Developing a Biological Approach for Chitin Extraction from *Homarus americanus* Shell Waste

by

Balaganesh Radhakrishnan

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
April 2021

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Lobster processing generates ~0.3M tons of shell wastes each year in Canada, which are rich in chitin, proteins, and minerals, making them valuable substrate. Among them, chitin, a natural biopolymer, has various applications in agriculture and, food. The current study focused on investigating the key parameters that impact the efficiency of fermentative extraction of chitin from American lobster shells. The optimal fermentation conditions in 250mL flasks were obtained with a combination of 5%w/v shell with a particle-size of 500µm-250µm, 10%w/v lactose with 3%w/v whey and 1%w/v inoculum, rest being water, showing an effective demineralization of 83.91±1.11%. This was further improved to 89.83% by staggered addition of carbon source and inoculum, with 88.87% decalcification and crude chitin yield of 28%. The bench scale fermentation was found to be reproducible up to 5L. The study strongly suggests optimizing parameters for biological extraction of chitin could yield a chitin product of commercial value.

Keywords: *Lobster shell, chitin, particle-size, nitrogen, fermentation, demineralization*

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
β	Beta
γ	Gamma
°C	Degree Celsius
%	Percent
<	Less than
>	Greater than
~	Approximate
µm	Micrometers
µl	Microlitre
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CAD	Canadian Dollars
Cfu	Colony forming unit
DC	Decalcification
DM	Demineralization
g	Gram
g/L	Gram per litre
h	hour(s)
H-bond	Hydrogen bond
HCl	Hydrochloric Acid
HCOOH	Formic acid
HNO_3	Nitric Acid
HPLC	High Pressure Liquid Chromatography
H_2SO_4	Sulphuric Acid
ICP MS	Inductively Coupled Plasma Mass Spectrometry

L	Litre
LAB	Lactic acid Bacteria
M	Molar
MAE	Microwave Assisted Extraction
min	Minute(s)
mg	Milligram(s)
mL	Millilitre(s)
mM	Millimolar
mm	Millimeter(s)
N	Normality
NaOH	Sodium hydroxide
ND	Not Disclosed
NH_2	Amino
NS	Nova Scotia
ppb	Parts per billion
psi	Pounds per square inch
r^2	Correlation Coefficient
rpm	Rotations per minute
S/I	Substrate/Inoculum
sp.	species
UAE	Ultrasound Assisted Extraction
U/ml	Units per millilitre
v/v	Volume per volume
W	Watts
w/v	Weight per volume
S1	Size - 2mm-500µm
S2	Size - 500µm-250µm
S3	Size - 250µm-125µm

S4	Size - 125µm-45µm
P1	Whey protein inclusion – 1%
P2	Whey protein inclusion – 1.5%
P3	Whey protein inclusion – 2%
P4	Whey protein inclusion – 3%
S2P4-A	72 Hour Staggered addition of Inoculum
S2P4-B	72 Hour Staggered addition of Carbon source and Inoculum
S2P4-C	48 Hour Staggered addition of Carbon source and Inoculum
S2P4-D	Microwave pre-treated, 48 Hour Staggered addition of Carbon source and Inoculum
A_s	Ash content of raw lobster shell
S	Weight of raw lobster shell sample before ashing
Ar	Ash content of the lyophilized insoluble fraction
R	Weight of the lyophilized insoluble fraction before ashing.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisors Dr. Beth Mason and Dr. Su-Ling Brooks for the continuous support and guidance through the learning process of this Master's program. I would like to thank my committee members Dr. Amyl Ghanem and Dr. Amina Stoddart for their valuable comments and feedback for improving my research project.

I am very grateful for the guidance, support, and mentorship from the Bio-products group at Verschuren centre – Aishwarya Mohan, Dr. Sathy Veeravalli, Subin Rajendran. Their advice and guidance were indispensable to conduct several experiments. I am thankful to Alison Wilson for her generous help.

I take this opportunity to thank Nova Scotia Graduate Scholarship (NSGS) and Mitacs Canada for providing financial support for my research.

I would like to thank the academic and technical support of Dalhousie University, Halifax, Nova Scotia and the faculty, staff who offered help and support.

A heartfelt thanks to my parents, Radhakrishnan and Indumathi and my brother Anandavignesh for their unconditional love, care, and encouragement throughout my life. Last but not least, I would like to thank all my friends and especially Mohan, Karthi and Satheesh for their moral support and motivation.

CHAPTER 1: INTRODUCTION

1.1 Thesis overview

The American lobster, *Homarus americanus,* is predominantly found in the Atlantic coast of North America (Stirn, 2012). Canada is the largest exporter of lobster in the world and during the processing of these lobsters a large amount of waste is produced (Fisheries and Oceans Canada, 2018). The growing production and consumption of lobster in seafood industries proportionately increases the quantity of waste. Disposal of lobster processing wastes has been a major coastal environmental problem and an obstacle for the shellfish processing industries. Every year, nearly 6-8 million tons of lobster shell wastes are produced around the world and at least 0.5 million ton is contributed by Atlantic Canada (Nguyen, Barber, Corbin, *et al.*, 2017). In lobster processing industries, nearly 40-50% of total mass of lobsters are wastes (Chakravarty *et al.*, 2018; Arbia *et al.*, 2013). These shell wastes are rich sources of various high value compounds such as chitin, proteins, minerals, lipids and astaxanthin (Kurita, 2006).

Chitin is one of the most valuable constituents of shell waste and a ubiquitous polysaccharide in nature. It is present in a matrix of proteins and calcium carbonate forming a hard-rigid shell material. Chitin, insoluble in aqueous solvents, can be converted into chitosan and chitooligosaccharides, with increased solubility and employed in many industries including food, pharmaceutical, agriculture, and wastewater treatment (Galanakis, 2015).

The extraction of chitin involves demineralization and deproteinization, removal of calcium carbonate and protein, respectively (Kaur and Dhillon, 2015a). The generation of by-products (solvent waste) from the conventional chemical extraction, along with the need for greener methods have paved the way for biological processes. Recently, there has been a growing

interest in biological extraction of chitin. It involves the use of lactic acid or non-lactic acid producing bacteria in the presence of a fermentable carbon source to carry out the demineralization and deproteinization simultaneously. The biological method allows recovery of other value-added products like minerals, astaxanthin and proteins (Lopes *et al.*, 2018).

Most studies on biological methods to extract chitin have been via lactic acid fermentation with added carbon source (Kaur and Dhillon, 2015a; Duan *et al.*, 2012). Due to low demineralization and deproteinization compared to the conventional chemical methods, the use of this method is still limited to laboratory-scale (Arbia *et al.*, 2013). Moreover, the majority of these studies have been carried out on shrimp or crab shell wastes (Arbia *et al.*, 2013; Pighinelli, 2019; Kaur and Dhillon, 2015). Although lobster shell wastes are generated in high quantity and are rich in chitin, there are only a few studies that have performed biological extraction on lobster shells (Chakravarty *et al.*, 2018; Bautista *et al.* (2001). There is a need for a scalable and effective bioprocessing approach to valorize the lobster shell waste into higher value co-products (chitin, protein and mineral). This research focuses on lactic acid bacterial fermentation on lobster shells for the optimization of select parameters to achieve demineralization of shells for chitin recovery.

1.2 Objectives

This project focuses on the development of an environmentally friendly method for the chitin extraction from American lobster (*Homarus americanus*) shell wastes through lactic acid bacterial fermentation using a fermentable carbon and nitrogen source. The objectives of the present study are:

1. Study the impact of shell particle size and added nitrogen source on the demineralization, decalcification, and yield of chitin from lobster shell fermentation.

2. Analyse the significance of staggered addition of lactic acid producing inoculum and carbon source on the demineralization, decalcification, and yield of chitin of lobster shells.
3. Study the impact of microwave pre-treatment of shell aqueous suspension prior to the fermentative process on the demineralization and decalcification.

1.3 Thesis organization

The thesis is organized into five chapters. Chapter 1 gives an introduction to the project and outlines the project objectives. Chapter 2 gives a review on the relevant works of literature in the field of study, including information on the lobster shell waste, pre-treatment of shells, different kinds of extraction methods and chitin. Chapter 3 provides information on the experimental overview for extraction process and procedure to investigate the chitin obtained. Chapter 4 presents the experimental results and examines the results with a comparison to available literature works. Chapter 5 concludes the research work and suggests insights into future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Lobster processing industry

Lobster is one of Canada's most valuable and profitable marine resource, belonging to the crustacean family. The American or Atlantic lobster, *Homarus americanus* is the predominant lobster species of Northern America (Stirn, 2012). The demand for lobsters continues to increase each year. The production of Atlantic lobster increased from 304,000 metric tonnes in 2014 to 364,000 metric tonnes in 2019, with an estimated value of CAD$9.32 billion (Fisheries and Oceans Canada, 2020). The lobsters are caught and processed for their edible meat while the inedible parts like shells, and claws are usually discarded with possible risk to the environment. Despite attempts to utilize these lobster by-products, the lack of efficient and viable valorization methods results in the industry disposing of lobster shell largely to landfill. In addition, the cost of waste management for the lobster processing industries is estimated at CAD$7.5 million per year, and the disposal of untreated lobster shell waste continues to be an environmental problem (Hamed, Özogul and Regenstein, 2016). A solution to this waste management challenge coupled with utilization of lobster by-products for the recovery of marine functional ingredients would benefit both the environment and lobster processing industries.

2.2 Lobster shell and lobster shell waste

Among lobster by-products, shell waste is considered a valuable resource, constituting 20-30% chitin, 20-40% protein, 30-60% minerals and 0-14% lipids. Of these, the component most commonly extracted from lobster shells is chitin (Arbia *et al.*, 2013; Kurita, 2006). Lobster shell waste has been used as animal feed or fertilizer, as the shells are rich in calcium and protein (Arbia *et al.*, 2013). Chitin and its derivatives extracted from crustacean shells have numerous

applications in water treatment, agriculture, food, pharmaceutical, and biomedicine (Nguyen, Barber, Corbin, *et al.*, 2017). Apart from chitin, the proteins derived from lobster by-products, are nutritionally of high value because of their nucleotide and peptide content and functional properties.

2.3 Chitin

Chitin is one of the most abundant biopolymers in nature and is a major constituent in building the carapace of crustaceans. Chitin is a cationic linear polysaccharide composed of β-(1-4) linked N-acetyl -D-glucosamine monomers (Nguyen, Barber, Corbin, *et al.*, 2017). Chitin is commonly found in cuticles of many invertebrates such as crab, lobster and shrimp and in cell walls of most fungi, yeast, insects and some algae (Rhazi *et al.*, 2000).

Chitin occurs in three forms, based on molecular arrangement (α, β, γ chitin). These three forms of chitin are structurally similar, with differences in the orientation of the micro-fibrils. The α chitin is the most stable and abundant form in crustacean shell wastes and consists of N-acetylglucosamine chains aligned in anti-parallel conformation (Sikorski, Hori and Wada, 2009), while the β and γ chitin are less common and less stable. Chitin is a natural biopolymer with high degradability, biocompatibility and non-toxicity and there is increasing global demand for chitin and its derivatives in textile for cotton fabrics as a durable antibacterial finish (Karagozlu and Kim, 2015).

Chitin is insoluble in polar solvents such as water and alcohols due to the presence of intermolecular H-bonds between chitin chains and hence is limited in commercial applications. To overcome this limitation, chitin is converted into more soluble chitosan through deacetylation, after which it can be dissolved in weak acids. The deacetylation causes N-Acetylglucosamine to

change into D-glucosamine units with free NH_2 as shown in Figure 2.1. The greater solubility of chitosan allows its use in various applications described elsewhere (Kurita, 2006). Chitin has been extensively studied and various methods have been explored to extract it and characterize its properties and potential. Currently, the most common methods to obtain chitin from lobster shell waste are using chemical methods.

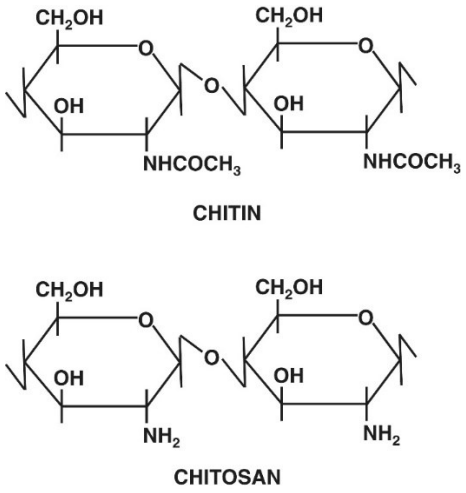


Figure 2.1: Structure of Chitin and Chitosan (Rhazi et al., 2000)

2.4 Chitin extraction methods

Presently, there are various techniques for extracting chitin described in the literature. Among the widely used chemical methods, ionic and deep eutectic liquids are also applied, while biological processes are accomplished with use of microorganisms and/or enzymes. The goal of both processes is to remove the minerals and proteins from shells to obtain the final chitin product.

2.4.1 Chemical extraction method

The traditional and most common technique for the commercial extraction of chitin from crustacean shells is the chemical extraction method. It consists of two fundamental steps, deproteinization by alkali treatment and demineralization by acidic treatment. Furthermore, the shells undergo a step of depigmentation to remove pigments. The fundamental steps can be performed in any order without affecting the final chitin purity or yield (Pighinelli, 2019). Demineralization is performed using a strong acid such as HCl, HNO_3, H_2SO_4, and HCOOH. HCl is the most commonly used acid at concentrations up to 2 M for 24 - 48 h at temperatures ranging from 75°C to 100°C (Yeul and Rayalu, 2013). Deproteinization is performed by alkali solutions such as NaOH and KOH, NaOH being the most adopted for commercial processes at concentrations up to 2.5 M for 24 hrs at temperature ranging from 65°C to 100°C (Yeul and Rayalu, 2013). After the removal of minerals and proteins from shells, organic solvents like acetone are used for depigmentation to obtain the final chitin.

Chemical treatment is highly effective in the demineralization and deproteinization of shells, however, the use of harsh chemicals can impact the environment and can result in inconsistent chitin properties such as varying molecular weight which can limit the resultant applications (Kaur and Dhillon, 2015). Ionic liquids and deep eutectic solvents have been used as a replacement for harsh chemicals to extract chitin (Barber *et al.*, 2013). These solvents are non-flammable, non-volatile and recyclable. Ionic liquids such as 1-allyl-methylimidazolium bromide and 1-ethyl-3-methyl-imidazolium have also been used to extract chitin prior to demineralization with citric acid (Setoguchi *et al.*, 2012).

Recently, techniques such as microwave-assisted extraction (MAE) and ultrasound extraction (UAE) have been applied for the extraction of biologically active compounds

(Thirugnanasambandham *et al*, 2015; Kadam *et al.*, 2015). These methods require less energy, solvents, and time to extract chitin from biomass. For example, in chitin extraction from shrimp, MAE had an advantage over UAE as the yield of chitin was higher without altering the nature of chitin. In the case of UAE, high energy input and pressure were used to extract chitin which could be solubilize or washed out with solvent due to depolymerization. UAE did not have significant improvement in demineralization (Knidri *et al.*, 2016; Way and Hall, 2006) . The process of extraction is further discussed in Section 2.6.

2.4.2 Biological extraction methods

The biological method has been found as an alternative way to overcome the challenges of chemical extraction, with the use of microorganisms and enzymes. The most commonly used biological process is fermentation of shells, involving the use of various microorganisms like *Lactobacillus spp., Streptococcus spp., Pseudomonas spp.* (Arbia *et al.*, 2013).

2.4.2.1 Enzymatic method

Proteolytic enzymes, like proteases and alkaline proteases are used for deproteinization to replace the alkali treatment. The enzymes hydrolyze the peptide bonds of the shell proteins and breakdown into amino acid components as shown in Figure 2.2 (Ilangumaran *et al.*, 2017). Commercial enzymes like Alcalase® (Gildberg and Stenberg, 2001) and crude protease extracted from *Bacillus spp.* (Younes *et al.*, 2012) have been widely used in various studies in shrimp, crab. The crude protease showed better deproteinization (70-88%) than commercial enzymes and a similar effect as alkali treatment (Younes *et al.*, 2012). However, the enzymes were used in combination with chemically demineralized shells. Although, the enzymatic method reduce the

usage of chemicals (alkalis), it does not completely eradicate the use of chemicals to be considered an environmentally friendly alternative for extraction of chitin (Younes *et al.*, 2012).

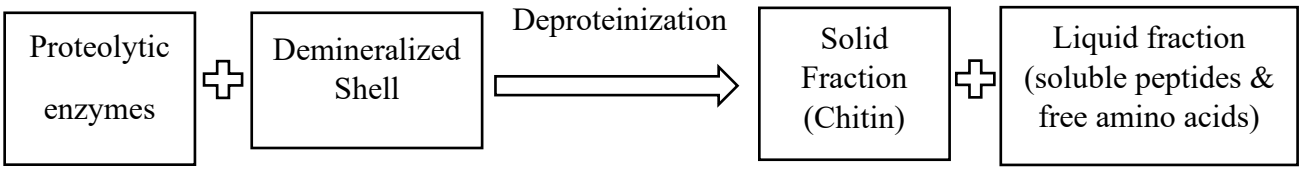


Figure 2.2: Enzymatic method- Deproteinization of shells

2.4.2.2 Microbial fermentation

Fermentative extraction is considered an economically and environmentally friendly alternative to chemical extraction methods (Pighinelli, 2019). This biological process for chitin extraction has been reported using bacteria that produce organic acids for the demineralization and protease for deproteinization of the crustacean shells (Arbia *et al.*, 2013). The microbial fermentation is generally carried out using a single organic acid producing bacteria or using co-culture fermentation (Kaur and Dhillon, 2015a).

Co-culture fermentation uses two kind of bacteria, the organic acid and protease producing bacteria. For example, Liu et al. (2014), used co-fermentation of *Bacillus licheniformis* and *Gluconobacter oxydans* in shrimp and achieved a demineralization (DM) and deproteinization (DP) of 93.5% and 87%, respectively. Similarly, Chakravarty et al. (2018), found a higher extraction efficiency with *Serratia marcescens* db11 and *Lactobacillus plantarum*, with DP and DM of 89.59% and 87.19%, respectively. Though the co-fermentation gave a higher extraction efficiency, the process optimization for multiple organisms would require more studies than with a single organism (Chakravarty *et al.*, 2018; Liu *et al.*, 2014).

The current trend in biological fermentation involves lactic acid fermentation of lobster shells with lactic acid bacteria and added carbon substrate. Lactic acid fermentation uses bacteria which are found in naturally fermented foods such as wine and cheese. The most common genera used in most crustacean shell fermentation is *Lactobacilli*, others include *Streptococcus spp.,* and *Pseudomonas spp.* (Arbia *et al.*, 2013). The crustacean shells are poor source of fermentable carbon, and thus require a carbon source like glucose or lactose for lactic acid production (Steeves, 2019). The lactic acid bacteria use the fermentable carbon to produce lactic acid, resulting in lowering the pH of the fermentation medium (Ding and Tan, 2006; Ilangumaran *et al.*, 2017). The lactic acid bacteria also serve as a source of proteases, the low pH of the medium activates the secretion of enzymes leading to DP (Ilangumaran *et al.*, 2017; Kaur and Dhillon, 2015). A schematic of the lactic acid fermentation process for chitin extraction from lobster shell is shown in Figure 2.3.

