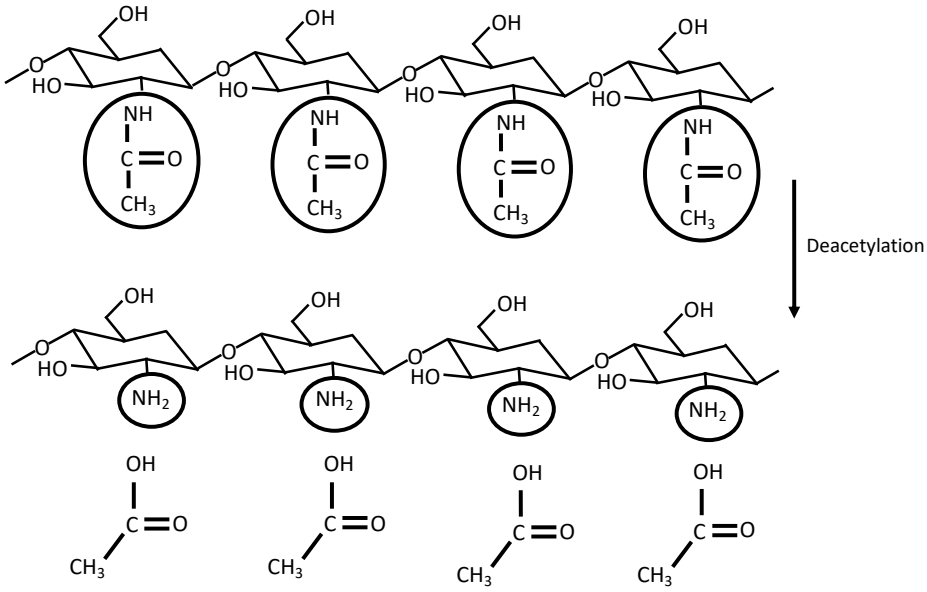


Figure 3.2: Chemical structure of chitin and chitosan adapted from Kaur & Dhillon (2015)

Chitin and its derivatives have a wide range of applications in industries such as agriculture, biomedicine, pharmaceuticals and food and beverage due to their biodegradable, non-toxic and antimicrobial properties. In agriculture, the polymers have been shown to stimulate plant growth while also functioning as a pesticide and fungicide (Sharp, 2013). Antimicrobial and non-toxic properties also make the biopolymers functional in pharmaceuticals and biomedicine for products such as wound dressings (Dai et al., 2011; Nguyen et al., 2017). Chitin and chitosan have also been used in the production of functional films and food packaging (Dutta, Tripathi, Mehrotra, & Dutta, 2009). Since the biopolymer has anti-microbial properties, it could preserve and extend the shelf-life of foods. Due to the higher solubility of chitosan, it can also be used directly as a preservative in some food products (Nguyen et al., 2017; No et al., 2007).

3.2.1 Lobster Shells as a Source of Chitin

Lobster shells contain approximately 30-50% minerals, 25-30% protein and 20-30% chitin by dry weight (Bolat et al., 2010; Nguyen, et al., 2017). The chitin polymer chains in the exoskeleton form a strong association with proteins through covalent bonds known as the chitin-protein complex. The carotenoid astaxanthin, which is a natural pigment, is also bound to the proteins giving the shell its colour.

As depicted in Figure 3.3, chitin composed of N-acetyl-D-glucosamine units makes up a chitin nanofibril that is on the scale of 3 μm in diameter. The chitin nanofibrils are coated with proteins and a cluster of typically 12 to 18 chitin nanofibrils make up one chitin fiber (~20 nm in diameter) within a horizontal layer of chitin fibers (Stirn, 2012).

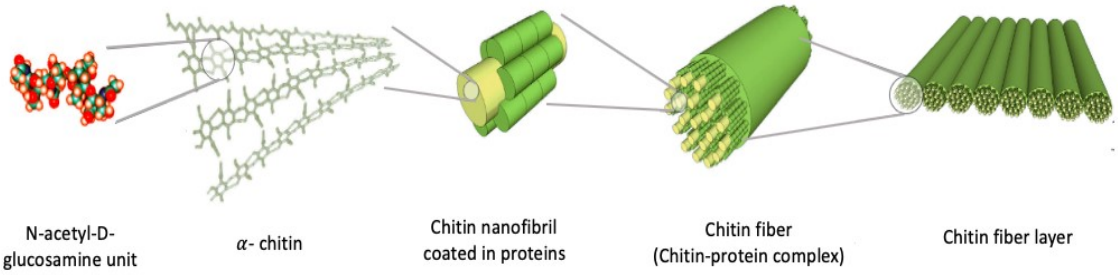


Figure 3.3: Nanoscale structure of chitin within crustacean shells adapted from Stirn (2012)

Multiple layers of chitin fibers stacked in a twisted formation form the cuticle of the shell, which gives the shells their mechanical stability. A pore canal system runs perpendicular to the chitin-protein layers and contains crystallized minerals, predominately calcium carbonate. The minerals in the canals are not bonded to the chitin-protein complex, and since they are woven within the complexed structure, are difficult to remove. The

presence of calcium carbonate hardens the shell, while crystalline calcite forms a thin but durable outermost protective layer of the shell (Stirn, 2012). Calcium makes up the majority of the mineral content in the shell at approximately 20% by dry weight followed by phosphate (2-6 wt%) and magnesium (~1%) (Boßelmann et al., 2007).

3.2.2 Conventional Chitin Extraction from Crustacean Shells

Demineralization and deproteinization are the two fundamental steps for the conventional chemical extraction of chitin from crustacean shell waste followed by decolourization for pigment removal. Demineralization is performed using a strong acid treatment, with hydrochloric (HCl) as the most commonly used acid at concentrations up to 2 M (Kaur & Dhillon, 2015; Tolaimate et al., 2003). The demineralization process can take up to 48 hours and is typically carried out at temperatures near 100℃ (Percot et al., 2003).

Deproteinization of crustacean shells is typically performed with 1 M NaOH, but studies have used concentrations ranging from 0.2-2.5 M to break the covalent bonds within the protein-chitin complex (Kaur & Dhillon, 2015; Percot et al., 2003; Tolaimate et al., 2003). Deproteinization can take up to 72 hours at temperatures ranging from 65 to 100℃ (Perot et al, 2003). The described conventional chitin extraction process is shown in Figure 3.4.

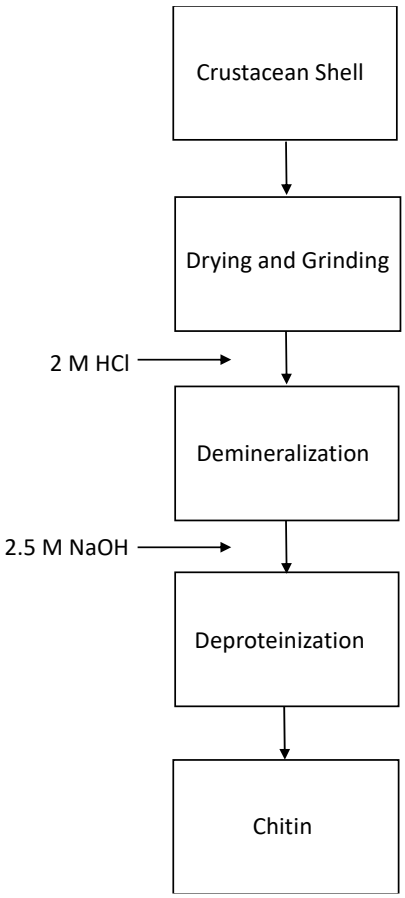


Figure 3.4: Conventional chitin extraction method

Mineral and protein removal can typically be performed in either order without any significant effect on the extraction efficiency or chitin product (Tolaimate et al., 2003; Younes & Rinaudo, 2015). However, one study saw a 9.81% increase in protein removal to 89.97% when deproteinization was performed following demineralization and only a 1% decrease in demineralization to 91.54% compared to the reverse treatment order (Chakravarty et al., 2018). This is likely due to the increased surface area of the substrate and availability of proteins once the protective layer of minerals is removed. Once the minerals and proteins are removed from the shell, the chitin product can then be

decolourized to remove astaxanthin with the use of an organic solvent such as acetone (Mohammed et al., 2013).

Conventional chitin extraction methods can achieve complete removal of both minerals and proteins and are well-established on both the lab and industrial scale (Younes & Rinaudo, 2015); however, the use of harsh acids and alkalis produce an inconsistent chitin product with varying molecular weights and undesired deacetylation and/or depolymerization (Hamed et al., 2016; Kaur & Dhillon, 2015). These methods are also disadvantageous for their potentially environmentally harmful effluents and high cost. The extraction methods do not allow for the production or salvation of co-products such as proteins, calcium and astaxanthin, since the components are denatured or difficult to extract from the harsh chemicals (Ilangumaran et al., 2017; Kaur & Dhillon, 2015). Alternative extraction methods that allow for the production of co-products would further valorize the biomass making alternative extraction methods desirable.

3.3 Alternative Chitin Extraction Methods

Due to the disadvantages of conventional chitin extraction using strong acids and bases, alternative methods have gained significant attention over the last fifteen years as effective and sustainable options. These methods include the use of alternative solvents, such as ionic liquids and deep eutectic solvents, as well as biological extraction methods, involving enzymes and microbes.

3.3.1 Ionic Liquids and Deep Eutectic Solvents

Ionic liquids are alternative solvents to the harsh acids and alkalis used in conventional extraction methods and have successfully been used to extract chitin from shrimp shells (Barber et al., 2013; Qin et al. , 2010; Setoguchi et al., 2012). The solvents are advantageous over acids and alkalis as they are non-flammable, non-volatile and recyclable (Ghandi, 2008). Ionic liquids are a combination of a solid anion and cation that when mixed have a melting point below 100℃. The mixed ions can be optimized for the extraction of specific macromolecules within the biomass by maximizing disruption of the hydrogen bonding (Barber et al., 2013).

1-allyl-methylimidazolium bromide (AMIMBr) was used for the deproteinization of shrimp shells prior to demineralization with citric acid (Setoguchi et al., 2012). After 24 hours of extraction, the protein content of the insoluble product was less than 0.1%. 1-ethyl-3-methyl-imidazolium acetate has also been used for chitin extraction and achieved a 94% chitin yield from shrimp shells (Barber et al., 2013).

Although there are advantages to ionic liquids, commonly used mixtures are toxic and costly (Phuong, Pham, Cho, & Yun, 2009) therefore deep eutectic solvents (DES) have also been studied. DES are commonly known as “green” ionic liquids as they share many characteristics and properties as ionic liquids however DES are biodegradable and non-toxic. DES are also advantageous over ionic liquids as they are typically cheaper and easier to prepare (Smith et al., 2014).

DES have been used for the extraction of chitin from lobster shells (Zhu et al., 2017), shrimp shells (Saravana et al., 2018) and crab shells (Setoguchi et al., 2012). DES consist of a hydrogen bond acceptor (HBA), typically a quaternary ammonium salt such as choline chloride, and a hydrogen bond donor (HBD) that when mixed have a lower melting

point than the two individual components due to strong hydrogen bonding between HBD and HBA (Chen & Mu, 2019).

A DES mixture of choline chloride (HBA) and malic acid (HBD) proved highly effective in multiple studies for the removal of proteins and minerals from shrimp shells (Saravana et al., 2018; Zhu et al., 2017). The mixture produced a higher purity chitin product compared to other tested DES mixtures as well as conventional chemical extraction. The high dissolution of chitin in both ionic liquids and DES is accredited to the solvent's capacity to break strong hydrogen bonds within the chitin complex and create new hydrogen bonds with the solvent (Chen & Mu, 2019; Dong, Zhang, & Wang, 2016; Sharma, Mukesh, Mondal, & Prasad, 2013; Smith et al., 2014).

3.3.2 Enzymatic Protein Hydrolysis

Proteolytic enzymes have been used as an alternative for NaOH treatment to deproteinize crustacean shell waste, thus decreasing the amount of harmful effluents associated with the extraction. The proteases work to hydrolyze peptide bonds within the shell and breakdown proteins into their amino acid components.

The commercial protease Alcalase® has been used in several studies for the deproteinization of shrimp shells (de Holanda & Netto, 2006; Gildberg & Stenberg, 2001; Synowiecki & Al-Khateeb, 2000; Valdez-Peña et al., 2010). When tested against other commercial proteases, Alcalase® was more effective than trypsin (Valdez-Peña et al., 2010) and pancreatin (de Holanda & Netto, 2006) for protein hydrolysis, however it was not as effective as bromelain (Jung et al.,2007) or an alkali treatment (de Holanda & Netto, 2006).

Although commercial enzymes are effective for protein removal, they are costly therefore crude proteases isolated from bacteria have also been tested as a cost-effective way to perform deproteinization. Crude enzymes have been found to achieve similar or improved deproteinization of shrimp shells compared to commercial proteases. One study found proteases isolated from *Bacillus mojavensis* A21 achieved higher deproteinization of shrimp shells than two commercial enzymes, bromelain and Alcalase®; thus showing costly commercial enzymes are not necessary to achieve high degrees of protein removal (Younes et al., 2012). As seen in Table 3.1, there is variability in the degrees of deproteinization achieved by enzymatic hydrolysis ranging from 54% (Younes et al., 2012) to 88.8% (Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010) as results are dependent upon the operating conditions as well as the enzyme used.

Table 3.1: Summary of enzymatic protein hydrolysis methods

Chitin Source	Enzymes	Temp. & Duration	DP (%)	Refs.
Shrimp shell (*Xiphopenaeus kroyeri)*	Alcalase®	60℃ 15 mins	64.6	de Holanda & Netto (2006)
	Pancreatin	40℃ 30 mins	57.5	
Shrimp shell	Alcalase®	40℃ 2 hours	70	Gildberg & Stenberg (2001)
Shrimp shell *(Crangon crangon)*	Alcalase®	55℃ 30 mins	N.D.	Synowiecki & Al-Khateeb (2000)
Shrimp shell	Alcalase®	37℃ 6 hours	N.D.	Valdez-Peña et al. (2010)
	Trypsin	37℃ 6 hours	N.D.	
Shrimp shell	*Bacillus cereus* SV1	40℃ 3 hours	88.8	Manni et al. (2010)
Shrimp and crab shell powder	*Pseudomonas aeruginosa* K-187	50℃ 7 days	77.5	Oh et al. (2000)
Shrimp shell	Alcalase®	50℃ 3.5 hours	54	Younes et al. (2012)
	Bromelain	50℃ 3.5 hours	67	
	Bacillus mojavensis A21	60℃ 6 hours	88	
Shrimp shell	*Bacillus mojavensis* A21	50℃ 3 hours	77	Younes et al. (2014)

DP - deproteinization
N.D. - Data not expressed in terms of deproteinization (%)

Proteolytic enzymes, although effective in deproteinization, are not capable of demineralizing the crustacean shells. Therefore, chemical demineralization is still required for chitin recovery, as a result singularly proteolytic enzymatic extraction is only a partial sustainable alternative to conventional chemical extractions.

3.3.3 Microbial Processes

Fermentative extraction methods employing microorganisms to demineralize and deproteinize crustacean shells have been considered as an economically and environmentally sustainable alternative to conventional methods. The fermentation slurry results in a liquid fraction containing solubilized minerals, proteins and pigments and an insoluble fraction containing chitin. Fermentative chitin extraction has potential for the co-production of proteins, calcium and astaxanthin as they can be separated from the fermentation slurry and used in supplements for human consumption or as an additive in animal feed (Rao et al., 2000). The minerals from the process can also be used in food preservation or de-icing agents (Ghaly et al., 2007; Kaur & Dhillon, 2015)

Fermentation using lactic acid bacteria, non-lactic acid bacteria and co-fermentative methods have all been explored (Arbia et al., 2013; Kaur & Dhillon, 2015). Depending on the inoculum used, demineralization and deproteinization can be achieved simultaneously or in a two-step process. Degrees of demineralization achieved by fermentative extraction vary from 61% (Zakaria, Hall, & Shama, 1998) to 99.6% (Castro, Guerrero-Legarreta, & Bórquez, 2018) while deproteinization ranges from 68.9% (Oh, Kim, Nguyen, Jung, & Park, 2007) to 96.5% (Duan et al., 2012).

Microbial methods may be an environmentally sustainable, lower-cost option that results in a high-quality chitin product however the methods require further optimization to achieve complete removal of minerals and proteins (Kaur & Dhillon, 2015).

3.3.3.1 Lactic Acid Fermentation

Lactic acid bacteria are commonly used in food preservation and can be found in naturally fermented foods such as wine, cheese and yogurt. There are many genera of lactic acid producing bacteria, including *Lactobacillus, Pediococcus* and *Streptococccus*. However, *Lactobacilli* strains are the most commonly used in lactic acid fermentation of crustacean shell waste. Lactic acid bacteria utilize fermentable carbon sources to produce lactic acid, which is their major end product (Goldstein et al., 2015). Crustacean shells are a poor source of fermentable carbon, therefore sources such as glucose, sucrose or lactose are added to the fermentation slurry.

The *in-situ* production of lactic acid reduces the pH of the fermentation medium, thus inhibiting the growth of competing microorganisms that can lead to spoilage (Alakomi et al., 2000). When fermented with crustacean shells, the lactic acid reacts with calcium carbonate present in the shell to produce calcium lactate. The calcium lactate can precipitate out of the slurry but can be easily removed by washing of the insoluble fraction (Bautista et al., 2001). Since the bacteria can also be a source of proteases, the pH of the fermentation activates the secretion of enzymes which promote deproteinization (Ilangumaran et al., 2017; Kaur & Dhillon, 2015). As seen in Figure 3.5, the fermented product can be separated into liquid and solid fractions. The liquid fraction contains minerals, pigments and solubilized proteins in the form of peptides and free amino acids while the insoluble fraction contains chitin.

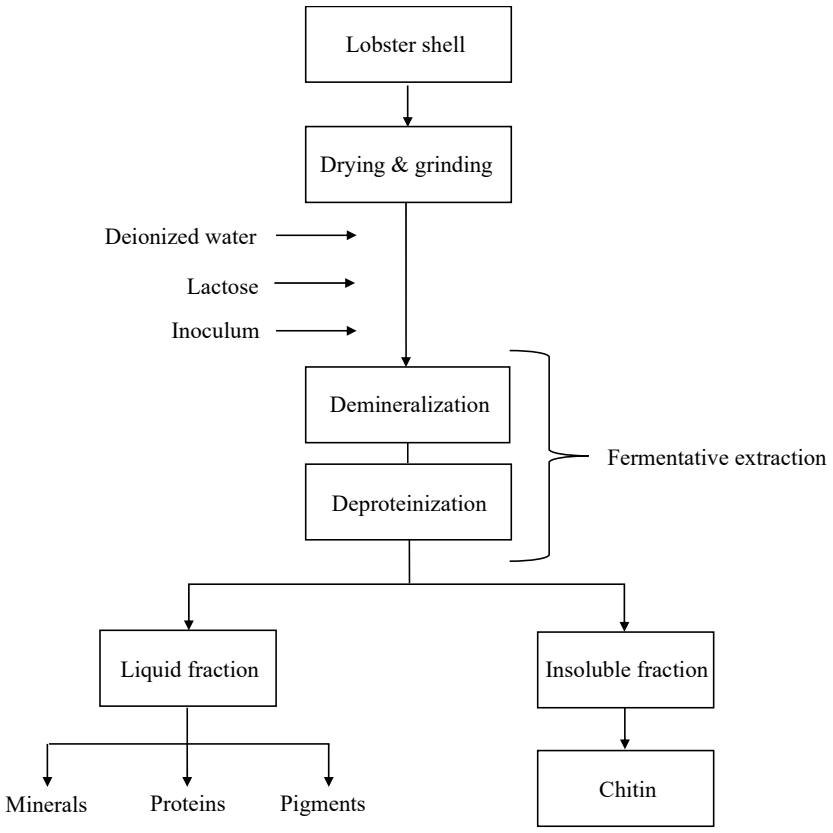


Figure 3.5: Lactic acid fermentative chitin extraction

Whey, date syrup and malt have been used as alternative carbon sources to make the process more cost efficient (Cira, 2002). Many studies have shown that the type of carbon source is among the critical factors for fermentation and is likely related to substrate preference of the bacterial strain used in the fermentation (Adour et al., 2008; Cira, 2002; Najafpour et al., 2011). For example, Najafpour et al. (2011) observed higher demineralization of shrimp shells when *Lactobacillus plantarum* PTCC 1058 was fermented with date syrup compared to glucose. Contrastingly, Adour et al. (2008) saw a lower degree of demineralization when *Lactobacillus helveticus* was fermented with date syrup compared to glucose for chitin extraction from shrimp shells.

The efficiency of the fermentation is indicated by the degree of demineralization and deproteinization as well as chitin yield. These responses are dependent upon many variables such as temperature, duration and pH as well as initial shell waste, carbon and inoculum quantities (Cira, 2002; Rao et al., 2000). Oh et al. (2008) found that higher degrees of demineralization were achieved at a lower initial shell concentration. Highest mineral removal of 90.9% was achieved when fermenting with *Lactobacillus paracasei tolerans* KCTC-3074 at a shell concentration of 5%. Slightly higher demineralization was also achieved in the study with shell particle sizes ranging from 3.35 to 20 mm compared to both larger and smaller particle sizes, but overall the particle size had a minor impact on the demineralization efficiency.

Fermentations are typically run for up to 7 days at temperatures between 30-37℃ as this is the optimal temperature range for the growth of *Lactobacillus* species (Ahmed et al., 2006). The pH of the fermentation can be left to reach acidic conditions solely by the natural production of lactic acid or it can be adjusted by supplementing the medium with organic acids. A pH of 6.0 or below is typically used for regulated pH fermentations while 4.0 is desired for fermentations with *in-situ* lactic acid production to inhibit the growth of competing microorganisms (Sini et al., 2007).

The type of acid used to adjust the pH of the fermentation medium can have a slight effect on the efficiency of the process. In a study by Rao et al. (2000), pH adjustment to 6.0 with citric acid achieved approximately 15% higher demineralization and deproteinization of shrimp shells than acetic acid and close to 20% higher than lactic acid. However, it is important to note that the study also found fermentations adjusted to a lower pH (5.5) resulted in higher demineralization but lower deproteinization than an unadjusted fermentation or a fermentation adjusted to a higher pH (6.0 or 6.5). Interestingly, the

unadjusted fermentation in this same study was considered spoiled due to a rancid smell and the absence of a quick pH drop resulting in the lowest demineralization (44.1%) but highest deproteinization (89.2%) of all trials. The findings of this study indicate that protein hydrolysis is more efficient at a higher pH but the opposite is seen for demineralization.

Lactic acid fermentation methods have been used to achieve simultaneous demineralization and deproteinization (summarized in Table 3.2) while other studies have used the process solely for demineralizing the shells (Choorit et al., 2008; Najafpour et al., 2011; Oh et al., 2008). To date, the highest degree of demineralization (99.6%) and deproteinization (95.3%) achieved through lactic acid fermentation was by Castro et al. (2018). Carbon source, incubation temperature and inoculum source were all optimized in the study to achieve high process efficiency. Sucrose was determined as the optimal carbon source as it achieved the highest bacteria growth rate compared to molasses and glucose. Optimal incubation temperature and inoculum were determined by conditions that resulted in maximum lactic acid production at 17 mg lactic acid per gram of the fermentation slurry.

Table 3.2: Summary of lactic acid fermentation processes with simultaneous demineralization and deproteinization

Chitin Source	**Inoculum Strains**	**Conditions**	**Temp. & Duration**	**DM (%)**	**DP (%)**	**Refs.**
Scampi shell (*Nephrops norvegicus*)	*Lactobacillus paracasei* A3	10% glucose (w/w) 10% inoculum (v/w)	30℃ 5 days	61.0	77.5	Zakaria et al. (1998)
Shrimp shell (*Penaeus sp*.)	*Lactobacillus spp* B2	10% sucrose (w/w) 5% inoculum (v/w)	30℃ 6 days	85	87.6	Cira et al. (2002)
Shrimp shell	*Lactobacillus plantarum* 541	5% glucose 6.7% inoculum pH 6.0 control	30℃ 2 days	86	75	Rao et al. (2000)
Crawfish shell (*Procambarus clarkia*)	*Lactobacillus paracasei* A3	10% glucose (w/w) 10% inoculum (v/w)	30℃ 3 days	97.2	94	Cremades et al. (2001)
Shrimp shell (*Penaeus merguiensis*)	*Pseudomonas aeruginosa*	5% shell 20% glucose 20% inoculum	37℃ 6 days	82	92	Sedaghat al. (2017)
Crab shell (Allopetrolisthes *punctatus*)	*Lactobacillus plantarum* 47	85% shell 15% sucrose 10% inoculum	32℃ 3 days	99.6	95.3	Castro et al. (2018)
Shrimp shell (P*enaeus vannamei*)	*Lactobacillus acidophilus* SW01	15% glucose (w/w) 5% inoculum (v/w	37℃ 7 days	96.7	96.5	Duan et al. (2012)
Shrimp shell (*Litopenaeus vannameii*)	*Lactobacillus plantarum*	10% sucrose (w/w) 5% inoculum (v/w)	35℃ 4 days	92	94	Pachecho et al. (2011)
Crayfish shell	*Lactobacillus pentosus* 4023	40 g/L lactose	35℃ 2 days	90.1	81.5	Bautista et al. (2001)

DM – demineralization
DP – deproteinization

Lactic acid fermentation is typically performed anaerobically however lactobacilli are facultative anaerobes therefore they can grow under aerobic conditions as well. For example, no significant change in deproteinization was seen when a commercial blend of

lactic acid bacteria, Stabisil®, was fermented with previously demineralized shrimp shells under aerobic or anaerobic conditions (Healy et al., 1994). Constant anaerobic conditions are difficult to achieve on an industrial scale therefore the use of lactobacilli is desirable as they can survive and adapt in a variety of environmental conditions.