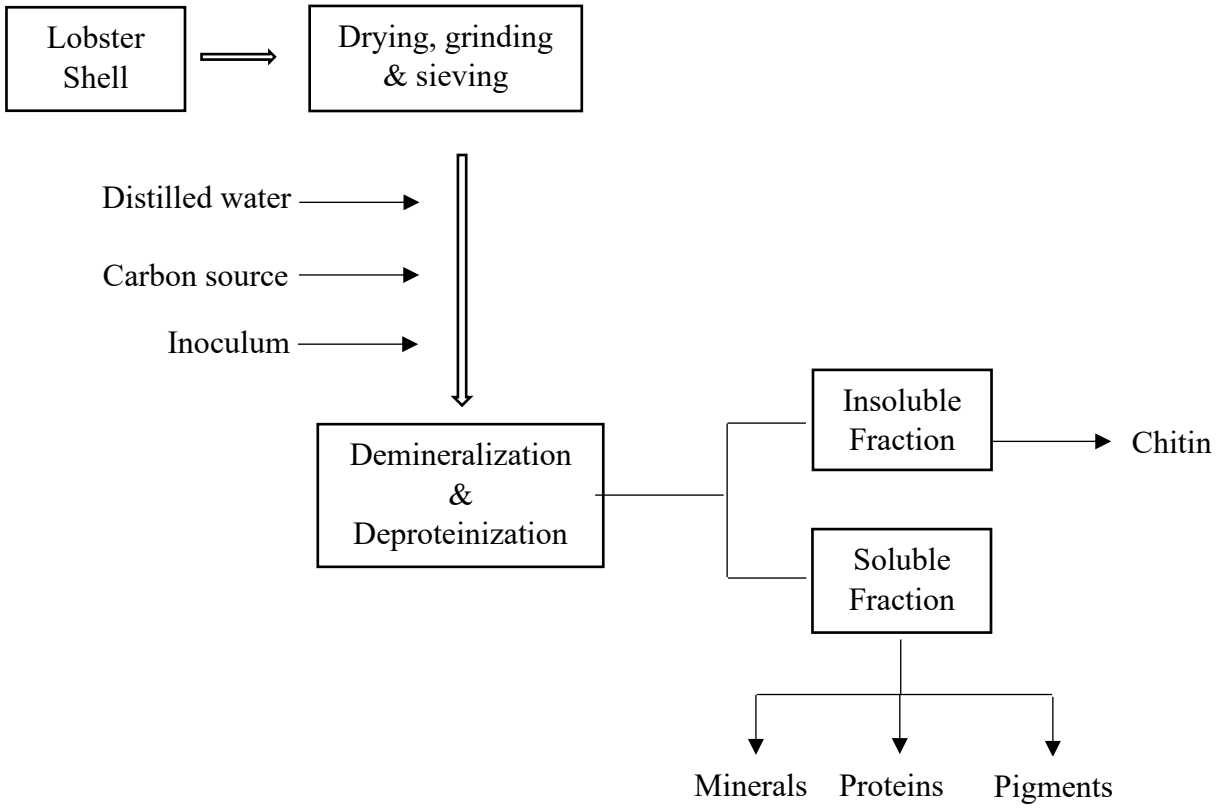


Figure 2.3: Lactic acid bacterial fermentation

Various sources of carbon and inoculum have been used in the literature for lactic acid fermentation and each carbon and inoculum source affects the demineralization and deproteinization efficiency to varying degrees. The carbon source used is typically matched to the inoculum used in the fermentation (Arbia *et al.*, 2013; Tan, Lee and Chen, 2020). Table 2.1 shows the use of different combinations of carbon source and inoculum used for the fermentation by various studies. The efficiency of the fermentation also depends on various factors such as temperature, time, pH, stirring speed, quantity of carbon source, shell waste and inoculum (Rao *et al.*, 2000).

Table 2.1: Lactic acid fermentation using different carbon source and inoculum strain to improve demineralization and deproteinization.

Marine Biomass	**Inoculum**	**Carbon Source**	**Fermentation efficiency (%)**		**References**
			DM	DP	
Shrimp shell	*Lactobacillus plantarum PTCC 1058*	Glucose	75	ND	Khorrami *et al.* (2011)
Scampi shell	*Lactobacillus paracasei* A3 (10%v/w)	Glucose (10%w/w)	61	77.5	Zakaria *et al.* (1997)
Shrimp shell	*Pediococcus* sp. L1/2	Sucrose	83.47	ND	Choorit *et al.* (2008)
Crab shell	*Lactobacillus plantarum* 47 (10%v/w)	Sucrose (15%w/w)	99.6	95.3	Castro *et al.* (2018)
Crayfish shell	*Lactobacillus pentosus 4023*	Lactose	90.1	81.5	Bautista *et al.* (2001)
Crawfish shell	*Lactobacillus paracasei* A3	Glucose	97.2	74	Cremades *et al.* (2001)

ND: Not Disclosed (Degree of Deproteinization)

The advantage of lactic acid producing microbial fermentation is that the DM and DP occur simultaneously. Fermentation has been usually performed between 3 to 7 days, at temperatures ranging between 30-37°C, which is the optimum temperature for *Lactobacillus* species (Wang, Corrieu and Be, 2005).

2.5 Fermentation parameters of microbial fermentation:

From previous studies, it is understood that fermentation is affected by several parameters and optimizing these parameters is recommended for improved results. For example, a maximum DM and DP of 99.6% and 95.3%, respectively was achieved through the lactic acid fermentation of shells from the crab species, *Allopetrolisthes punctatus,* by optimizing the carbon source (sucrose 15% w/w), inoculum (10% w/w) and temperature (32°C) (Castro, Guerrero-legarreta and Bórquez, 2018). Similarly, Oh et al. (2007) performed lactic acid fermentation on crab shell waste based on parameters such as shell size, carbon source and temperature. The general media components required in a fermentation are, a carbon source, a nitrogen source, along with micro and macro nutrients. In case of crustacean shell biomass, the macro and micro-nutrients are often naturally present in the substrate/biomass or in the added carbon sources. Moreover, crustacean shell wastes have varying levels of protein present in the shell and tissue remnants, therefore an additional protein source is often not included. However, in the current research, an additional protein source in the form of whey protein isolate is used as an added nutrient for the growth of bacteria. The success of the fermentation is determined by the degree of demineralization, degree of deproteinization and chitin yield. The degree of DM and DP depends on various factors such as carbon source, microbial strain, pH, temperature, nitrogen source, shell size.

2.5.1 Effect of carbon source:

The most commonly used carbon sources are glucose and lactose (Tan, Lee and Chen, 2020). The concentration of carbon has a significant effect on the organic acid production of lactic acid bacteria sp. with an optimal concentration of fermentable carbon results in higher organic acid production (Bautista *et al.*, 2001). Cira et al (2002) studied different carbohydrate sources such as sucrose, lactose, cassava, malt, and glucose to find a suitable source for the fermentation of shrimp.

The sucrose, lactose and glucose showed similar effects on the fermentation by lowering the pH to 4.4-4.8 at 96 h, while cassava and malt showed no improved effect in pH reduction for production of lactic acid. Duan et al (2012) performed lactic acid fermentation for extracting chitin from shrimp using glucose as the carbon source. These studies used 15% (w/v) of glucose for the fermentation which lowered the pH to 3.73 with production of 18mL/g of lactic acid resulting in 96.7% and 96.5 DM and DP, respectively.

A similar efficiency of fermentation has been reported for glucose and lactose with proteolytic enzyme producing bacteria (Healy, Green and Healy, 2003; Chakravarty *et al.*, 2018). An important factor for organic acid production is the presence of adequate quantity of fermentable carbon. Limited or inadequate levels of carbon results in reduced acid production. The lactic acid reacts with calcium carbonate in the shell thus affecting the reduction in pH and DC (Levin, 1994). Rao et al., (2000) optimized the glucose concentration (1.75-7% w/w) for *Lactobacillus plantarum* and achieved a DM and DP of 86% and 75%, respectively, using 5% w/w glucose. In another study by Aytekin and Elibol (2010), glucose concentrations of 0 to 10% w/w were studied with constant 10% *Lactococcus lactis* inoculum, where a pH of 5.5 was achieved within 12 h with 5% w/w glucose, improving the reduction of pH in less time. According to these authors, excess concentration of carbon source did not have significant reduction in the pH and produced low levels of lactic acid. The concentration of the carbohydrate source also depends on the inoculum used, for example, Zakaria, Hall and Shama, (1997) used 10% w/w glucose for 10% w/v of *Lactobacillus paracasei* A3 and achieved a DM and DC of 80% and 79.2%, respectively.

2.5.1.1 Staggered addition of carbon source:

Staggered addition is a new technique which has been followed in alcoholic beverage production (Ribeiro *et al.,* 2020) and involves the addition of energy sources fractionally, i.e.,

portions of the nutrients added at different time intervals, during fermentation. Studies investigating the staggered addition of a carbon source have not been reported frequently in the literature for chitin extraction, however staggered nutrient addition in alcoholic beverage production has been compared with a fed-batch fermentation (Ribeiro, Olivo and Eller, 2020). In the case of chitin fermentative extraction, the portion of carbon source that is fractionally added depends on the pH of the medium during the course of fermentation (Chakravarty *et al.*, 2018). Apart from the substrate source and feeding, like any other fermentation, the process efficiency is dependent on inoculum growth and acid production (Khanafari *et al.*, 2008). For example, Ribeiro et al (2020) performed a staggered nutrient addition and showed an increase of 33% in lactic acid production. The staggered addition of nutrient also depends on the inoculum quantity and strain used for the fermentation, as the strain uses the carbon source for the lactic acid production and lowering the pH of the fermentation. Inadequate carbon source in a fermentation medium leads to reduced bacterial growth, affecting the lactic acid production which in turn results in a poor fermentation efficiency (Chakravarty *et al.*, 2018).

2.5.2 Effect of inoculum:

Kaur and Dhillon (2015) found that the best biological method for extracting chitin uses microorganisms, which can replace the expensive and environmentally unfriendly chemical extraction (Kaur and Dhillon, 2015). Different species of microorganisms have been investigated including *Lactobacillus plantarum, Pseudomonas aeruginosa, Pseudomonas maltophila, Bacillus subtilis, Penicillium chrysogenum,* and *Lactobacillus paracasei* (Oh *et al.*, 2007). The use of microbial treatment allows the activation of proteases at low pH enabling the recovery of additional value-added co-products from the shell, whereas, the chemical extraction does not allow the recovery of other valuable products (Chakravarty *et al.*, 2018). Table 2.2 shows the comparison

between the *Lactobacillus* sp. and non-lactic acid bacteria with respect to the fermentation efficiency. *Lactobacillus* sp. have been widely used for ensilation due to their ability to produce lactic acid and effective pH reduction which enables higher DM and DP (Kaur and Dhillon, 2015).

Table 2.2: Comparison between *Lactobacillus* sp. and non-lactic acid sp. fermentation with regard to demineralization and deproteinization

Marine Biomass	**Strain**	**Carbon Source**	**Fermentation efficiency (%)**		**References**
			Demineralization	**Deproteinization**	
Lactic acid bacterial fermentation					
Shrimp shell	*Lactobacillus plantarum 541*	Glucose	81.4	59.8	Rao *et al.* (2006)
Lobster shell	*L. plantarum*	Glucose	73.02	ND	Chakravarty *et al.*, (2018)
Crab shell	*Lactobacillus plantarum* 47	Sucrose	99.6	95.3	Castro *et al.* (2018)
Non- Lactic acid bacterial Fermentation					
Shrimp shell	*Pseudomonas aeruginosa A2*	Glucose	85	56	Ghorbel-Bellaaj *et al*. (2011)
Crab shell	*Serratia marcescens*	ND	84	47	Jo *et al.* (2008)
Shrimp shell	*Bacillus cereus*	ND	ND	68	Sini *et al*. (2007)

ND: Not Disclosed

In general, lactic acid producing bacterial fermentations had greater efficiency in converting fermentable carbon to acid compared to non-lactic acid bacterial fermentation. The lactic acid bacteria has a conversion efficiency to produce at least 85% of lactic acid from the carbohydrate source and most of *Lactobacillus* sp. such as *Lactobacillus plantarum, Lactobacillus helveticus* can reduce pH up to 3.7 (Levin, 1994). For example, Castro *et al.* (2018) performed lactic acid bacterial fermentation and achieved a high DM and DP of 99.6% and 95.3%, respectively while most of the non-lactic acid bacterial fermentation achieved lower DP (Sini *et al.*, 2007; Jo *et al.*, 2008). A correlation to carbon source and different levels of inoculum have been used to find an optimum inoculum level for certain *Lactobacillus* sp. with respect to the carbohydrate source (Chakravarty *et al.*, 2018). The optimum level is not a standardized relationship, it changes according to the biomass, inoculum, and the carbon source. Apart from carbon source, a nitrogen source (amino acids) would also be required for the microorganism growth. A nitrogen source was not included in any of the fermentation studies in the literature, but an addition of nitrogen could improve the process (Kampen, 2014). The added nitrogen source improves the productivity of secondary metabolites and thus supplying more energy to bacteria and driving the cell growth phase (Barrios-González, 2018).

2.5.2.1 Staggered addition of inoculum:

There is limited information on staggered addition of inoculum for chitin extraction, but there are a number of studies using co-culture of dual strains with staggered addition of these microbial cultures. These studies have shown a higher degree of DM and DP than the use of single strain in marine biomass. Chakravarty et al. (2018) performed the co-culture fermentation using the organic acid and protease producing bacteria in lobster shell. *Lactobacillus plantarum* and *Serratia marcescens* db11 were used for the fermentation, and the pH of the medium lowered from

6.5 to 4.2 in 3 days and measured proteolytic activity of 575 U/ml at day 2. The simultaneous inoculation of microorganisms showed DM of 60.65% and DP of 54.54%, while the staggered addition of co-culture had a demineralization of 89.59% and deproteinization of 87.19%. In staggered addition, the *S. marcescens* was added at 0 h and *L. plantarum* was added at 72 h. Aytekin et al. (2010) showed a similar effect on DM and DP of 80% and 77.8%, respectively using co-culture fermentation using *T. turnirae* at 0 h and *L. lactis* at 96 h.

One drawback of staggered co-culture fermentation is the need for extensive optimization studies for both organisms, which is time consuming and resource intensive, requiring more analyses and may not be cost efficient (Liu *et al.*, 2014). Thus, the staggered addition of a single organism with optimum level of carbon source would be easier to optimize in order to increase the efficiency of the fermentation. The staggered addition would possibly increase the interaction between the shell and inoculum and may stimulate proteolytic activity during the initial stage, where staggered addition at day 2, 3 or 4 would help in lowering the pH effectively by producing high lactic acid. The timing of staggered addition can be optimized by measuring the time course of pH reduction, lactic acid production and proteolytic activity (Liu *et al.*, 2014; Chakravarty *et al.*, 2018; Aytekin *et al.*, 2010).

The staggered addition of carbon source and inoculum are related, the pH, protease activity and organic acid production are closely monitored so that the appropriate time for the staggered addition can be estimated. To date, the simultaneous staggered addition of carbon source and inoculum has been not studied in the literature.

2.5.3 Effect of substrate particle size:

Substrate particle size has a significant effect on the fermentation efficiency (Tetteh., 1991). However only a few research studies have explained the impact of the particle size on biological or chemical extraction methods. These studies have been conducted on crab, snail, or prawn shells of irregular particle sizes or particle size less than specific size (Oh *et al.*, 2007).

A few studies have evaluated the impact of particle size on the chemical and fermentative extraction of chitin. Tetteh (1991) studied the effect of particle size (ranging from 2 mm, 1.7 mm, 1.41 mm, 1 mm) on the chemical extraction of chitin and its yield from lobster, crab, and shrimp. The chemical extractions were carried out using HCl and NaOH for 8 hrs each, and chitin extraction with respect to particle size differs with the type of crustacea. The particle size had a significant effect on the chitin extraction from lobster shell compared to crab and shrimp. In lobster shells, the 1mm sized shells yielded higher chitin yield of 29% while the yield ranged from 21-24% for the other particle sizes. Similarly, Alves *et al.* (2017), studied the effect of particle size of shell on the degree of deacetylation for size ranging from >106 µm, 106-63 µm, 62-43 µm, <43 µm and found that the efficiency of deacetylation was highest as particle size decreased. It was reported that reduction in the particle size provides increased surface area of the shells for the action of lactic acid and shell interaction (Alves *et al.*, 2017). Oyekunle et al. (2019), performed chemical treatment on different particles size ranging 6.3-4.75 mm, 4.75-2 mm, 2-1 mm and 600-300 µm based on DM, and observed higher DM of 80% for particle size of 600-300 µm.

Santos et al. (2019) performed microwave treatment on different particle size of shrimp ranging from 60 (250 µm), 32 (500 µm), 16 (1080 µm) mesh size based on chitin yield and degree of deacetylation efficiency to determine the effective size. From the study, it was found that the particle size of 32 mesh (500µm) had greatest chitin yield of 74% and 92% degree of deacetylation. In another study by Mahmoud and Ghaly (2015), it was found that particle size reduction had a

significant effect in improving the efficiency of DP and quality of chitin extracted from shrimp shell using *Aspergillus niger*. A 33.23% increase in DP efficiency was seen in size reduced shells compared to unsized shells and also the chitin yield increased from 16.59% to 22.68% compared to the unsized shells (Mahmoud and Ghaly, 2015)

From the literature studies, the particle size significantly influences the rate of demineralization and chitin yield on chemical and microwave treatment. The reduction in particle size resulted in requiring lower amounts of chemicals compared to large sized biomass (Hou et al. 2016). The biological treatment showed an improved impact on size reduction on the growth and production of lactic acid and achieved a DM of 88%. Reduced particle size resulted in higher specific growth of inoculum and enhanced protease production. The smaller sized shells was described as having a greater surface area for the growth of microorganism and for enzyme production (Mahmoud and Ghaly, 2015). From the literature data, it can be seen that reduction in particle size may increase the efficiency of fermentation with respect to DM and yield of chitin. In the same regard, the lactic acid bacterial fermentation on different particle size ranges may improve the efficiency of the fermentation.

2.6 Pre-treatment of biomass:

Pre-treatment techniques such as the application of microwaves and ultrasonication are routinely used to improve the extraction efficiency of compounds from various types of biomass through cell membrane disruption. The exoskeleton of crustaceans is a biological composite material constituted of chitin, proteins, and minerals. The chitin is generally complexed within a protein-chitin fibre network, where the network is protected by amorphous minerals (Raabe *et al.*, 2006). The disruption of the complex network and amorphous layer would likely increase the extraction efficiency of chitin. The commonly used pre-treatment for crustacean shells has been

microwave irradiation technique as it reduces the excessive use of chemicals (Leke-Aladekoba, 2018).

Microwave-assisted extraction is commonly used along with chemical treatment, where the shells are pre-treated with NaOH for DP and HCl for DM (Santos *et al.*, 2019). Microwave treatment induces electromagnetic radiation with frequencies range between 100 W to 1500 W and are passed through a medium forming a dielectric gradient inside the biomass. The dielectric gradient creates heat, heating the solvent (NaOH or HCl) which leads to the dissolution of the analyte causing increased rate of mass transfer from solution to the biomass, and leading to disruption of cell structure (Wang and Yin, 2017).

Microwave treatment helped in reducing the extraction time and use of solvent after pre-treatment in shrimp and crab shells (Kaur and Dhillon, 2015). Enzymatic hydrolysis was improved by pre-treatment with microwave irradiation in crab shells and achieved a DP of 85.8% (Nguyen *et al.*, 2016). The efficiency of the pre-treatment depends on the duration of extracting the final product, the microwave assisted chemical extraction of chitin in shrimp shell reduced the extraction time from 4 hours to 16 minutes in which the DM was performed at 500 W for 8 min and deproteinization at 160 W for 5 min followed by 350 W for 3 minutes (Knidri *et al.*, 2016). The combination of chemical and microwave pre-treatment in lobster shells improved the efficiency of DM, DP, and chitin yield with less consumption of chemicals. The microwave pre-treatment combined with microbial extraction showed an improvement in chitin extraction by reducing the pH of the medium in a shorter period of time (Steeves, 2019).

Ultrasonication pre-treatment is similar to microwave, in case of ultrasound, it improved the process by cavitation (Leke-Aladekoba, 2018). The process involves the collision of microbubbles leading to an increase in the solution temperature and pressure. The increased

temperature and pressure creates cavitation with high mechanical waves and turbulence and thus facilitating the heat and mass transfer in the chemicals (Frydenberg *et al.*, 2013). Ultrasonication have shown to decrease the extraction time (Leke-Aladekoba, 2018). Microwave and ultrasonication pre-treatment had similar effects in chemical extraction of chitin. In both treatments the power, processing time, and temperature must be optimized for improved extraction efficiency.

2.7 Summary:

Crustacean species have been the principal source for commercial production of chitin, mainly shrimp, crab and lobsters, which generate by-products in sea food processing industries. Various methods of extraction of chitin from crustaceans, starting from traditional chemical method to advanced ultrasonic assisted extraction have been described in this review. Lactic acid bacterial fermentation has been widely studied as an alternative method to extract chitin without affecting the final product characteristics and providing a more environmentally friendly option.

Lactic acid bacterial fermentations have been successfully used for the extraction of chitin in crab and shrimp wastes. However, there is a dearth of literature on the extraction of chitin from lobster shell waste, particularly, using lactic acid bacterial fermentation. Further research on lobster wastes using lactic acid bacterial fermentation with certain changes such as particle size, carbon source and addition of pre-treatment, protein inclusion and staggered addition in the technique may increase the extraction efficiency of chitin. It will also lead to development of a biological method that is not only eco-friendly but also employment of extracted product in various applications in agriculture, pharmaceutical, wastewater treatment and utilization of by-products such as proteins, minerals, astaxanthin, lipids during extraction.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental design:

Different weight percent combinations of shell loading, initial lactose in the medium and inoculum volume were studied based on preliminary work described in Steeves (2019) to identify the optimal combination for effective reduction of pH during the fermentation process. The experimental overview shown in Figure 3.1 was designed based on the determining effective pH lowering combination and on different shell particle size and whey protein inclusion to develop a fermentative chitin extraction process from lobster shells. Initial test scenarios used a fixed carbon/inoculum rate (per Steeves 2019). The experimental design was divided into two phases. In Phase 1, the primary set of fermentations were carried out using a factorial design to establish the significance of two variables (Whey protein inclusion and shell particle size) on degree of DM. The best formulation from Phase 1 showing highest DM rate was chosen for further testing in Phase 2. In Phase 2, two approaches were studied for enhancing the DM achieved by the optimal formulation identified in Phase 1: (i) a staggered addition of fermentable carbon and inoculum, (ii) pre-treatment of shell with microwave. The most effective condition from Phase 2 was then scaled up to 5 L in a bench scale bioreactor, to evaluate the scalability of the process.

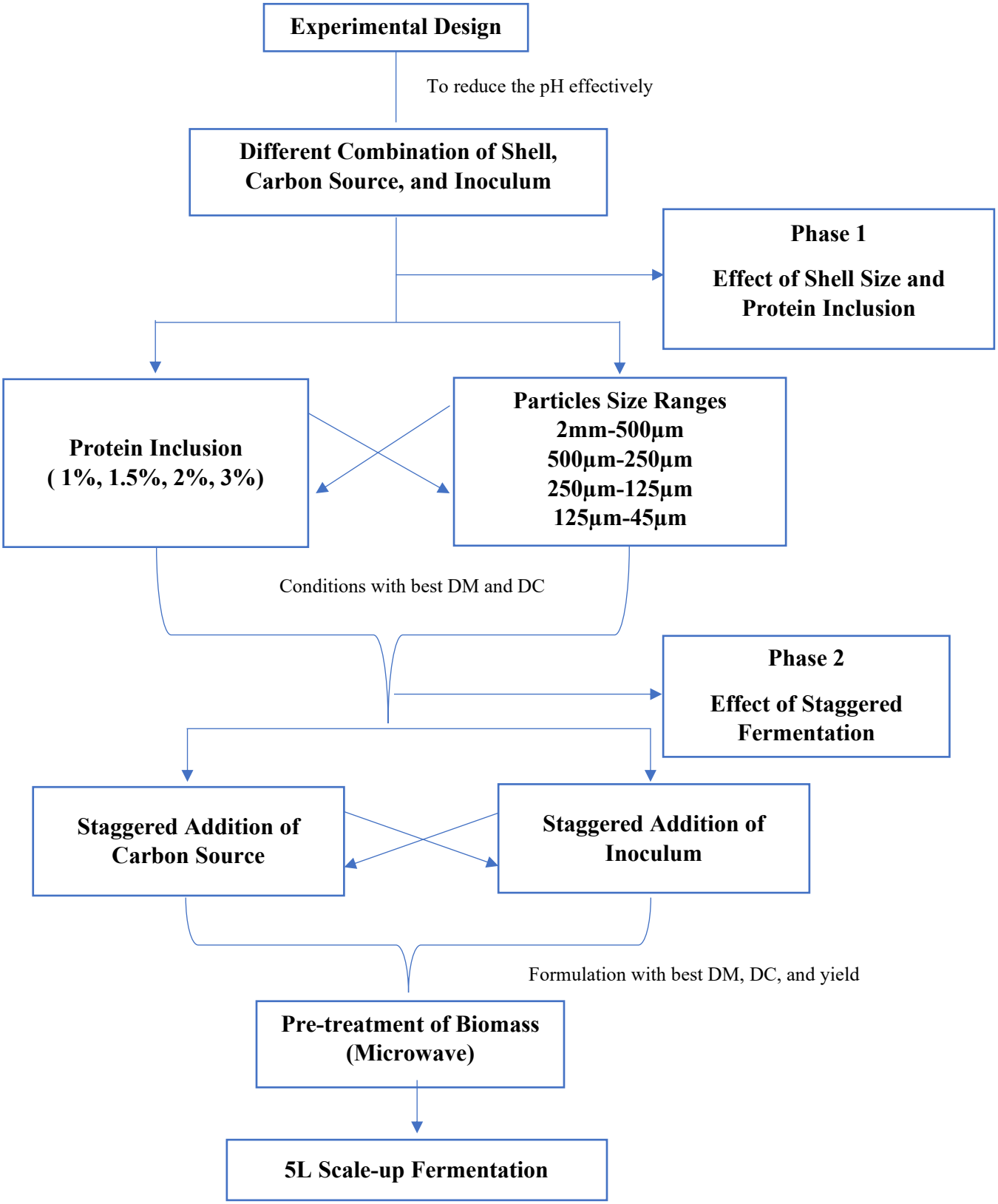


Figure 3.1: Two- Phase experimental design for developing a fermentative chitin extraction process from lobster shells. DM: Demineralization, DC: Decalcification

3.2 Shell preparation:

A volume of shells, *Homarus americanus* were procured from largest lobster processor in Nova Scotia and stored in a deep freezer at -20 °C, until further use. The shells were dried at 65°C in an air convection oven for 72 hours and then the dried lobster shells were crushed using a stainless-steel hammer mill (Dwyer Manufacturing Inc.) at 3600 rpm for 15-20 minutes. The non-uniform crushed shells were further crushed into smaller particle size using a coffee grinder and the ground shell powder was sieved using a mesh, in order to characterize the particle size distribution of the shells. As shown in Figure 3.2, a wide range of mesh sizes (2 mm-500 µm, 500 µm-250 µm, 250µm-125 µm, 125 µm-45 µm) were used to sieve the shells into different size fractions. The sieved shell particles were then stored in a sealed container at room temperature.

(a) (b)

(c) (d)

Figure 3.2: Sieved shell particle sizes used in the fermentation (a) 2000µm-500µm (b) 500µm-250µm (c) 250µm -125µm (d) 125µm -45µm.

3.3 Proximate composition of lobster shell:

The proximate analyses were performed by following the methods described by AOAC (1990), the moisture content of all sieved shell particles was determined by drying in a forced air convection oven at 105°C for 24 hours. The ash content was determined by heating the shell particles in a muffle furnace at 500°C for 4 hours (Tan, Lee and Chen, 2020). Lipid content was determined by Soxhlet extraction (SOXTHERM ®, C. Gerhardt UK Ltd, UK) using petroleum

ether as extraction solvent (AOAC, 1990). The moisture content of biomass was calculated by the weight removed by drying using the Equation (3.1),

$$\text{Moisture content (\%)} = 100 - \left(\frac{\text{Sample weight after drying}}{\text{sample weight before drying}}\right) \times 100 \quad \text{Eq. (3.1)}$$

The ash and lipid content of shell was determined by weighing the sample left after the treatment by using the Equation (3.2),

$$\text{Ash or lipid content (\%)} = \left(\frac{\text{Sample weight after process}}{\text{sample weight before process}}\right) \times 100 \quad \text{Eq. (3.2)}$$

The protein content (Total nitrogen) of lobster shells was determined using an elemental analyzer (Perkin Elmer series II CHNS/O Analyzer Perkin Elmer, USA). The chitin and protein adds up to the total nitrogen content of lobster shell, thus it makes difficult to differentiate between the protein nitrogen and chitinous nitrogen (Steeves, 2019). The raw lobster shells were chemically treated to isolate chitin and the protein content was measured using the Eq.3.3,

$$\text{Protein content (\%)} = \left(\frac{\text{protein content after process (isolated chitin)}}{\text{protein content before process (raw lobster shell)}}\right) \times 100 \quad \text{Eq. (3.3)}$$

All the proximate analyses were performed in duplicates for all the four different particle shell sizes.

3.4 Fermentation studies

3.4.1 Preliminary screening: Influence of Carbon: Inoculum ratio on fermentation efficiency

The initial medium combination was based on the data chosen from Steeves (2019). Each variable was given as a percentage composition (w/v): shells (5 to 10%), lactose (10%) and inoculum (1 to 2.5%) and rest percentage being water. These were varied one factor at a time to

find a best combination that would efficiently reduce the pH of the fermentation medium. The preliminary fermentations were performed on shell size > 2mm in experimental duplicates.

3.4.2 Phase 1: Influence of shell size and whey protein inclusion on fermentation efficiency.

As shown in Figure 3.3, the fermentations were performed with four different particle size ranges of shells and whey protein inclusion. A factorial design was used to study the effect of these variables to determine their influence on response variable, DM. The particle size range of the shells were chosen based on the previous studies of chitin extraction from crab and shrimp shells (Alves *et al.*, 2017; Tetteh., 1991). The added whey protein (Bulk Barn Foods Limited) levels were chosen depending on carbon source (lactose) concentration. The whey protein was added in powdered form and it helps in the production of secondary metabolites for the growth of microorganism and also makes the fermentation more rapid and efficient (Barrios-González, 2018; Kampen, 2014).

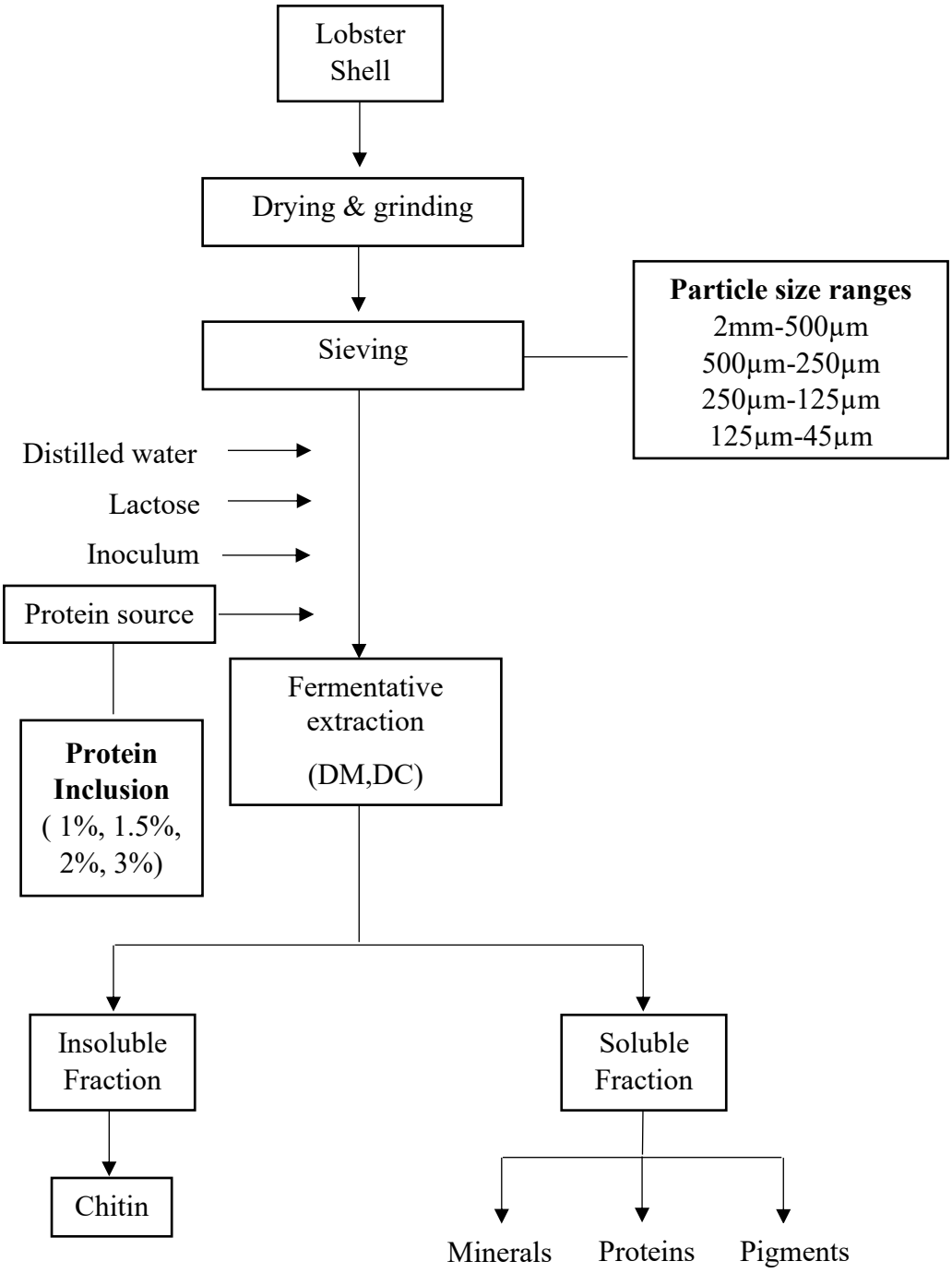


Figure 3.3: Flow process of fermentation during the extraction of chitin in Phase 1

The different sizes of sieved lobster shells (biomass) and lactose (Sigma-Aldrich, Oakville, ON, Canada), varied levels of whey protein inclusion according to the experimental design was

mixed with 100 mL of distilled water in a 250 mL Erlenmeyer flask. To this mixture a commercial inoculum (Lallemand Canada Inc., QC, Canada) consisting of *Lactobacillus* strain was added at 1% w/v and covered with a parafilm. The fermentation was carried out for 5 days where the temperature of the fermentation medium was maintained at 37°C with a constant magnetic stirring of 180 rpm on a hot plate (Fisher Scientific, Isotemp, USA). During the course of fermentation, samples were collected at intermediate time points to analyze for pH and metabolites. All fermentations were performed in duplicates.

3.5 Phase 2: Staggered addition fermentation trials

The best conditions from Phase 1 was chosen to be further improved, i.e., to increase the efficiency of the fermentation as determined by the DM and chitin yield. In phase 2, the effect of staggered addition of carbon source and inoculum source and microwave pre-treatment were studied on improving the DM and chitin yield.

During the course of fermentation, the samples were collected at specified intervals of time for pH and metabolite analysis. The time at which the carbon source and inoculum were added are shown in Figure 3.4.

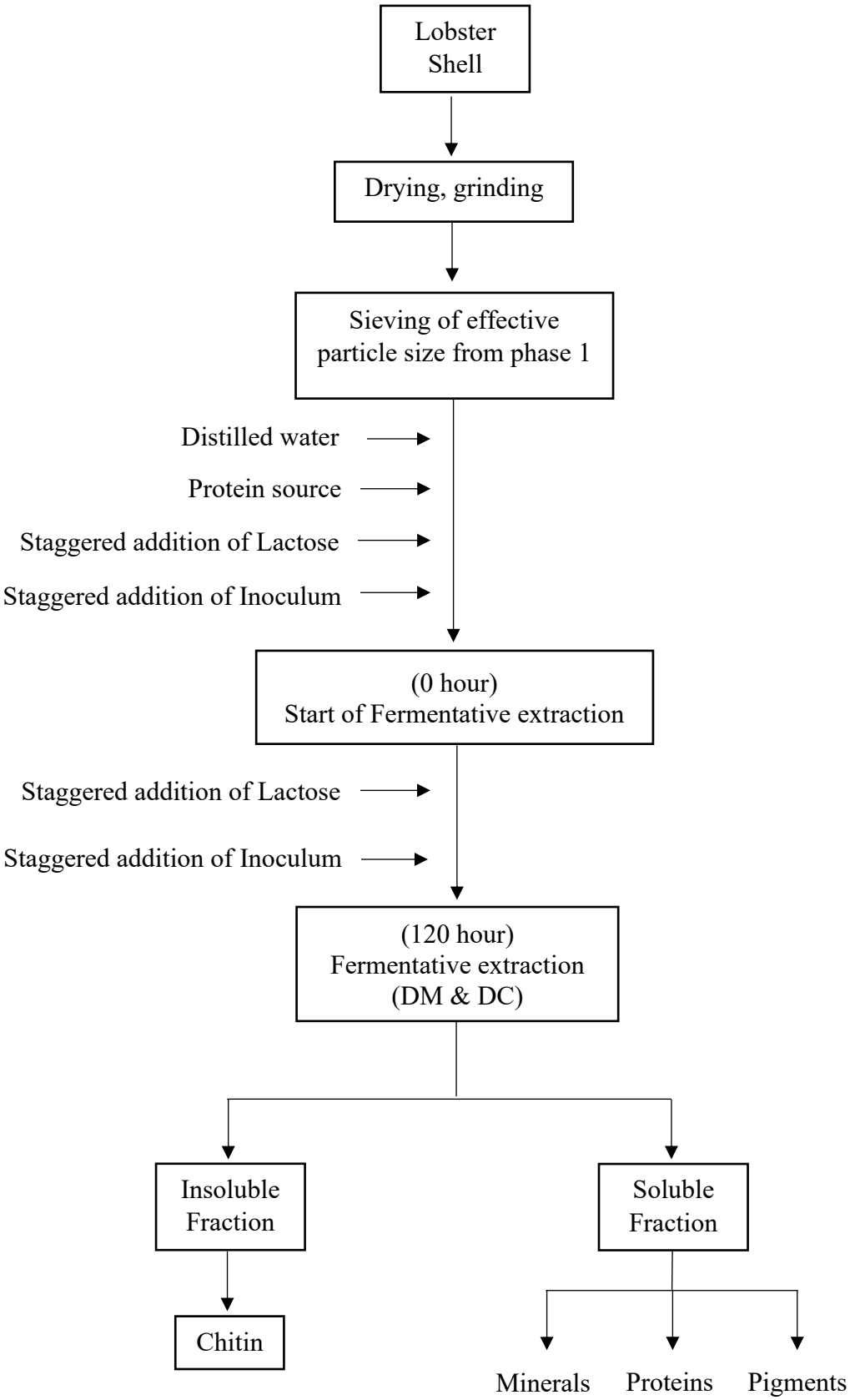


Figure 3.4: Flow process of staggered addition during the extraction of chitin in phase 2

In the case of staggered addition, a part of total lactose and inoculum are added at the start of fermentation (0 hour) and the remaining lactose and inoculum are added after 48 hour or 72 hours depending on the lactic acid production and lactose consumption.

3.5.1 Influence of microwave pre-treatment on fermentative efficiency:

Microwave was employed as a pre-treatment (on the shell) in combination with the best formulation of staggered addition of carbon and inoculum to further increase the fermentation efficiency. The starting shell material suspended in distilled water (100 mL) was pre-treated using MARS 6 230/60 910900 (CEM Co., NC, USA), according to the protocol described by Steeves (2019), where the shells were pre-treated at 70°C, 700 W for 30 minutes at 800 psi. The microwave pre-treated shells were subjected to staggered addition fermentation in a 250 mL Erlenmeyer flask as represented in Figure 3.4. The samples were collected at regular time intervals to measure the pH and metabolites (t – 0, 16, 24, 48, 72, 96 and 120 hour)

3.5.2 Replicational 5L fermentation:

Optimum conditions from Phase 2 were chosen to be scaled up to a 7 L benchtop bioreactor system (Eppendorf, Bioflo® 115, USA) with a 5 L working volume. The fermentation with staggered addition of inoculum and lactose was carried out at 37°C controlled by a heating blanket with continuous stirring at 300 rpm for 5 days. The pH was continuously monitored by the system throughout the fermentation and the samples for metabolite analysis were collected at intermediate time points (t= 0, 16, 24, 48, 72, 96 and 120 hour)

3.6 Fermentate metabolite analysis:

3.6.1 Soluble and insoluble fractions:

At the end of the 5-day fermentation, the fermentate from the flasks were transferred into two 50mL falcon tubes and were centrifuged for 15 minutes at 3600 rpm. The supernatant (soluble liquid fraction) was collected separately in another falcon tube. This liquid fraction was frozen at -20°C and freeze dried using free zone freeze dryers (Labconco, US) and further analyzed for lactose and lactic acid. The insoluble fraction was washed with distilled water until the process water reached a neutral pH. The washed insoluble fraction was then freeze-dried and further analyzed for DM and DC.

3.6.2 Concentration of lactose and lactic acid in supernatant:

The samples collected during the fermentation at 0, 16, 24, 72 and 120 hours were analyzed using High Performance Liquid Chromatography (HPLC) (PerkinElmer, Shelton, CT, USA) to estimate the residual lactose and lactic acid present in all fermentations of Phase 1 and Phase 2. The collected samples were diluted at 5× in nano pure water. The diluted samples were acidified with 0.14N H_2SO_4 and centrifuged at 12,700 rpm for 20 minutes at room temperature.

The chromatographic column used for separation of compounds was Aminex HPX-87H (300 × 7.8mm), an organic acid column (Bio-Rad Laboratories, CA, USA). The standard peak area compound identification and quantification of lactose and lactic acid was performed at concentration 2mM – 50mM and 2mM- 100mM, respectively. The diluted samples (20 µL) were eluted in an isocratic mode with 0.014N H_2SO_4 solution at 0.6 mL/min flow rate at 35°C.

3.6.3 Degree of DM:

The DM is the quantity of minerals removed from the lobster shell and it was calculated by determining the ash content of the freeze-dried insoluble fraction of the fermentation. The DM analysis was performed by ashing the lyophilized sample (500 mg) in ceramic crucibles in a muffle furnace (Yamato F0410CR, Yamato Scientific Co., Ltd.) at 500°C for 4 hours (Steeves, 2019). The ashes were weighed immediately after taking the samples out of the furnace, to avoid sample loss. From the weight loss, the DM can be calculated using Equation (3.3) as shown below,

$$\text{DM (\%)} = \frac{[A_s \times S) - (A_r \times R)]}{(A_s \times S)} \times 100 \qquad \text{Eq. (3.4)}$$

Where, A_s denotes the ash content of raw lobster shell, S denotes the weight of raw lobster shell sample before ashing. Ar represents the ash content of the lyophilized insoluble fraction and R is the weight of the lyophilized insoluble fraction before ashing.