3.3.3.2 Non-Lactic Acid Fermentation

Aside from lactic acid producing bacteria, other microorganisms such as species of *Bacillus* and *Pseudomonas* have been used for the extraction of chitin. The bacteria typically have high protease activity suitable for deproteinization therefore they are desirable for chitin extraction from crustacean shells. The bacteria use the shell as an energy source suggesting that chitin present in the shell is used as a carbon source to stimulate the bacterial growth (Chang, Chen, & Jao, 2007; Cheba, Zaghloul T., & El-Mahdy, 2018). The use of chitin by the microbes does not govern direct dissolution of the biopolymer but will likely result in undesired deacetylation of the chitin product (Beier & Bertilsson, 2013).

Although lactobacilli can be a source of proteases to facilitate deproteinization, it is not their main end product and vice versa for non-lactic acid bacteria. Non-lactic acid producing bacteria can also produce organic acids to lower the pH of the medium and achieve high degrees of demineralization however, the organic acids are not their main end-product. For example, *Pseudomonas aeruginosa F722* isolated from soil was able to achieve 92% demineralization and 63% deproteinization of crab shells under optimal conditions (Oh et al., 2007). Similarly, *Bacillus subtilis* fermented with shrimp shells saw high degrees of both demineralization (84%) and deproteinization (72%) due to its sufficient *in-situ* production of organic acids and proteases (Sini et al., 2007).

Since crustacean shells and fungi are both sources of chitin, the biopolymer can be concurrently extracted through fungal fermentation. In the fungal fermentation process, proteases produced by the fungi lead to deproteinization of the shrimp shells. The proteins solubilized in the slurry can then be used as a nitrogen source for the fungi, promoting fungal growth, lowering the pH and thus leading to the demineralization of the crustacean shells. A study successfully performed this extraction using a strain of *Aspergillus niger* and shrimp shells. When separated, the shrimp shell derived chitin product had less than 5% protein while the fungal derived chitin contained 10-15% residual protein (Teng et al., 2001).

Although single strains of microorganisms have been used for fermentative chitin extraction, using a combination or sequential application of these microorganisms could potentially increase demineralization and deproteinization of crustacean shells and therefore extraction efficiency.

3.3.3.3 Two-Step & Co-Fermentative Extraction

Two-step and co-fermentative methods employ one lactic acid producing bacteria, predominately for mineral removal, and one with high protease activity, predominately for protein removal. The use of two different bacteria is hypothesized to achieve higher degrees of removal compared to mono-culture fermentation, however the methods to date (summarized in Table 3.3) achieved similar degrees of removal as those achieved by mono-culture fermentations.

As was found in the study by Rao et al. (2000), demineralization requires a lower pH while deproteinization is more efficient at a near neutral pH. The benefit to a two-step

fermentation is the ability to operate each fermentation step at optimal conditions for the respective bacteria. The order in which the fermentations are carried out in two-step fermentations may have an effect on the overall efficiency of the process. Multiple studies have shown a higher extraction efficiency when fermentation with a protease rich inoculum is performed first. For example, fermentation of crab shell waste showed a 17.4% increase in deproteinization to 68.9% and only a 3.8% decrease in demineralization to 94.3% when fermented with *Serratia marcescens* FS-3 prior to Lactobacillus *paracasei* KCTC-3074 compared to the reverse fermentation order (Jung et al., 2007). A study by Chakravarty et al. (2018) also found a consistently higher extraction efficiency when lobster shells were fermented with *Serratia marcescens* db11 or *Bacillus negaterium* NH21 prior to *Lactobacillus plantarum*. Fermentation with *S. marcescens* followed by *L. plantarum* resulted in 89.6% demineralization and 87.2% deproteinization while the reverse order achieved only 68.8% demineralization and 76.3% deproteinization. Similarly, *B. negaterium* prior to *L. plantarum* resulted in 82.9% demineralization and 78.6% deproteinization while only 63.7% demineralization and 65.5% deproteinization was achieved by the reverse order.

Although the majority of studies use successive fermentations when utilizing multiple microorganisms, co-fermentation employing both bacteria simultaneously could be beneficial. Chakravarty et al. (2018) compared the individual and co-fermentations of lobster shells with *Lactobacillus plantarum*, *Bacillus negaterium* NH21 and *Serratia marcescens* db11(Chakravarty et al., 2018). Results consistently showed higher degrees of demineralization, deproteinization and chitin yield when two strains of bacteria were used compared to one strain, validating the benefits of employing multiple bacteria strains.

Similarly, another study compared both the successive and simultaneous fermentation of shrimp shells with *Gluconobacter oxydans* and *Bacillus licheniformis*. Results showed a 4.1% increase in demineralization and only 1.5% decrease in deproteinization when using simultaneous fermentation over successive (Liu et al., 2014). High protein and mineral removal in less time and fewer processing steps make co-fermentative methods a desirable option for chitin extraction however the use of multiple microorganisms would likely be costly. A higher cost process that achieves similar results to mono-culture fermentation would not be economical, furthermore multiple microorganisms would require more studies for optimization compared to a process using one microorganism.

Table 3.3: Summary of two-step and co-fermentative chitin extraction methods

Chitin Source	Inoculum Strains	Conditions	DM (%)	DP (%)	Refs.
Scampi shell	Protease cultures isolated from sewage sludge & ground meat *Lactobacillus case* MRS1	Successive fermentation	99	93	Xu et al. (2008)
Shrimp shell	*Serratia marcescens* B742 *Lactobacillus plantarum* ATCC 8014	Successive fermentation	93	94.5	Zhang et al. (2012)
Crab shell	*Serratia marcescens* FS-3 *Lactobacillus paracasei* KCTC-3074	Successive fermentation	94.3	68.9	Jung et al. (2007)
Shrimp shell	*Bacillus licheniformis* *Gluconobacter oxydans*	Successive fermentation Simultaneous fermentation	95 93.5	83.1 87	Liu et al. (2014)
Lobster shell	*Bacillus negaterium* NH21 *Lactobacillus plantarum*	Simultaneous fermentation	89.9	87.2	Chakravarty et al. (2018)

DM – demineralization
DP – deproteinization

3.4 Pre-Treatment Processes for Extraction

Heat treatments such as microwave and ultrasound are commonly used to improve the extraction efficiency of compounds from several biomass substrates such as seaweed, microalgae and lignocellulosic biomass through cell membrane disruption. Crustacean shells are largely chemical in nature therefore, the goal of these treatments when applying them to chitin extraction processes is to disrupt the structure of the shell without the negative effects of conventional heating, such as the denaturing of proteins. Novel pre-treatments can be used to break down bonds within the chitin complex causing separation

and degradation of the structure (Kaufmann & Christen, 2002; Taurozzi, Hackley, & Wiesner, 2011). Microwave and ultrasound treatments have both been used in the extraction of chitin from crustacean shells through chemical methods (El Knidri, El Khalfaouy, Laajeb, Addaou, & Lahsini, 2016; Kjartansson, Zivanovic, And, & Weiss, 2006) while only one study used microwave treatments with a biological chitin extraction method (Nguyen, Zhang, Barber, Su, & He, 2016).

3.4.1 Microwave-Assisted Extraction (MAE)

In MAE, electromagnetic radiation is absorbed by the extraction medium and is rapidly converted into heat. The heating of the solvent (added or inherent water content) increases dissolution of the analyte and therefore the rate of mass transfer to the extraction medium. Pressure changes within the cells can occur due to evaporation leading to rupturing of cell membranes accelerating the release of compounds from the cells (Poojary et al., 2016).

Benefits of using microwave irradiation include a reduction in the amount of solvent required as well as extraction time (El Knidri et al., 2016; Eskilsson & Bjorklund, 2000). In a study using a chemical chitin extraction method, total extraction time was dramatically reduced from 4 hours to 16 minutes with the use of MAE (El Knidri et al., 2016).

The impact of the treatment is dependent upon its duration as shown in a study by Nguyen et al. (2016). Microwave irradiation was used during the enzymatic deproteinization of lobster shells at 55℃ for 30, 60 and 90 minutes. The highest degree of deproteinization was achieved after 90 minutes of treatment at 85.8%. However, there was no significant difference between deproteinization after 60 or 90 minutes of treatment. This

shows that the effects of the treatment would likely plateau at a certain time point after which extraction efficiency would no longer be greatly increased.

3.4.2 Ultrasound-Assisted Extraction (UAE)

UAE uses the frequencies of sound waves to create variations in pressure throughout the extraction medium. The alternating pressure causes bubble cavities to form and implode, resulting in localized high temperature and pressure (O'Donnell et al., 2010). The implosions cause cell disruption, thinning of the cell membrane and increased surface area allowing for better penetration of the solvent and therefore increased mass transfer of target compounds. Low frequency ultrasound, between 18-200 kHz, is typically used for extraction processes (Poojary et al., 2016).

UAE was shown to slightly increase the chemical deproteinization efficiency of shrimp shells. Treatment duration was an important factor as there was no increase in deproteinization after 1 hour however a 3.7% increase was achieved after 4 hours of treatment at 41 W/cm^2 (Kjartansson et al., 2006). In this study, UAE was found to have no significant effect on demineralization efficiency irrespective of duration.

To date, ultrasound pre-treatments have not been used in conjunction with biological methods and only one study has combined MAE with enzymatic deproteinization (Nguyen et al., 2016). There is a lack of research pertaining to pre-treatments prior to fermentative extraction therefore the impact of these pre-treatments on the particular method is difficult to predict.

Chapter 4 : Materials and Methods

4.1 Project Flow Schematic

Figure 4.1 shows the three phases of the experimental design used in the present study to develop a fermentative chitin extraction method from lobster shells. In Phase 1, preliminary testing was carried out using a factorial design to determine the significance of three factors: shell, lactose and inoculum content on two responses: demineralization and deproteinization. The two most effective formulations from Phase 1 were chosen based on demineralization, deproteinization and cost effectiveness to be tested further in Phase 2. Trials in Phase 2 were designed to assess the impact of microwave and ultrasound pre-treatments on demineralization and deproteinization with and without lactose in the pre-treated mixture. In Phase 3, the two most efficient conditions from Phase 2 were then scaled up for further characterization. Fermentations in all three phases were performed in experimental duplicates.

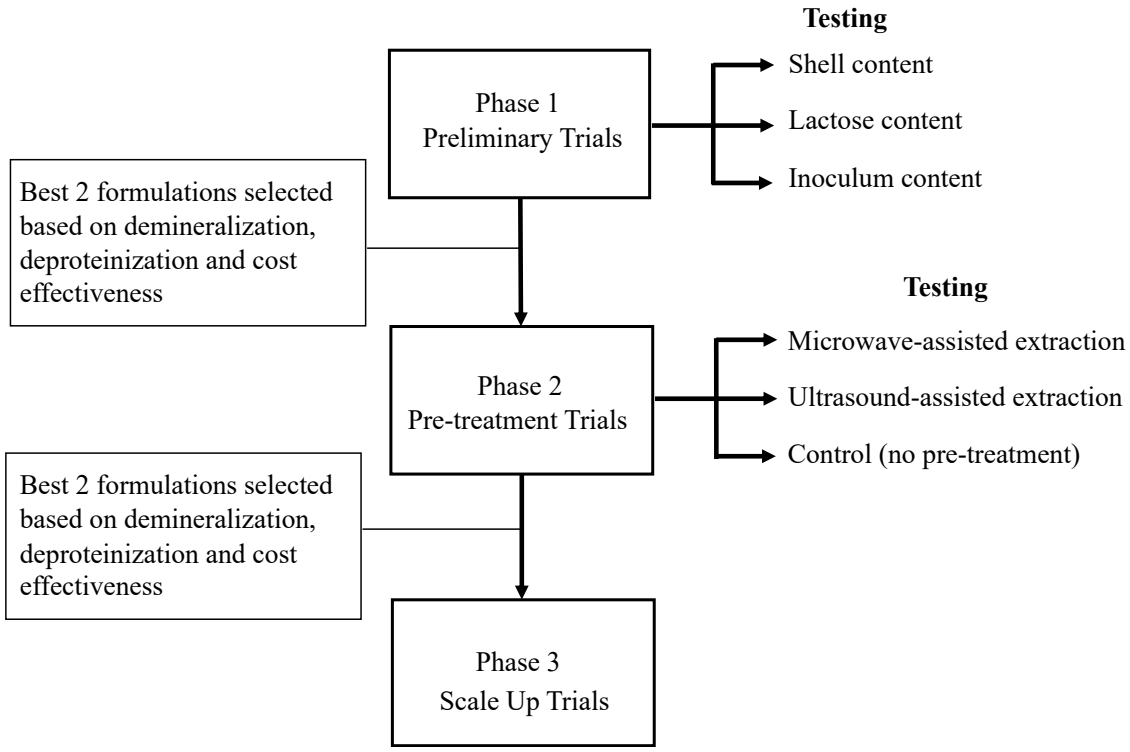


Figure 4.1: Flow diagram of the three phases of experimental trials for developing a fermentative chitin extraction process from lobster shells

4.2 Preparation of Starting Materials

Lobster shells (tails and claws) were obtained from Clearwater Seafoods Inc. (Nova Scotia, Canada). The shells were dried in a forced air convection oven at 50℃ for 72 hours and ground in a high-speed household blender. The shells were further ground using a ball mill and sieved to a particle size of < 2 mm. The shell powder was stored in a sealed container at room temperature.

4.3 Proximate Lobster Shell Composition

Following the methods described by AOAC (1990), moisture content was determined by drying the prepared shell powder in a forced air convection oven at 105℃ for 24 hours. Lipid content was determined by Soxhlet extraction (SOXTHERM®, C.

Gerhardt UK Ltd, UK) using petroleum ether as the extraction solvent for 3 hours (AOAC, 1990). Ash content was determined by treating the shell powder in a muffle furnace at 500℃ for 4 hours (Chakravarty et al., 2018). Ash and lipid contents of the shell were calculated by measuring what was remaining after extraction while moisture content was determined by the mass removed by drying using the following equations.

$$\text{Ash or lipid content (\%)} = \frac{\text{Sample weight after treatment}}{\text{Sample weight before treatment}} \times 100$$

$$\text{Moisture content (\%)} = 100 - \left(\frac{\text{Sample weight after treatment}}{\text{Sample weight before treatment}} \times 100\right)$$

4.3.1 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed on the untreated shell powder using a Perkin Elmer STA 8000 system (Perkin Elmer Inc, USA). A sample of the ground shells (40 mg) was heated in a nitrogen atmosphere from 35℃ to 900℃ in increments of 20℃ per minute to determine the temperatures at which mass loss occurred.

4.4 Phase 1: Preliminary Trials

As shown in Table 4.1, an initial factorial design was performed with a high and low level of three variables: shell, lactose and inoculum to determine their influence on two responses: demineralization and deproteinization. The high and low levels for each variable were chosen based on trends seen in the literature for successful lactic acid fermentations of other crustacean shells such as crab and shrimp (Arbia et al., 2013; Kaur & Dhillon, 2015). The ratios given in Table 4.1 are relative to the volume of deionized water used in the fermentations.

Table 4.1: High and low levels of variables tested in phase one trials

Variable	High	Low
Shell (w/v %)	20	5
Lactose (w/v %)	20	5
Inoculum (w/v %)	5	1

Ground lobster shells, lactose (Sigma-Aldrich, Oakville, Ontario, Canada) and a purchased inoculum powder composed of *Lactobacillus plantarum* and *Pediococcus acidilactici* strains (Lallemand Canada Inc., QC, Canada) known to be high in lactic acid production were mixed thoroughly with 400 mL of deionized water in covered 1 L Erlenmeyer flasks. The temperature of the mixture was maintained at 37℃ by a hot plate with constant magnetic stirring (450 rpm) for a maximum of 8 days. Samples (50 mL) and pH readings were collected at 0, 6 and 24 hours followed by 24-hour intervals for up to 192 hours (8 days). All fermentations were performed in experimental duplicate.

4.5 Phase 2: Pre-Treatment Trials

Two formulations from Phase 1 were chosen to be tested further based on their high degree of demineralization, deproteinization and cost effectiveness. In Phase 2, the impact of novel heat pre-treatments (MAE and UAE) on demineralization and deproteinization efficiency were studied on these formulations.

Fermentations in Phase 2 were prepared using the same methods as Phase 1 with shell, lactose and inoculum mixed with 400 mL of deionized water in a covered 1 L Erlenmeyer flask maintained at 37℃ by a hot plate with constant magnetic stirring (450 rpm). Slight adjustments were made however to fermentation length and sampling frequency for Phase 2 trials. A fermentation length of 120 hours was used in Phase 2 rather

than 192 hours. Since the desired pH drop was observed to stabilize after 48 to 72 hours and no significant increase in degree of demineralization or deproteinization was observed in the results, fermentation length of 120 hours was used for remaining trials. Similar to Phase 1 trials, pH readings were taken at 0, 8, 24, 48, 72, 96 and 120 hours. The second pH reading was shifted to 8 hours due to an insignificant change in pH between 0 and 6 hours in Phase 1 trials. A small sample (3 mL) was taken at Time 0 in Phase 2 trials to measure initial lactose and lactic acid content of the liquid fraction. No further samples were collected until the final time point of 120 hours to get a better representation of the final chitin yield and chitin product.

Pre-treatments were performed on mixtures that contained lactose as well as mixtures that did not contain lactose to determine if this would have an impact on the demineralization and deproteinization efficiency. Figure 4.2 shows the flow of the fermentation trials when pre-treatment was performed on a lactose containing mixture (i.e. lobster shells + lactose + deionized water). Following pre-treatment, inoculum was added to the mixture once cooled to 37℃ after which the fermentation was started immediately.

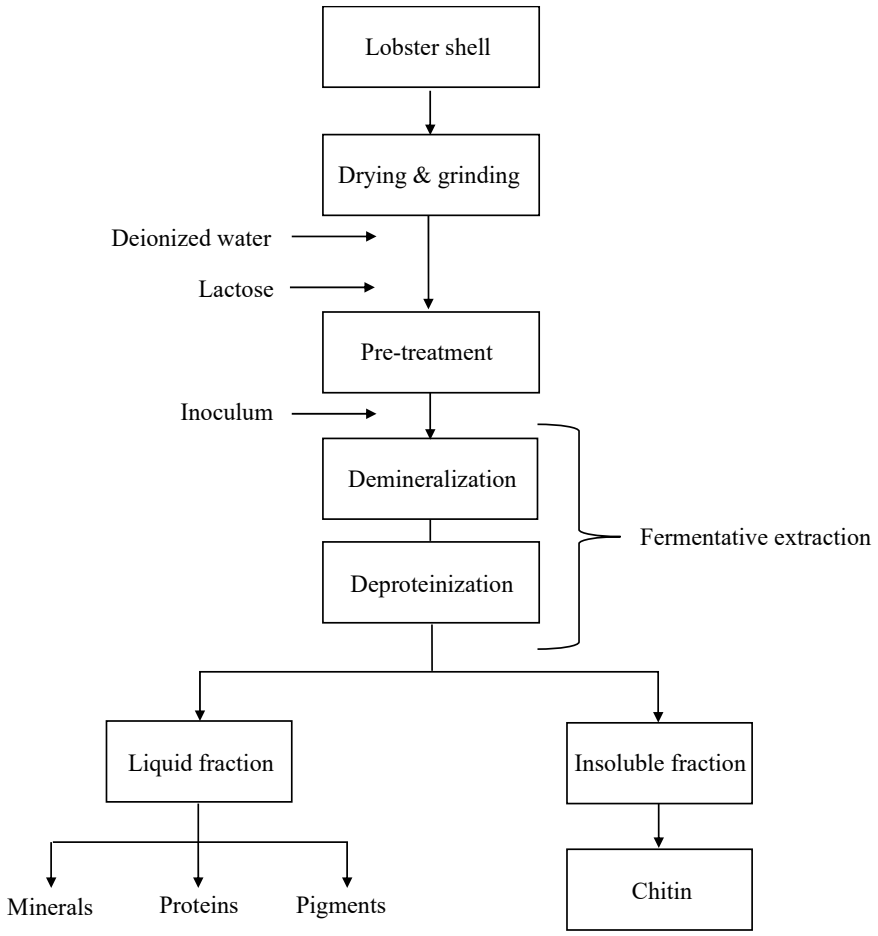


Figure 4.2: Flow diagram of fermentations in Phase 2 when pre-treatment was performed following the addition of lactose

Figure 4.3 shows the preparation steps for trials when the pre-treatment was performed before the addition of lactose. Following pre-treatment, lactose and inoculum were added to the mixture once it cooled to 37°C after which the fermentation was started immediately.

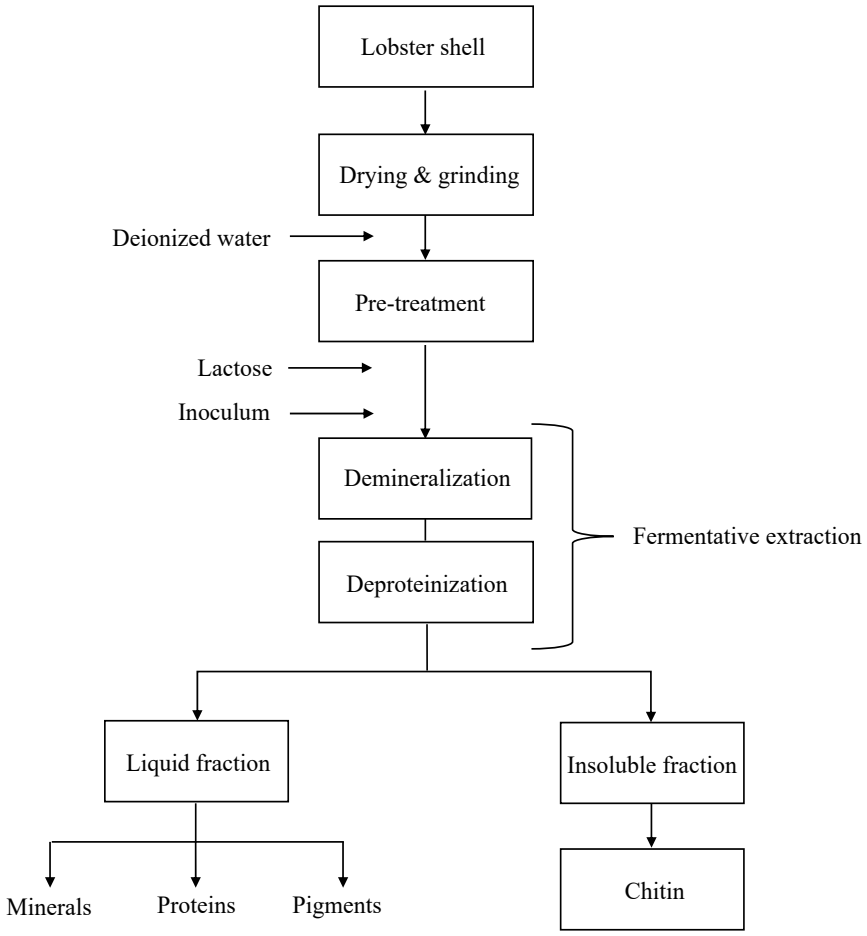


Figure 4.3: Flow diagram of fermentations in Phase 2 when pre-treatment was performed prior to the addition of lactose

4.5.1 Microwave-Assisted Extraction (MAE)

The components to be pre-treated were mixed thoroughly and divided evenly amongst 8 closed Teflon extraction vessels (50 mL/tube). Microwave irradiation (MARS 6 230/60 910900, CEM Co., NC, USA) was performed at 70°C, 700 W for 30 minutes with extraction vessels pressurized to 800 psi. The parameters were chosen based on similar values used in other studies found in the literature regarding crustacean shells (Leke-Aladekoba, 2018; Nguyen et al., 2016; Valdez-Peña et al., 2010). Following pre-treatment,

the contents of each vessel were combined in a 1 L Erlenmeyer flask and remaining components for the fermentation were added.

4.5.2 Ultrasound-Assisted Extraction (UAE)

The components to be pre-treated were mixed thoroughly in a 1 L plastic beaker and sonicated (VCX750 Ultrasonic Processor, Sonics and Materials Inc., Connecticut, USA) for 30 minutes at 20 kHz and an amplitude of 80%. The parameters were chosen based on similar values used in other studies found in the literature regarding crustacean shells (Kjartansson et al., 2006; Leke-Aladekoba, 2018). A half-inch (13 mm diameter) removable probe was used and the sample beaker was placed in an ice bath during the treatment to prevent overheating of the mixture. An average temperature of 40°C to 45°C was observed for the mixture during treatment.

4.6 Phase 3: Measurement of Fermentation Dynamics

Phase 3 trials were performed in a bioreactor (AMPTS II light, Bioprocess Control, Sweden) at a volume of 1 L in 2 L glass bottles. The temperature of the mixture was maintained at 37℃ by a controlled water bath and the fermentation mixture had constant stirring (200 rpm) based on limitations from the apparatus. pH readings were taken at 0, 12, 24, 48, 72, 96 and 120 hours. Due to an insufficient change in pH after 8 hours in Phase 2, the second pH reading was shifted to 12 hours. Samples were drawn at 0, 12, 24, 72 and 120 hours to ensure sufficient material for chitin extraction and insoluble material for analysis.