3.6.4 Degree of DC:

The degree of DC was determined to evaluate the calcium removal from the insoluble fraction. The calcium content of the residue was analyzed using ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) (NexION 300D, PerkinElmer, USA). For the analysis, the insoluble fraction (25mg) was microwave digested (MARS 6, USA) in 8 M HNO_3 (10mL) solution for 30 minutes at 200°C at 1200W and 800 psi. A calibration standard containing calcium from concentration 10 – 1000 ppb diluted in 2M HNO_3 was used to determine the calcium concentration in the residue.

The DC is estimated by differentiating the calcium present in the insoluble faction and the raw lobster shell. The following equations were used to determine the degree of DC,

$$\text{Calcium in insoluble fraction (mg)} = \frac{\text{Ca2+ concentration of insoluble fraction}\left(\frac{\text{mg}}{\text{L}}\right)\times 0.01\text{L}}{\text{sample digested (25mg)}} \quad \text{Eq. (3.5)}$$

$$\text{Ca}^{2+}\text{ in raw lobster shell} = \frac{\text{Ca2+ concentration of raw shells}\left(\frac{\text{mg}}{\text{L}}\right)\times 0.01\text{L}}{\text{sample digested (25mg)}} \quad \text{Eq. (3.6)}$$

$$\text{Degree of DC} = \frac{\text{Calcium in raw shell (mg)} - \text{Calcium in insoluble fraction (mg)}}{\text{Calcium in raw lobster shell (mg)}} \times 100 \quad \text{Eq. (3.7)}$$

3.6.5 Yield of chitin (Insoluble residue):

The yield of chitin was determined by the weight of lyophilized insoluble fraction remaining after the fermentative process and it was calculated by the equation below,

$$\text{Yield of Chitin (\%)} = \left(\frac{\text{Weight of lyophilized insoluble fraction}}{\text{Weight of inital shell biomass}}\right) \times 100 \quad \text{Eq. (3.8)}$$

The yield of chitin as determined by the above equation, would still have excipients present in the insoluble fraction such as minerals and proteins. Therefore, the reported yield of chitin may anticipate being higher in the current extraction process than the theoretical chitin content of lobster shells.

3.6.6 Statistical analysis:

Mean values and standard deviations were calculated from the experimental data obtained from all the experiments. The Analysis of Variance (ANOVA) was performed using Minitab 19 statistical software (Minitab Inc., USA) at a 95% confidence interval ($\alpha = 0.05$). The comparison

of means was implemented using the Tukey's method at a significance level of 95% ($\alpha = 0.05$). All the Phase 1 and Phase 2 fermentations were performed in experimental duplicates.

Chapter 4: RESULTS AND DISCUSSION

4.1 Lobster shell composition:

The lobster shell composition of the four different particle sizes used in the current research are shown in Table 4.1.

Table 4.1: Lobster Shell composition of four different particle sizes on dry weight basis.

Shell Size	Moisture (%)	Ash (%)	Lipid (%)	Chitin (%)	Protein (%)
2mm - 500µm	5.62 ±0.10	63.82±0.45	0.21±0.01	20.2±0.28	10.15±0.83
500µm - 250µm	5.33±0.25	63.26±0.53	0.22±0.02	20.3±0.14	10.89±0.16
250µm - 125µm	5.62±0.15	63.04±0.49	0.19±0.04	20.1±0.14	11.04±0.44
125µm - 45µm	5.75±0.22	63.97±0.18	0.2±0.02	20.1±0.14	09.98±0.12

Note: The data shown above is from three replicates of different shell size obtained from an individual batch of lobster shells

The compositional analysis on the four different particle sizes of the lobster shell indicated no significant variation in moisture, ash, lipid, protein, and chitin. This indicates that shells of different particle size are similar in composition. The lobster shells are predominantly made of minerals and thus the ash content of the shell is more than half of their dry weight. The reported mineral data agree with those reported in other studies with a mineral content of 50% by weight (Boßelmann *et al.*, 2007; Kaur and Dhillon, 2015). The lipid content in these shells was found to be present in trace amount of <0.3%, which is similar to the lipid content observed in the lobster

shells from *Homarus americanus* by Xu et al. (2020). The drying of shells at 60°C for 24 hours resulted in low moisture content prior to grinding and sieving.

The remaining composition of lobster shell is made up of organic material such as chitin and protein. Chitin and protein content were calculated on an ash-free basis and they did not vary with respect to the particle size (data not shown). The composition of chitin was similar to findings of other studies with chitin content of 20 - 25 % dry weight (Nguyen *et al.*, 2017; Toliba, Rabie and El-Araby, 2016, Morrow, 2002). Among these, Morrow (2002), performed a complete analysis on different seasonal, batch and particle size variations on Nephrops a Norwegian lobster and observed no variations due to season or batches, but however, observed differences in chitin and protein. The protein content of nephrops with particle size <0.5mm was higher compared to that of chitin and increasing particle size showed decreased protein content. In the current study, protein constituents were observed to be ~10%. This value is low compared to those reported in the literature. For example, Hong et al. (2019) reported a protein content of 25.83%, while, Chakravarty et al. (2018) reported 23.54%. The lower protein content can be due to removal of meat from shells (Gulf Nova Scotia Fleet Planning Board, 2019). Note, the protein determination value arises as the difference between the total nitrogen in dried lobster shells determined by a CHNS-analyser (accuracy of this method is ±0.11 of the mean) and the nitrogen content in the isolated chitin (Steeves, 2019).

4.2 Influence of combination of shell, carbon source, and inoculum on pH

A preliminary study was conducted to determine the influence of different ratios of shell, and inoculum (lactic acid bacteria) on pH reduction efficiency, at a constant carbon load. The combinations of shell, lactose, and inoculum for the experiments are shown in Table 4.2.

Table 4.2: pH of different combination of shell, lactose, and inoculum

Exp. No.	Shell (w/v%)	Lactose (w/v%)	Inoculum (w/v%)	pH on Day 5
1	10	10	2.5	6.05±0.54
2	5	10	2.5	5.79±0.86
3	10	10	1	6.15±1.24
4	5	10	1	4.90±0.21

The combinations were selected based on studies conducted by Steeves (2019), and lactose was used a carbon source. The lactose level was kept constant to avoid a substrate limiting condition as the inoculum depends on lactose for energy to grow and produce enough reactant species i.e., lactic acid which in turn reacts with the shell.

The shell size used for the preliminary experiments was <2mm. From the initial experiments, a combination of 5% w/v shell, 10% w/v lactose, and 1% w/v inoculum reduced the pH effectively to 4.90 after 120 h, while the other combinations resulted in a final pH ranging between 5.75 to 6.15. Ineffective acidification at the 10% w/v shell inclusion level could be due to increased inhibition from constituents of the shell. Jung et al. (2006), studied 0-10% glucose and 0-10% v/v of starter culture in crab shell fermentation, these authors observed a decrease in pH concomitant with an increase in the inoculum and substrate level. The authors reported that initial decrease in pH was rapid i.e., pH reduced from 8 to 6 within first 24 h of fermentation, after which the drop in pH was around 1.0 unit, over the next 96 h of fermentation. Reduction in pH is not only important to the acidification but is also desired for the process to be free from spoilage microorganisms in the liquid broth containing the lobster shell waste and other nutrients. A quick reduction in pH is a good indicator of the success of fermentation. Numerous studies have shown that an increased acidification rate at the beginning of the process helps to suppress the growth of

spoilage organisms (Healy, Green and Healy, 2003; Aytekin and Elibol, 2010). In the current study, the best performing combinations were shown to reduce pH by ~ 2 units within the first 36 h. Thus, for all further experimentation the combination ratio of 5:10:1 (shell: lactose: inoculum) was used to study the fermentative extraction of chitin from lobster shells.

4.3 Phase 1: Effect of shell size and protein inclusion on DC and DM

In Phase 1, the effect of shell sizes and whey protein inclusion on lobster shell fermentation was studied to find suitable conditions for maximizing the response variables of DM and DC. Two different kinds of protein source (yeast extract and whey protein) were studied to determine their influence on their efficiency of the fermentation process. An intermediate 2% w/v of yeast and whey protein was added in the fermentation, the pH attained in the fermentation was 4.38 and 4.20, respectively. The presence of lactic acid was found to be higher in whey supplemented fermentation of 6.41 mg/ml with yeast supplementation producing lactic acid of 6.67 mg/ml. On determining the fermentation efficiency, a higher demineralization of 77.27% was achieved in whey assisted fermentation than that with yeast extract, showed demineralization of 68.28%. Comparing the efficiencies, whey protein supplementation was found to be an effective protein source. The Lobster shells of varying size range from 2 mm to 125 μm were studied for fermentative chitin extraction with the different whey protein inclusion levels as shown in Figure 4.1.

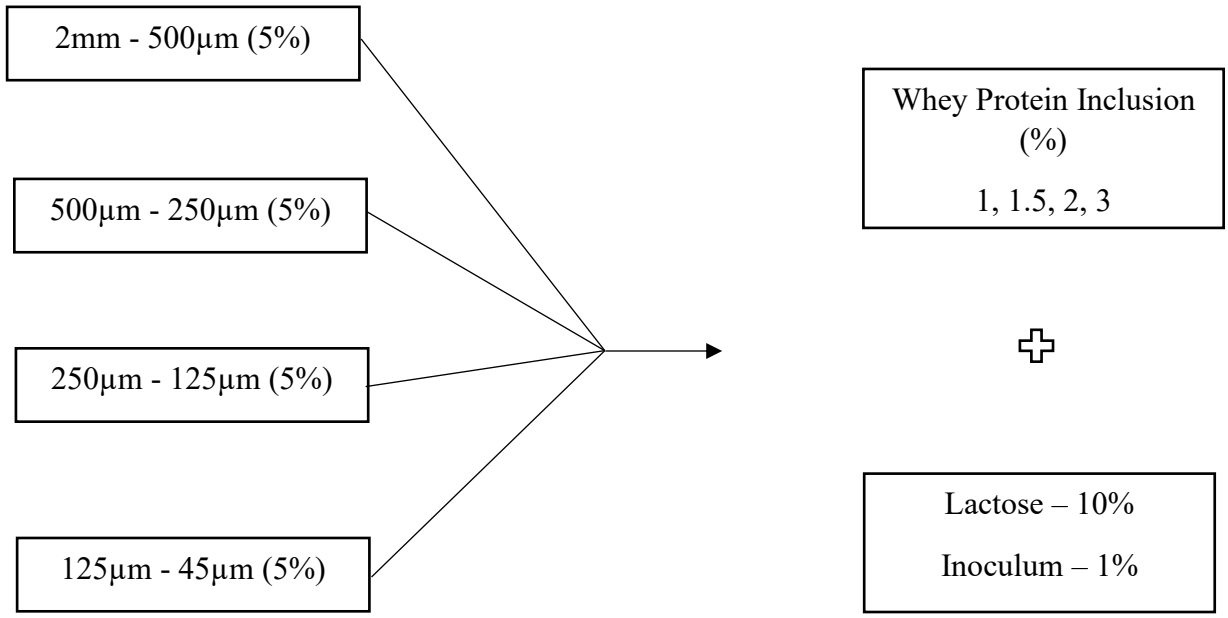


Figure 4.1: Phase 1 fermentation of different shell size and whey protein inclusion with selected combination of shell, lactose, and inoculum.

4.3.1 pH change:

The final pH at 120 hours of the Phase 1 fermentations are shown in Figure 4.2. An acidic pH of ≤4.8 was achieved across different experimental conditions, indicating an effective fermentation process. No spoilage microbes were observed in plating different spent media samples and at least 100 million CFU of Lactic acid bacteria (LAB) was observed across different experimental conditions tested (Figure A 10). According to Rao and Stevens (2006), an acidic medium averts the growth of spoilage microorganisms by making the media selective against these microbes there by controlling the perishability of the waste. A pH of ~4.5±0.5 was achieved with the particle size S1 (2 mm to 500 µm) and S3 (250 µm to 125 µm) after 120 hours while, in the case of particle size S4 (125 µm to 45 µm) the pH drop was up to 4.80±0.26 at all protein inclusion

level between 1 to 3%.

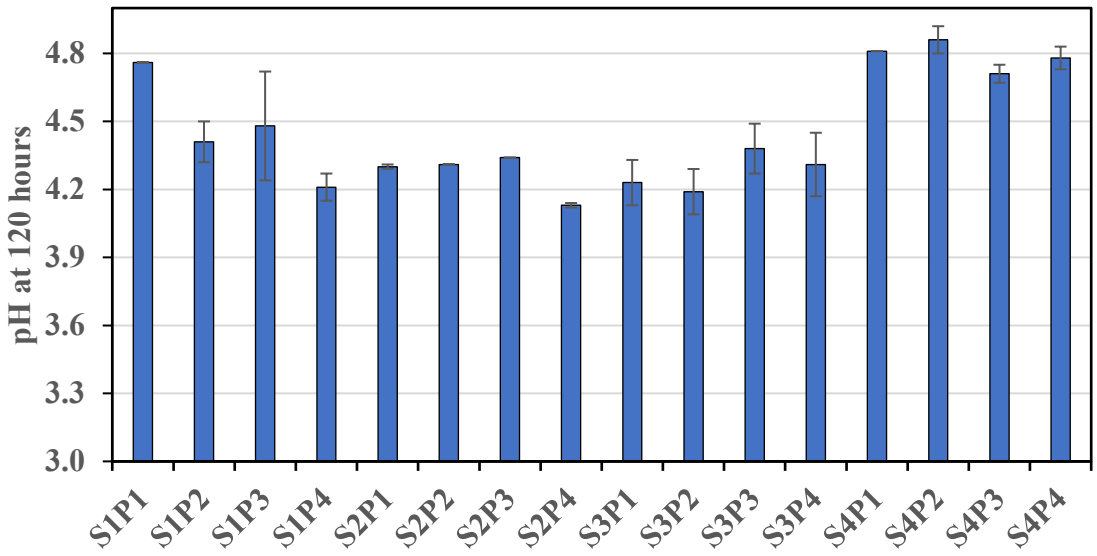


Figure 4.2: Final pH of Phase 1 fermentation experiments. Legends for size: S1 - 2mm - 500µm, S2-500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

A pH of <4.3 was attained in fermentations using particle size S1 (2mm - 500µm) with all protein inclusion rates and pH as low as 4.13 was attained using particle size S2 (500µm - 250µm) at protein inclusion of 3% (P4) (S2P4). The difference in the end pH across various experimental conditions of phase 1 fermentations was due to the changes in growth of LABs and the production of lactic acid. A reduced particle size of shells is said to exhibit high production of organic acid and higher microorganism growth (Mahmoud and Ghaly, 2015). In the current study, this pattern was not observed as the reduced particle size (250 µm – 125 µm, S4 – 125 µm – 45 µm) produced lower lactic acid and lower microorganism growth (Figure A11).

The porosity of the different shell sizes may influence the reaction between the shell and lactic acid produced to alter the microstructure of the lobster shell to breakdown the complex

network (Chakravarty *et al.*, 2018). A significant pH drop was observed in S2P4 fermentation with low level of lactic acid production compared to S1 fermentations which had higher lactic acid.

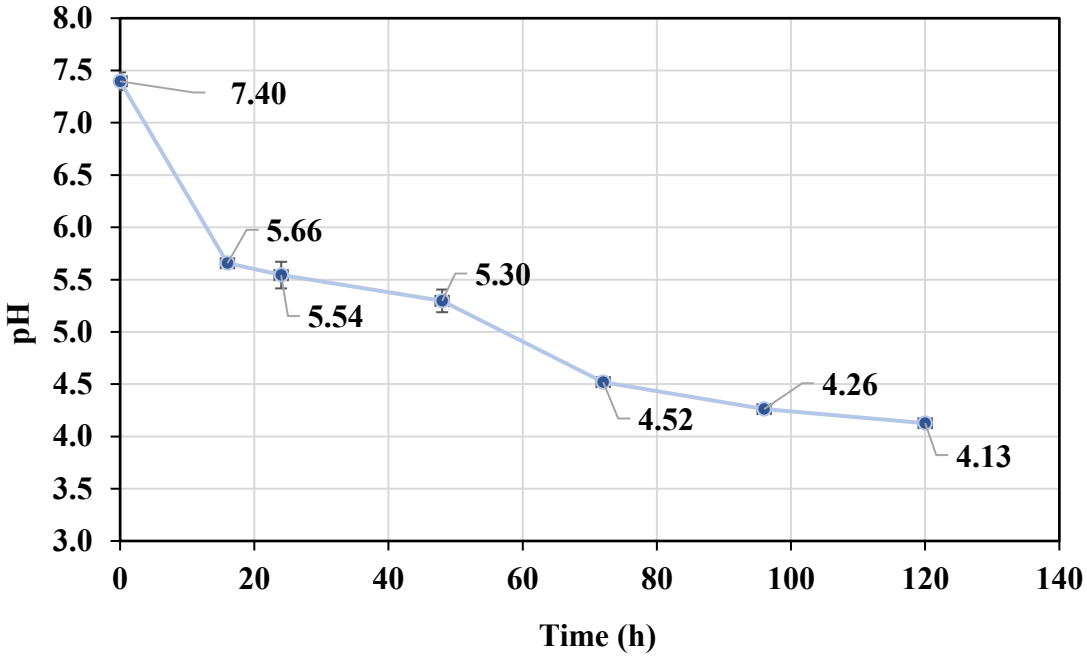


Figure 4.3: pH drop during fermentation of S2P4. Legend: S2 – 500 µm – 250 µm. P4: 3%.

4.3.2 Concentration of lactose and lactic acid

The residual levels of lactose unutilized after 120 hours are shown in Figure 4.4 and the chromatogram are shown in Appendix (Figure A11-A16). An optimum substrate to inoculum ratio (S/I) is critical for an effective anaerobic fermentation (Tan, Lee and Chen, 2020). In general, a lower substrate to inoculum ratio favors anaerobic fermentation because, for an S/I over 1.0 a higher accumulation of volatile fatty acid is observed due to high conversion of initial substrate, which in turn inhibits the fermentation (Tan, Lee and Chen, 2020). In contrast, in the current study, S/I ratio of 10 was used to achieve a higher conversion rate of lactose to lactic acid, which in turn results in a lower pH and an increased DM. Steeves (2019), observed that S/I ratio of 10 gave

superior results in terms of pH reduction and lactate production over S/I ratio of 2, where 1% inoculum was used in the former and a 5% inoculum was used in the latter. The data shown in the Figure 4.4 presents the three conditions under which the residual lactose was detected. These data show that there were trace amount of residual lactose of less than 5 mM in the spent medium after 120 hours of fermentation.

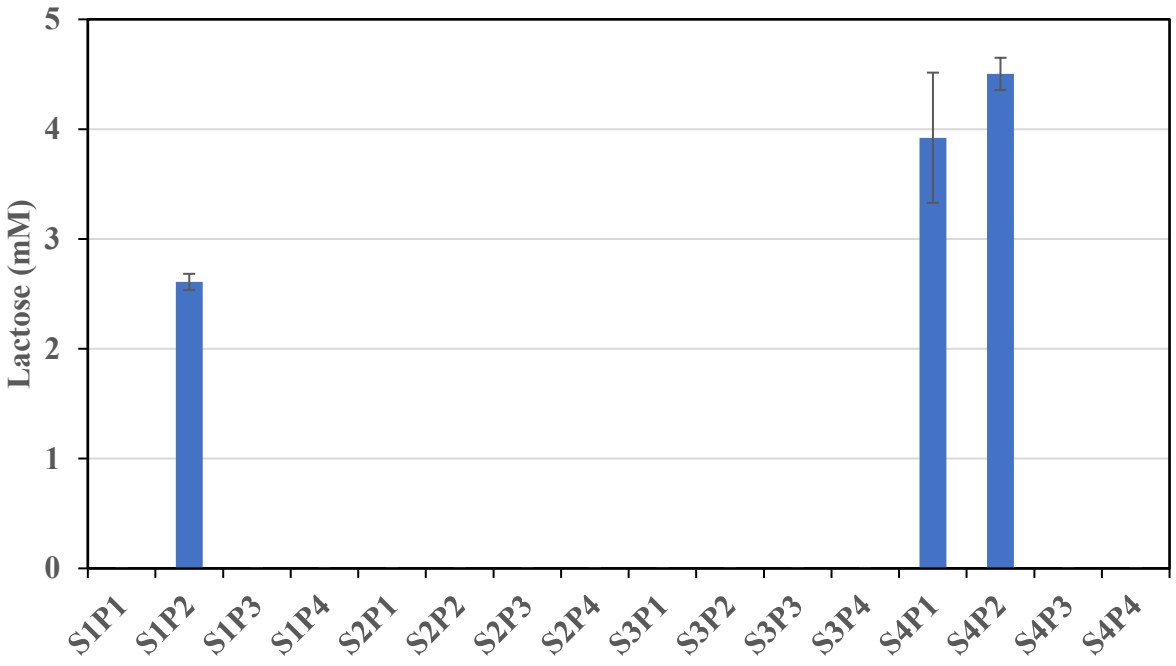


Figure 4.4: Concentration of Lactose (mM) unutilized in the Phase 1 fermentation after 120 hours. Legends for size: S1 – 2 mm – 500 µm, S2- 500µm – 250 µm, S3 – 250 µm – 125 µm, S4 – 125 µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

Figure 4.5 shows the concentration of lactic acid present at the end of fermentation. Higher production of lactic acid tends to result in a lower pH medium (Yadav *et al.*, 2019) but contrastingly the fermentations using S1 particle size had higher end pH with higher production of lactic acid. On the other hand, the pH of fermentations with particle size S2 and S3 were lower than S1 though the lactic acid production were low. Chakravarthy et al (2018), suggested that the

shells after treatment with inoculum in presence of nutrients, would have increased the porosity of the shell by fracturing the complex structure of shell and improved the fermentation efficiency (Chakravarty *et al.*, 2018). The reaction between calcium and lactic acid produced in S2 fermentation helped in the reduction of pH to 4.13 in presence of higher nitrogen content which further improved the bacterial metabolism to increase the process efficiency (Kemsawasd *et al.*, 2015).

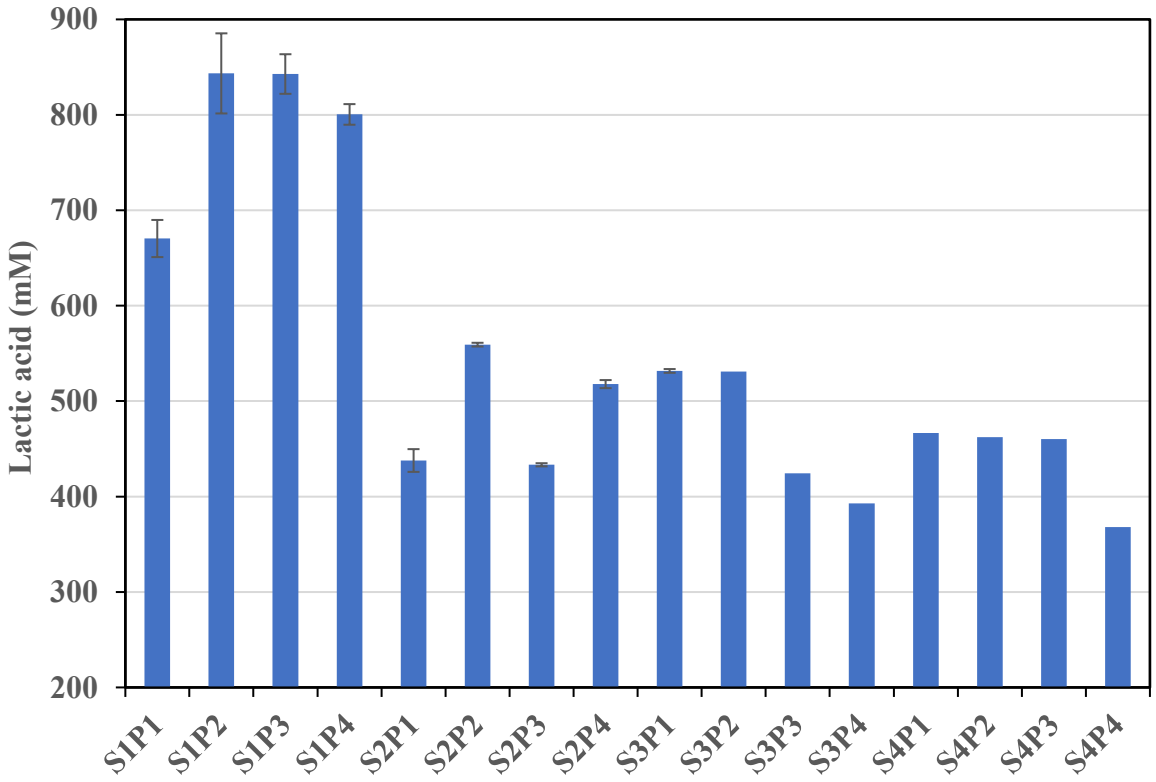


Figure 4.5: Concentration of Lactic acid (mM) produced in the Phase 1 fermentation after 120 hours. Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

4.3.3 Degree of DM:

The degree of DM achieved in the Phase 1 fermentations are shown in Figure 4.6. The degree of DM depends on the reaction of lactic acid with calcium carbonate resulting in formation

of calcium lactate (Kaur and Dhillon, 2015a). The calcium precipitate present in the insoluble fraction is then removed by washing to neutral pH. From the Figure 4.6, a high degree of DM was achieved in S2P4 of 83.91%. A larger surface area favors rapid reaction of lactic acid with reactants in this case the shells, while larger particle sizes result in the low conversion (DM) of reactants. In the current study, particle size S2 has shown higher degree of DM in the presence of 3% nitrogen inclusion. As mentioned in section 4.3.2, larger surface area of shell S2 (500 µm-250 µm,) may have resulted in an increased interaction between the lactic acid and the shells and thereby forming calcium lactate precipitate.

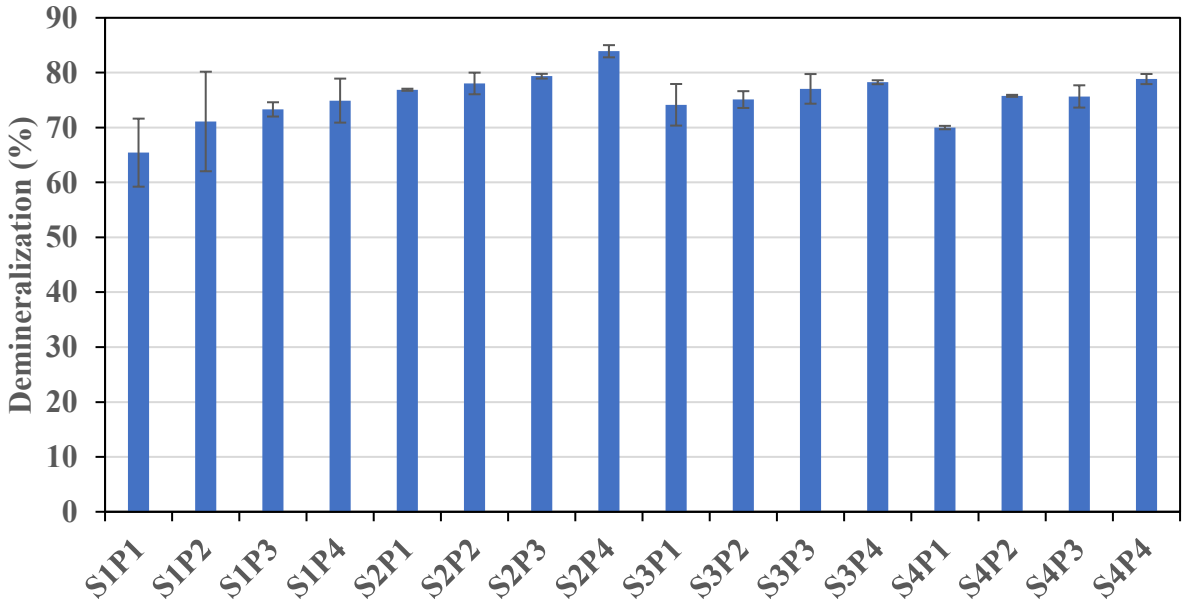


Figure 4.6: Degree of Demineralization of phase 1 fermentations. Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

The degree of DM attained in all phase 1 fermentations are comparable to demineralization data of crustaceans found in the literature studies, ranging from 55.2 – 97.2% using lactic acid bacterial fermentation (Arbia *et al.*, 2013; Kaur and Dhillon, 2015; Castro, Guerrero-legarreta and Bórquez, 2018). The DM of shells with particle size S1, S2, S3 and S4 was ranging from 65.43 –

83.91%. A higher DM was obtained with shell size S2 at all protein inclusion (P1- P4) varying between 76 – 83%.

4.3.4 Degree of DC:

The DC is a part of the DM, where DC specifically assesses the removal of calcium, as the shells are predominantly made of calcium constituting approximately 60% dry weight (Nguyen, Barber, Corbin, *et al.*, 2017). The degree of DC of phase 1 fermentations are shown in Figure 4.7. The degree of DC is similar to the degree of DM with high a DC of 84.56% achieved in S2P4 fermentation as a result of increased surface area.

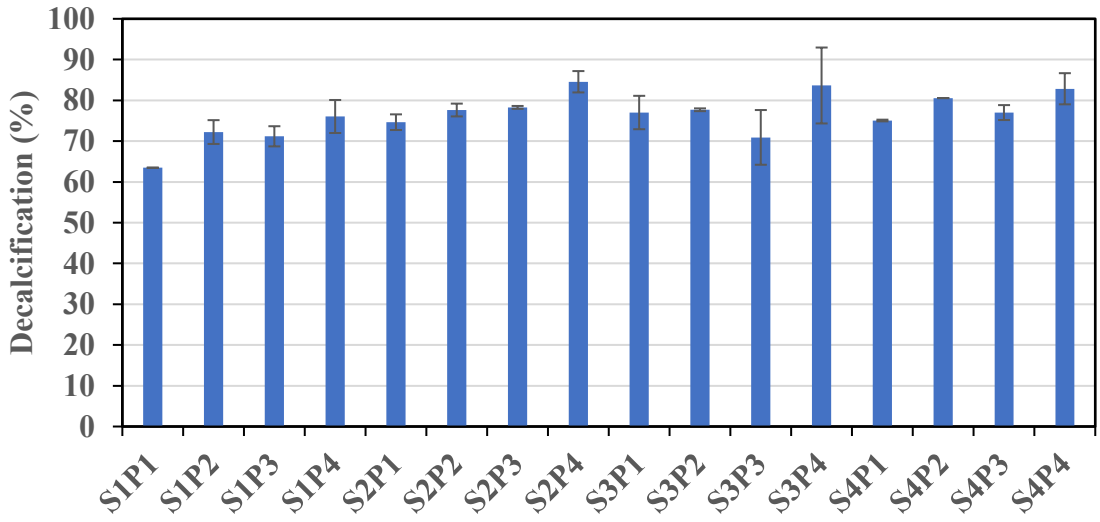


Figure 4.7: Degree of Demineralization of phase 1 fermentations. Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

The DC was ranging from 63.47 – 84.56%, similar to the range of DM as majority of shell are composed of calcium. The equivalent data of DM and DC signifies that the calcium is the significant portion of the mineral component present in lobster shells and the calcium was removed by the lactic acid bacterial fermentation process.

4.3.5. Chitin yield

The percent recovery of the insoluble fraction was determined as the chitin yield, shown in Figure 4.8.

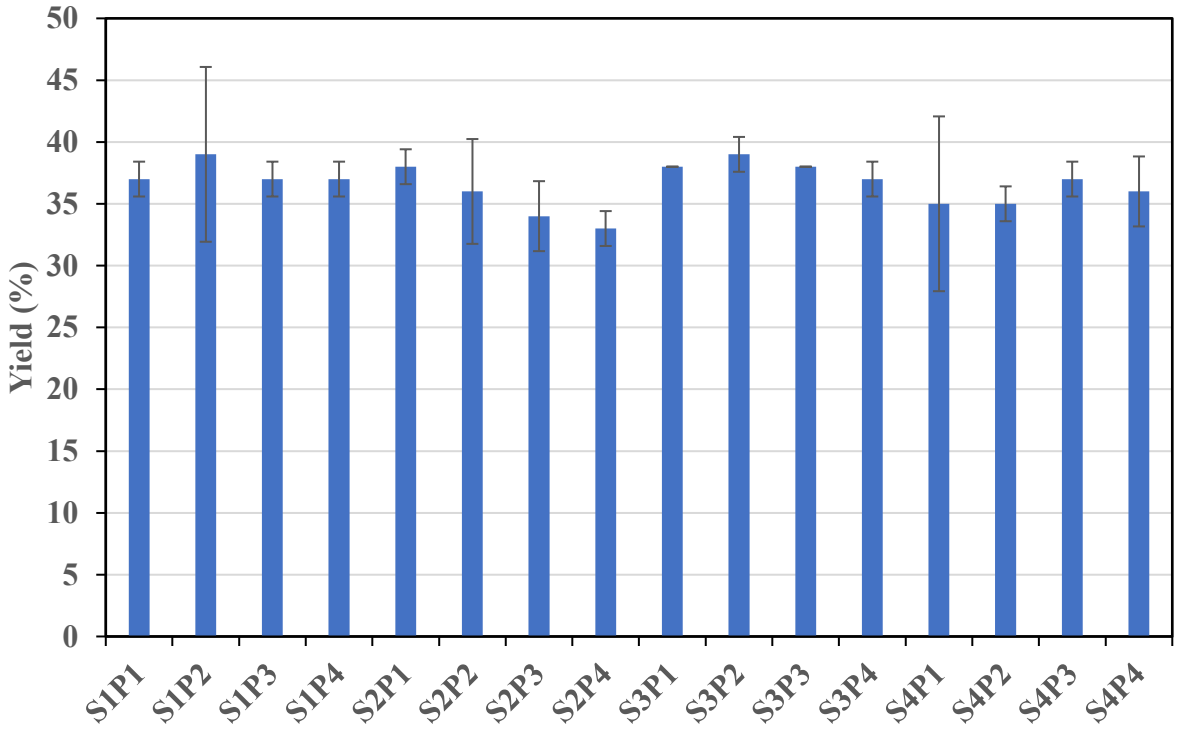


Figure 4.8: Chitin yield of phase 1 fermentations. Legends for size: S1 - 2mm - 500µm, S2-500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

The chitin yield achieved ranged from 33% to 39% which are comparable with the values of chitin yield reported in case of lobster shells, ranging from 20 – 30% dry weight (Chakravarty *et al.*, 2018). The reported chitin yield in the current study is marginally higher than the literature values due to presence of proteins or minerals in the insoluble fraction that were not thoroughly removed in the fermentation process (Lopes *et al.*, 2018).

A yield of 33% was achieved in S2P4 as a result of higher DM as discussed in the above sections. A yield closer to the proximate number signifies high purity of chitin with the presence of low residual proteins or minerals in the final product (Chakravarty *et al.*, 2018). As shown in the Table 4.1, the proximate content of chitin was 20% in the lobster shell used in the current study with complete removal of proteins and minerals. A 39% crude chitin yield obtained in the fermentation using particle size S3 and a nitrogen inclusion of 1% indicates unsuccessful fermentation process, with less removal of proteins and minerals from the shell waste. This could be due to lower nitrogen inclusion or a combination of low nitrogen inclusion and the shell size.

4.3.6. Statistical analysis

A factorial design analysis was performed at a confidence interval of 95% to determine the significance of two independent factors (size and protein inclusion) on the response variable, in this case, degree of DM. The f-values and p-values for particle size and protein inclusion in the regression model for DM are summarized in the Table 4.3. Based on the p- values, the shell size and protein inclusion are both statistically significant factors with respect to DM ($\alpha \leq 0.05$). The model signifies that shell size and protein inclusion has direct influence on DM. The R^2 value of the regression model is 81.92%, representing the data fit to the model.

Table 4.3: Analysis of Variance (ANOVA) of the regression model from the factorial design on response to demineralization. Model summary: S = 1.83, R^2 = 81.92%, R^2 (adjusted) = 74.69%, R^2 (predicted) = 60.58%, P<0.05 significant level.

	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	304.095	38.012	11.33	0.000
Linear	4	272.481	68.120	20.30	0.000
Protein	1	137.571	137.571	41.00	0.000
Size	3	168.828	56.276	16.77	0.000
Square	1	2.828	2.828	0.84	0.370
Protein*Protein	1	2.828	2.828	0.84	0.370
2-Way Interaction	3	9.324	3.108	0.93	0.446
Protein*Size	3	9.324	3.108	0.93	0.446
Error	20	67.115	3.356		
Lack-of-Fit	6	16.780	2.797	0.78	0.601
Pure Error	14	50.334	3.595		
Total	28	371.210			

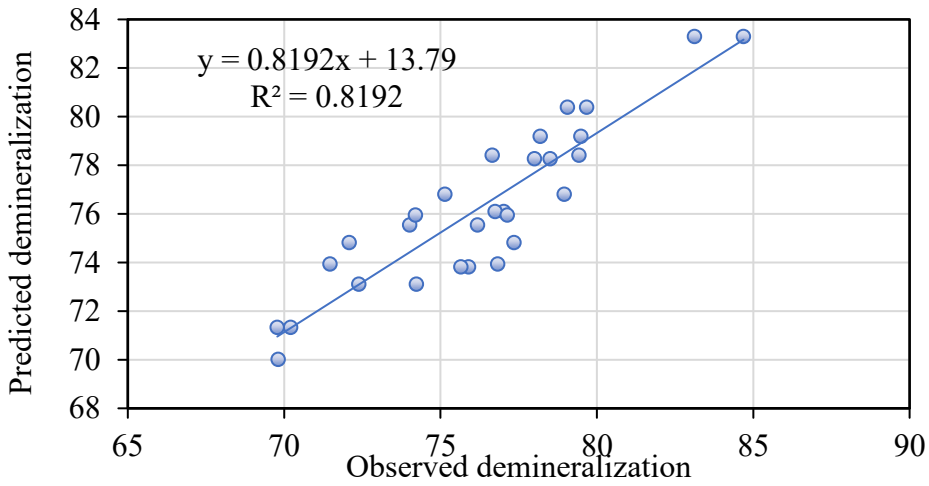


Figure 4.9: The coefficient of determination for the predicted demineralization vs. observed demineralization.

The p- value of 'lack of fit' is insignificant representing that the model does not detect any lack of fit and thus the model was genuinely able to specify the relationship between the observed and predicted DM as shown in Figure 4.9. The insignificance in 'lack of fit' also indicates that the experimental factors and the factor levels chosen are able to determine the predicted DM and fit well within the model.

The contour plot determines the suitable or optimal factor range of size and protein inclusion in response to DM as shown in Figure 4.10. The shell particle sizes are represented as a categorical factor, as the sizes are not a single value, but a range of values. It is seen that high DM of >80% is attained at size S2 and S3 with protein inclusion of 3%.

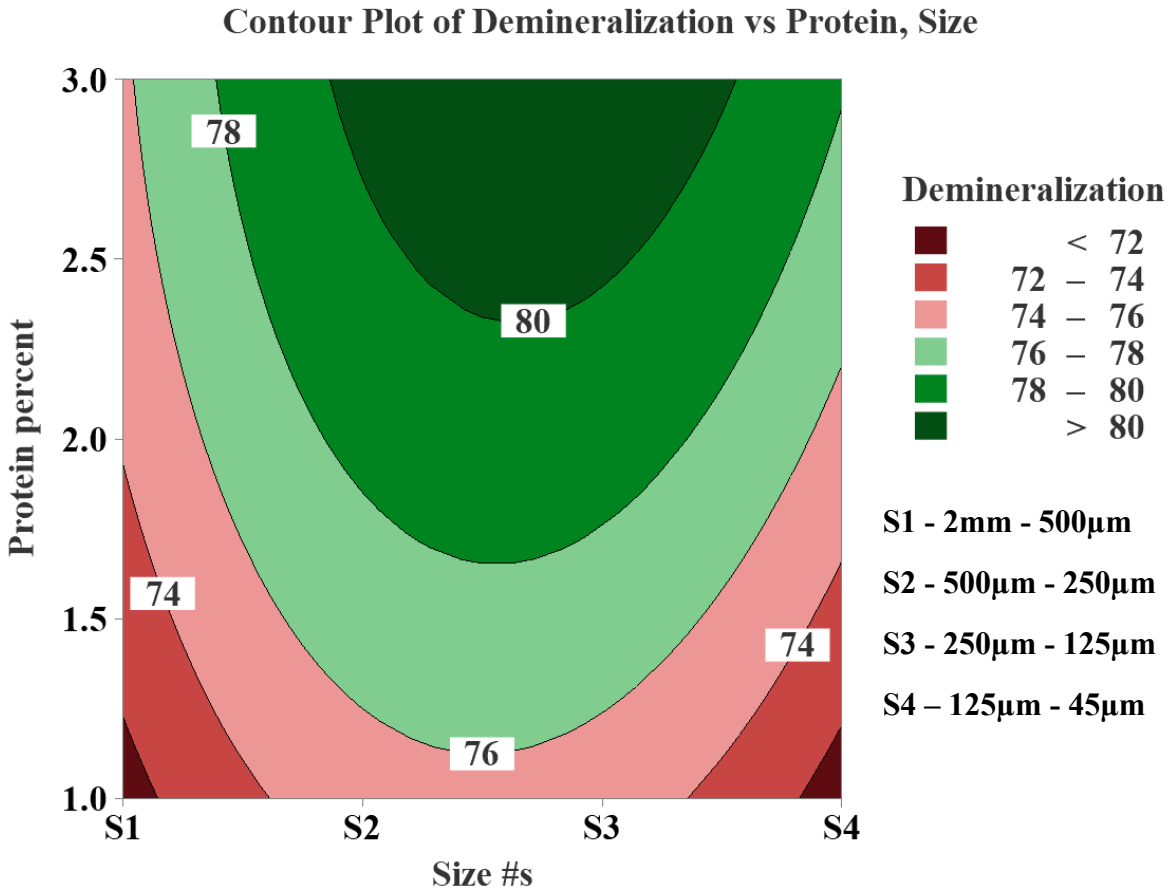


Figure 4.10: Contour plot (Size, protein inclusion vs. Demineralization)

The main effect plot shows a clear representation on the best shell size and protein inclusion with response to DM as seen in Figure 4.11. A high DM of >80% (fitted mean) was achieved at shell size S2 (500 µm – 250 µm) with protein inclusion of 3%.