4.7 Characterization of Bioprocess

4.7.1 Sampling

All collected samples were weighed and centrifuged for 15 minutes (4700 rpm in 50 mL centrifuge tubes or 3600 rpm in 750 mL centrifuge containers). The liquid and insoluble fractions were separated into previously weighed centrifuge tubes (50 mL). All insoluble fractions were washed with deionized water until the wash water reached a neutral pH. The washed insoluble fractions were then lyophilized for further analysis. Liquid fractions and wash waters were stored at -20 °C until further analysis.

4.7.2 Solubilized Calcium

Solubilized calcium of the liquid fraction was determined by atomic absorption spectroscopy (AAS) (Thermo Fisher Scientific, MA, USA) in Phase 1 trials. Liquid fractions were diluted 1000× in 1 M nitric acid (HNO_3) and an external curve of calcium chloride solution (concentration ranging from 5 - 100 ppm) in 1 M HNO_3 was used to quantify the calcium content in the samples.

In Phases 2 and 3, total solubilized calcium in the liquid fractions was determined by measuring the calcium content by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (NexION 300D, PerkinElmer, USA). Liquid fractions were diluted 25,000× in 2 M HNO_3 while wash waters were diluted 1000× in 2 M HNO_3. A mixed external calibration standard containing calcium (NexION Standard Mode Instrument Calibration Standard 2, PerkinElmer, USA) in a concentration range from 10 - 1000 ppb diluted in 2 M HNO_3 was used to quantify calcium concentration of the samples.

4.7.3 Chitin Yield

The total chitin yield (%) obtained from the fermentative extraction process was estimated as follows:

$$\text{Yield of Chitin (\%)} = \left(\frac{\text{Weight of dried insoluble fraction}}{\text{Weight of starting shell material}}\right) \times 100$$

Chitin yield is estimated as there is potential for other components to remain in the insoluble fraction such as lactose, inoculum or insolubilized minerals and proteins. Therefore, the estimation may result in a chitin yield slightly higher than expected for the theoretical chitin content of lobster shells.

4.7.4 Lactose and Lactic Acid Concentration

High Performance Liquid Chromatography (HPLC) equipped with refractive index detection (PerkinElmer, Shelton, CT, USA) was used to qualitatively and quantitatively detect lactose and lactic acid in Phase 2 and Phase 3 samples. Liquid fractions were diluted (5% lactose diluted 10×, 20% lactose diluted 100×) in deionized water. The liquid samples (1.5 mL) were acidified with 1 M H_2SO_4 (7.5 μL) and centrifuged at 27,000 rpm for 20 minutes.

Chromatographic separations were performed with an Aminex HPX-87H (300 mm x 7.8 mm) Ion Exclusion Column (Bio-Rad Laboratories, Hercules, CA, USA). Injected samples (50 μL) were eluted in an isocratic mode with 5 mM H_2SO_4 solution at a flow rate of 0.5 mL/min. Compound identification and quantification was performed using external standard curves for lactose (0.02 – 10 mg/mL) and lactic acid (0.02 - 3 mg/mL).

Lactose consumed in the fermentation at the final time point in Phase 2 trials was calculated using the following equations:

$$\text{Lactose remaining (\%)} = \frac{\text{Final lactose concentration (mg/L)}}{\text{Inital lacotse concentration (mg/L)}} \times 100$$

$$\text{Lactose consumption(\%)} = 100 - \text{Lactose remaining (\%)}$$

$$\text{Lactose consumption(g)} = \text{Inital lactose (g)} \times \frac{\text{Lactose consumption (\%)}}{100}$$

4.7.5 Degree of Demineralization

The degree of demineralization of the lobster shells was determined by ash content of the lyophilized insoluble product. Ash content was determined by treating samples (0.5 g) in ceramic crucibles in a muffle furnace (Yamato FO410CR, Yamato Scientific Co., Ltd.) at 500°C for 4 hours (Chakravarty et al., 2018). Samples were weighed directly after to determine the organic mass loss. The following equation was used to calculate the degree of demineralization (Mukku Shrinivas Rao & Stevens, 2005).

$$\text{Demineralization (\%)} = \frac{[(A_o \text{ x } O) - (A_R \text{ x } R)]}{(A_o \text{ x } O)} \text{x } 100$$

Where, A_o is the ash content of the untreated shell, O is the weight of the untreated shell sample before ashing, A_R is the ash content of the residue of the fermentation and R is the weight of the treated sample before ashing.

4.7.6 Degree of Decalcification

Degree of decalcification was used in Phase 2 and 3 as a method to quantify and characterize calcium removal specifically as calcium is the predominant mineral component of the shell. Calcium content of the insoluble fraction was determined by ICP-MS (NexION 300D, PerkinElmer, USA). Samples of the insoluble fraction (25 mg) were microwave digested (MARS 6 230/60 910900, USA) in 8 M HNO_3 (10 mL) at 200°C for 30 minutes, 1200 W with vessels pressurized to 800 psi. Prior to ICP-MS analysis, the digested samples were diluted 1000× with 2 M HNO_3. A mixed external calibration standard containing calcium (NexION Standard Mode Instrument Calibration Standard 2, PerkinElmer, USA) in a concentration range from 10 - 1000 ppb diluted in 2 M $HNO_3$was used to quantify calcium concentration of the samples.

Degree of decalcification was calculated by comparing the calcium content of the treated sample with the calcium content of the untreated ground lobster shell. The untreated shell was found to have an average calcium content of 3.3 mg Ca^{2+} per 25 mg shell. The degree of decalcification was calculated using the equations as follows:

$$1)\ Ca^{2+}\ \text{in ins. frac (mg)} = Ca^{2+}\ \text{concentration (mg/L)} \times\ 0.01\ \text{L}$$

$$2)\ \text{Normalized } Ca^{2+}\ \text{content} = \frac{25\ \text{mg of shell powder}}{\text{Ins. frac digested (mg)}} \times Ca^{2+}\ \text{in sample (mg)}$$

$$3)\ Ca^{2+}\ \text{remaining in ins. frac (\%)} = \frac{\text{Normalized } Ca^{2+}\ \text{content}}{3.3\ \text{mg } Ca^{2+}} \times 100$$

$$4)\ \text{Degree of decalcification (\%)} = 100 - Ca^{2+}\ \text{remaining in ins. frac (\%)}$$

*ins. frac = insoluble fraction

4.7.7 Degree of Deproteinization

Nitrogen content of the lyophilized insoluble fraction (2 mg) determined using an elemental analyzer (Perkin Elmer series II CHNS/O Analyzer 2400 Perkin Elmer, USA) was used to calculate the degree of deproteinization. Both chitin and protein contribute to the total nitrogen content of the shell (N_T) therefore it is difficult to distinguish between protein nitrogen or chitinous nitrogen. Due to this, protein content is typically calculated using the theoretical percentage of nitrogen in fully acetylated chitin (6.9%) and in proteins (6.25%) resulting in the following equation (Percot et al., 2003; Tshinyangu & Hennebert, 1996):

$$\text{Protein content (\%)} = (N_T - 6.9) \text{ x } 6.25$$

Total percentage of nitrogen in the ground untreated lobster shells (N_T) was 5.5% as determined by elemental analysis. This value is lower than the theoretical percentage of nitrogen in chitin therefore protein content of the ground shells could not be calculated using the above equation. The low nitrogen content could be due to slightly lower levels of chitin or protein in the untreated shell than those found in literature. Due to this, protein content and thus degree of deproteinization was estimated based on the theoretical chitin and protein content of lobster shells at approximately 20% and 25% dry weight respectively (Bolat et al., 2010; Nguyen, Trung T; Barber, Andrew R; Corbin, Kendall; Zhang, 2017). Degree of deproteinization was estimated using the following equations:

$$1)\ \text{Inital chitin (g)} = \text{Weight of starting shell powder (g)} \times\ 0.2$$

$$2)\ \text{Chitin in ins. frac (g)} = \text{Weight of dry ins. frac (g)} \times\ 0.2$$

$$3)\ \text{Chitinous nitrogen } (\mathrm{N_c}) = \left(\frac{\text{Chitin in ins. frac (g)}}{\text{Weight of dry ins. frac (g)}}\right) \times 6.9$$

$$4)\ \text{Protein in ins. frac (\%)} = (N_T - N_c) \times 6.25$$

$$5)\ \text{Initial protein (g)} = \text{Weight of starting shell material} * 0.25$$

$$6)\ \text{Protein in ins. frac (g)} = \frac{\text{Weight of dry ins. frac (g)} \times \text{Protein in ins. frac (\%)}}{100}$$

$$7)\ \text{Protein in ins. frac (\%)} = \frac{\text{Protein in ins. frac (g)}}{\text{Initial protein (g)}} \times 100\%$$

$$8)\ \text{Deproteinization} = 100\% - \text{Protein remaining in ins. frac (\%)}$$

*ins. frac = insoluble fraction

In Phase 3, elemental analysis was also performed on lyophilized samples of the liquid fraction to measure the change in nitrogen content over time due to the solubilization of proteins. The protein content of the liquid fraction was also calculated using the theoretical nitrogen content in proteins.

$$\text{Protein content (\%)} = N_T * 6.25$$

4.7.8 Lactic Acid Bacteria Growth

In Phase 3 trials, inoculum growth over the course of fermentation was determined by colony counting on DeMan-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, MO, USA) plates. Samples of the liquid fraction were serially diluted (x 10^{-3}) in 0.9% sodium chloride (NaCl) solution and spread (100 μL) on the MRS agar plates. The plates were incubated

for 48 hours at 37°C after which colonies were counted and expressed as colony forming units per mL (cfu/mL).

4.7.9 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR (Nicolet 6700 Spectrometer, Thermo Instruments, Canada) was used to characterize chitin in the insoluble fraction based on the presence of characteristic functional groups.

A mild acid wash using 0.5 M HCl was performed on the pellet prior to FTIR analysis for 2 hours at room temperature to remove any residual minerals or impurities in the sample that could lead to interferences in the spectra. Following the acid wash, the insoluble fraction was washed with distilled water until the wash water reached a neutral pH and was then lyophilized.

The lyophilized samples of the insoluble fraction (1.5 mg) were finely ground with potassium bromide (KBr) (300 mg) using a mortar and pestle. The ground powder was formed into a transparent disc under vacuum and 20,000 psi for 15 minutes. A KBr disk was used as a reference blank and duplicate pellets were made for each sample. A total of 64 scans were taken for each FTIR spectra over the frequency range of 4000 – 400 cm^{-1} at a resolution of 8.

4.7.10 Statistical Analysis

Mean values and standard deviations were calculated for all experimental data obtained. All Analysis of Variance (ANOVA) were carried out using Minitab 18.1 software

(Minitab Inc., USA) at a 95% confidence interval (α = 0.05). Comparison of means was performed using the Tukey method at a 95% confidence interval (α = 0.05).

Chapter 5 : Results and Discussion

5.1 Proximate Composition of Lobster Shells

The proximate composition of the lobster shells used in the present study is summarized in Table 5.1.

Table 5.1: Proximate composition of ground lobster shells on dry matter basis

Moisture (%)	13.1 ± 0.04
Lipid (%)	0.08 ± 0.02
Ash content (%)	50.8 ± 0.40

The high moisture content of the dried ground shells could be due to incomplete drying or the uptake of moisture by the shells during storage. The shell powder was found to have a very low lipid content as determined by petroleum ether extraction. These results agree with the findings of other studies with low lipid content of shrimp shells at 0.5% dry weight (Mukku Shrinivas Rao & Stevens, 2005). Nguyen et al., (2017) also found that lobster carapace had a lipid content of less than 2%.

Ash content of the lobster shells showed that half of the dry weight of lobster shells is mineral content. These results are consistent with the findings of Boßelmann et al. (2007) who also found lobster shells had a mineral content of 50% by weight. The remaining 50% of the shell is likely organic material such as chitin and protein each making up approximately 20 – 30% by dry weight of the shell (Bolat et al., 2010; Nguyen et al., 2017).

5.2 Thermogravimetric Analysis (TGA)

TGA was performed on the untreated shell powder heating from 35℃ to 900℃ in increments of 20℃ per minute to determine the temperatures at which mass loss occurs. The TGA results are shown in Figure 5.1.

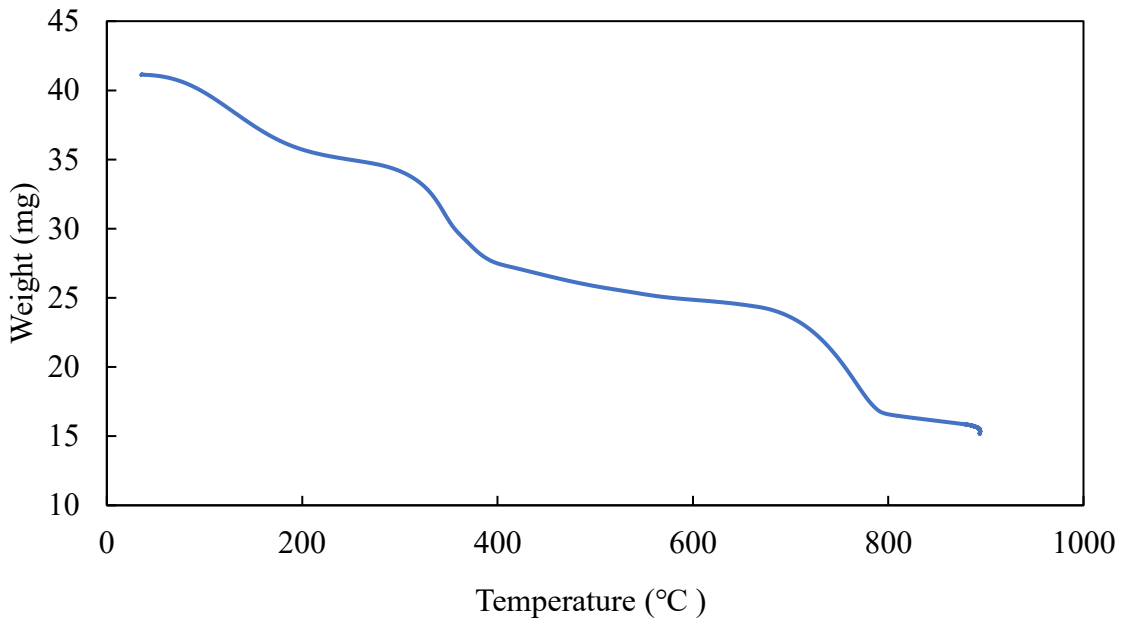


Figure 5.1: TGA data of ground lobster shell showing mass loss (mg) by heating from 35℃ to 900℃ in increments of 20℃ per minute

Mass loss was seen at three notable phases with the first phase between 100℃ and 200℃, which can be attributed to the evaporation of residual water in the lobster shell powder resulting in 13.2% mass reduction. The second mass loss phase is between 300℃ and 580℃ degrees, which can be attributed to the loss of organic matter such as chitin and proteins with a 26.1% mass reduction. The third mass loss phase is between 680℃ and 800℃ which is the decarboxylation of calcium carbonate as observed by Boßelmann et al. (2007) with a 20.4% mass reduction. The TGA data and percentage of mass loss at each phase was very similar to the TGA results of lobster shells in a study by Boßelmann et al.

(2007) with mass loss occurring at similar temperatures suggesting that they are the same components being lost as those in the present study.

5.2 Phase 1: Preliminary Trials

Phase 1 of the process development consisted of initial screening experiments to assess the influence of shell, lactose and lactic acid bacteria (inoculum) ratios on demineralization and deproteinization efficiency. A factorial design was performed with a high and low level for each factor as previously discussed in Section 4.4. The full experimental design for Phase 1 is shown in Table 5.2 with each formulation performed in experimental duplicate.

Table 5.2: Full factorial design for Phase 1 of fermentative chitin extraction method development

Formulation Coding	Shell (w/v%)	Lactose (w/v%)	Inoculum (w/v%)
A	5	5	1
B	5	5	5
C	5	20	1
D	5	20	5
E	20	5	1
F	20	5	5
G	20	20	1
H	20	20	5

5.2.1 Change in pH

A rapid drop in pH to acidic conditions within the first 24 to 48 hours is an indication that the fermentation is viable (Duan et al., 2012; Rao et al., 2000; Zakaria et al., 1998). A low pH is desirable as acidic conditions prevent the growth of spoilage microorganisms (Alakomi et al., 2000; Rao et al., 2000). The change in pH over the course of all fermentations in Phase 1 trials are shown in Figure 5.2.

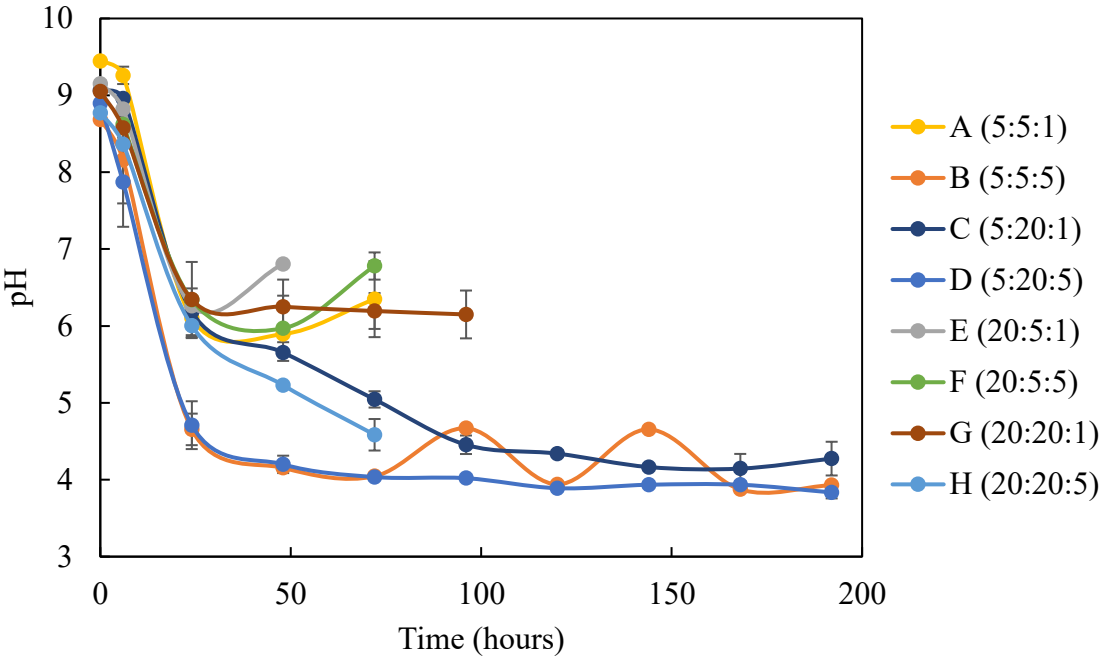


Figure 5.2: Change in pH over the duration of fermentation with standard deviations represented as error bars for all tested formulations in Phase 1 trials. Legend key for composition of each formulation - Shell (%): Lactose (%): Inoculum (%).

As expected, the pH decreased to an acidic range for all trials within the first 24 hours however after 48-72 hours, the pH of A, E and F began to increase. This is likely due to insufficient amounts of initial inoculum and/or lactose for adequate lactic acid production. The formulations were considered failed fermentations once the pH of the

mixture increased and were ended at that time point due to their rancid smell caused by spoilage microorganisms.

Although the pH of G and H did not begin to increase, the mixtures were also considered failed fermentations because the mixtures became a thick sludge after 120 and 72 hours respectively. The thickening of the mixture is expected to be due to the crystallization of calcium lactate which is further discussed in Section 5.1.3. The mixtures could no longer achieve stirring and therefore were ended.

Formulations B, C and D were the only trials that had a sufficient pH drop and reached the final time point of 192 hours without spoiling. B and D had the most significant pH drop with both mixtures reaching a pH of 4.20 within the first 48 hours. Formulation C had a slower pH drop to 4.46 at 96 hours due to the lower initial bacteria content. At 1% inoculum, more growth is required to produce the same amount of lactic acid as 5% inoculum therefore the pH decline was delayed for 1% inoculum trials. Once the pH dropped to approximately 4.0 for B, C and D the readings remained relatively constant for the remainder of the fermentations.

5.2.2 Solubilized Calcium

In lactic acid fermentation, calcium carbonate is removed from the shell when it reacts with lactic acid to produce calcium lactate (Kaur & Dhillon, 2015). The calcium lactate is solubilized into the liquid fraction therefore calcium concentration of the liquid fraction is expected to increase over time. Figure 5.3 shows the concentration of elemental calcium (mg/mL) in the liquid phase over the time course of fermentation in Phase 1 trials.

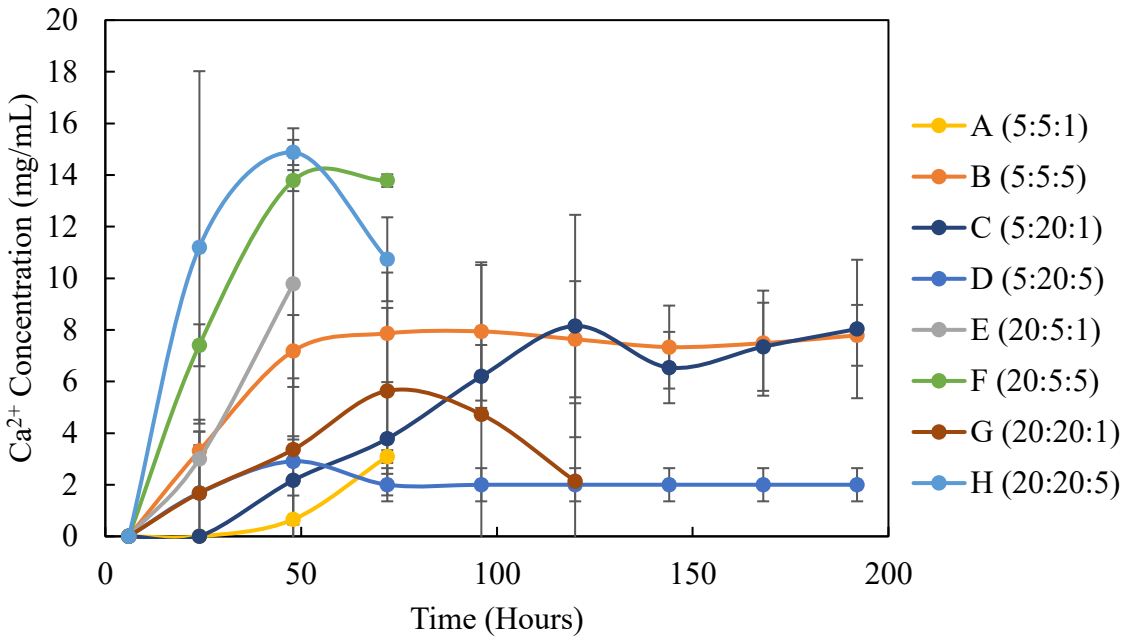


Figure 5.3: Concentration of solubilized calcium over the course of the fermentation with standard deviation represented as error bars for all tested formulations in initial trials. Legend key for composition of each formulation - Shell (%): Lactose (%): Inoculum (%).

As expected, the calcium content of the liquid fractions increased over time for all formulations showing that calcium was solubilized into the liquid fraction likely in the form of calcium lactate. Solubilized calcium for 5% shell trials reached a plateau around 8 mg/mL for formulations B and C. The saturation of solubilized calcium does not necessarily mean that demineralization is no longer occurring, however the remaining calcium removed is likely precipitated in the insoluble fraction.

The drastic drop of solubilized calcium content seen at 120 hours for formulation G and 72 hours for H is likely caused by crystallization of calcium lactate. The crystallization occurs when calcium lactate concentrations exceed the solubility limit of the solute and form filamentous structures thus thickening the mixture (Kubantseva, Hartel, & Swearingen, 2004; Mimouni, Bouhallab, Famelart, Naegele, & Schuck, 2007). Multiple

studies have investigated the solubility of calcium lactate and trends show that the solubility increases with an increase in temperature and decreases with a decrease in pH (Kubantseva et al., 2004; Vavrusova, Liang, & Skibsted, 2014). Based on data from tests performed by Kubantseva et al. (2004), the solubility limit of calcium lactate in water at 37℃ was found to be approximately 8.5 g of CaL_2/100 mL. Studies have yet to report the solubility limit at 37℃ with an acidic pH however the solubility limit in the present study is expected to be lower than 8.5 g of CaL_2/100 mL based on the trends found in the literature.

A study by Mimouni et al. (2007) found that the crystallization process was highly dependent on calcium and lactose concentration as well as pH. The initial concentrations of calcium and lactose were the same for G and H at 20% shell powder and 20% lactose therefore the results show that the combination of these ratios is undesirable and likely led to crystallization.

5.2.3 Degree of Demineralization and Deproteinization

In Phase 1 trials, demineralization and deproteinization of the insoluble fraction at the final time point was analyzed. The nitrogen content (%) was used for calculating the degree of deproteinization. The results for Phase 1 trials are summarized in Table 5.3.

Table 5.3: Summary of demineralization and deproteinization results with standard deviations from Phase 1 trials

Formulation	Ratio	DM (%)	DP (%)
A	5% shell 5% lactose 1% inoculum	38.6 ± 3.2	56.1 ± 3.2
B	5% shell 5% lactose 5% inoculum	77.6 ± 4.2	77.0 ± 21.8
C	5% shell 20% lactose 1% inoculum	66.8 ± 6.2	68.8 ± 8.3
D	5% shell 20% lactose 5% inoculum	67.1 ± 7.7	67.2 ± 7.9
E	20% shell 5% lactose 1% inoculum	55.4 ± 3.8	54.2 ± 25.4
F	20% shell 5% lactose 5% inoculum	21.0 ± 5.7	24.8 ± 22.1
G	20% shell 20% lactose 1% inoculum	31.0 ± 13.0	N.D.
H	20% shell 20% lactose 5% inoculum	57.0 ± 17.4	N.D.

DM - Demineralization
DP - Deproteinization

Formulations with 5% shell generally achieved higher levels of demineralization compared to 20% shell showing that the lower shell ratio was more efficient for higher degrees of mineral removal. Similar results were found in a study fermenting crab shell by Oh et al. (2007) where 5% shell waste was also found to be the optimal shell ratio for demineralization when compared to 10% shell waste.

Formulation B achieved the highest extraction efficiency while A, E, F, G and H were not desirable as they achieved low degrees of removal compared to the other tested

formulations as well as values found in the literature (Arbia et al., 2013; Kaur & Dhillon, 2015). The low levels of demineralization and deproteinization achieved by these formulations is due to insufficient lactic acid production and incompatible ratios of shell, lactose and inoculum. Deproteinization could not be accurately estimated for formulations G and H due to the sludge formation.

To determine the significance of the three independent factors (shell, lactose and inoculum) on the responses of demineralization and deproteinization a factorial regression ANOVA was performed at a confidence interval of 95% ($\alpha = 0.05$). No significant relationships were initially formulated due to inconsistent data across the formulations therefore the results were separated into 20% shell trials and 5% shell trials for statistical analysis.

The factorial regression ANOVA showed significant relationships based on 5% shell trials however significant relationships were still not formulated for 20% shell trials. Due to a lack of significant relationships concluded from 20% shell trials and consistent spoilage in the formulations, the 20% shell trials were determined to be undesirable. Therefore, the remainder of the experimental design focused on developing a chitin extraction method at a 5% shell ratio.

Tables 5.4 summarizes the f-values and p-values for all three variables in the factorial regression model for demineralization. Based on p-values, inoculum and its interaction with lactose are both statistically significant factors in the demineralization response ($\alpha = 0.05$). These results confirm that lactic acid directly facilitates demineralization and lactic acid is produced when inoculum consumes lactose. Therefore, the inoculum and its two-way interaction with lactose is expected to be significant for

demineralization. The regression model has an R^2 value of 93.06% representing how well this data fits the linear model.

Table 5.4: Analysis of Variance (ANOVA) of the regression model from the factorial design for factor contribution to demineralization response. Model summary: S = 5.59, R^2 = 93.06%, R^2(adjusted) = 87.86%, R^2 (predicted) = 72.25%, P < 0.05 significance level

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	1677.1	559.02	17.89	0.009
Linear	2	929.9	464.97	14.88	0.014
Lactose	1	155.1	155.14	4.96	0.090
Inoculum	1	774.8	774.80	24.79	0.008
2-Way Interactions	1	747.1	747.10	23.91	0.008
Lactose*Inoculum	1	747.1	747.10	23.91	0.008
Error	4	125.0	31.25		
Total	7	1802.0			

Table 5.5 summarizes the f-values and p-values for the factors in the factorial regression model for deproteinization. Based on p-values, inoculum is the only statistically significant factor. Proteases are responsible for deproteinization of the shell, which are produced by the inoculum without directly interacting with lactose therefore, it is expected that only inoculum would be significant for the deproteinization response. The model has an R^2 value of 71.16% representing the fit of the data in the linear model.

Table 5.5: Analysis of Variance (ANOVA) of the Factorial Regression model for factor contribution to deproteinization response. Model summary: S = 6.87, R^2 = 71.16%, R^2 (adjusted) = 49.54%, P < 0.05 significance level

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	466.323	155.441	3.29	0.140
Linear	2	466.228	233.114	4.93	0.083
Lactose	1	67.338	67.338	1.43	0.298
Inoculum	1	398.890	398.890	8.44	0.044
2-Way Interactions	1	0.095	0.095	0.00	0.966
Lactose*Inoculum	1	0.095	0.095	0.00	0.966
Error	4	188.952	47.238		
Total	7	655.275			

The interaction plot for the response of demineralization (Figure 5.4) shows that demineralization increased as lactose increased from 5% to 20% at 1% inoculum. This shows that an increase in available lactose for 1% inoculum increased the demineralization efficiency. The opposite is seen for 5% inoculum, where demineralization decreases with an increase of lactose from 5% to 20%. These results were unexpected as it was hypothesized a positive correlation would exist between available lactose and demineralization efficiency at all inoculum levels. The decrease in efficiency may be due to the combination of high lactose and high inoculum ratios causing a stress response from the lactic acid bacteria. When the lactic acid bacteria are in an environment with a very high growth rate and quick acidification, the inoculum may attempt to stabilize the growth rate by modifying their metabolism to decrease lactic acid production through carbohydrate starvation (de Alteriis et al., 2018; Molenaar et al., 2009; Papadimitriou et al., 2016). Due to a decrease in lactic acid production efficiency, the 5% inoculum ratio could achieve similar degrees of removal to 1% inoculum.

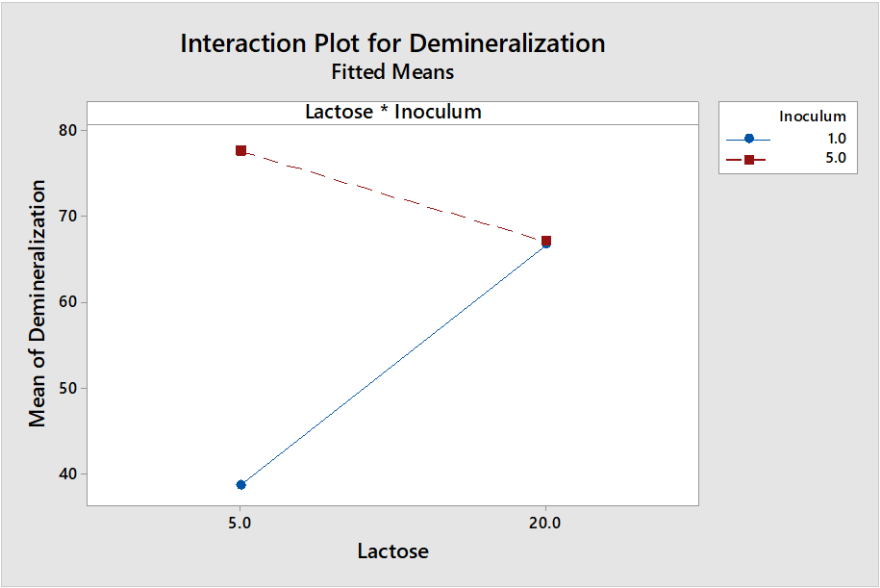


Figure 5.4: Interaction plot for demineralization response based on the two variables analyzed in Minitab 18

The interaction plot for the response of deproteinization (Figure 5.5) shows that deproteinization is significantly higher for both 5% inoculum trials (B and D) compared to 1% inoculum trials (A and C). These results confirm that inoculum is the most significant factor for the response of deproteinization.

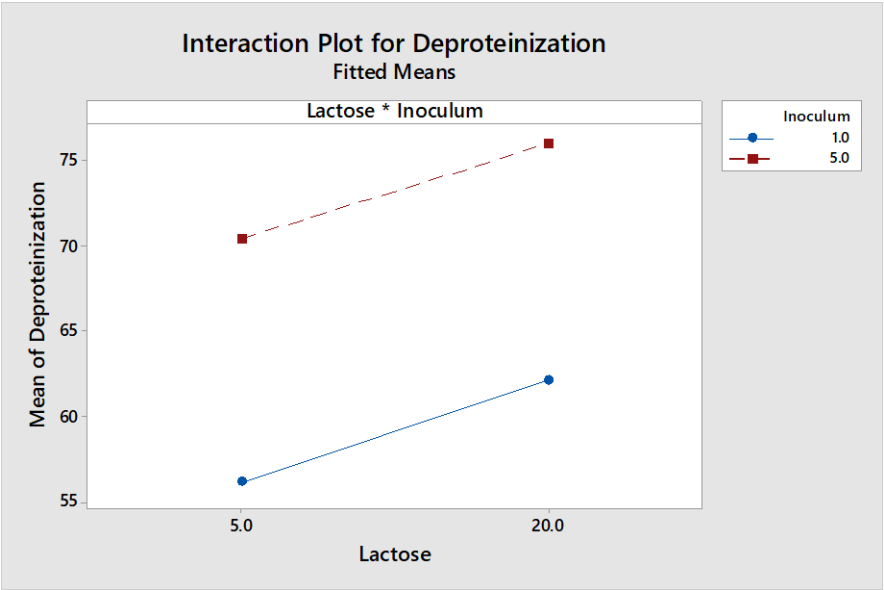


Figure 5.5: Interaction plot for deproteinization response based on the two variables analyzed in Minitab 18

Table 5.6 is a condensed version of Table 5.3 where demineralization and deproteinization results for only trials with 5% shell are summarized.

Table 5.6: Summary of demineralization and deproteinization results with standard deviation of only 5% shell trials from Phase 1

Formulation	Ratio	DM (%)	DP (%)
A	5% shell 5% lactose 1% inoculum	38.6 ± 3.2	56.1 ± 3.2
B	5% shell 5% lactose 5% inoculum	77.6 ± 4.2	77.0 ± 21.8
C	5% shell 20% lactose 1% inoculum	66.8 ± 6.2	68.8 ± 8.3
D	5% shell 20% lactose 5% inoculum	67.1 ± 7.7	67.2 ± 7.9

DM - Demineralization
DP - Deproteinization

Formulation B was the most efficient formulation of the 5% shell trials due to its high degrees of mineral and protein removal compared to other tested formulations making it desirable for further investigation. Formulation A was not desirable due its relatively low levels of demineralization and deproteinization when compared to other 5% shell trials.

Interestingly, both formulations C and D, which had high lactose levels (20%), achieved lower degrees of demineralization and deproteinization than formulation B (5% lactose). Initially it was theorized that more lactose available to the inoculum would increase lactic acid production and therefore increase mineral removal however, this was not seen in the present trials. As previously mentioned, this could be due to the high growth rate inducing a stress response by the bacteria decreasing lactic acid production through carbohydrate starvation (Papadimitriou et al., 2016). The high lactose concentration in the medium may also have caused osmotic stress for the bacteria therefore the lactose intake was decreased resulting in lower degrees of removal (Glaasker, Tjan, Ter Steeg, Konings, & Poolman, 1998). Although formulations C and D achieved degrees of demineralization and deproteinization about 10% lower than those of B, C would be a more economical option than B or D based on the cost of inputs. A lower inoculum ratio is desirable as inoculum is more expensive than lactose therefore formulation C was also desirable for further investigation.

It was hypothesized that the use of a pre-treatment on the fermentation mixture could potentially improve the extraction efficiency of C and B. This hypothesis was tested in Phase 2 of the process development.

5.3 Phase 2: Pre-Treatment Trials

Phase 2 trials were performed on formulations B and C to assess the impact of novel heat pre-treatments (MAE and UAE) on the demineralization and deproteinization efficiency with and without lactose in the pre-treated mixture. Table 5.7 shows all experimental trials performed in Phase 2 with the formulation coding used in the following sections. To date, no previous studies have tested microwave or ultrasound pre-treatments on lactic acid fermentation of crustacean shells.

Table 5.7: Summary of formulations tested in Phase 2 with MAE and UAE pre-treatments with and without lactose in the mixture (B – 5% shell: 5% lactose; 5% inoculum; C – 5% shell: 20% lactose: 1% inoculum)

Formulation	Pre-treatment type	Lactose in pre-treated mixture	Formulation Coding
B	Control	N/A	B
B	MAE	+	BM+L
B	MAE	-	BM-L
B	UAE	+	BU+L
B	UAE	-	BU-L
C	Control	N/A	C
C	MAE	+	CM+L
C	MAE	-	CM-L
C	UAE	+	CU+L
C	UAE	-	CU-L

M = Microwave assisted extraction;
U = Ultrasound-assisted extraction
+L = Pre-treatment performed with lactose
-L = Pre-treatment performed without lactose

5.3.1 Change in pH

The change in pH over the course of the fermentation for each treatment tested in Phase 2 is shown in Figure 5.6.

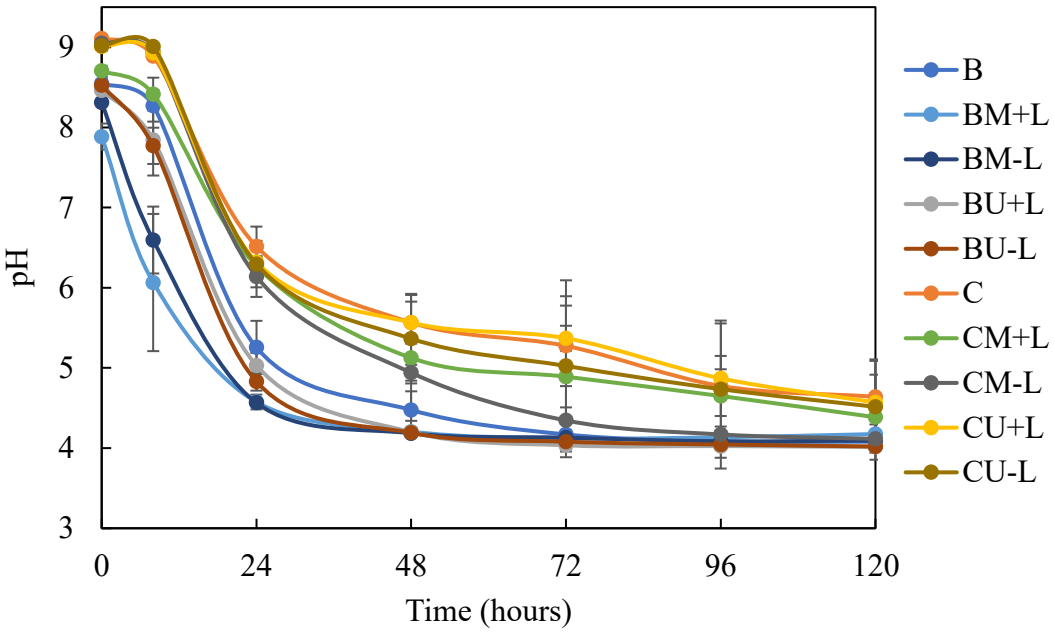


Figure 5.6: Change in pH over time with standard deviations represented as error bars for all tested formulations in Phase 2 trials (B – 5% shell: 5% lactose; 5% inoculum; C – 5% shell: 20% lactose: 1% inoculum)

All formulations were carried out for 120 hours without any spoilage. The pH drop was consistently faster for B formulations compared to C formulations regardless of the pre-treatment used. As seen in Phase 1 results, this is expected to be due to the lower initial amount of inoculum in formulation C therefore more time was needed for inoculum growth to acidify the mixture. Formulation BM-L had the fastest drop in pH over the first 12 hours however both BM+L and BM-L reached a pH of 4.57 after 24 hours. All B formulations had a final pH between 4.12 and 4.02 while the final pH for all C formulations were slightly higher at 120 hours ranging from 4.57 to 4.39.

5.3.2 Solubilized Calcium

The total solubilized calcium concentration was determined by summing the calcium content of the liquid fraction and the wash waters from each trial measured by ICP-MS. The highest solubilized calcium was achieved by BM-L (6.98 mg/mL) while C had the lowest (5.14 mg/mL). According to Figure 5.7, there was no significant increase in solubilized calcium amongst B formulations with the use of either pre-treatment however, CM-L had a significant increase compared to its control and was not statistically different from B formulations. This indicates that the pre-treatments did not significantly impact calcium removal for any formulations except for CM-L.

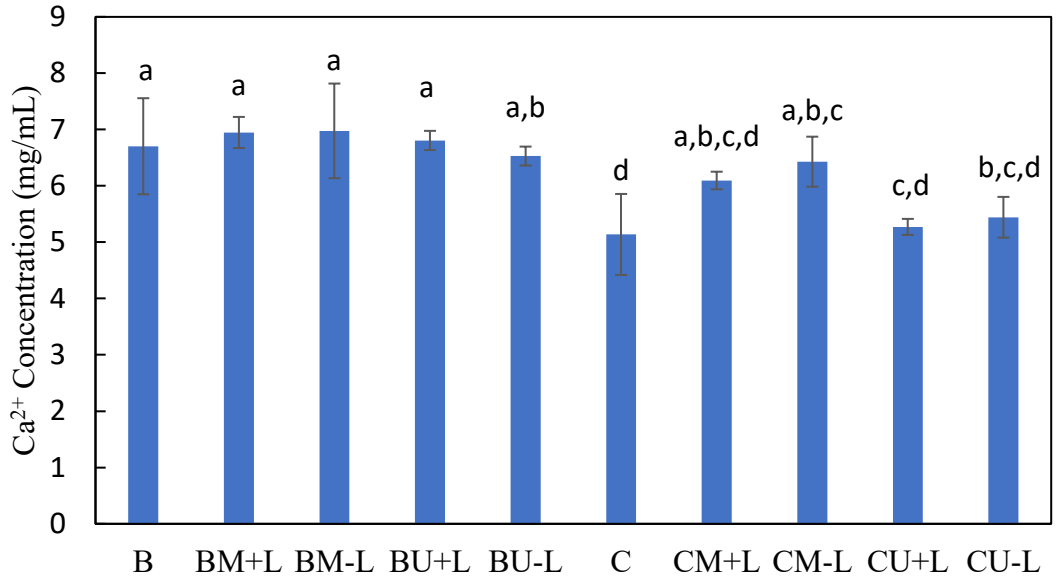


Figure 5.7: Sum of solubilized calcium in liquid fractions and wash waters for each formulations tested in Phase 2 with standard deviations represented by error bars and statistical comparison of means. Data points with the same lettering show no significant difference based on the Tukey method.

5.3.3 Chitin Yield

Chitin yield could be estimated in Phase 2 trials as subsamples were not taken throughout the fermentation therefore the end product was taken to represent the overall process chitin yield. The chitin yields as determined by the total weight of the insoluble product for all formulations in Phase 2 trials are summarized in Table 5.8.

Table 5.8: Final chitin yield results for all formulations tested in Phase 2 with standard deviations and statistical comparison of means. Yields with the same superscript letter show no significant difference based on the Tukey method.

Formulation Code	Chitin yield (%)
B	28.8 ± 0.35^{a}
BM+L	32.3 ± 3.89^{a}
BM-L	31.8 ± 0.35^{a}
BU+L	31.0 ± 0.70^{a}
BU-L	32.8 ± 1.06^{a}
C	34.3 ± 4.60^{a}
CM+L	32.3 ± 3.89^{a}
CM-L	31.3 ± 3.18^{a}
CU+L	37.3 ± 3.18^{a}
CU-L	34.0 ± 4.24^{a}

The chitin yields achieved ranges from 28.8% to 37.3% which correlate well with the chitin content of lobster shells found in the literature (20-30 wt%) (Bolat et al., 2010;

Nguyen et al. , 2017). Similar yields of chitin were achieved in other studies using lactic acid fermentation of shrimp shells at a 21.35% chitin yield (Zhang et al., 2017) as well as crab shells with chitin yields ranging from 30.0% to 38.6% (Jung et al., 2007). Due to a lack of statistical difference between the chitin yield from all formulations, it can be concluded that the pre-treatments at the tested parameters did not significantly impact the chitin yield. However, this could be dependent upon the intensity or duration of the pre-treatment and different results may be achieved at different pre-treatment parameters (i.e. longer duration and/or higher intensity).

The chitin yields are similar for all trials however some formulations are slightly higher than the expected literature values, predominately the C formulations. This is likely due to residual proteins and minerals in the insoluble fraction that were not removed in the fermentation process thus increasing the weight of the total insoluble product. This is reflected in decreased degrees of mineral and protein removal by C formulations in Tables 5.9 and 5.10.

5.3.4 Lactose and Lactic Acid Concentration

Lactose consumption results after 120 hours of fermentation (Figure 5.8) show that C formulations consistently consumed more lactose than B formulations. This data confirms that 1% inoculum required higher lactose consumption for sufficient growth compared to 5%. However, the carbon source was not depleted for any trial with the highest percent consumption at 55% by B. Since the lactose was consistently in excess, both 5% and 20% lactose were too high of an initial lactose content. No other studies were found where the consumption of lactose or other fermentable carbon sources by the inoculum was quantified.

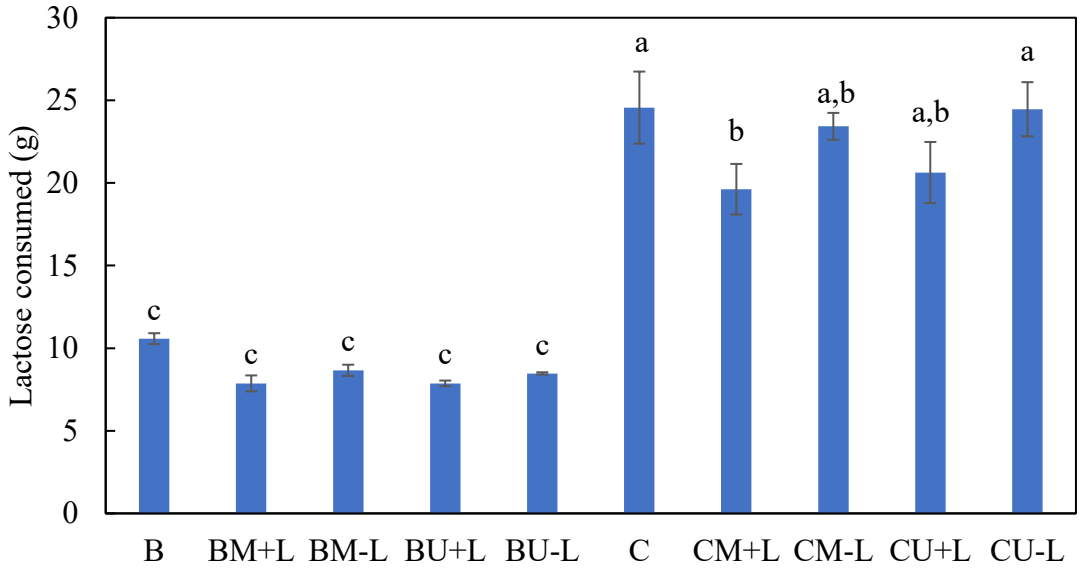


Figure 5.8: Lactose consumed (g) after 120 hours of fermentation for all formulations tested in Phase 2 with standard deviations represented by error bars and statistical comparison of means. Data points with the same lettering show no significant difference based on the Tukey method.

Based on lactic acid concentrations of the liquid fraction (Figure 5.9), it is clear that B formulations (composition of 5% shell: 5% lactose: 5% inoculum) were far superior in lactic acid production regardless of whether microwave or ultrasound pre-treatments were used when compared to C formulations (composition of 5% shell: 20% lactose: 1% inoculum). Lactic acid production was also more consistent between replicates across B trials. B and BM-L had the highest lactic acid concentrations of 4.4 mg/mL after 120 hours while CU+L had the lowest lactic acid production with 0.99 mg/mL after 120 hours. One study that quantified the lactic acid content optimized a fermentation using crab shells to a maximum lactic acid concentration of 17 mg/g of fermentation slurry (Castro et al., 2018). Comparing the results to this study, all trials have relatively low concentrations of lactic acid yet achieved a sufficient pH drop.

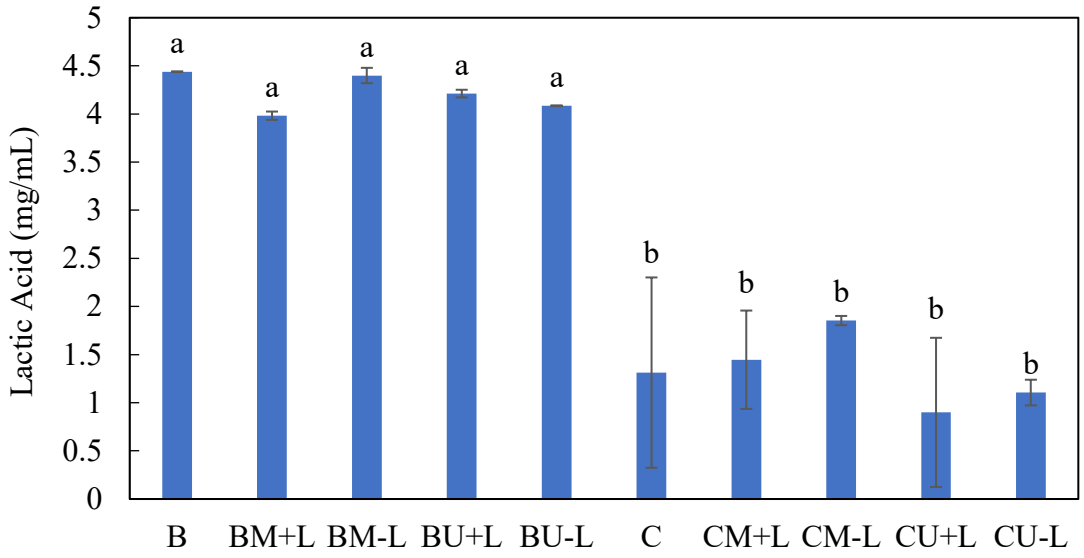


Figure 5.9: Lactic acid concentration in liquid fraction (mg/mL) after 120 hours for all formulations tested in Phase 2 with standard deviations represented by error bars and statistical comparison of means. Data points with the same lettering show no significant difference based on the Tukey method.

When comparing results from Figures 5.8 and 5.9, it can be concluded that although total grams of lactose consumed by all B formulations was consistently lower than C formulations, the higher initial inoculum ratio was still able to produce significantly higher levels of lactic acid.