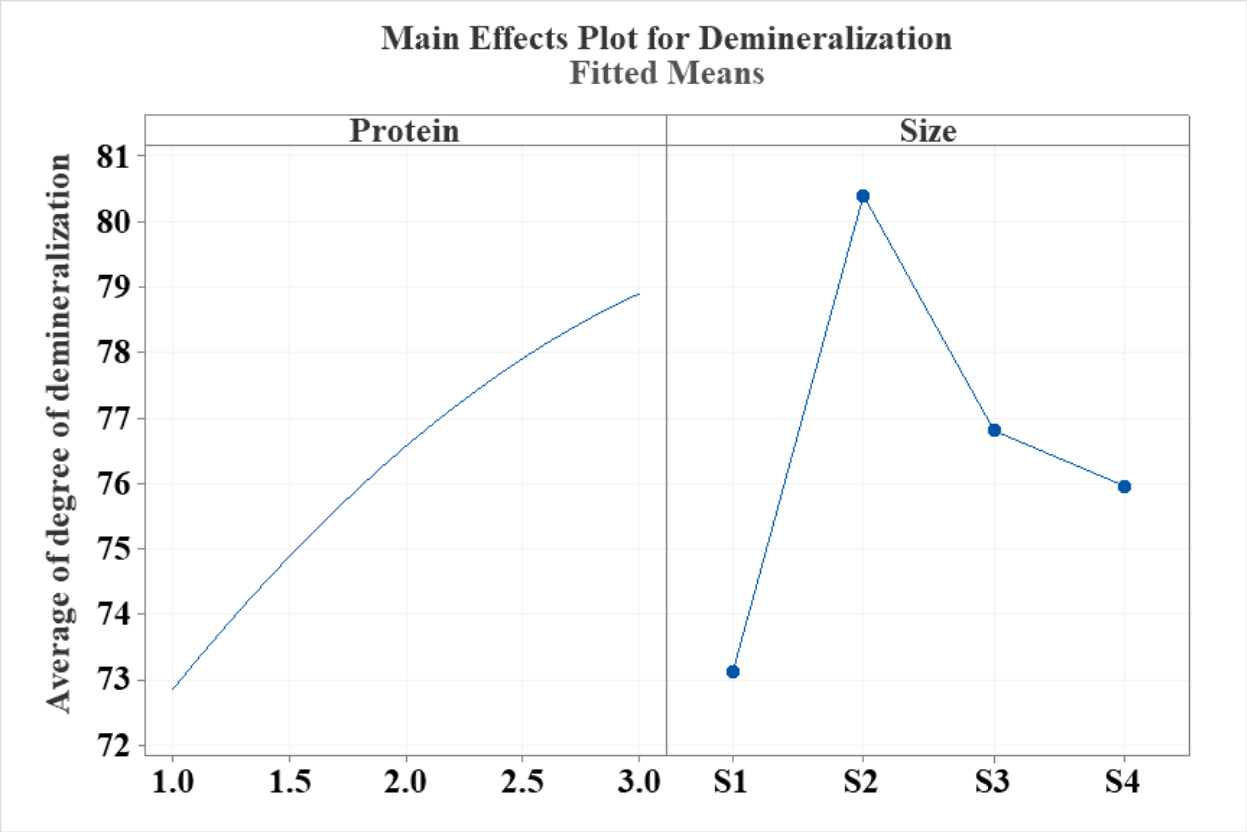


Figure 4.11: Main effect plot of demineralization vs. shell size and protein inclusion

The statistical analysis of the Phase 1 fermentations showed that S2P4 had a maximum effect on DM. Oh et al (2007), studied the relationship between DM and carbon source, pH, and protein individually and found the DM has a strong relationship with protein in the medium and pH with a correlation coefficient (r^2) of 0.620 and 0.793, respectively. Similarly, a higher DM percent was achieved in the current study with a high protein inclusion in the medium and a low pH at the end of fermentation.

From the experimental data and statistical analysis of Phase 1 fermentation, the best efficient size range and appropriate protein inclusion for the fermentation in response to DM was found to be 500µm-250µm with 3% protein in the medium. The formulation of S2P4 was further tested in the Phase 2 fermentation with staggered addition of carbon source and inoculum, where the primary intent was to further increase the efficiency of the fermentation process and purity of chitin.

4.4 Phase 2: Effect of staggered addition of carbon source and inoculum

The Phase 2 fermentation was performed using the shell size 500µm - 250µm with protein inclusion of 3% on four different variations along with microwave pre-treatment and staggered fermentation. The different variations of staggered addition fermentation are described below,

i. 72 - hour staggered addition of inoculum, (S2P4-A).
ii. 72 - hour staggered addition of inoculum and carbon source, (S2P4-B).
iii. 48 - hour staggered addition of inoculum and carbon source, (S2P4-C).
iv. Microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

The different time intervals at which staggered addition were based on lactic acid concentration in the medium during the fermentation (refer to Section 4.4.1 and 4.4.2). The Phase 2 of the fermentations with the best fermentation efficiency was then scaled-up to 5L to further validate as the results in a larger scale.

4.4.1 Change in pH

The final pH (120 hour) of the phase 2 fermentations are shown in Figure 4.12. A final pH of 4.2 was obtained with S2P4-A fermentation, which is marginally higher than S2P4

fermentation, where a final pH of 4.13 was obtained. Possibly, the high lactose concentration with S/I ratio of 20 caused osmotic stress to the lactic acid bacteria which in turn triggered the bacteria to stabilize their growth by adjusting the metabolism for an increased substrate inhibition. A similar phenomenon was observed in studies conducted by Alteriis *et al.*, 2018 and Papadimitriou *et al.*, 2016, where these authors incorporated high glucose level in fermentation in the presence of *Lactobacillus* sp. which caused a metabolic shift in the glucose consumption to survive the carbon source rich environment and produced low lactic acid. To avoid stress response, staggered additions of both carbon source and inoculum were incorporated (S2P4-B). In this case, a pH of 4.36 was achieved in S2P4-B which was higher than S2P4-A due to complete depletion of lactose at 72 hours in response to which the pH did not drop between 60 - 72 hours (Figure 4.14) and thus the fermentation efficiency was not up to other phase 1 and phase 2 fermentation. In fermentations S2P4-C and S2P4-D, staggered addition time was conducted at 48 hours to overcome the depletion of lactose, and by doing so, a low pH of 4.07 and 4.10 was achieved, respectively (Figure 4.13).

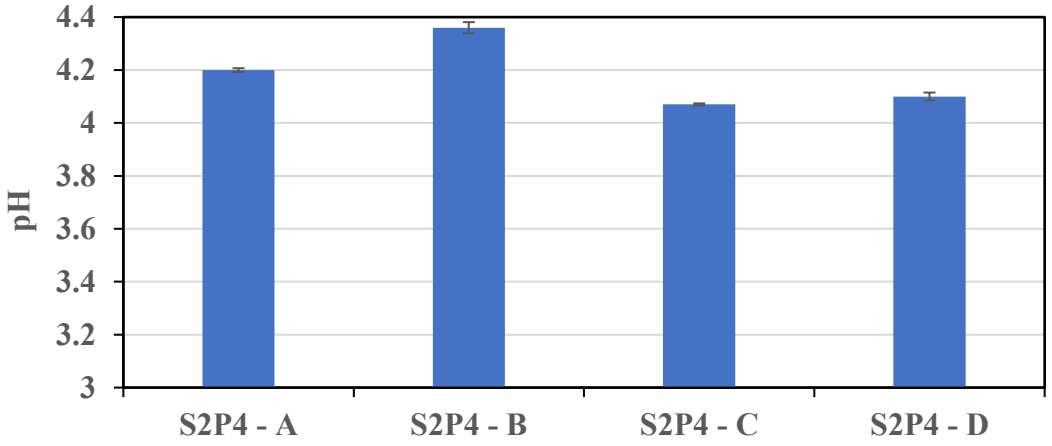


Figure 4.12: Final pH of Phase 2 fermentations. , (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

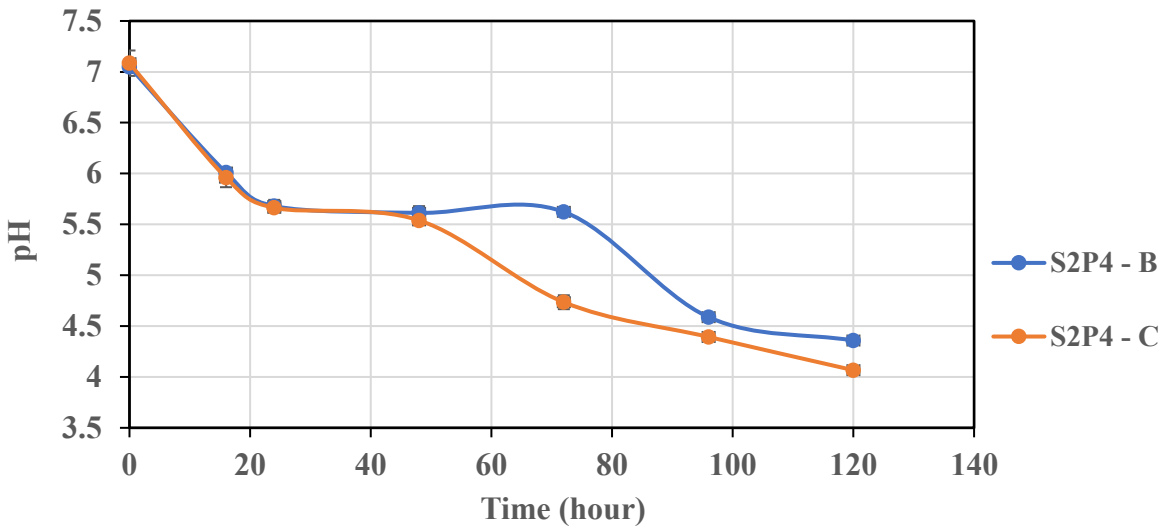


Figure 4.13: pH drop of S2P4-B and S2P4-C during the course of fermentation. (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C),

4.4.2 Concentration of lactose and lactic acid

The concentration of lactic acid produced during staggered addition fermentation is shown in Figure 4.14. In the case of staggered addition, the production of lactic acid was higher while the pH was reduced as low as 4.07. It is postulated that the production of lactic acid is higher in staggered addition because of lower osmotic stress due to less lactose on the bacteria and thus lactose is consumed by bacteria to produce high lactic acid (Glaasker *et al.*, 1998; Steeves, 2019). Comparing the lactic acid production of S2P4 in Phase 1 and Phase 2, the production increased from 517.85 mM (S2P4) to 858.82 mM (S2P4-C) indicating that staggered addition improved the lactic acid production and thereby other fermentation characteristics. Liu, Liao and Chen (2008), studied the comparison between the batch and fed batch fermentation for lactic acid production and chitin co-production and found production of lactic acid of 140g/l in fed batch whereas batch production yielded 113g/l resulting higher chitin production from 31% to 86% for fed batch fermentation.

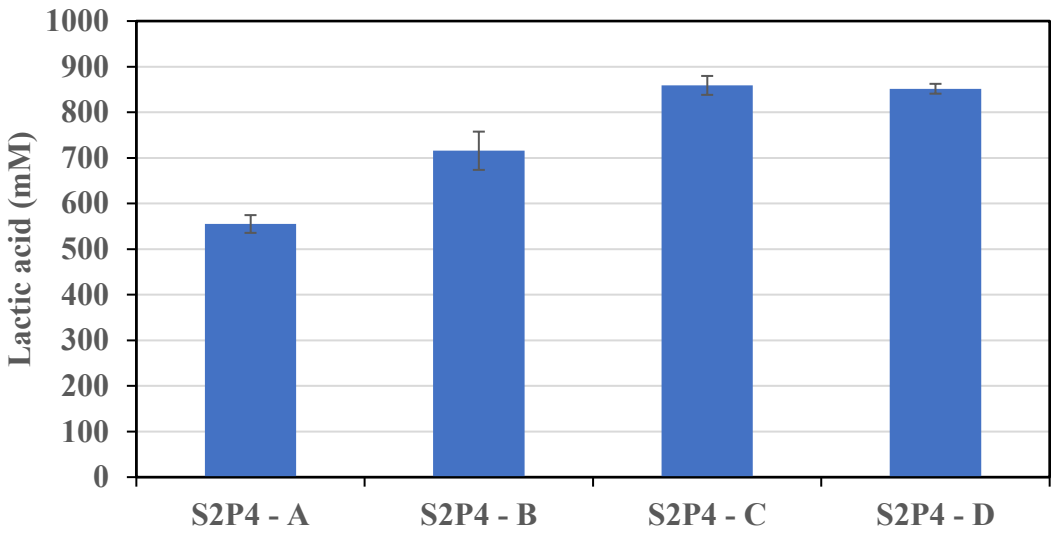


Figure 4.14: Concentration of Lactic acid (mM) produced in the Phase 2 fermentation after 120 hours. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

The concentration of lactose remaining in the fermentation after 120 hours is shown in Figure 4.15. A trace amount of lactose was unconsumed by the bacteria in all Phase 2 fermentations but the concentration of lactose unused in S2P4-B was highest with 8.73 mM due to complete depletion of lactose after 72 hours, which probably negatively affected the vitality of cells in the absence of nutrients and leading to a slower metabolic rate in Phase 2 of the fermentation. This delayed or poor consumption rate of lactose resulted in a marginally higher end pH and high residual lactose in the spent medium. Though the lactose remaining in S2P4-A medium is lower than S2P4-B, the lactic acid production was higher in S2P4-B than S2P4-A due to the staggered addition of lactose and inoculum which reduced the stress of bacteria for lactose consumption, whereas only the inoculum was staggered in S2P4-A fermentation.

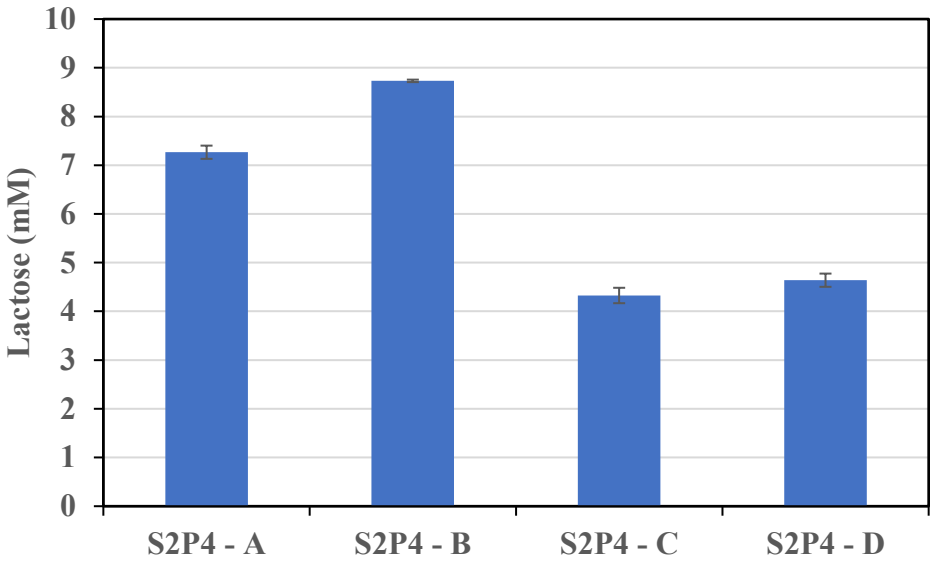


Figure 4.15: Concentration of Lactose (mM) unutilized in the Phase 2 fermentation after 120 hours. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

Figure 4.16 shows the lactose consumption in S2P4-B during the course of fermentation. The staggered addition of lactose and or inoculum resulted in less consumption of lactose compared to Phase 1 fermentation trials, as nearly all the fermentation trials in Phase 1 had <5.0 mM of residual lactose present in the system while at least two trials in phase 2 fermentation still had significantly increased level of lactose unused in the medium. As discussed in section 4.4.1, presence of high lactose at the start of fermentation in Phase 1 did not produce high amounts of lactic acid due to metabolic shift in lactose consumption while the phase 2 staggered addition lowered the stress of the lactose and produced higher lactic acid.

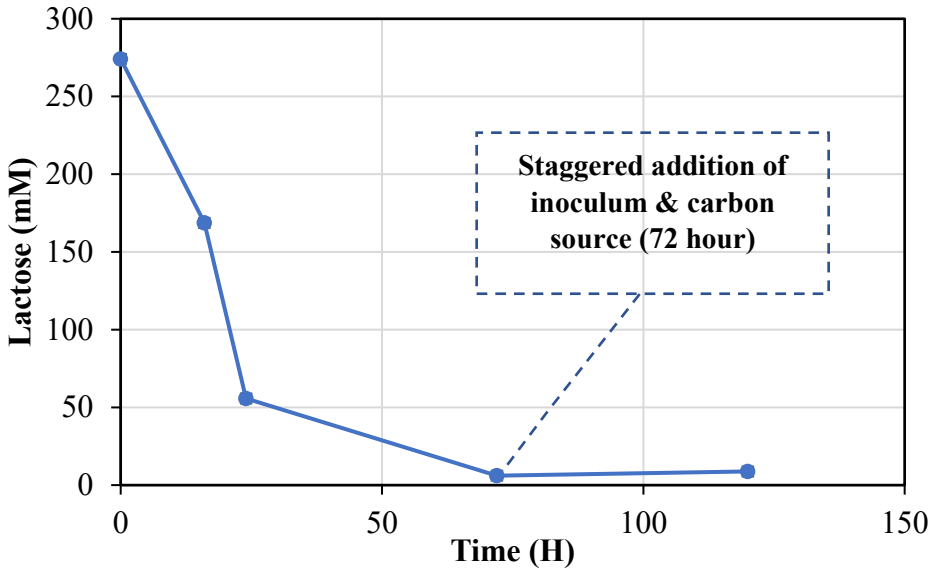


Figure 4.16: The lactose consumption of S2P4-B during the course of fermentation.

4.4.3 Degree of DM and DC

The degree of DM achieved in the Phase 2 fermentations are shown in Figure 4.17. The higher production of lactic acid and high surface area of the shell facilitated increased interaction between the lactic acid and calcium carbonate allowing a high DM of 89.83% for S2P4-C. An equivalent DM of 89.55% was also achieved for S2P4-D, signifying that microwave pre-treatment did not have any effect on DM in comparison to 48 - hour staggered addition of inoculum and carbon source, (S2P4-C). The fermentations S2P4-A and S2P4-B attained DM values of 87.08% and 83.62 %, respectively. Due to the complete depletion of lactose during the fermentation of 72 - hour staggered addition of inoculum and carbon source (S2P4-B), the pH was not reduced effectively due to low lactic acid production which in turn resulted in a lower DM of 83.62%. The degree of DM achieved in Phase 2 experiments are comparable to literature values ranging between 60.0 – 99.6% DM (Adour, Arbia and Mameri, 2008;Castro, Guerrero-legarreta and Bórquez, 2018). Adour et al (2008) studied chitin extraction of shrimp waste using lactic acid fermentation

on shrimp waste using date juice as the carbon source and *Lactobacillus* Sp. but due to presence of calcium content in the date juice, the calcium from the shells were not diffused to the medium which reduced the acidification process contributing to lower DM of 60%. Similarly, Castro et al (2018) extracted chitin using same process but using sucrose as carbon source, the extracted chitin was further purified with chemical to attain higher DM of 99.6% but in current study a high DM of 89.83% was attained using fermentative lactose and without use of chemicals for improving the purity of final product.