5.3.5 Degree of Demineralization

From Figure 5.10, it can be noted that the degrees of demineralization are comparable after 120 hours to the results of Phase 1 ($\pm$ 5% of each other) therefore fermentation beyond 120 hours did not greatly improve demineralization.

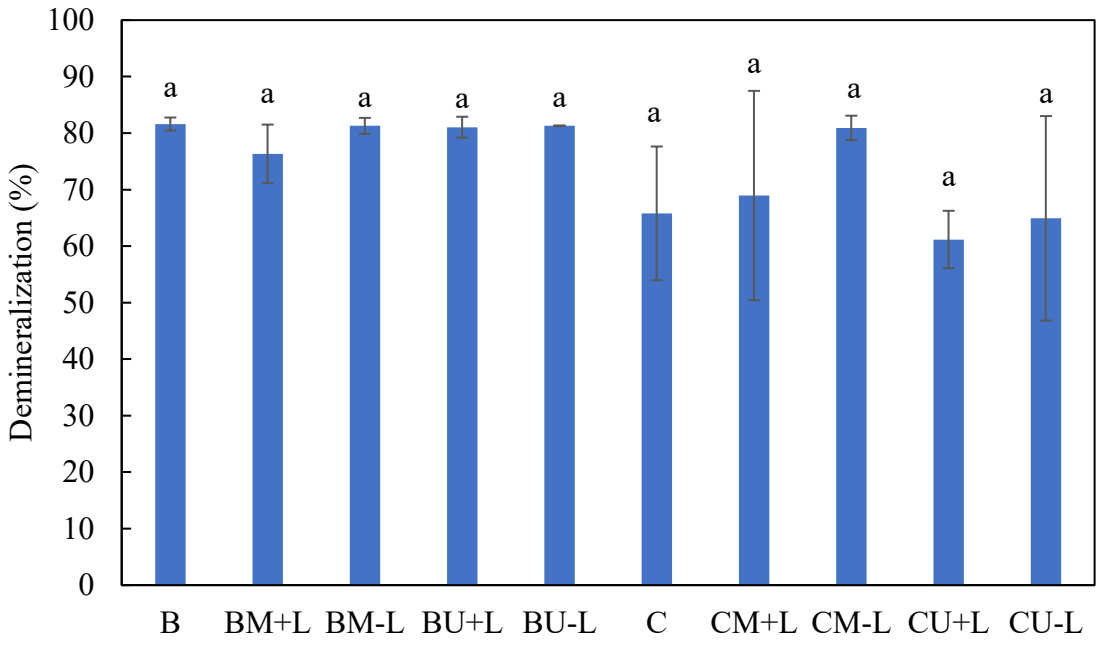


Figure 5.10: Demineralization results for Phase 2 trials with standard deviations represented by error bars and statistical difference. Data points with the same lettering show no significant difference based on the Tukey method.

The achieved degrees of demineralization for all formulations are comparable to values found in the literature as literature values range from 61.0 – 99.6% demineralization (Castro et al., 2018; Zakaria et al., 1998). Similar trends were seen in demineralization data as those for decalcification (Table 5.9) however there was no statistical difference between the means for the response of demineralization. Based on statistical analysis, the use of a pre-treatment under the tested conditions did not significantly impact the degree of demineralization regardless of the presence of lactose.

5.3.6 Degree of Decalcification

Decalcification was used in Phase 2 trials along with demineralization to assess mineral removal since calcium is the predominant component of the ground lobster shells

at approximately 50%. Table 5.9 summarizes the degrees of decalcification for all formulations in Phase 2.

Table 5.9: Summary of decalcification results for all formulations tested in Phase 2 with standard deviations and statistical comparison of means. Data points with the same lettering show no significant difference based on the Tukey method.

Formulation Code	Decalcification (%)
B	93.5 ± 2.2^{a}
BM+L	94.5 ± 1.1^{a}
BM-L	95.5 ± 0.6^{a}
BU+L	94.1 ± 3.2^{a}
BU-L	92.8 ± 7.8^{a}
C	76.2 ± 12.3^{c}
CM+L	89.0 ± 7.1^{abc}
CM-L	91.2 ± 4.0^{ab}
CU+L	77.3 ± 4.0^{bc}
CU-L	86.0 ± 8.1^{abc}

As expected, a positive trend can be seen between lactic acid concentration and decalcification efficiency within trials of the same formulation (Figure 5.9 and Table 5.9). It is notable to mention that lower lactic acid concentration at the final time point from CM+L, CM-L and CU-L (1.5 mg/mL, 1.9 mg/mL and 0.9 mg/mL respectively) resulted in comparable degrees of decalcification to B formulations.

The comparison of means at a significance of 95% showed that B formulations are not statistically different from one another therefore pre-treatments nor the presence of

lactose in the pre-treated mixture significantly impacted the outcomes of B formulations. It is important to note that although C (control) is statistically different from B formulations, CM+L, CM-L and CU-L are not statistically different from B formulations. These results show that the use of a pre-treatment on formulation C could increase the decalcification of C to degrees comparable to B. The C formulations however were generally more variable between replicates compared to B.

5.3.7 Degree of Deproteinization

The nitrogen content (%) of the insoluble fraction from the final time point was used to calculate the degree of deproteinization for each trial which is summarized in Table 5.10. Similar to demineralization results, similar degrees of deproteinization were achieved after 120 hours (Phase 2) and 192 hours of fermentation (Phase 1) ($\pm$5% of each other) therefore fermentation after 120 hours did not greatly impact deproteinization. The degrees of deproteinization achieved in Phase 2 are also comparable to those found in literature pertaining to shrimp shells, which range from 68.9% to 96.5% (Duan et al., 2012; Jung et al., 2007).

Table 5.10: Degree of deproteinization for Phase 2 trials with standard deviation and statistical significance. Data points with the same lettering show no significant difference based on the Tukey method.

Formulation Code	Deproteinization (%)
B	83.6 ± 4.6^{a}
BM+L	70.5 ± 2.6^{b}
BM-L	74.6 ± 5.1^{ab}
BU+L	73.4 ± 1.1^{b}
BU-L	68.9 ± 1.0^{b}
C	68.7 ± 6.2^{b}
CM+L	70.5 ± 6.1^{b}
CM-L	71.3 ± 2.6^{b}
CU+L	71.2 ± 5.5^{b}
CU-L	65.8 ± 8.7^{b}

According to the Tukey method, only B achieved a statistically different degree of deproteinization amongst all other trials. Interestingly, these results show that both MAE and UAE at the tested conditions significantly decreased the efficiency of deproteinization for B and had no significant impact on C regardless of lactose in the pre-treated mixture. The decrease in deproteinization could be due to the denaturing of proteins during pre-treatment however this would be unexpected due to the heating methods of the pre-treatments and the lack of significant decrease for C formulations.

The duration of the pre-treatments likely plays a role with the degree to which they impact the process efficiency. A study found that deproteinization efficiency was not increased with the use of MAE (maximum of 1200 W) until 90 minutes of treatment when

compared to 30 and 60 minutes for enzymatic deproteinization of lobster shells (Nguyen et al., 2016). While a another study only saw a 3.7% increase in deproteinization with UAE employed for 4 hours with the chemical extraction of chitin from shrimp shells (Kjartansson et al., 2006) therefore a longer duration of pre-treatment may make a more significant impact.

Based on the results from all trials in Phase 2, the pre-treatments at the tested conditions did not have a significant impact on the process efficiency and the inclusion of lactose did not greatly impact pre-treatment efficiency. Due to a high degree of decalcification achieved by B at 93.5% and a statistically different degree of deproteinization at 83.5%, it was chosen for further characterization in Phase 3. BM-L achieved the second highest degree of deproteinization at 74.6% and achieved a slightly higher degree of decalcification than B at 95.5%. To determine if pre-treatment could potentially decrease extraction time BM-L was also tested for further characterization in Phase 3.

5.4 Phase 3: Measurement of Fermentation Dynamics

In Phase 3 trials, microwave pre-treatment was performed at a 10% shell ratio due to a higher volume required to be pre-treated and the size limitations of the equipment used. Shell powder (50 g) and distilled water (500 mL) were divided evenly amongst 8 Teflon tubes (6.25 g shell/ tube and 62.5 mL water) and microwaved at the same conditions as the previous trials. Following pre-treatment, the contents of each tube were combined in a 2 L glass bottle and an additional 500 mL of water was added to bring the shells to a 5% ratio (w/v). The remaining components for the fermentation were then added to the bottle and the fermentation was started immediately.

Phase 3 results followed the same trends as those seen in Phase 2 trials with regards to changes in pH and degrees of mineral and protein removal. The fermentation conditions for Phase 3 trials were more controlled (temperature and anaerobic conditions) than Phases 1 and 2 as the trials were performed in a bioreactor under completely anaerobic conditions apart from when sampling occurred. Since Phase 1 and 2 trials were performed in Erlenmeyer flasks covered with parafilm, there was likely disruptions to complete anaerobic conditions. Although completely anaerobic conditions may not have been achieved, high degrees of mineral and protein removal still occurred since lactobacillus strains are facultatively anaerobic, meaning the bacteria can still function in oxygen rich environments (Goldstein et al., 2015; Watanabe, van der Veen, & Abee, 2012). A study using a commercial blend of lactobacillus strains for shrimp shell fermentation also found no significant difference between process efficiency when comparing anaerobic and aerobic conditions (Healy et al., 1994).

5.4.1 Change in pH

As shown in Figure 5.11, the drop in pH was relatively consistent between the control (B) and MAE treated trial (BM-L) over the course of the fermentation. The pH drop was slightly quicker for the pre-treated mixture within the first 24 hours compared to the control. After 48 hours, both fermentations consistently had very similar pH readings for the duration of the fermentation.

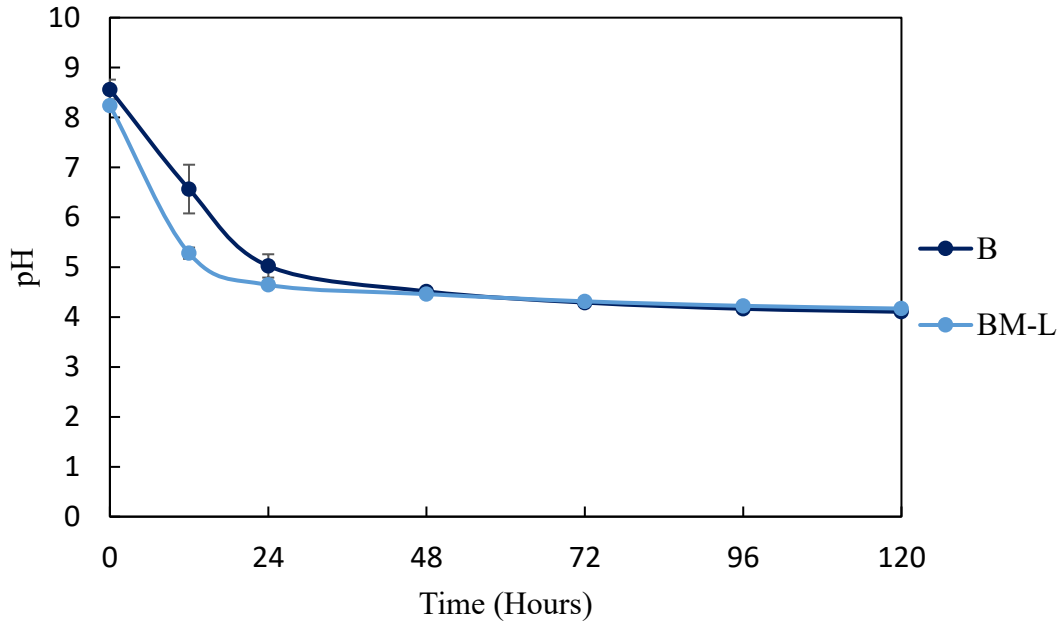


Figure 5.11: Change in pH over the course of fermentation for Phase 3 trials with standard deviations represented by error bars.

5.4.2 Solubilized Calcium

The time course of solubilized calcium content for B and BM-L are shown in Figure 5.12. Within the first 24 hours, the pre-treated mixture had a higher solubilized calcium content which could be due to the pre-treatment degrading the shells allowing for an increased accessibility of calcium to the inoculum. However, after 72 hours the control reached the same calcium concentration as the pre-treated mixture and slightly surpassed the concentration at 120 hours with a final solubilized calcium concentration of 6.29 mg/mL.

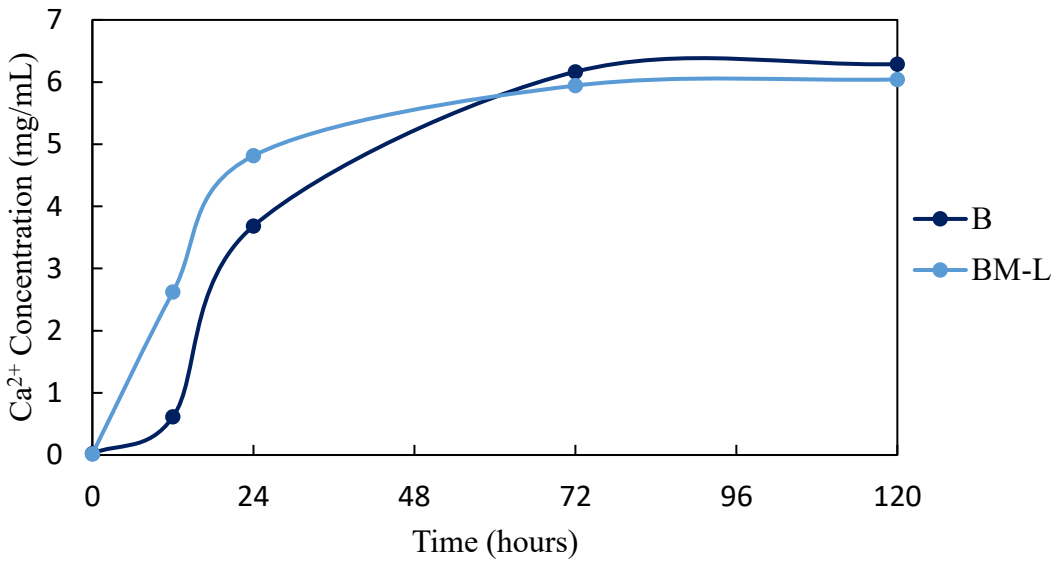


Figure 5.12: Time course of total solubilized calcium content (mg/mL) in Phase 3 trials.

5.4.3 Lactose and Lactic Acid Concentration

The lactose and lactic acid concentration of the liquid fraction (mg/mL) measured for both tested formulations are shown in Figure 5.13 and Figure 5.14 respectively. According to Figure 5.13, approximately half of the initial lactose is consumed in both formulations after 120 hours which was also seen in Phase 2 results. This shows that the initial ratio of 5% lactose exceeds the requirements of 5% inoculum and initial lactose content could likely be decreased to approximately 2.5%. Similar amounts of lactic acid were also produced by both trials by the final time point however, the pre-treated mixture produced lactic acid at a slightly faster rate than the control over the first 72 hours. Based on these results, overall the MAE pre-treatment at the tested conditions did not greatly impact the consumption of lactose or production of lactic acid.

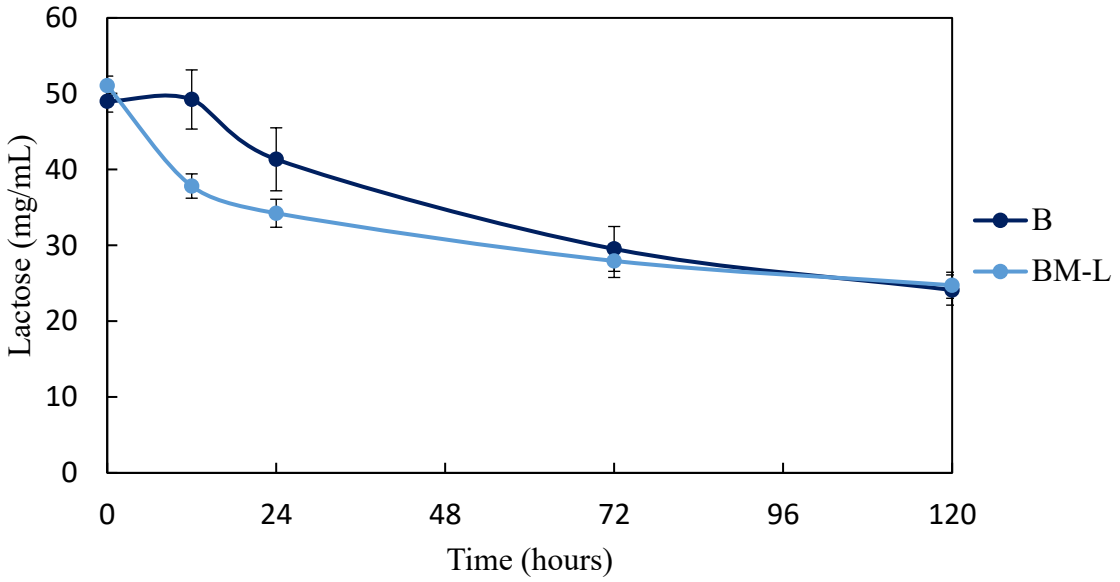


Figure 5.13: Change in lactose concentration (mg/mL) of liquid fractions over 120 hours with standard deviations represented by error bars for Phase 3 trials.

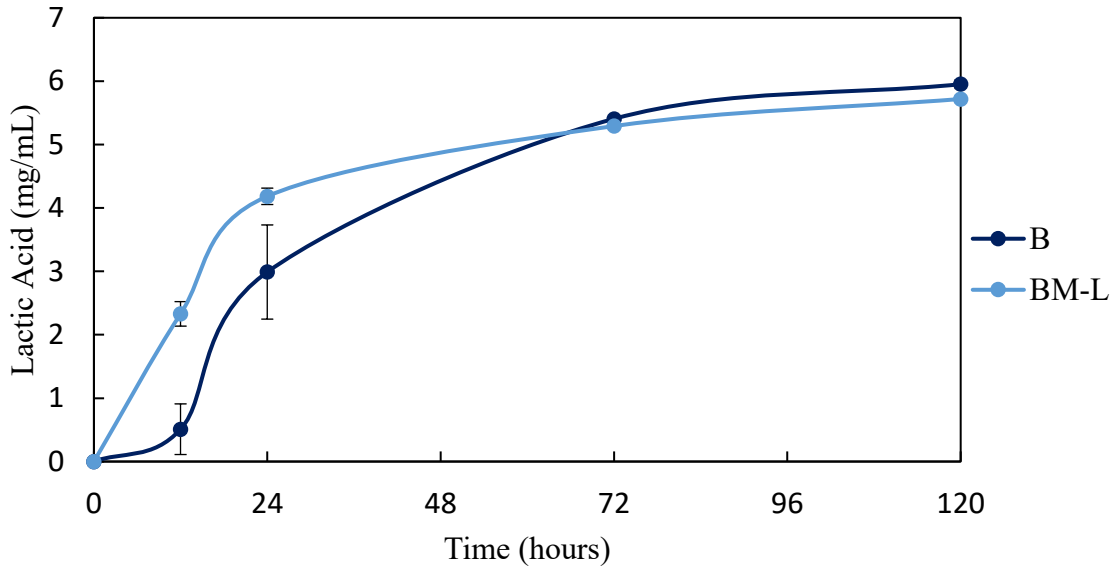


Figure 5.14: Change in lactic acid concentration (mg/mL) of liquid fractions over 120 hours with standard deviations represented by error bars for Phase 3 trials.

5.4.4 Degree of Demineralization and Decalcification

As shown in Figure 5.15, the degree of mineral removal shows a similar trend to lactic acid production (Figure 5.14). As lactic acid is produced, decalcification is occurring

at a similar rate. The same trend was also seen in the demineralization data. Decalcification and demineralization results are similar to those in Phase 2 showing the results were reproducible in a relative scale up condition. As per the trend seen with previously discussed results, the pre-treated mixture had a higher degree of mineral removal over the first 72 hours however the control reached the same degree of mineral removal by the final time point. The increased efficiency within the first 24 hours is likely due to the increased accessibility of calcium to the inoculum from shell degradation during pre-treatment. Based on the decalcification and demineralization results, the microwave pre-treatment did not decrease extraction time, nor did it significantly impact the degree of calcium removal.

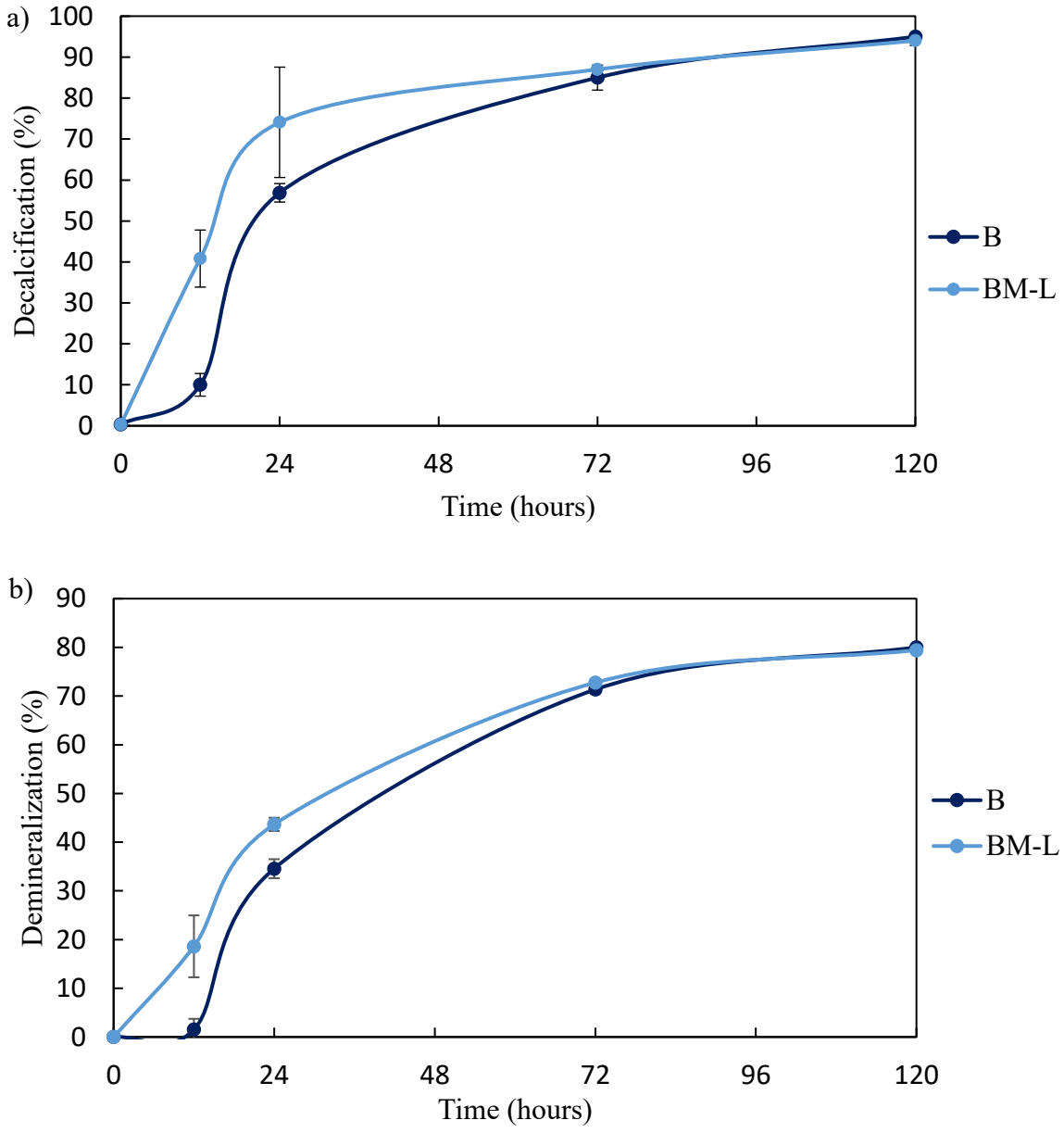


Figure 5.15: a) Degree of decalcification of insoluble fractions across 120 hours of fermentation with standard deviations represented as error bars for Phase 3 trials b) Degree of demineralization of insoluble fractions across 120 hours of fermentation with standard deviations represented by error bars for Phase 3 trials.

5.4.5 Degree of Deproteinization

As shown in Figure 5.16, deproteinization results are comparable between the two trials however the control (B) was slightly higher (72.7% ± 2.95%) compared to the pre-treated trial (BM-L) (69.3% ± 2.42%). High mineral content remaining in the insoluble fraction of samples taken in the early stages of fermentation (0-24 hours) led to nitrogen percentages of the insoluble fractions which were lower than the nitrogen content (%) of the insoluble fraction at the final time point. The low nitrogen contents (%) therefore skewed elemental analysis results and an accurate deproteinization could not be calculated over the time course of the fermentation. Compared to Phase 2 trials, there was a decrease in deproteinization for both B and BM-L in Phase 3 trials. This could be due to slightly different conditions with the bioreactor compared to the flask trials performed in previous phases such as slower stirring speed, heating method (water bath vs. hot plate) and running at a larger volume.