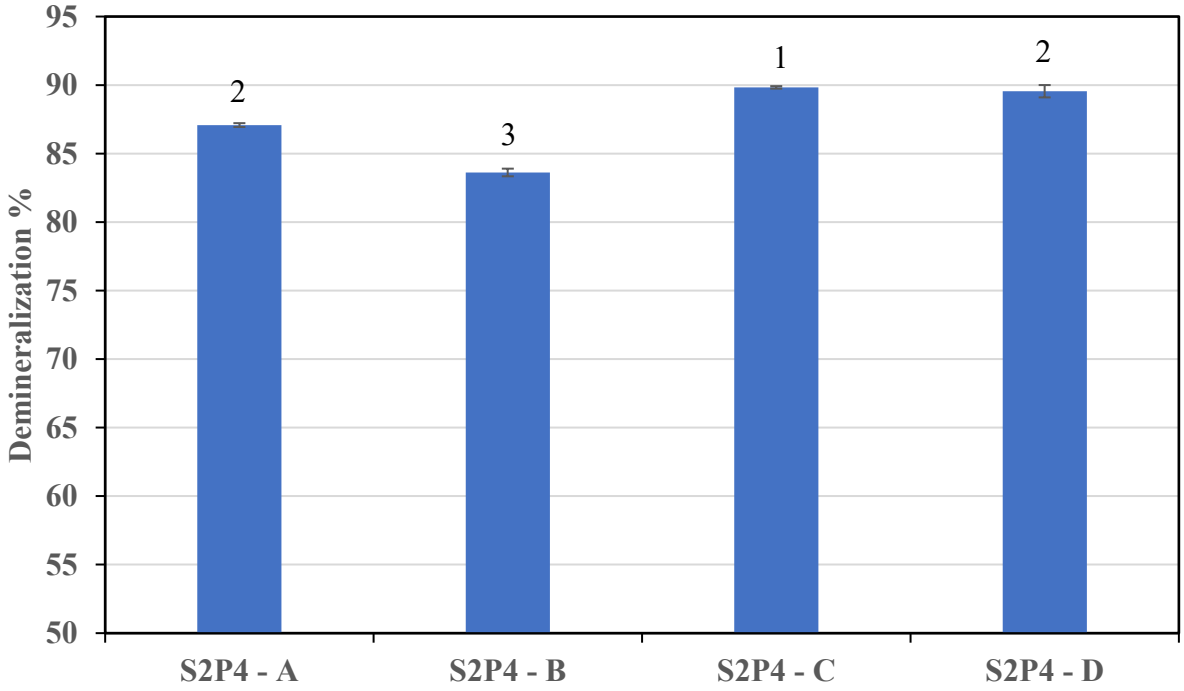


Figure 4.17: Degree of Demineralization in phase 2 fermentation. Data points with different numeric show significant difference based on Tukey method. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

Similar to the results for DM efficiency, degree of DC as observed in the fermentations is shown in Figure 4.18. A high DC of 88.67% and 88.23% were achieved in S2P4 – C and S2P4 – D, respectively. The S2P4 – A and S2P4 – B attained a DC of 86.89% and 82.70%, respectively.

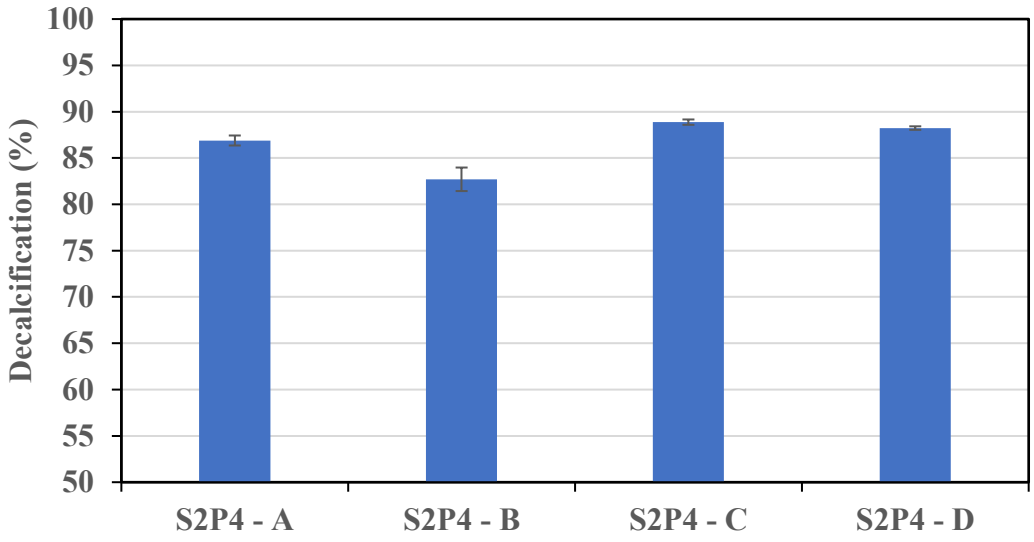


Figure 4.18: Degree of Decalcification in phase 2 fermentation. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

4.4.4 Chitin yield

The total weight of the insoluble fraction (dry weight) at the end of the fermentations is designated as the yield of chitin which is shown in Figure 4.19.

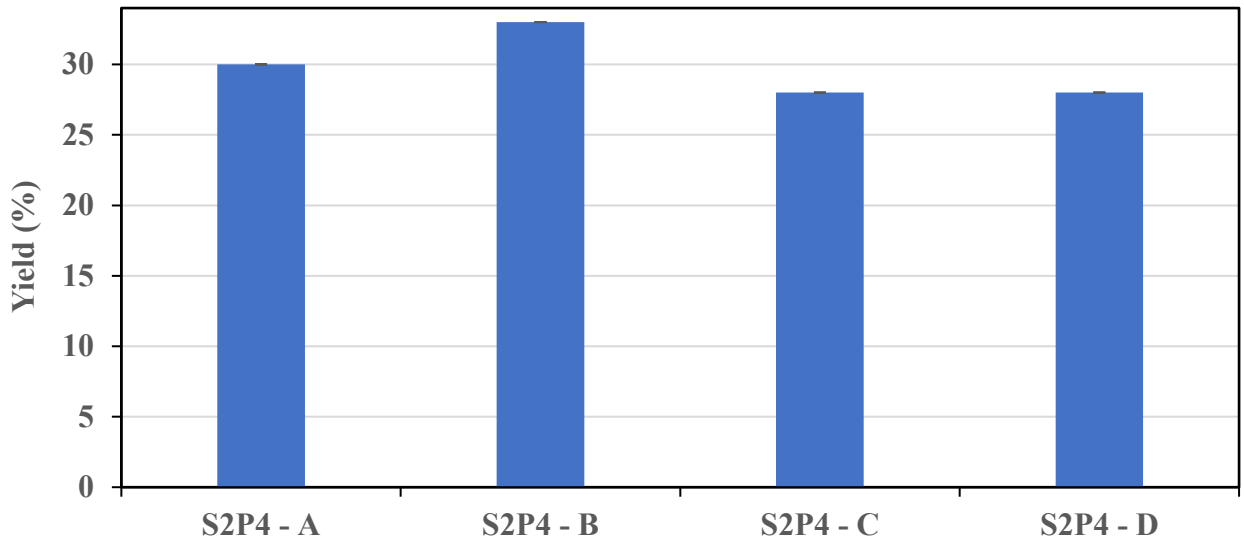


Figure 4.19: Chitin yield of Phase 2 fermentations. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – hour staggered addition of inoculum and carbon source, (S2P4-D).

The chitin yield of the Phase 2 fermentations ranges from 28% to 32% which corresponds well with the chitin content of lobster shells found in the literature (Chakravarty *et al.*, 2018; Nguyen *et al.*, 2017). As shown in Table 4.1, the actual chitin yield reported in pre-fermentation samples was 20%, while a yield of 28% was achieved in both S2P4-C and S2P4-D indicating that the remaining portions of the insoluble fraction are likely excipients such as residual proteins and minerals, which were neither demineralized nor deproteinized during the fermentation. A 32% yield in the Phase 2 fermentation was attained in S2P4-B. This higher value of chitin yield was due to marginally low degree of DM as compared to other fermentations in Phase 2. In general, the higher the degree of DM, the lower the presence of excipients in the insoluble fraction and vice-versa.

4.4.5 Scale-up fermentation

In the scaled-up fermentation, staggered addition of inoculum and carbon source at 48 hours (S2P4-C) was performed, as the DM and DC was 89.83% and 88.67%, respectively, in the small-scale Erlenmeyer flasks. The fermentation S2P4-D also achieved a similar degree of demineralization and deproteinization but since the addition of microwave pre-treatment on shells did not improve outcomes in comparison to the staggered addition at 48 hours, it was not scaled up.

The S2P4-C was scaled- up to 5 L with the same ratio of shell, lactose, inoculum, and nitrogen source as mentioned in staggered fermentations (being 5%, 10%, 1% and 3%). The fermentation conditions in the scale-up fermentation were able to be more consistently controlled (temperature and anerobic conditions) than Phase 1 and staggered addition fermentations at the small scale. However, a complete anaerobic condition was not achieved since staggered addition of lactose and carbon source required opening of ports during the fermentation in the fermenters. After 24 hour of fermentation, production of foam was observed in the bioreactor due to release of carbon dioxide by the dissolution of carbonate in the lobster shell with lactic acid.

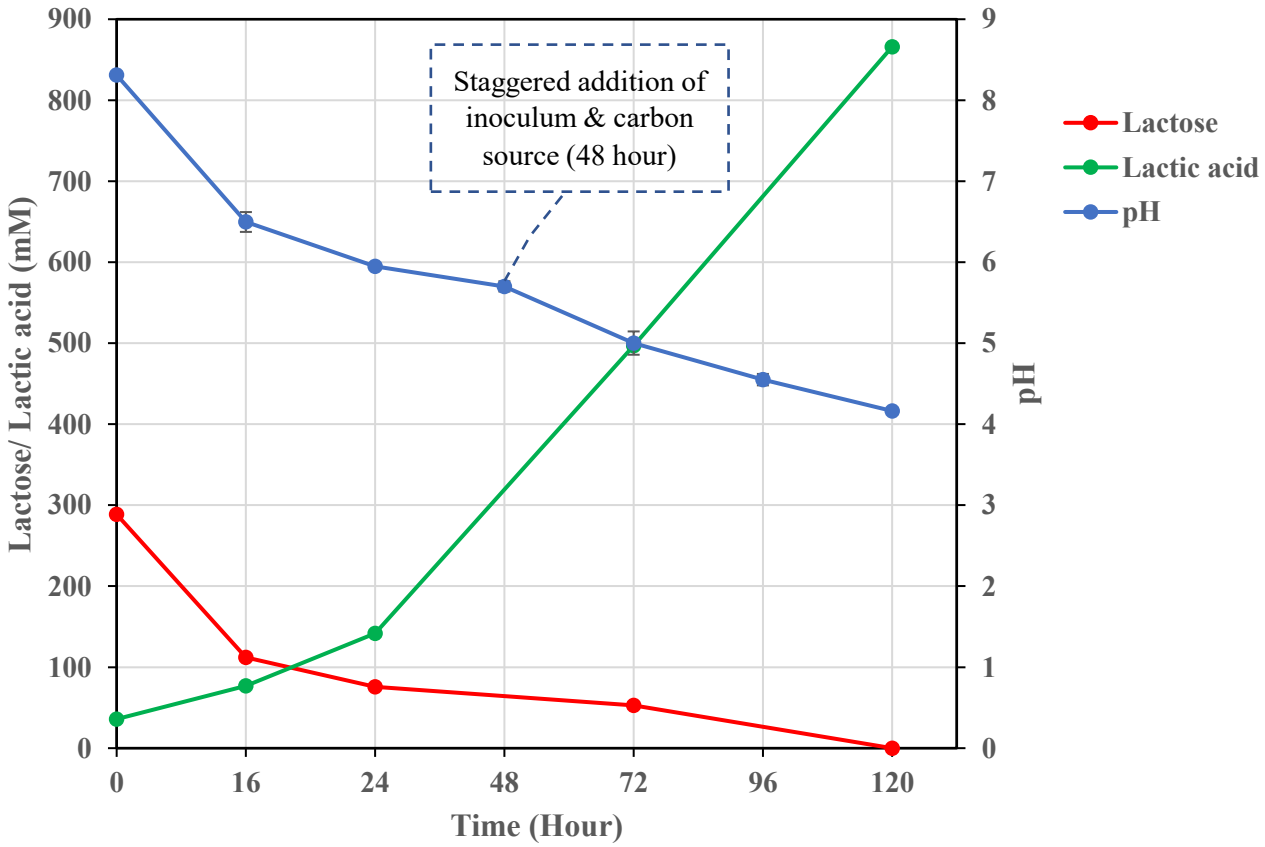


Figure 4.20: Fermentation profile of 48 - hour staggered addition of inoculum and carbon source, S2P4-C scale-up at 5 L

The fermentation profile of the 5L fermentation of S2P4-C is shown in Figure 4.20 which includes pH, lactose consumption and lactic acid production. There was a uniform decline of pH to a final pH of 4.16 at the end of fermentation comparable to end pH of flask fermentation of S2P4-C.

Lactose consumed, and lactic acid produced are shown in Figure 4.20 (standard deviation not visible due to higher scale units). A high production of lactic acid was attained in 5 L scale-up of 865.92 mM which is equivalent to that of the flask fermentation 858.82 mM. The residual lactose in 5 L fermentation was significantly low at 1.2 mM than in flask fermentation, indicating maximum utilization of lactose by the bacteria during the fermentation, compared with equivalent

flask fermentation which did not consume the lactose completely having residual lactose level of 4.3 mM. Though the pH of the 5L fermentation is a little higher than S2P4-C flask fermentation, the high production of lactic acid assisted in a successful fermentation as measured by HPLC. In comparison, Duan et al (2012) and Cira et al (2002) conducted pilot scale production of chitin from shrimp wastes at 200 L and 30 L capacity, respectively using *Lactobacillus* sp. These authors observed a similar fermentation efficiency as flask fermentation with pH reducing to 3.86 and leaving 1% of mineral in the final product. Fermentation conditions in scale-up such as low moisture content and low oxygen concentration are advantageous in reproducing similar or greater efficiency as flask fermentation (Cira *et al.*, 2002). The high production of lactic acid increased the reaction between the lactic acid and shells (calcium carbonate) which in turn resulted in a high DM and DC of 89.66% and 89.24%, respectively and chitin yield of 28.4% (Table 4.4).Thus, lobster shell chitin can be effectively extracted from shell material with a combination of grinded size, inoculum carbon source, and protein inclusion in a staggered fermentation. The purity of chitin and the degree of DP was also calculated using chemical method as shown in Appendix (Figure A17 and A18).

Table 4.4: Fermentation efficiency and Yield of 48 - hour staggered addition of inoculum and carbon source, S2P4-C scale-up

DC (%)	DM (%)	Yield (%)
89.24 ± 0.04	89.66 ± 0.19	28.4 ± 0.11

CHAPTER 5: CONCLUSION

Lobsters are an excellent source of chitin and are among the less explored crustaceans to produce industrially important ingredients which can be used to generate products of practical application. This study investigated the extraction of crude chitin (insoluble fraction) from lobster shells (*Homarus americanus*) through lactic acid bacterial fermentation fed with carbon and nitrogen sources. The primary focus of this research was to study the factors influencing and optimize the conditions for the biological recovery of chitin from the lobster shells. The objectives of the research study were accomplished using appropriate experimental designs at the screening and optimization stage (Phase 1) and through the staggered addition of inoculum and/or carbon source for process improvement (Phase II), both conducted at the flask level. The following conclusions were determined from the study outcomes:

1. An optimal weight percent combination of shell loading, initial lactose, and inoculum volume in the fermentation medium for effective reduction of pH up to 4.90 were found to be 5 w/v% shell, 10 w/v% lactose and 1 w/v% inoculum.
2. **Phase 1:** The optimal combination was employed in Phase 1 fermentations for studying the effect of shell particle size and nitrogen inclusion using the full factorial design in response to degree of DM. The results showed a significant positive effect of nitrogen inclusion and a significant variable effect of particle size on degree of DM. A lobster shell size range of 500 μm – 250 μm and nitrogen inclusion of 3% resulted in high DM and DC of 83.91% and 83.56%, respectively and a crude chitin yield of 33%.
3. **Phase 2:** The best formulation from Phase 1 was further tested using two approaches, (i) staggered addition of carbon and inoculum, (ii) pre-treatment of shells with microwave.

A combined staggered addition of carbon and inoculum at 48 hours showed an improvement in the fermentation efficiency with a DM and DC rate of 89.83% and 88.87%, respectively and a crude chitin yield of 28%. The metabolic profile during the course of fermentation was consistent with a steady reduction in pH and increased lactic acid production.

The second approach of Phase 2 trials with microwave pre-treatment of shell and staggered addition of carbon and inoculum at 48 hours did not have any effect on the fermentation efficiency and achieved a similar efficiency to that of staggered addition alone with DM and DC of 89.55% and 88.23%, respectively and a crude chitin yield of 28%.

4. The validation of trials in a scale up to 5L using the best conditions from Phase 2 trials showed similar efficiency as flask fermentation with DM and DC of 89.66% and 89.24%, respectively and a crude chitin yield of 28.4%.

Overall, the lactic acid bacterial fermentation with staggered addition of lactose and inoculum with added nitrogen source paved a way for the development of chitin extraction in the current study. However, the low shell load of 5% is unlikely to be cost-effective as the final insoluble fraction (chitin) is approximately 20% in dry weight. Therefore, it would be reasonable to have future studies focusing on increased shell ratio in the fermentation to increase the productivity of chitin produced per batch. Studies on measurement of surface area or porosity of the shell material may give a view on the impact of particle size on fermentation efficiency changes . In future, studies focusing on optimizing the microwave or ultrasound pre-treatment could be a valuable addition in the biological extraction of chitin. Chitin production via biological methods has been explored in the past decade and is not yet commercially available. This study provides more insights on the factors affecting the fermentative extraction

of chitin and how the existing process might be improved. The results from this study have shown crustacean waste as a valued feed stock and the potential to upscale the microbial bioconversions of these crustacean waste to value added products.

REFERENCES

Adour, L., Arbia, W. and Mameri, N. (2008) 'Combined use of waste materials – recovery of chitin from shrimp shells by lactic acid fermentation supplemented with date juice waste or glucose'. *Journal of Chemical Technology & Biotechnology*: 1664-1669 doi: 10.1002/jctb.1980

Elisabetta de, A ., Fabrizio, C., Palma, P., Jacinta, S., Stefano, M (2018) 'Revisiting the Crabtree / Warburg effect in a dynamic perspective : a fitness advantage against sugar-induced cell death'. Taylor & Francis: 688-701 doi: 10.1080/15384101.2018.1442622.

Helton José, A., Maristela, F., Cristie Luis, K., Clayton Rodrigues de, O., Vanessa Rossato, B., Karine Natani, L., Andressa Caroline, N., and Mabel Karina, A (2017) 'Effect of shrimp shells milling on the molar mass of chitosan', Polimeros, 27(1): 41–47.doi: 10.1590/0104-1428.2354.

AOAC. (1990) 'Official Methods of Analysis (Volume 1)', 15th ed. *Association of Official Analytical Chemists*: 246. Washington, DC.

Wassila, A., Leila, A., Lydia, A., and Abdelti, A (2013) 'Chitin Extraction from Crustacean Shells Using Biological Methods – A Review'. *Food Technology and Biotechnology*, 51 (1): 12-25.

Ayothiraman, N. S. S. E. J. S. (2019) 'Marine shell industrial wastes – an abundant source of chitin and its derivatives : constituents , pretreatment , fermentation , and pleiotropic applications - a revisit', *International Journal of Environmental Science and Technology*. Springer Berlin Heidelberg, 16(7): 3877–3898. doi: 10.1007/s13762-018-02204-3.

Aytekin, O. and Elibol, M. (2010) 'Cocultivation of *Lactococcus lactis* and *Teredinobacter turnirae* for biological chitin extraction from prawn waste', *Bioprocess and Biosystems Engineering*, 33(3): 393-399: doi: 10.1007/s00449-009-0337-6.

Patrick S. Barber, Chris S. Griggs, Jonathan R. Bonner, and Robin D. Rogers (2013) 'Green Chemistry'. RSC Publishing: 601-607: doi: 10.1039/c2gc36582k.

Barrios-González, J. (2018) 'Secondary Metabolites Production', *Current Developments in Biotechnology and Bioengineering*: 257–283: doi: 10.1016/b978-0-444-63990-5.00013-x.

Bautista J, Jover M, Gutierrez J, Corpas R, Cremades O, Fontiveros E, Iglesias F and Vega J. (2001) 'Preparation of crayfish chitin by in situ lactic acid production'. *Process Biochemistry,* 37: 229–234: doi:10.1016/S0032-9592(01)00202-3.

F.Boßelmann, P.Romano, H.Fabritius, D.Raabe, M.Epple (2007) 'The composition of the exoskeleton of two crustacea: The American lobster *Homarus americanus* and the edible crab Cancer pagurus', *Thermochimica Acta*, 463(1–2): 65–68: doi: 10.1016/j.tca.2007.07.018.

Castro, R., Guerrero-legarreta, I. and Bórquez, R. (2018) 'Chitin extraction from Allopetrolisthes punctatus crab using lactic fermentation', *Biotechnology Reports*. Elsevier B.V, 20: e00287: doi: 10.1016/j.btre.2018.e00287.

Jayashree, C., Chen-Lu, Y., Jacob, P., Christoper J Brigham (2018) 'Chitin extraction from lobster shell waste using microbial culture-based methods', *Applied Food Biotechnology*, 5(3): 141-154: doi: 10.22037/afb.v%vi%i.20787.

Choorit, W., Patthanamanee, W. and Manurakchinakorn, S. (2008) 'Use of response surface method for the determination of demineralization efficiency in fermented shrimp shells'. *Bioresource Technology* 99: 6168-6173: doi: 10.1016/j.biortech.2007.12.032.