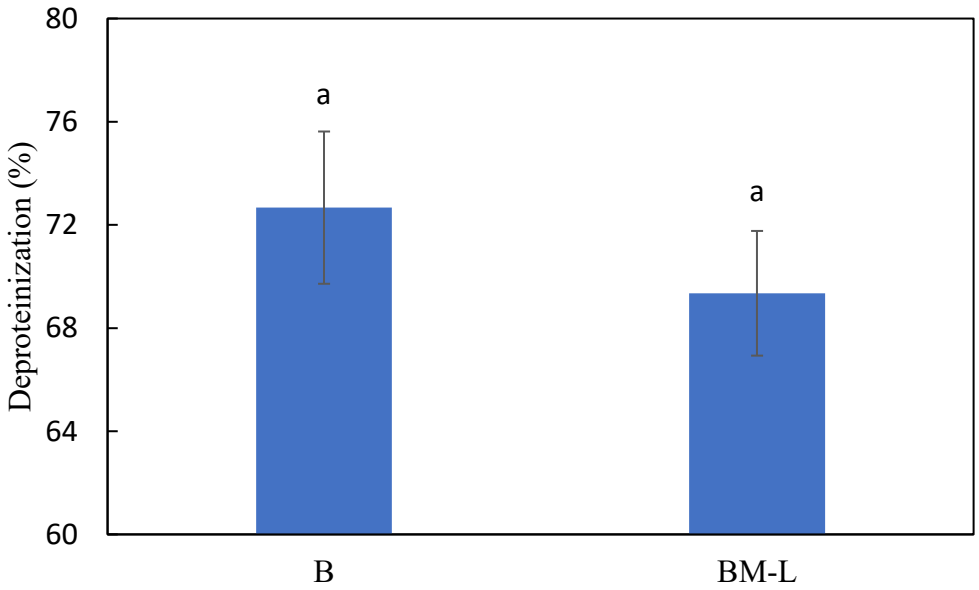


Figure 5.16: Degree of deproteinization (%) of insoluble fraction at 120 hours with standard deviations represented as error bars for Phase 3 trials. Data points with the same lettering show no significant difference based on the Tukey method.

Figure 5.17 shows the protein content of the liquid fraction based on results from elemental analysis. Although the concentration of protein is increasing over time in the liquid fraction, the data is not an accurate representation of the degree of protein removal from the shell or the rate at which deproteinization occurred. This is due to high amounts of calcium also being solubilized into the liquid fraction over time. Since the shell has a higher mineral content than protein content, calcium will also make up a higher percentage of the liquid fraction. This results in a low nitrogen content (%) for the liquid as it is based on the percentage of all liquid fraction constituents (minerals, pigments and proteins).

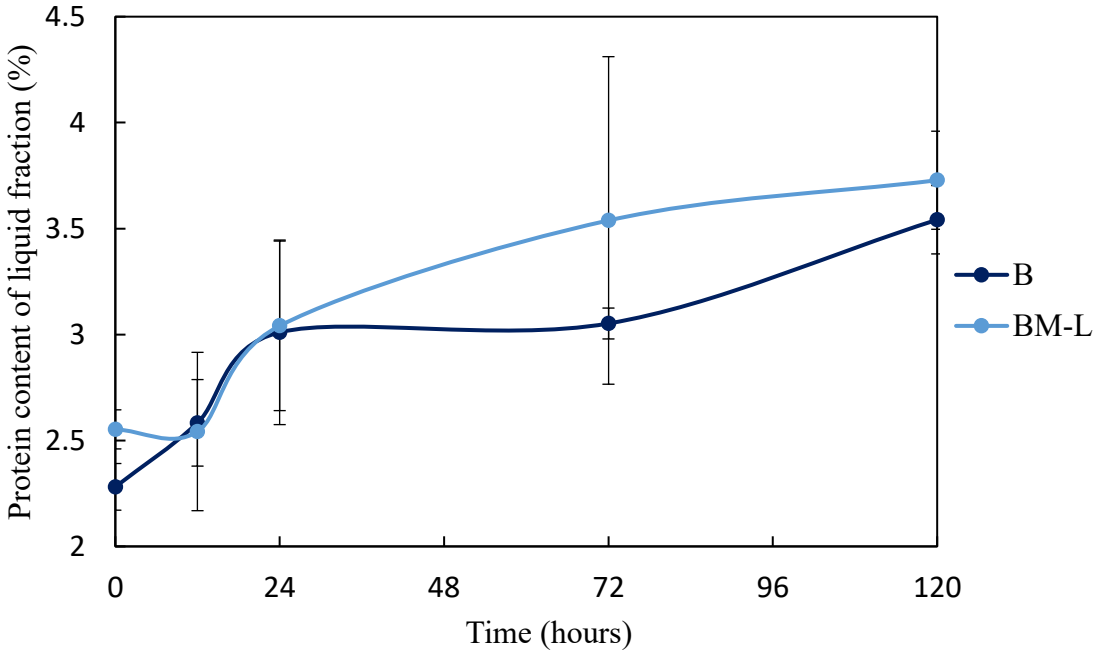


Figure 5.17: Change in solubilized protein content of liquid fractions (%) over 120 hours with standard deviations represented as error bars for Phase 3 trials.

Although it was initially hypothesized that the nitrogen content of the liquid fraction would only be attributed to solubilized protein from the shell, the initial nitrogen content was above zero. It is hypothesized that the initial nitrogen content could be attributed to soluble proteins in the lobster shell powder or proteins present in the lactobacillus strains

of the purchased inoculum. Lactobacillus have a bilayer lipid cell membrane with embedded proteins which contain nitrogen and therefore could increase the nitrogen content of the liquid fraction (Górska et al., 2016).

Interestingly, solubilized protein is the only response to which BM-L is not superior for the first 24 to 48 hours which correlates with the lower degree of deproteinization achieved compared to the control. However, after 24 hours BM-L has a slightly higher percentage of solubilized protein. This is a reasonable response as decalcification was slightly higher for B meaning that the liquid had a higher calcium concentration than BM-L and subsequently the percentage of nitrogen of the overall liquid would be lower. From Figure 5.17, we can see that there is an upward trend in the nitrogen content of the liquid over the course of the fermentation suggesting the solubilization of proteins occurred.

5.4.6 Lactic Acid Bacteria Growth

The estimation of bacteria growth as measured by colony count over 120 hours of fermentation (Figure 5.18) shows rapid growth within the first 12 to 24 hours followed by a steep decline in colonies which agrees with the kinetic modelling of lactic acid bacteria growth by Passos et al. (1994). The kinetic modelling by Passos et al. (2014) however showed a plateau after the steep decline where a second growth phase was seen after 72 hours in the current trials (Figure 5.18). As previously discussed, bacteria can initiate a stress response to regulate growth through metabolic pathways when they are in an environment with very high growth rates. Although it was expected that a high lactose ratio of 20% could be causing a stressful environment, the 5% lactose and 5% inoculum may also induce the stress response. If this were the case, the high growth rate within the first 12 to 24 hours could cause carbohydrate starvation under which lactic acid bacteria lose

the ability to form colonies leading to the dramatic drop in colony counts (Papadimitriou et al., 2016). Under carbohydrate starvation, lactic acid bacteria begin to catabolize amino acids as an alternative energy source (Papadimitriou et al., 2016; Pessione, Lamberti, & Pessione, 2010) which could be the cause of the second growth phase. Due to shell mineral and protein removal during fermentation, amino acids and proteins would be more available later on in the fermentation thus leadings to a quick spike in colony counts.

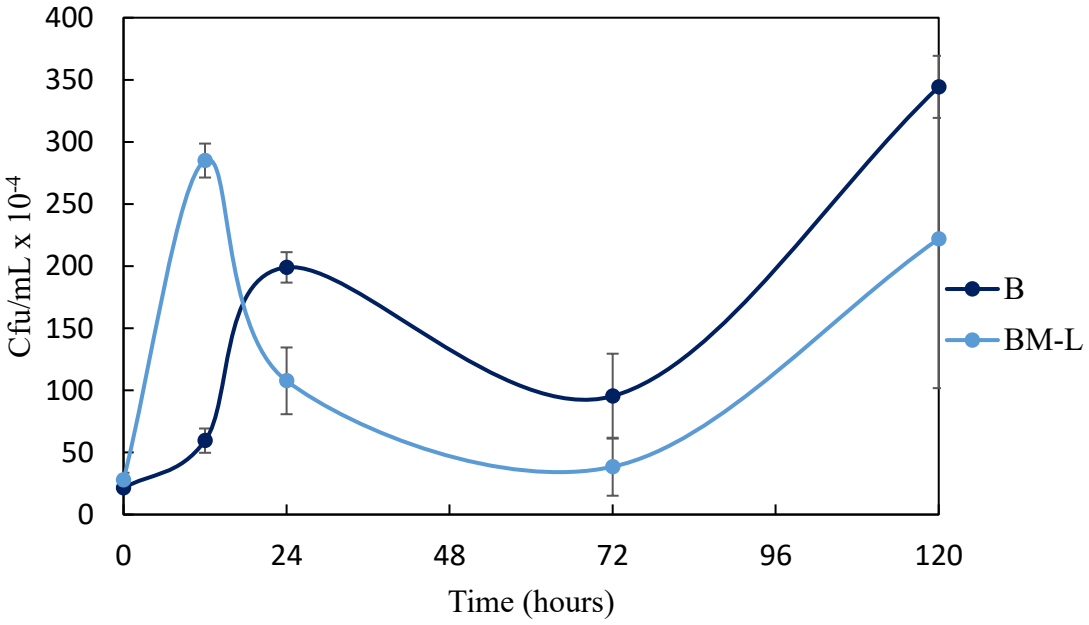


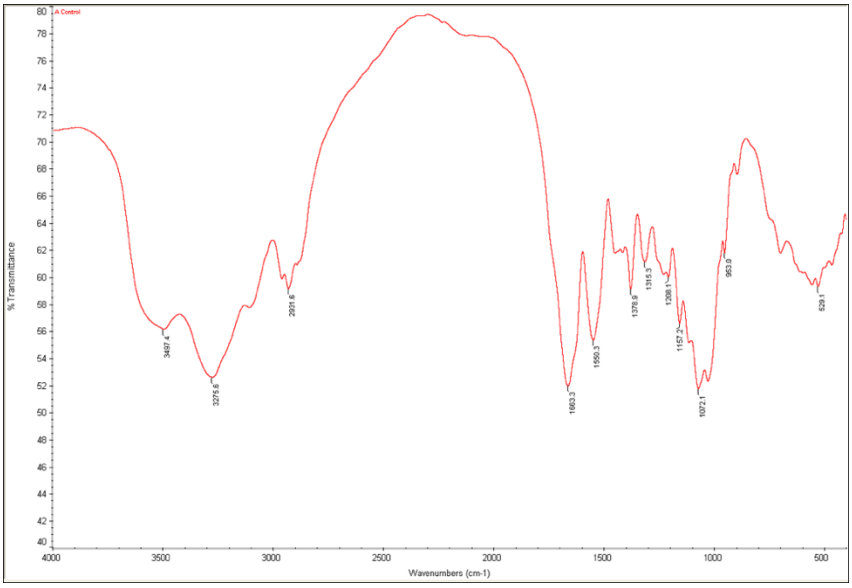
Figure 5.18: Bacteria growth as estimated by colony counts (cfu/mL x 10^{-4}) with standard deviations represented as error bars for Phase 3 trials.

5.4.7 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR is a common qualitative method used for characterizing biomolecules by determining functional groups present in the sample. An example of the FTIR spectra of the insoluble chitin product for the control (B) and pre-treated trials (BM-L) are shown in

Figure 5.19. All absorption bands in the FTIR spectra corresponding to characteristic functional groups of chitin are summarized in Table 5.11.

a)



b)

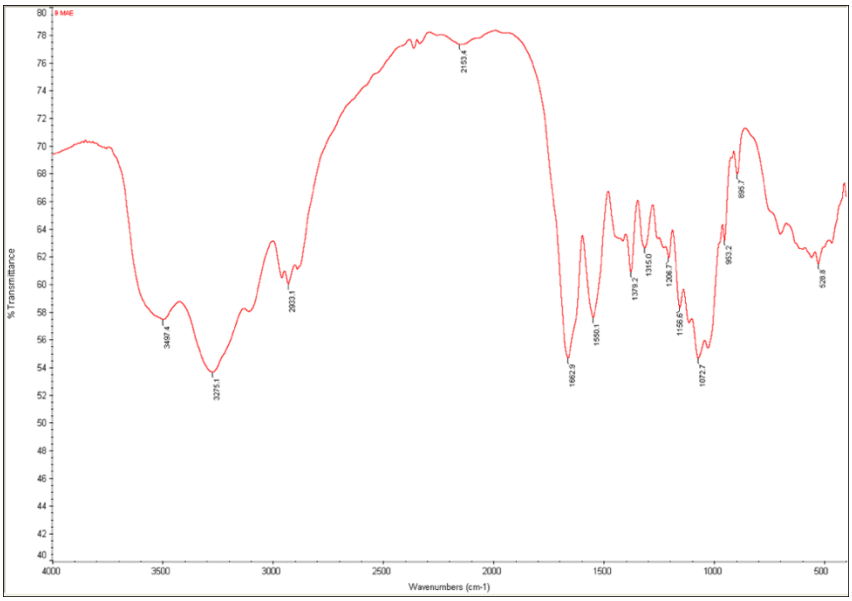


Figure 5.19: FTIR spectra of a) control b) MAE pre-treated final chitin product after 120 hours of fermentation for Phase 3 trials.

Table 5.11: Characteristic FTIR spectra bands of chitin

Band Assignment (Kaya et al., 2014)	**Wavelength (cm^{-1})**
OH Stretching	3497
NH stretching	3275
CH_3 sym. And CH_2 asym. stretching	2931
C=O secondary amide stretching (Amide I)	1663
NH bending and CN stretching (Amide II)	1557
CH bending and sym. CH_3 deformation	1379
CH_2 wagging (Amide III)	1315
Asym. bridge oxygen stretching	1157
C-O-C asym. stretching in phase ring	1072
C-O asym. in phase ring	1027
CH_3 wagging	953
CH ring stretching	895

Sym = symmetrical
Asym = asymmetrical

There are three significant spectra bands characteristic to α-chitin at 1652, 1620 and 1556 cm^{-1} (Kaya et al., 2014; Prabu & Natarajan, n.d.). The bands at 1652 cm^{-1} and 1620 cm^{-1} both correspond to the C=O secondary amide stretch (Amide I) as the functional group shows up as two peaks for α-chitin. The band at 1556 cm^{-1} corresponds to the N-H bend and C-N stretch (Amide II).

When analyzing the chitin product from both the fermentative extractions, the C=O secondary amide stretch showed only one peak at 1663cm^{-1} while the N-H bend and C-N stretch was shown at 1557cm^{-1}. A second distinct amide stretch was not seen at 1620 cm^{-1} however this is likely due to the presence of impurities in the chitin product such as residual proteins and minerals that were not removed in the fermentative extraction process. Another characteristic peak of α-chitin is CH ring stretching shown at 895cm^{-1} which was found in the spectra of the chitin product thus it can be concluded that α-chitin is present in the insoluble fermentation product.

In general, the FTIR spectra between the two formulations had only slight differences in the presence of absorption bands concluding that chitin was present in the insoluble product. The slight variations between spectra are likely due to the presence of impurities that were not removed in the fermentative extraction process or during the weak acid wash.

Chapter 6: Conclusions and Future Work

The overall goal of this study was to develop an environmentally and economically sustainable chitin extraction method from lobster shell waste using lactic acid fermentation. Through this research the following conclusions can be made:

1. A factorial design testing the influence of three factors - shell, lactose and inoculum on the responses of demineralization and deproteinization showed that only 5% shell trials had significant relationships between factors and responses compared to 20% shell. Statistical analysis of the results from 5% shell trials determined that inoculum and its interaction with lactose plays a significant role in the demineralization efficiency while only inoculum is a significant factor for deproteinization efficiency.
2. MAE and UAE pre-treatments did not significantly increase mineral or protein removal under the present experimental conditions regardless of whether lactose was included in the pre-treated mixture.
3. Levels of demineralization and deproteinization achieved in the present study are comparable to studies found in the literature using other crustacean shell wastes. Formulation B (5% shell:5% lactose: 5% inoculum) without the use of a pre-treatment was the most efficient formulation of the tested formulations achieving 72.7% demineralization and 95% decalcification at a volume of 1L at 37℃ with constant stirring (200 rpm) for 120 hours.
4. Chitin was present in the insoluble fraction of the fermentation product as characterized by FTIR.

Lactic acid fermentation has potential as a sustainable chitin extraction method however future development and optimization of the process developed in the current study is required. The trends seen in the bacteria colony growth indicate that the lactobacilli may be stressed under the tested conditions of 5% shell, 5% lactose an 5% inoculum. Optimizing bacteria growth by adjusting lactose and inoculum ratios could increase process efficiency as there is potential for improved mineral and protein removal by the process.

Despite a 5% shell ratio being significantly more effective in the tested fermentations than 20% shell, the shell ratio is another important factor for future work. Using a low ratio of 5% shell is unlikely to be cost-effective for the process as available chitin is approximately 20% by dry weight of the shell (Bolat et al., 2010; Nguyen et al., 2017). Therefore, it is desirable to maximize shell ratio in the fermentation in order to maximize the amount of chitin produced per fermentation.

Although pre-treatments did not show any significant impact on process efficiency under the conditions tested in the present study, future work assessing the impact of pre-treatments at varying temperatures and durations may yield different results. Once the factors of the lactic acid fermentation process are optimized to achieve the highest degree of mineral and protein removal in a cost-effective way, the process could be scaled up for use as an alternative method for chemical chitin extraction from lobster shell waste on an industrial scale.

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Appendix

Table A 1: Mean elemental analysis results for insoluble fractions in Phase 1

Formulation	Carbon (%)	Hydrogen (%)	Nitrogen (%)
A	42.85	5.86	9.69
B	44.07	6.19	8.37
C	44.27	5.96	9.56
D	44.02	6.03	8.28
E	37.71	5.06	8.19
F	45.39	6.19	8.98

Table A 2: Mean elemental analysis results for insoluble fractions in Phase 2

Formulation	Carbon (%)	Hydrogen (%)	Nitrogen (%)
B	41.98	6.28	7.16
BM+L	42.09	6.27	7.53
BM-L	44.28	6.72	8.18
BU+L	43.89	6.43	7.99
BU-L	43.87	6.58	8.13
C	41.38	6.07	7.86
CM+L	42.09	6.27	7.53
CM-L	44.69	6.63	8.22
CU+L	37.76	5.52	6.94
CU-L	43.78	6.50	8.19

Table A 3: Mean elemental analysis results of insoluble fractions in Phase 3

Formulation	Carbon (%)	Hydrogen (%)	Nitrogen (%)
B	42.92	6.72	7.38
BM-L	42.45	6.64	7.30

Table A 4: Mean elemental analysis data of liquid fractions in Phase 3

Formulation	Time (hours)	Carbon (%)	Hydrogen (%)	Nitrogen (%)
B	0	39.16	6.84	0.37
B	12	38.00	6.74	0.41
B	24	36.49	6.64	0.48
B	72	34.15	6.64	0.49
B	120	34.00	6.59	0.57
BM+L	0	38.68	6.94	0.41
BM+L	12	37.05	6.86	0.41
BM+L	24	33.85	6.63	0.49
BM+L	72	32.44	6.72	0.57
BM+L	120	34.30	6.49	0.60

Factorial Regression: Demineralization versus Lactose, Inoculum

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	1677.1	559.02	17.89	0.009
Linear	2	929.9	464.97	14.88	0.014
Lactose	1	155.1	155.14	4.96	0.090
Inoculum	1	774.8	774.80	24.79	0.008
2-Way Interactions	1	747.1	747.10	23.91	0.008
Lactose*Inoculum	1	747.1	747.10	23.91	0.008
Error	4	125.0	31.25		
Total	7	1802.0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.59014	93.06%	87.86%	72.25%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		62.55	1.98	31.65	0.000	
Lactose	8.81	4.40	1.98	2.23	0.090	1.00
Inoculum	19.68	9.84	1.98	4.98	0.008	1.00
Lactose*Inoculum	-19.33	-9.66	1.98	-4.89	0.008	1.00

Regression Equation in Uncoded Units

Demineralization = 16.29 + 2.520 Lactose + 12.97 Inoculum - 0.644 Lactose*Inoculum

Alias Structure

Factor	Name
A	Lactose
B	Inoculum

Aliases
I
A
B
AB

Effects Pareto for Demineralization

Figure A 1: Factorial regression from Minitab testing the factors of lactose and inoculum against the response of demineralization based on Phase 1 data

Factorial Regression: Deproteinization versus Lactose, Inoculum

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	466.323	155.441	3.29	0.140
Linear	2	466.228	233.114	4.93	0.083
Lactose	1	67.338	67.338	1.43	0.298
Inoculum	1	398.890	398.890	8.44	0.044
2-Way Interactions	1	0.095	0.095	0.00	0.966
Lactose*Inoculum	1	0.095	0.095	0.00	0.966
Error	4	188.952	47.238		
Total	7	655.275			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
6.87299	71.16%	49.54%	0.00%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		66.18	2.43	27.23	0.000	
Lactose	5.80	2.90	2.43	1.19	0.298	1.00
Inoculum	14.12	7.06	2.43	2.91	0.044	1.00
Lactose*Inoculum	-0.22	-0.11	2.43	-0.04	0.966	1.00

Regression Equation in Uncoded Units

Deproteinization = 50.48 + 0.409 Lactose + 3.62 Inoculum - 0.007 Lactose*Inoculum

Alias Structure

Factor	Name
A	Lactose
B	Inoculum

Aliases
I
A
B
AB

Figure A 2: Factorial regression from Minitab testing the factors of lactose and inoculum against the response of deproteinization based on Phase 1 data

One-way ANOVA: Soluble Calcium versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	18.035	2.0039	8.16	0.000
Error	30	7.369	0.2456		
Total	39	25.404			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.495607	70.99%	62.29%	48.43%

Means

Formulation	N	Mean	StDev	95% CI
B	4	6.703	0.853	(6.197, 7.209)
BM-L	4	6.976	0.840	(6.469, 7.482)
BM+L	4	6.948	0.276	(6.442, 7.454)
BU-L	4	6.5285	0.1680	(6.0224, 7.0346)
BU+L	4	6.8060	0.1703	(6.2999, 7.3121)
C	4	5.137	0.719	(4.631, 5.643)
CM-L	4	6.429	0.444	(5.923, 6.935)
CM+L	4	6.0950	0.1565	(5.5889, 6.6011)
CU-L	4	5.442	0.362	(4.936, 5.948)
CU+L	4	5.2713	0.1423	(4.7652, 5.7773)

Pooled StDev = 0.495607

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping			
BM-L	4	6.976	A			
BM+L	4	6.948	A			
BU+L	4	6.8060	A			
B	4	6.703	A			
BU-L	4	6.5285	A	B		
CM-L	4	6.429	A	B	C	
CM+L	4	6.0950	A	B	C	D
CU-L	4	5.442		B	C	D
CU+L	4	5.2713			C	D
C	4	5.137				D

Means that do not share a letter are significantly different.

Figure A 3: ANOVA and Tukey test from Minitab testing solubilized calcium against formulation based on Phase 2 data

One-way ANOVA: Chitin Yield versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	92.95	10.328	1.13	0.423
Error	10	91.50	9.150		
Total	19	184.45			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.02490	50.39%	5.75%	0.00%

Means

Formulation	N	Mean	StDev	95% CI
B	2	28.750	0.354	(23.984, 33.516)
BM-L	2	31.750	0.354	(26.984, 36.516)
BM+L	2	32.25	3.89	(27.48, 37.02)
BU-L	2	32.750	1.061	(27.984, 37.516)
BU+L	2	31.000	0.707	(26.234, 35.766)
C	2	34.25	4.60	(29.48, 39.02)
CM-L	2	31.25	3.18	(26.48, 36.02)
CM+L	2	37.25	3.18	(32.48, 42.02)
CU-L	2	34.00	4.24	(29.23, 38.77)
CU+L	2	32.25	3.89	(27.48, 37.02)

Pooled StDev = 3.02490

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping
CM+L	2	37.25	A
C	2	34.25	A
CU-L	2	34.00	A
BU-L	2	32.750	A
CU+L	2	32.25	A
BM+L	2	32.25	A
BM-L	2	31.750	A
CM-L	2	31.25	A
BU+L	2	31.000	A
B	2	28.750	A

Means that do not share a letter are significantly different.

Figure A 4: ANOVA and Tukey test from Minitab testing chitin yield against formulation based on Phase 2 data

One-way ANOVA: Lactose versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	1010.46	112.273	78.23	0.000
Error	10	14.35	1.435		
Total	19	1024.81			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.19797	98.60%	97.34%	94.40%

Means

Formulation	N	Mean	StDev	95% CI
B	2	10.575	0.332	(8.688, 12.462)
BM-L	2	8.660	0.339	(6.773, 10.547)
BM+L	2	7.870	0.481	(5.983, 9.757)
BU-L	2	8.4700	0.0707	(6.5826, 10.3574)
BU+L	2	7.870	0.170	(5.983, 9.757)
C	2	24.56	2.18	(22.67, 26.45)
CM-L	2	23.425	0.813	(21.538, 25.312)
CM+L	2	19.62	1.53	(17.73, 21.51)
CU-L	2	24.46	1.64	(22.57, 26.35)
CU+L	2	20.63	1.85	(18.74, 22.52)

Pooled StDev = 1.19797

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping		
C	2	24.56	A		
CU-L	2	24.46	A		
CM-L	2	23.425	A	B	
CU+L	2	20.63	A	B	
CM+L	2	19.62		B	
B	2	10.575			C
BM-L	2	8.660			C
BU-L	2	8.4700			C
BM+L	2	7.870			C
BU+L	2	7.870			C

Means that do not share a letter are significantly different.

Figure A 5: ANOVA and Tukey test from Minitab testing lactose consumption against formulation based on Phase 2 data

One-way ANOVA: Lactic Acid versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	43.413	4.8236	25.89	0.000
Error	10	1.863	0.1863		
Total	19	45.276			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.431671	95.88%	92.18%	83.54%

Means

Formulation	N	Mean	StDev	95% CI
B	2	4.440	0.000	(3.760, 5.120)
BM-L	2	4.400	0.000	(3.720, 5.080)
BM+L	2	3.9800	0.0424	(3.2999, 4.6601)
BU-L	2	4.0850	0.0778	(3.4049, 4.7651)
BU+L	2	4.2100	0.0424	(3.5299, 4.8901)
C	2	1.3150	0.0495	(0.6349, 1.9951)
CM-L	2	1.850	0.990	(1.170, 2.530)
CM+L	2	1.450	0.509	(0.770, 2.130)
CU-L	2	1.1050	0.1344	(0.4249, 1.7851)
CU+L	2	0.895	0.771	(0.215, 1.575)

Pooled StDev = 0.431671

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping	
B	2	4.440	A	
BM-L	2	4.400	A	
BU+L	2	4.2100	A	
BU-L	2	4.0850	A	
BM+L	2	3.9800	A	
CM-L	2	1.850		B
CM+L	2	1.450		B
C	2	1.3150		B
CU-L	2	1.1050		B
CU+L	2	0.895		B

Means that do not share a letter are significantly different.

Figure A 6: ANOVA and Tukey test from Minitab testing lactic acid concentration against formulation based on Phase 2 data

One-way ANOVA: Demineralization versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	2425	269.44	3.08	0.010
Error	30	2624	87.48		
Total	39	5049			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
9.35316	48.02%	32.43%	7.60%

Means

Formulation	N	Mean	StDev	95% CI
B	4	81.608	1.156	(72.057, 91.158)
BM-L	4	81.293	1.415	(71.742, 90.843)
BM+L	4	76.34	5.16	(66.79, 85.90)
BU-L	4	81.2975	0.0650	(71.7467, 90.8483)
BU+L	4	81.043	1.850	(71.492, 90.593)
C	4	65.80	11.83	(56.25, 75.35)
CM-L	4	80.93	2.15	(71.38, 90.48)
CM+L	4	68.95	18.53	(59.40, 78.50)
CU-L	4	64.92	18.09	(55.37, 74.47)
CU+L	4	61.17	5.09	(51.61, 70.72)

Pooled StDev = 9.35316

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping
B	4	81.608	A
BU-L	4	81.2975	A
BM-L	4	81.293	A
BU+L	4	81.043	A
CM-L	4	80.93	A
BM+L	4	76.34	A
CM+L	4	68.95	A
C	4	65.80	A
CU-L	4	64.92	A
CU+L	4	61.17	A

Means that do not share a letter are significantly different.

Figure A 7: ANOVA and Tukey test from Minitab testing demineralization against formulation based on Phase 2 data

One-way ANOVA: Decalcification versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	1795	199.41	5.31	0.000
Error	30	1127	37.55		
Total	39	2921			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
6.12811	61.43%	49.86%	31.44%

Means

Formulation	N	Mean	StDev	95% CI
B	4	93.54	2.17	(87.28, 99.80)
BM-L	4	95.524	0.639	(89.266, 101.782)
BM+L	4	94.475	1.058	(88.218, 100.733)
BU-L	4	92.77	7.76	(86.51, 99.02)
BU+L	4	94.14	3.18	(87.88, 100.40)
C	4	76.16	12.27	(69.90, 82.41)
CM-L	4	91.16	3.98	(84.90, 97.41)
CM+L	4	89.03	7.08	(82.77, 95.29)
CU-L	4	86.03	8.14	(79.78, 92.29)
CU+L	4	77.32	4.05	(71.06, 83.58)

Pooled StDev = 6.12811

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping		
BM-L	4	95.524	A		
BM+L	4	94.475	A		
BU+L	4	94.14	A		
B	4	93.54	A		
BU-L	4	92.77	A		
CM-L	4	91.16	A	B	
CM+L	4	89.03	A	B	C
CU-L	4	86.03	A	B	C
CU+L	4	77.32		B	C
C	4	76.16			C

Means that do not share a letter are significantly different.

Figure A 8: ANOVA and Tukey test from Minitab testing decalcification against formulation based on Phase 2 data

One-way ANOVA: Deproteinization versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	10	B, BM-L, BM+L, BU-L, BU+L, C, CM-L, CM+L, CU-L, CU+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	9	1244	138.26	5.69	0.000
Error	50	1216	24.31		
Total	59	2460			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.93088	50.58%	41.69%	28.84%

Means

Formulation	N	Mean	StDev	95% CI
B	6	83.58	4.57	(79.54, 87.62)
BM-L	6	74.64	5.12	(70.59, 78.68)
BM+L	6	70.47	2.57	(66.43, 74.51)
BU-L	6	68.945	0.990	(64.902, 72.988)
BU+L	6	73.365	1.052	(69.322, 77.408)
C	6	68.66	6.17	(64.61, 72.70)
CM-L	6	71.30	2.64	(67.26, 75.34)
CM+L	6	70.51	6.07	(66.47, 74.55)
CU-L	6	65.80	8.65	(61.75, 69.84)
CU+L	6	71.18	5.53	(67.14, 75.23)

Pooled StDev = 4.93088

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping	
B	6	83.58	A	
BM-L	6	74.64	A	B
BU+L	6	73.365		B
CM-L	6	71.30		B
CU+L	6	71.18		B
CM+L	6	70.51		B
BM+L	6	70.47		B
BU-L	6	68.945		B
C	6	68.66		B
CU-L	6	65.80		B

Means that do not share a letter are significantly different.

Figure A 9: ANOVA and Tukey test from Minitab testing deproteinization against formulation based on Phase 2 data

One-way ANOVA: Deproteinization versus Formulation

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Formulation	2	B, BM+L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Formulation	1	33.08	33.082	4.24	0.067
Error	10	78.04	7.804		
Total	11	111.12			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.79350	29.77%	22.75%	0.00%

Means

Formulation	N	Mean	StDev	95% CI
B	6	72.67	2.58	(70.13, 75.21)
BM+L	6	69.35	2.99	(66.81, 71.89)

Pooled StDev = 2.79350

Tukey Pairwise Comparisons

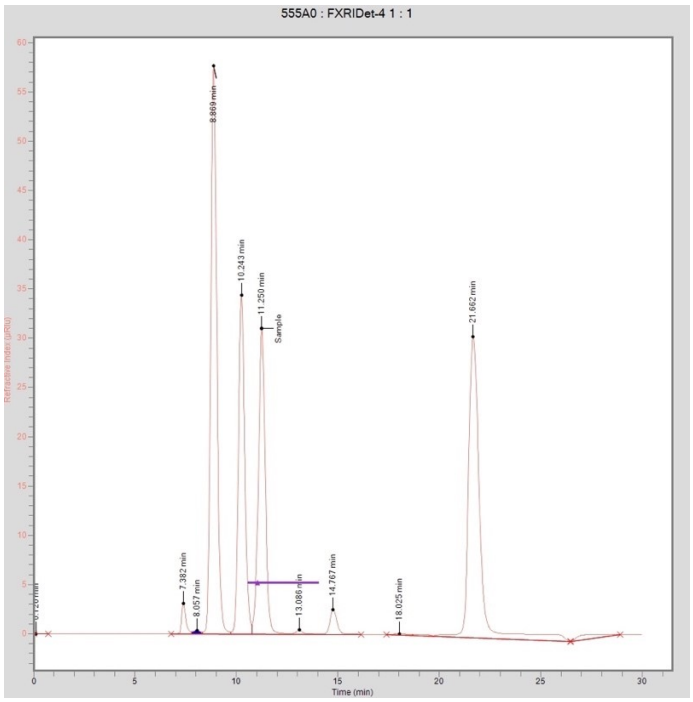
Grouping Information Using the Tukey Method and 95% Confidence

Formulation	N	Mean	Grouping
B	6	72.67	A
BM+L	6	69.35	A

Means that do not share a letter are significantly different.

Figure A 10: ANOVA and Tukey test from Minitab testing deproteinization against formulation based on Phase 3 data

a)



b)

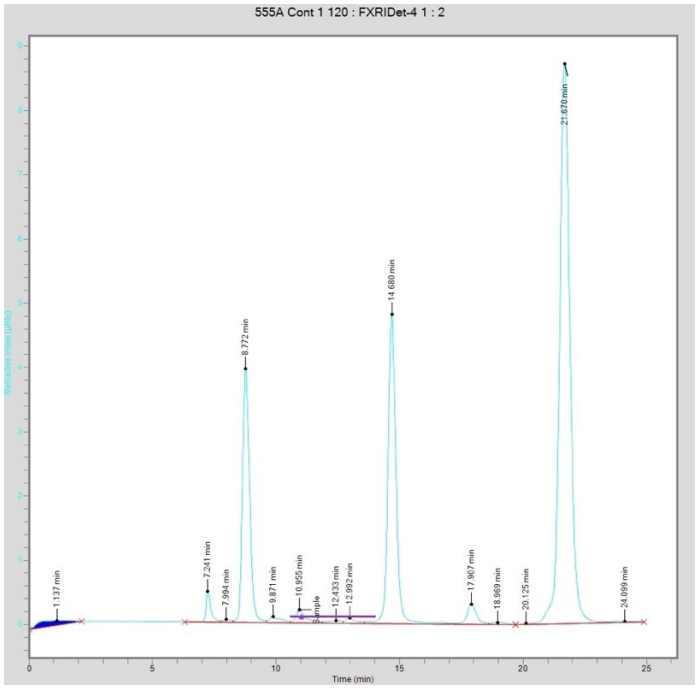
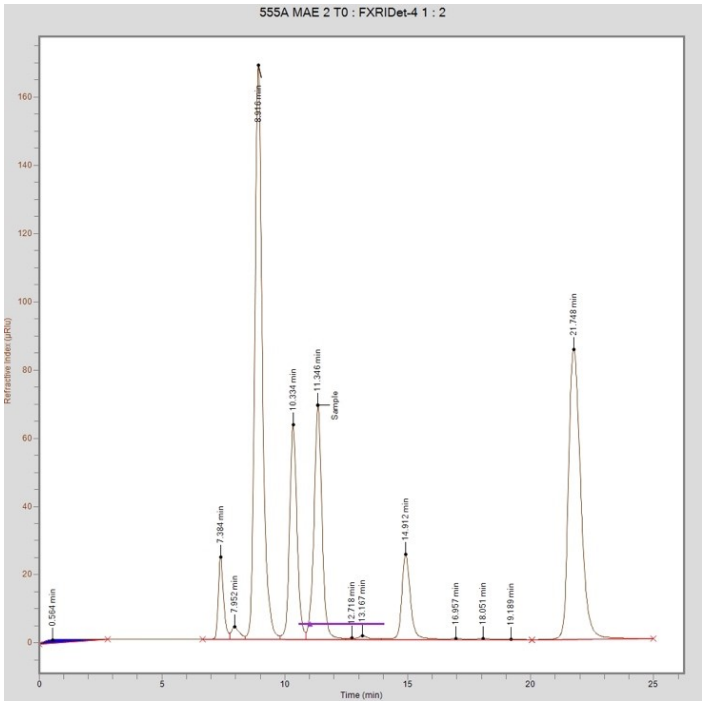


Figure A 11: B Lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



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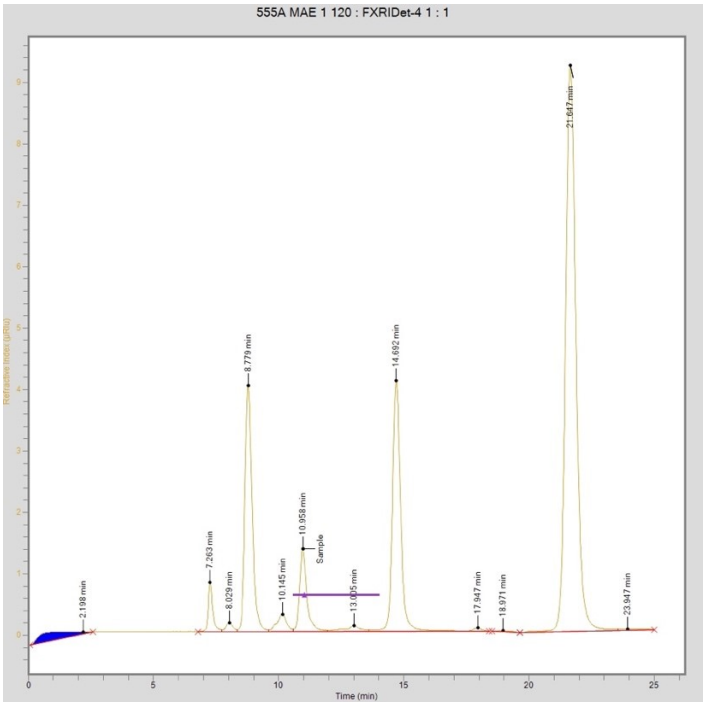
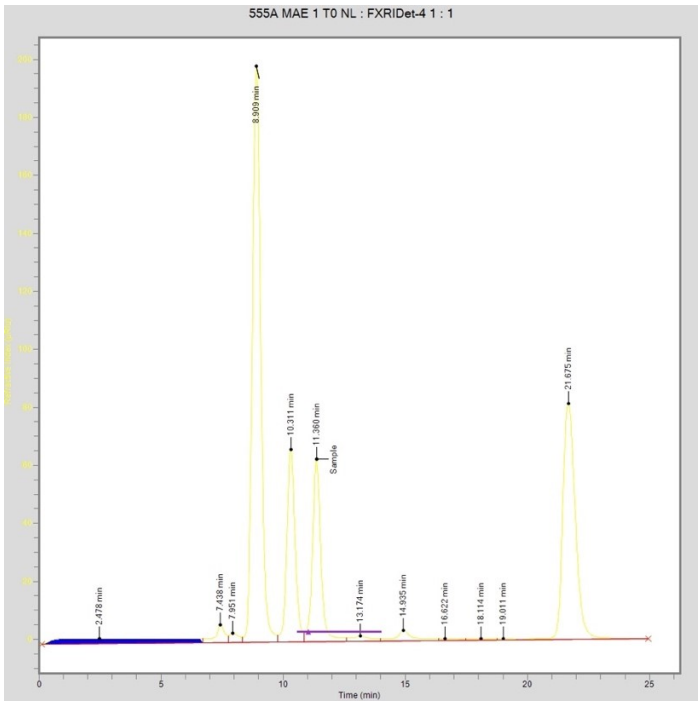


Figure A 12: BM+L lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



b)

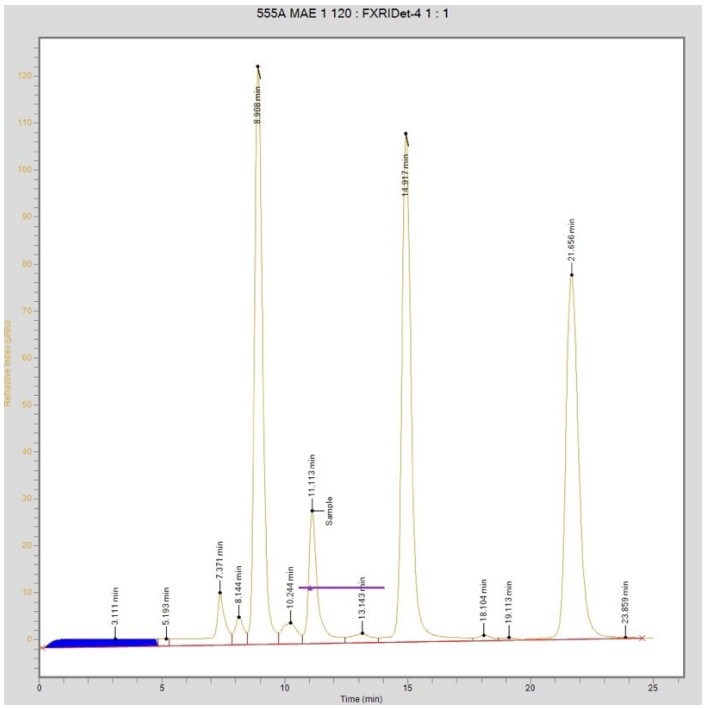
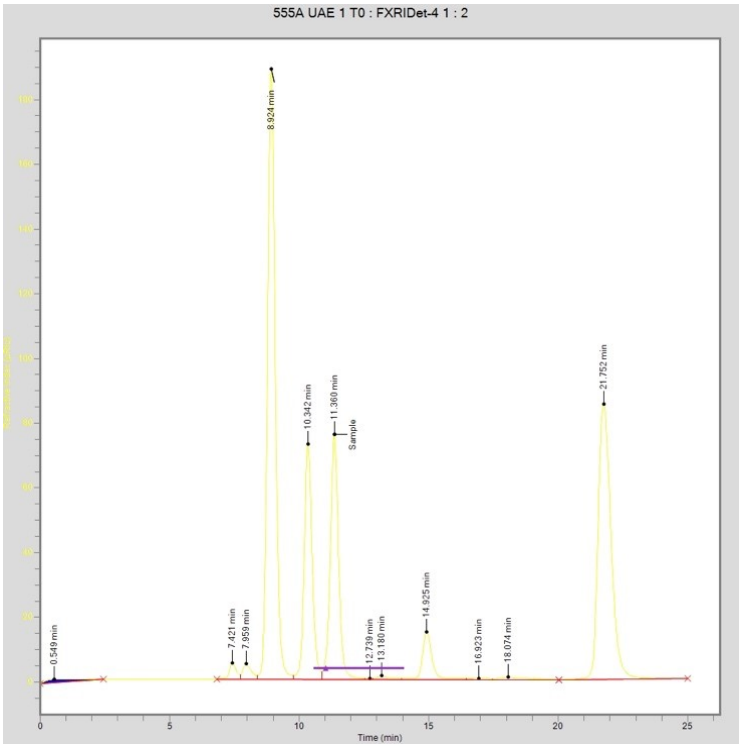


Figure A 13: BM-L Lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



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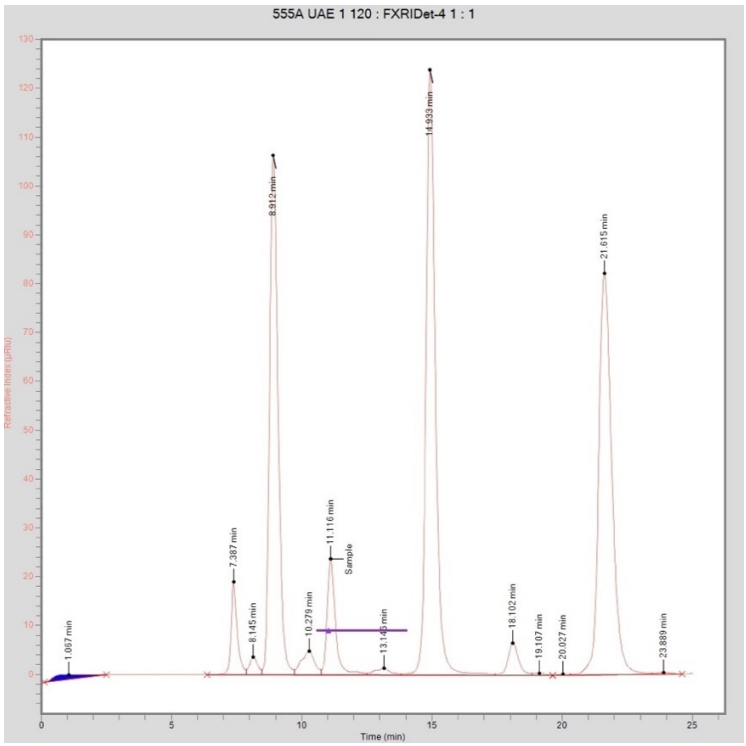
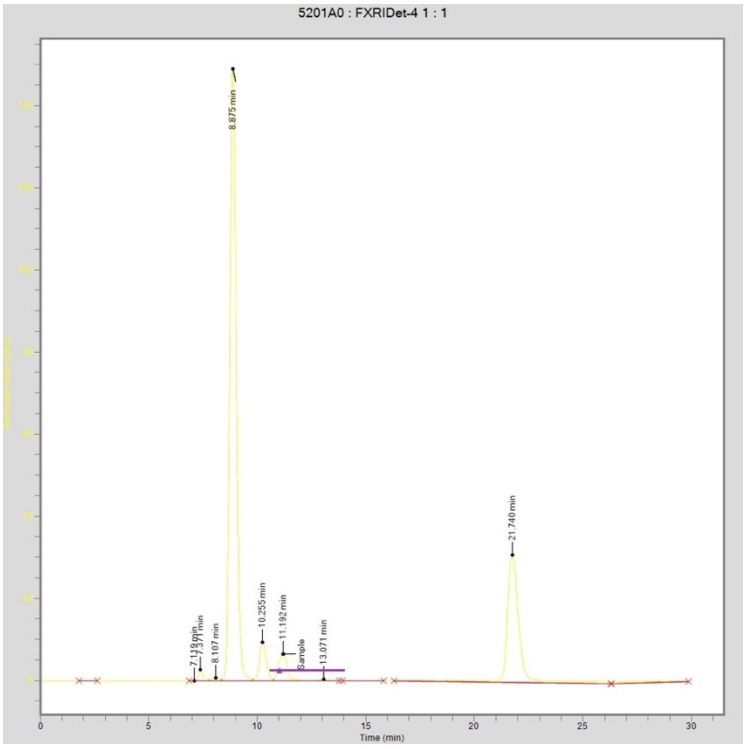


Figure A 14: BU+L lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



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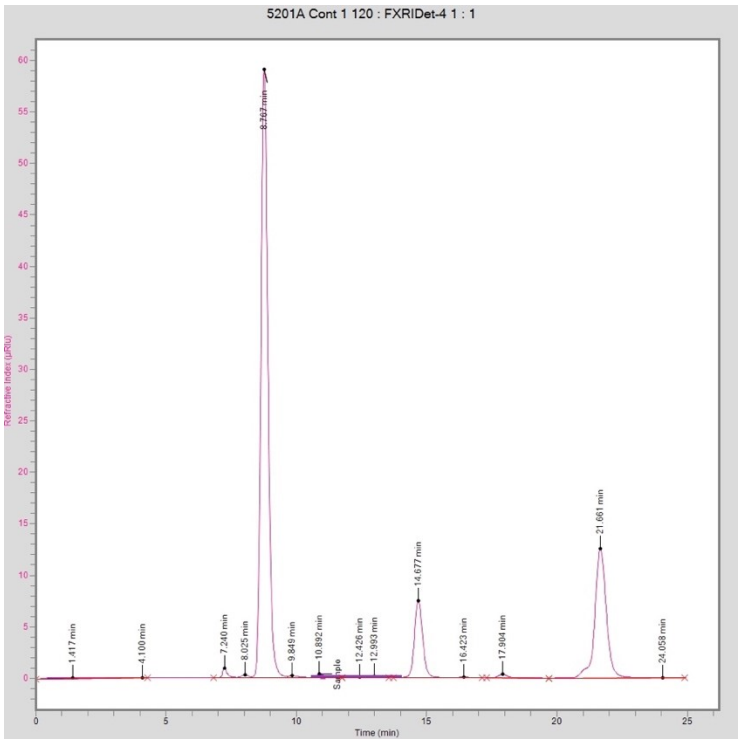
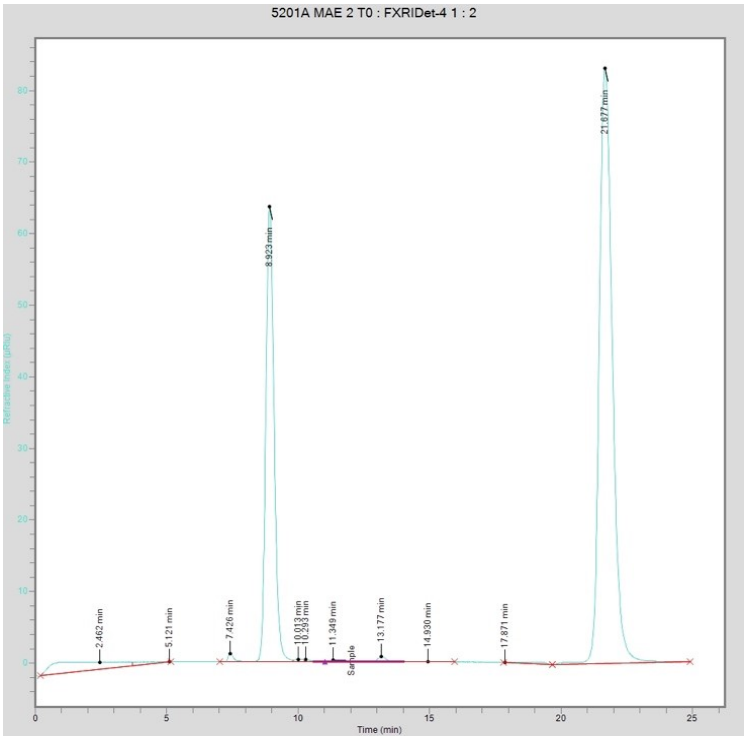


Figure A 15: C Lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



b)

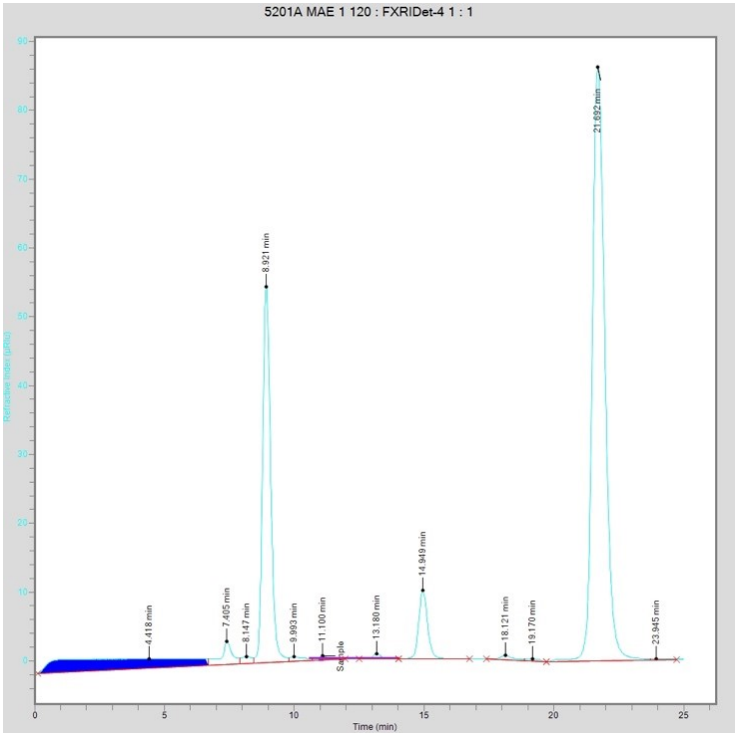
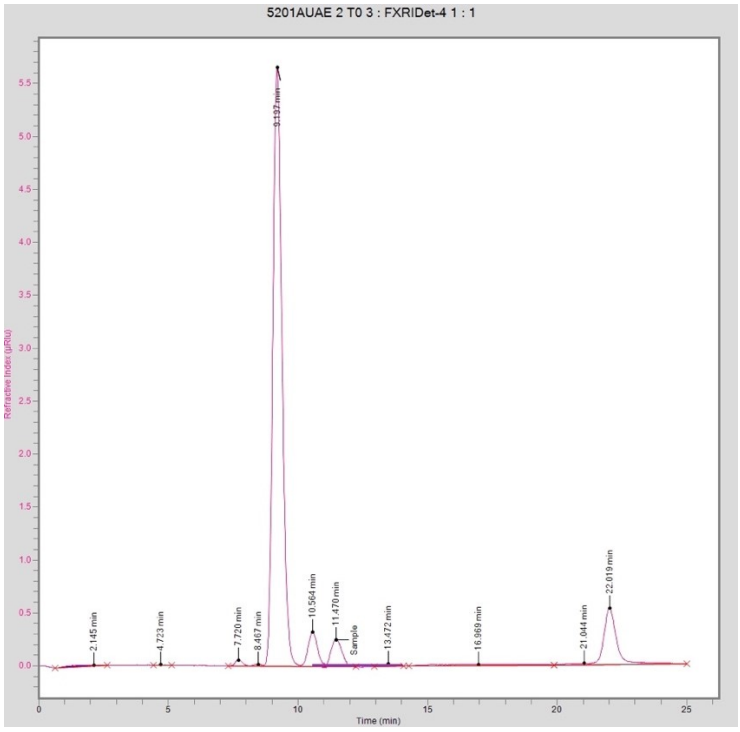


Figure A 16: CM-L lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

a)



b)

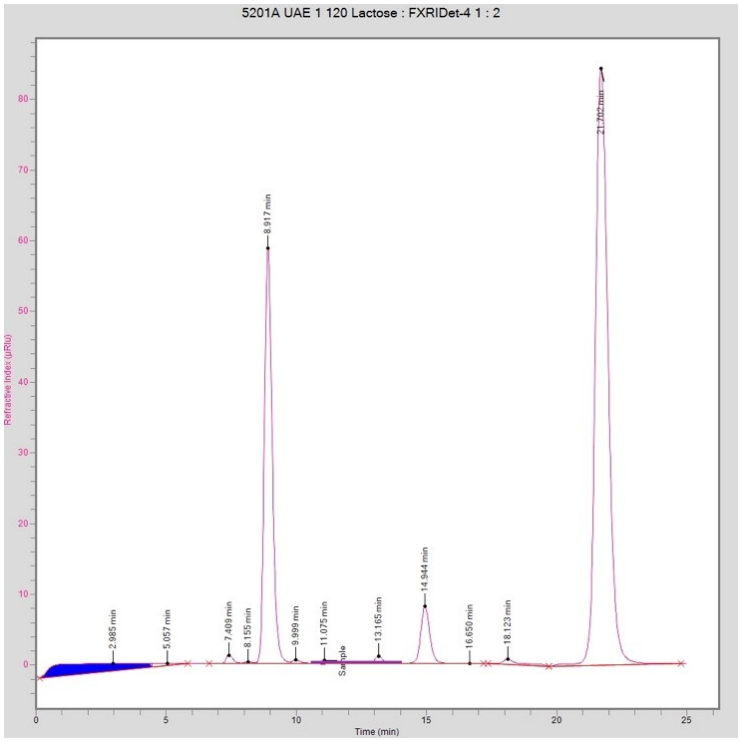
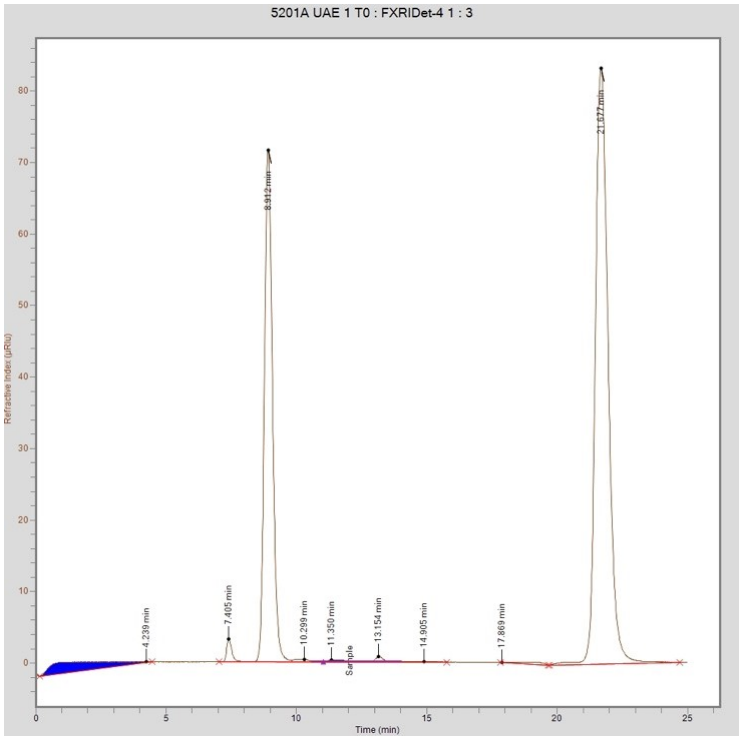


Figure A 17: Lactose and Lactic acid chromatogram at CU+L a) 0 hours b) 120 hours

a)



b)

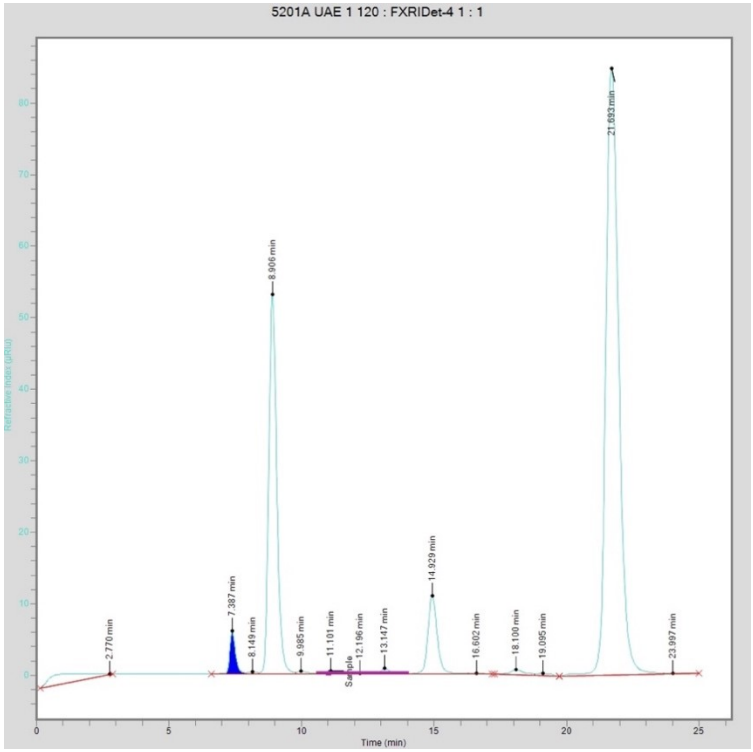


Figure A 18: CU-L lactose and lactic acid chromatogram at a) 0 hours b) 120 hours

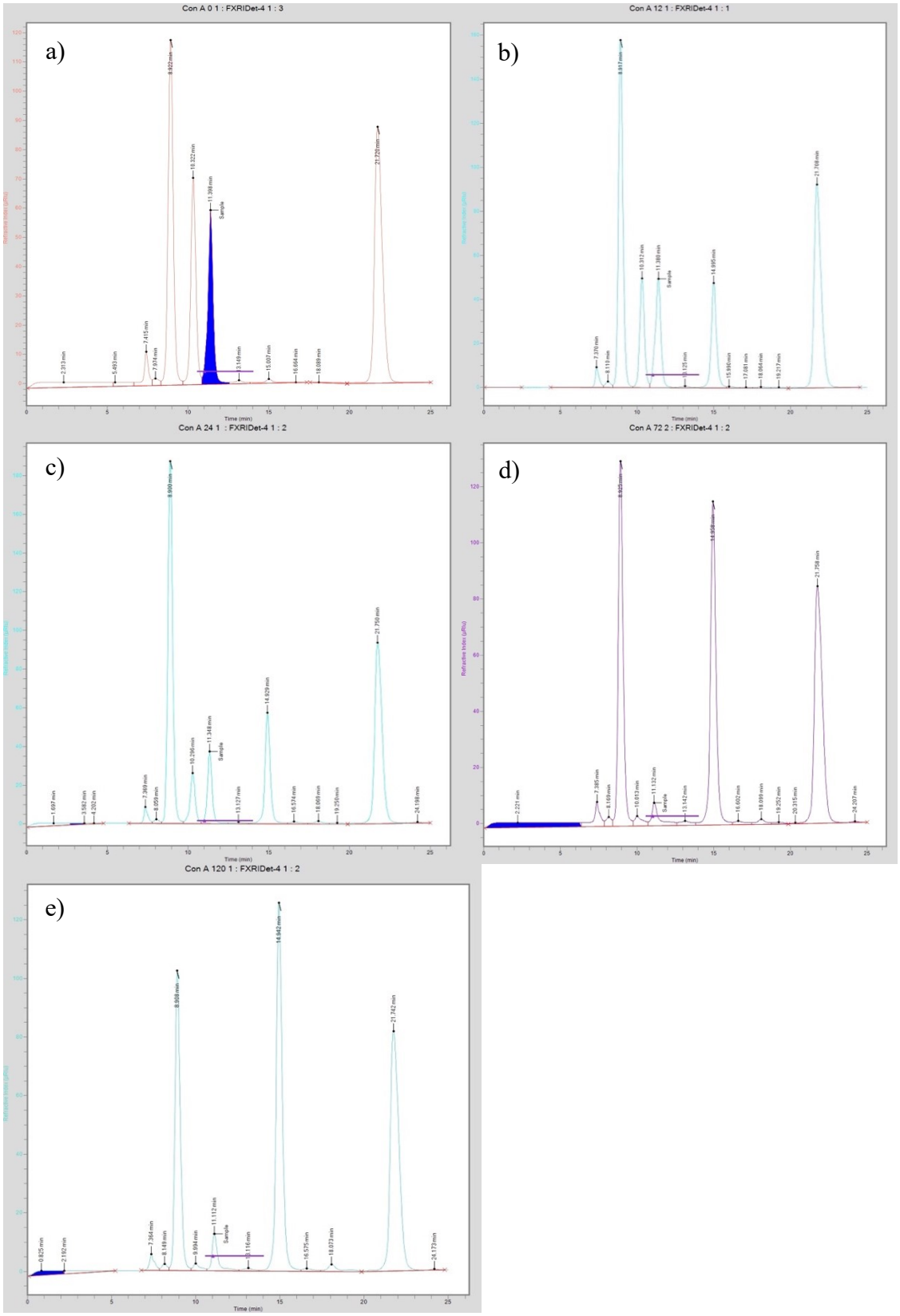


Figure A 19: Phase 3 trials HPLC chromatograms for B a) Time 0 b) 12 hours c) 24 hours d) 72 hours e) 120 hours

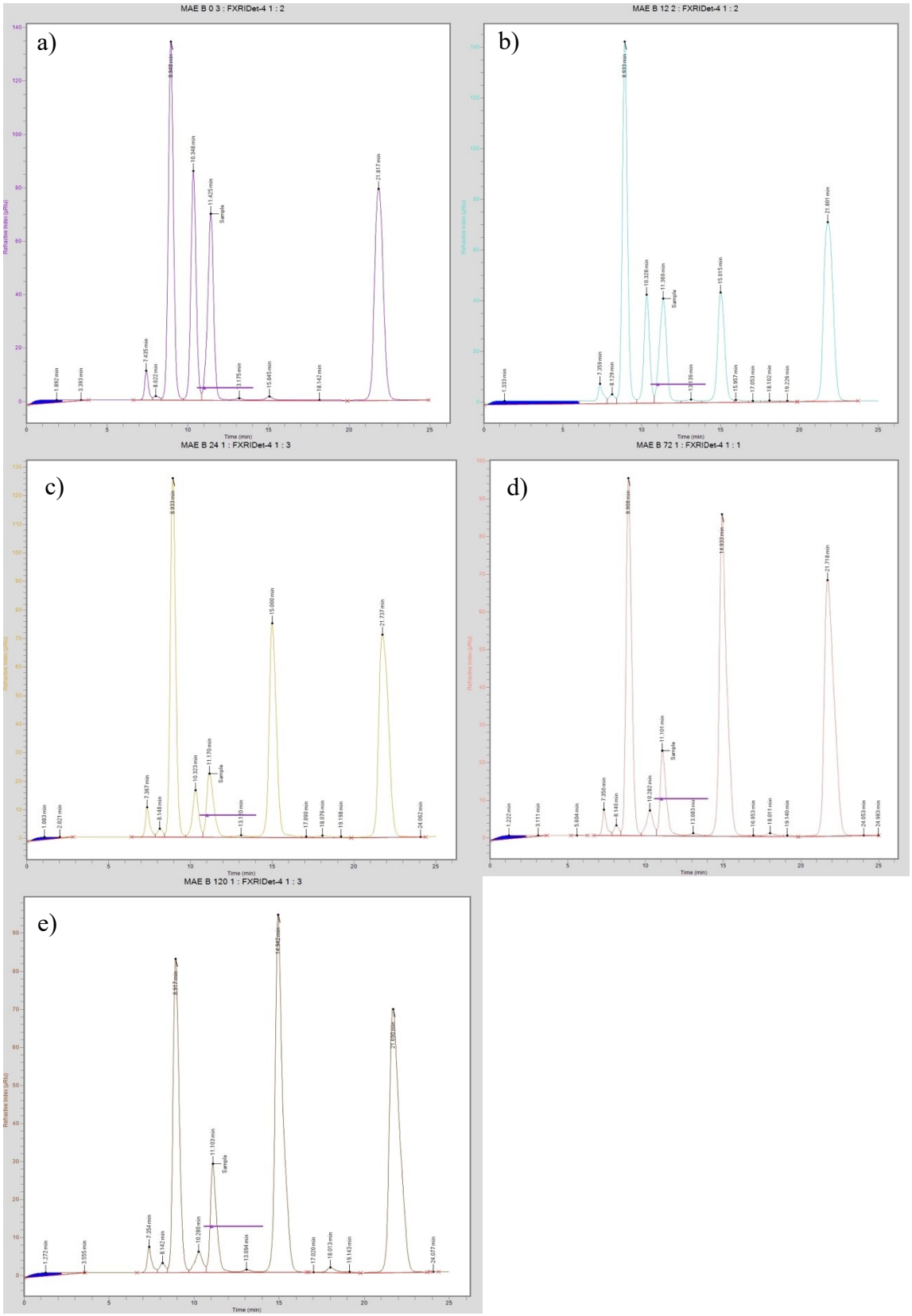
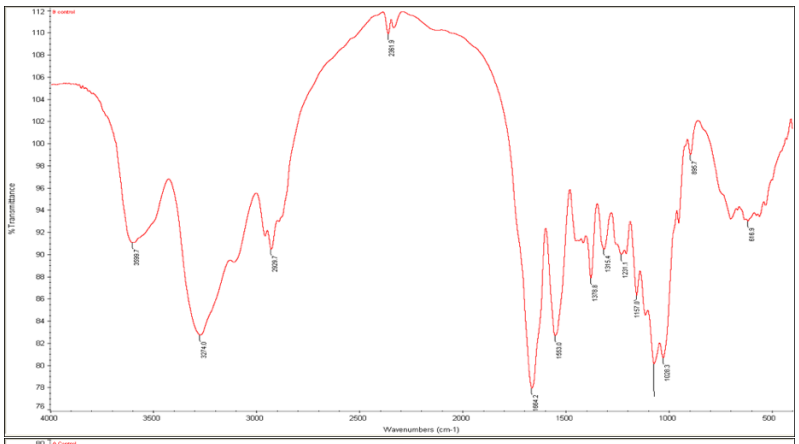
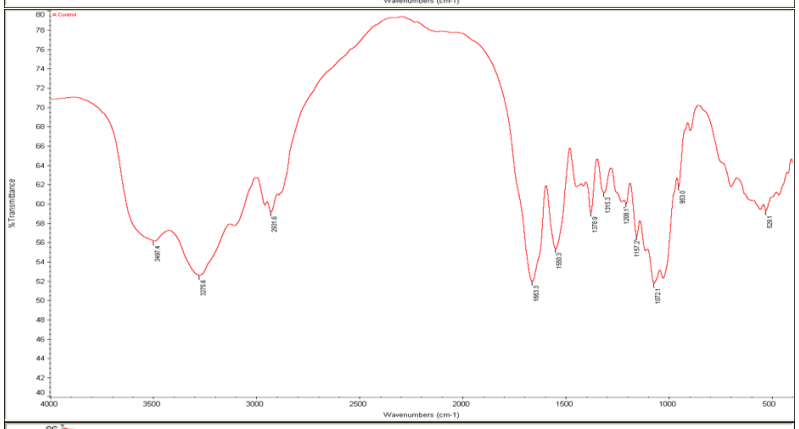


Figure A 20: HPLC chromatograms for BM-L a) 0 hours b) 12 hours c) 24 hours d) 72 hours e)120 hours

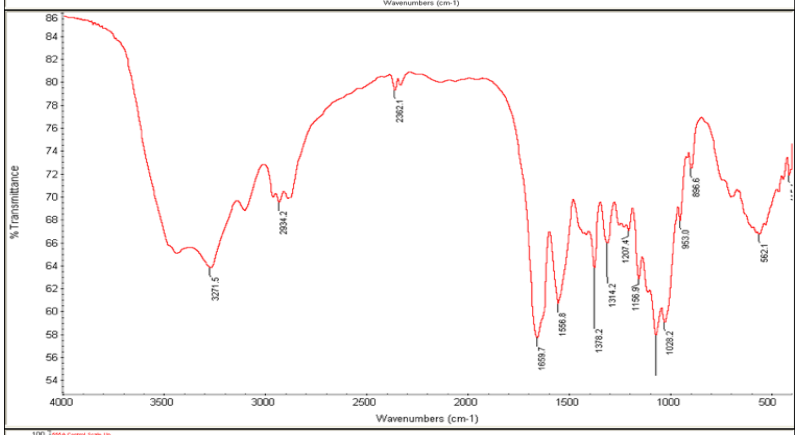
a)



b)



c)



d)

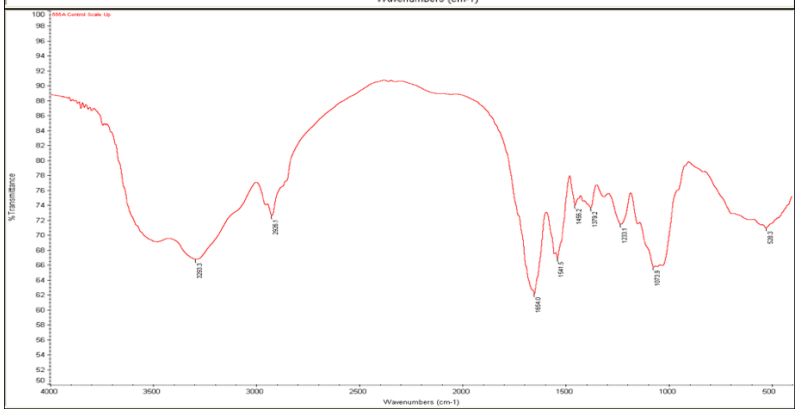
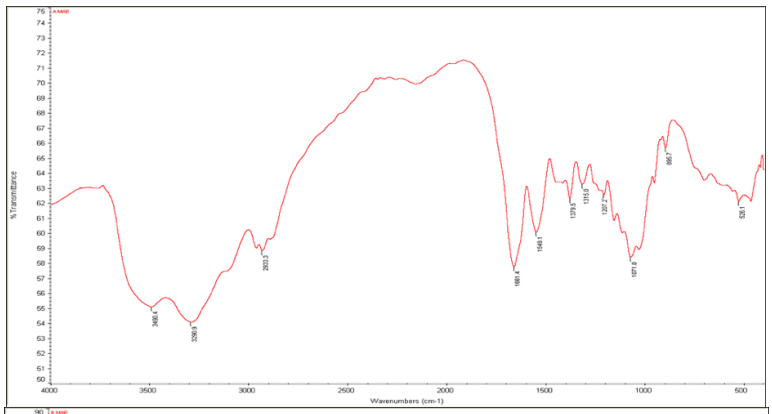
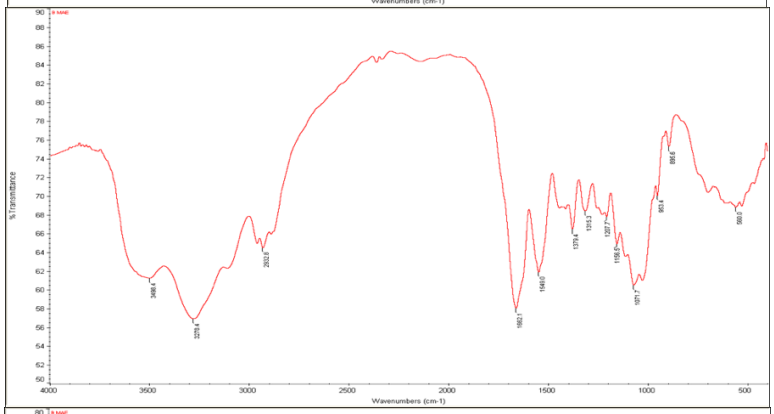


Figure A 21: FTIR spectra of control (B) from scale up trials (replicate sample pellets)

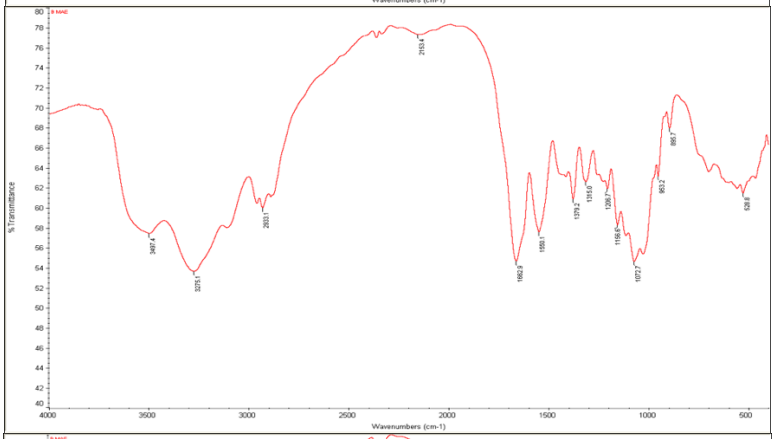
a)



b)



c)



d)

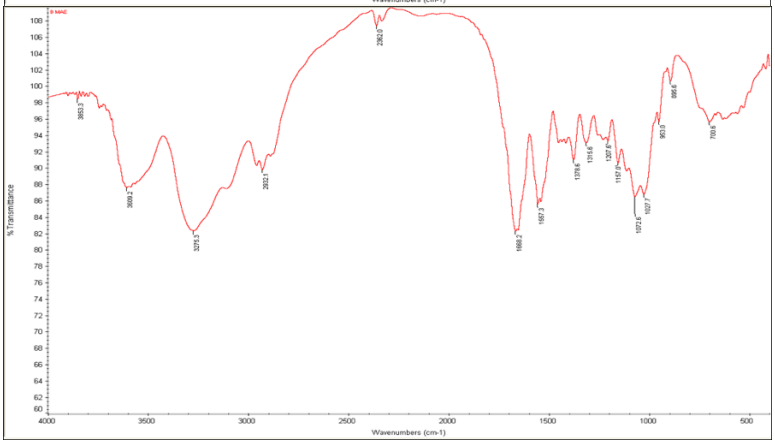


Figure A 22: FTIR spectra of BM-L from scale up trials (replicate sample pellets)