Luis A Cira , Sergio H , George M Hall , Keiko S (2002) 'Pilot scale lactic acid fermentation of shrimp wastes for chitin recovery', *Process Biochemistry*, 37(12): 1359-1366: doi: 10.1016/S0032-9592(02)00008-0.

O Cremades, E Ponce, R Corpas, J F Gutierrez, M Jover, M C Alvarez-Ossorio, J Parrado, J Bautista (2001) 'Processing of Crawfish (Procambarus clarkii) for the Preparation of Carotenoproteins and Chitin'. *Journal of Agricultural and Food Chemistry*, 49(11): 5468-5472: doi: 10.1021/jf0104174.

M.S. Rao, J. Munoz and W.F. Stevens 'Critical factors in chitin production by fermentation of shrimp biowaste' (2000) *Applied Microbiology and Biotechnology*, 54(6): 808-813: doi: 10.1007/s002530000449.

Ding, S. and Tan, T. (2006) 'Short communication L -lactic acid production by Lactobacillus casei fermentation using different fed-batch feeding strategies'. *Process Biochemistry* 41(6): 1451-1454: doi: 10.1016/j.procbio.2006.01.014.

Duan, S., Li, L., Zhuang, Z., Wu, W., Hong, S., & Zhou, J. (2012) 'Improved production of chitin from shrimp waste by fermentation with epiphytic lactic acid bacteria', *Carbohydrate Polymers*. Elsevier Ltd., 89(4): 1283-1288: doi: 10.1016/j.carbpol.2012.04.051.

Ealy, H., Reen, G. and Ealy, H. (2003) 'Bioprocessing of Marine Crustacean Shell Waste', *Acta Biotechnologica* 2(3): 151-160: doi: 10.1002/abio.200390023.

Fisheries and Oceans Canada (2019). *Fisheries and Oceans Canada. Canadian Trade.* Retrived December 11, 2020 , from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/fishers-pecheurs/fp18-eng.htm.

Frydenberg, R. P., Hammershøj, M., Andersen, U., & Wiking, L. (2013) 'Ultrasonication Affects Crystallization Mechanisms and Kinetics of Anhydrous Milk Fat'. *Crystal Growth & Design*, 13(12): doi: 10.1021/cg4012923.

Galanakis, C. M. (2015) Food Waste Recovery: Processing Technologies and Industrial Techniques, *Food Waste Recovery: Processing Technologies and Industrial Techniques*. doi: 10.1016/C2013-0-16046-1.

Ghorbel-Bellaaj, O., Jellouli, K., Younes, I., Manni, L., Ouled Salem, M., & Nasri, M. (2011) 'A Solvent-Stable Metalloprotease Produced by Pseudomonas aeruginosa A2 Grown on Shrimp Shell Waste and Its Application in Chitin Extraction. *Applied Biochemistry and Buiotechnology*, 164(4): 410-425: doi: 10.1007/s12010-010-9144-4.

Gildberg, A. and Stenberg, E. (2001) 'A new process for advanced utilisation of shrimp waste', *Process Biochemistry* 36: 809-812: doi: 10.1016/S0032-9592(00)00278-8.

Erwin Glaasker, Frans S. B. Tjan, Pieter F. Ter Steeg, Wil N. Konings, Bert Poolman (1998) 'Physiological response of Lactobacillus plantarum to salt and nonelectrolyte stress', *Journal of Bacteriology*, 180(17). 4718–4723: doi: 10.1128/jb.180.17.4718-4723.1998.

Gulf Nova Scotia Fleet Planning Board (2019) 'Lobster blood protein and moult cycle analysis', 1-28.

Haddar, A., Hmidet, N., Ghorbel-Bellaaj, O., Fakhfakh-Zouari, N., Sellami-Kamoun, A., & Nasri, M. (2011) 'Alkaline Proteases Produced by Bacillus licheniformis RP1 Grown on Shrimp Wastes : Application in Chitin Extraction , Chicken Feather- degradation and as a Dehairing Agent', *Biotechnology and Bioprocess Engineering*, 16(4): 669-678: doi: 10.1007/s12257-010-0410-7.

Hamed, I., Özogul, F. and Regenstein, J. M. (2016) 'Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review', *Trends in Food Science and Technology*, 48: 40-50: doi: 10.1016/j.tifs.2015.11.007.

Healy, M., Green, A. and Healy, A. (2003) 'Bioprocessing of marine crustacean shell waste', *Acta Biotechnologica*, 23:(2–3): 151-160: doi: 10.1002/abio.200390023.

Hong, S., Yang, Q., Yuan, Y., Chen, L., Song, Y., & Lian, H. (2019) 'Sustainable co-solvent induced one step extraction of low molecular weight chitin with high purity from raw lobster shell', *Carbohydrate Polymers*. Elsevier: 236-243: doi:

10.1016/j.carbpol.2018.10.045.

Ilangumaran, G., Stratton, G., Ravichandran, S., Shukla, P. S., Potin, P., Asiedu, S., & Prithiviraj, B. (2017) 'Microbial Degradation of Lobster Shells to Extract Chitin Derivatives for Plant Disease Management', *Frontiers in Microbiology*, 8: 1-14: doi: 10.3389/fmicb.2017.00781.

Jo, G. H., Jung, W. J., Kuk, J. H., Oh, K. T., Kim, Y. J., & Park, R. D. (2008). Screening of protease-producing Serratia marcescens FS-3 and its application to deproteinization of crab shell waste for chitin extraction. *Carbohydrate Polymers*: 74(3): 504-508: doi:10.1016/j.carbpol.2008.04.019.

Jung, W. J., Jo, G. H., Kuk, J. H., Kim, K. Y., & Park, R. D. (2006) 'Extraction of chitin from red crab shell waste by cofermentation with Lactobacillus paracasei subsp. tolerans KCTC-3074 and Serratia marcescens FS-3', *Applied Microbiology and Biotechnology*, 71(2): 234-237: doi: 10.1007/s00253-005-0126-3.

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015) 'Trends in Food Science & Technology Ultrasound applications for the extraction , identification and delivery of food proteins and bioactive peptides', *Trends in Food Science and Technology*, 46(1): 60-67: doi: 10.1016/j.tifs.2015.07.012.

Kampen, W. H. (2014) Nutritional Requirements in Fermentation Processes. Third Edit, Fermentation and Biochemical Engineering Handbook: Principles, Process Design, and Equipment: Third Edition. Elsevier Inc: doi: 10.1016/B978-1-4557-2553-3.00004-0.

Kandra, P., Challa, M. M. and Kalangi Padma Jyothi, H. (2012) 'Efficient use of shrimp waste: Present and future trends', *Applied Microbiology and Biotechnology*, 93(1): 17-29: doi: 10.1007/s00253-011-3651-2.

Karagozlu, M. Z. and Kim, S. (no date) *Anticancer Effects of Chitin and Chitosan Derivatives*. 1st edn, *Marine Carbohydrates: Fundamentals and Applications, Part A*. Elsevier Inc. doi: 10.1016/B978-0-12-800269-8.00012-9.

Kaur, S. and Dhillon, G. S. (2015) 'Recent trends in biological extraction of chitin from marine shell wastes: A review', *Critical Reviews in Biotechnology*, 35(1): 44-61: doi: 10.3109/07388551.2013.798256.

Kemsawasd, V., Viana, T., Ardö, Y., & Arneborg, N. (2015) 'Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation', *Applied Microbiology and Biotechnology,* 99(23): 10191-10207: doi: 10.1007/s00253-015-6835-3.

Khanafari, A. and Marandi, R. (2008) 'Recovery of Chitin and Chitosan From Shrimp Waste By', *Journal of Environmental Health Science and Engineering*, 5(1): 19-24.

Khorrami, M, G.D. Najafdour, H. Younesi and G.H. Amini (2011) 'Growth Kinetics and Demineralization of Shrimp Shell Using Lactobacillus plantarum PTCC 1058 on Various Carbon Sources', *Journal of Energy and Environment* 2(4): 320-325: doi: 10.5829/idosi.ijee.2011.02.04.2391.

El Knidri, H., El Khalfaouy, R., Laajeb, A., Addaou, A., & Lahsini, A. (2016) 'Eco-friendly extraction and characterization of chitin and chitosan from the shrimp shell waste via microwave irradiation', *Process Safety and Environmental Protection*. Institution of Chemical Engineers, 104: 395-405: doi: 10.1016/j.psep.2016.09.020.

Kurita, K. (2006) 'Chitin and chitosan: Functional biopolymers from marine crustaceans', *Marine Biotechnology*, 8(3): 203-226: doi: 10.1007/s10126-005-0097-5.

Levin, R. E. (1994) 'Lactic acid and propionic acid fermentations of fish hydrolyzates', *Fisheries Processing*: 273-310: doi: 10.1007/978-1-4615-5303-8_12.

Liu, P., Liu, S., Guo, N., Mao, X., Lin, H., Xue, C., & Wei, D. (2014) 'Cofermentation of Bacillus licheniformis and Gluconobacter oxydans for chitin extraction from shrimp waste', *Biochemical Engineering Journal*. Elsevier B.V., 91:10-15: doi: 10.1016/j.bej.2014.07.004.

Liu, Y., Liao, W. and Chen, S. (2008) 'Co-production of lactic acid and chitin using a pelletized filamentous fungus Rhizopus oryzae cultured on cull potatoes and glucose', *Journal of Applied* Microbiology,105: 1521-1528: doi: 10.1111/j.1365-2672.2008.03913.x.

Lopes, C., Antelo, L. T., Franco-Uría, A., Alonso, A. A., & Pérez-Martín, R. (2018) 'Chitin production from crustacean biomass: Sustainability assessment of chemical and enzymatic processes', *Journal of Cleaner Production*, 172: 4140-4151: doi: 10.1016/j.jclepro.2017.01.082.

Morrow, J. C. (2002) 'Biotechnological utilisation of Nephrops shell waste'. A thesis submitted for the degree of Doctor of Philosophy. *Faculty of veterinary medicine, University of Glasgow*.

Nguyen, T. T., Zhang, W., Barber, A. R., Su, P., & He, S. (2016) 'Microwave-Intensified Enzymatic Deproteinization of Australian Rock Lobster Shells (Jasus edwardsii) for the Efficient Recovery of Protein Hydrolysate as Food Functional Nutrients', *Food and Bioprocess Technology*, 9(4): 628-636: doi: 10.1007/s11947-015-1657-y.

Nguyen, T. T., Barber, A. R., Smith, P., Luo, X., & Zhang, W. (2017) 'Application and optimization of the highly efficient and environmentally-friendly microwave-intensified lactic acid demineralization of deproteinized Rock lobster shells (Jasus edwardsii) for chitin production', *Food and Bioproducts Processing*. Institution of Chemical Engineers, 102: 367-374: doi: 10.1016/j.fbp.2017.02.005.

Nguyen, T. T., Barber, A. R., Corbin, K., and Zhang, W. (2017) 'Lobster processing by-products as valuable bioresource of marine functional ingredients, nutraceuticals, and pharmaceuticals', *Bioresources and Bioprocessing*. Springer Berlin Heidelberg, 4(1): doi: 10.1186/s40643-017-0157-5.

Oh, K.-T., Kim, Y.-J., Nguyen, V. N., Jung, W.-J., & Park, R.-D. (2007) 'Demineralization of crab shell waste by Pseudomonas aeruginosa F722', *Process Biochemistry*, 42(7): 1069–1074: doi: 10.1016/j.procbio.2007.04.007.

Oyekunle, D. T. and Omoleye, J. A. (2019) 'Extraction, characterization and kinetics of demineralization of chitin produced from snail shells of different particle sizes using 1.2 M HCl', *International Journal of Mechanical Engineering and Technology*, 10(1): 2011-2020.

Konstantinos, P., Ángel, A., Peter A. Bron, Maria de Angelis, Marco, G., Michiel, K., José A. Lemos, Daniel M. Linares, Paul, R., Catherine, S., Francesca, T., Douwe van Sinderen, Pekka, V., Marco, V., Manuel, Z., Effie, T., and Jan, K. (2016) 'Stress Physiology of Lactic Acid Bacteria', *Microbiology and Moecular Biology* Reviews, 80(3): 837-890: doi: 10.1128/MMBR.00076-15.

Pighinelli, L. (2019) 'Methods of Chitin Production a Short Review', *American Journal of Biomedical Science & Research*, 3(4): 307-314: doi: 10.34297/ajbsr.2019.03.000682.

Raabe, D., Romano, P., Sachs, C., Fabritius, H., Al-Sawalmih, A., Yi, S.-B., Hartwig, H. G. (2006) 'Microstructure and crystallographic texture of the chitin-protein network in the biological composite material of the exoskeleton of the lobster Homarus americanus', *Materials Science and Engineering*, 421(1–2): 143-153: doi: 10.1016/j.msea.2005.09.115.

Rao, M. S. and Stevens, W. F. (2006) 'Fermentation of Shrimp Biowaste under Different Salt Concentrations with Amylolytic and Non-Amylolytic Lactobacillus Strains for Chitin Production', 44(1): 83-87.

Rhazi, M., Desbrieres, J., Tolaimate, A., Alagui, A., & Vottero, P. (2000) 'Investigation of different natural sources of chitin: Influence of the source and deacetylation process on the physicochemical characteristics of chitosan', *Polymer International*, 49(4): 337-344: doi: 10.1002/(SICI)1097-0126(200004)49:4<337::AID-PI375>3.0.CO;2-B.

Ribeiro, M. V. D. S., Olivo, J. E. and Eller, M. R. (2020) 'Fed-Batch and Staggered Nutrient Addition: An Improved Method for Mead Production', *Industrial Biotechnology*, 16(2), pp. 133-138: doi: 10.1089/ind.2019.0038.

Santos, V. P. *et al.* (2019) 'Recovery of chitin and chitosan from shrimp waste with microwave technique and versatile application', *Arquivos do Instituto Biológico*, 86,1-7: doi: 10.1590/1808-1657000982018.

Setoguchi, T., Kato, T., Yamamoto, K., & Kadokawa, J. (2012) 'International Journal of Biological Macromolecules Facile production of chitin from crab shells using ionic liquid and citric acid', *International Journal of Biological Macromolecules*. Elsevier B.V., 50(3): 861-864: doi: 10.1016/j.ijbiomac.2011.11.007.

Sikorski, P., Hori, R. and Wada, M. (2009) 'Revisit of α-chitin crystal structure using high resolution X-ray diffraction data', *Biomacromolecules*, 10(5): 11000-1105: doi: 10.1021/bm801251e.

Sini, T. K., Santhosh, S. and Mathew, P. T. (2007) 'Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation', 342: 2423-2429: doi: 10.1016/j.carres.2007.06.028.

Steeves, H. (2019) 'Developing a Sustainable Bioprocess for Chitin Extraction from Atlantic Lobster Shell Waste'. A thesis submitted for the degree of Master of Applied Science. *Faculty of Engineering, Department of Process Engineering and Applied Science, Dalhousie University,* Halifax, NS, Canada: uri: http://hdl.handle.net/10222/80181

Stirn, A. (2012) 'The Formula for Lobster Shell', *Max Planck Research*: 72-79: Retrieved from https://www.mpg.de/5720889/W004_Materials-Technology_072-079.pdf

Tan, Y. N., Lee, P. P. and Chen, W. N. (2020) 'Microbial extraction of chitin from seafood waste using sugars derived from fruit waste-stream', *AMB Express*. Springer Berlin Heidelberg, 10(1): doi: 10.1186/s13568-020-0954-7.

Tetteh, A. Y. (1991) 'Optimization Studies on Chitin Extraction from Crustaceans Silid Wastes'. M.Sc. thesis, McGill University, Montreal, Canada.

Thirugnanasambandham, K., Sivakumar, V. and Maran, J. P. (2015) 'International Journal of Biological Macromolecules Microwave-assisted extraction of polysaccharides from mulberry leaves', *International Journal of Biological Macromolecules*. Elsevier B.V., 72: 1-5: doi: 10.1016/j.ijbiomac.2014.07.031.

Toliba, A. O., Rabie, M. A. and El-Araby, G. M. (2016) 'Extending the Shelf-Life of Cold Stored Strawberry By Chitosan and Carnauba Coatings', *Zagazig Journal of Agricultural*

Research, 41(5): 1067-1076. Retrieved from http:/www.journals.zu.edu.eg/journalDisplay.aspx?Journalld=1&queryType=Master.

Wang, J. and Yin, Y. (2017) 'ScienceDirect Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures', *International Journal of Hydrogen Energy*. Elsevier Ltd, 42(8): 4804-4823: doi: 10.1016/j.ijhydene.2017.01.135.

Wang, Y., Corrieu, G. and Be, C. (2005) 'Fermentation pH and Temperature Influence the Cryotolerance of Lactobacillus acidophilus RD758', *Journal of Dairy Science,* 88 (1): 21–29. doi: 10.3168/jds.S0022-0302(05)72658-8.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K., & Weiss, J. (2006) 'Sonication-Assisted Extraction of Chitin from North Atlantic Shrimps, *Journal of Agricultural and Food Chemistry*, 54 (16): 5894-5902: doi: 10.1021/jf060646w.

Xu, W., Mohan, A., Pitts, N. L., Udenigwe, C., & Mason, B. (2020) 'Bile acid-binding capacity of lobster shell-derived chitin, chitosan and chitooligosaccharides', *Food Bioscience*., 33:100476: doi: 10.1016/j.fbio.2019.100476.

Yadav, M., Goswami, P., Paritosh, K., Kumar, M., Pareek, N., & Vivekanand, V. (2019) 'Seafood waste: a source for preparation of commercially employable chitin/chitosan materials', *Bioresources and Bioprocessing*. Springer Berlin Heidelberg, 6(1): doi: 10.1186/s40643-019-0243-y.

Yeul, V. S. and Rayalu, S. S. (2013) 'Unprecedented Chitin and Chitosan: A Chemical Overview', *Journal of Polymers and the Environment*, 21(2): 606-614. doi: 10.1007/s10924-012-0458-x.

Younes, I., Ghorbel-Bellaaj, O., Nasri, R., Chaabouni, M., Rinaudo, M., & Nasri, M. (2012) 'Chitin and chitosan preparation from shrimp shells using optimized enzymatic deproteinization', *Process Biochemistry*. Elsevier Ltd, 47(12): 2032-2039: doi: 10.1016/j.procbio.2012.07.017.

Zakaria, Z., Hall, G. M. and Shama, G. (1998) 'Lactic acid fermentation of scampi waste in a rotating horizontal bioreactor for chitin recovery'. *Process Biochemistry,* 33(1): 1-6. Retrieved from https://doi.org/10.1016/S0032-9592(97)00069-1.

APPENDIX

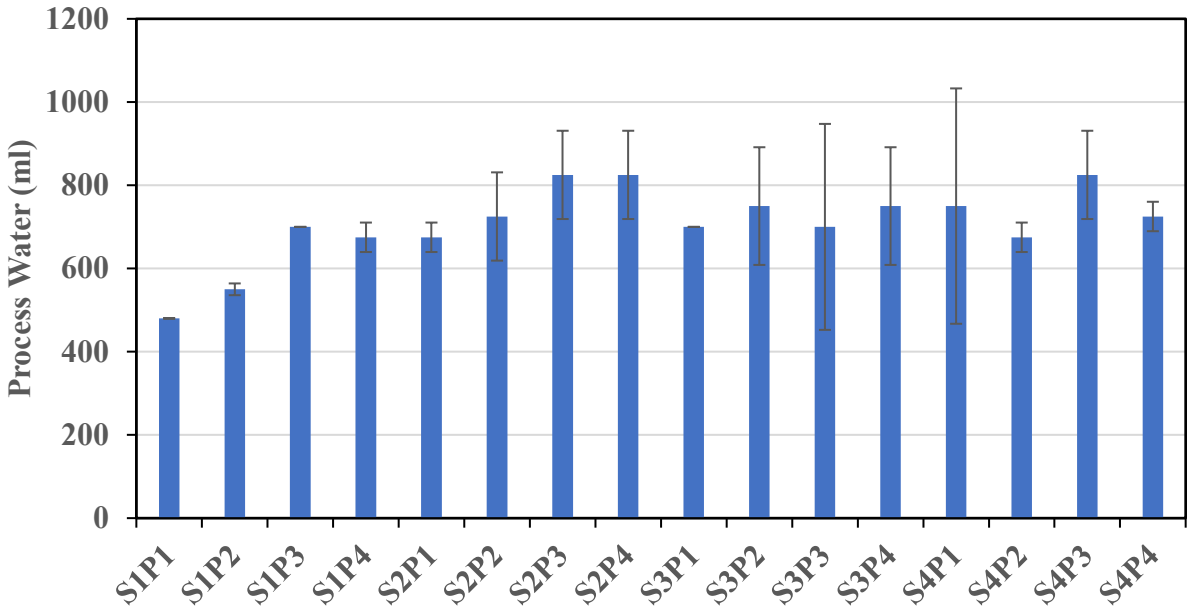


Figure A 1: Process water used by phase 1 fermentations. Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm -45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

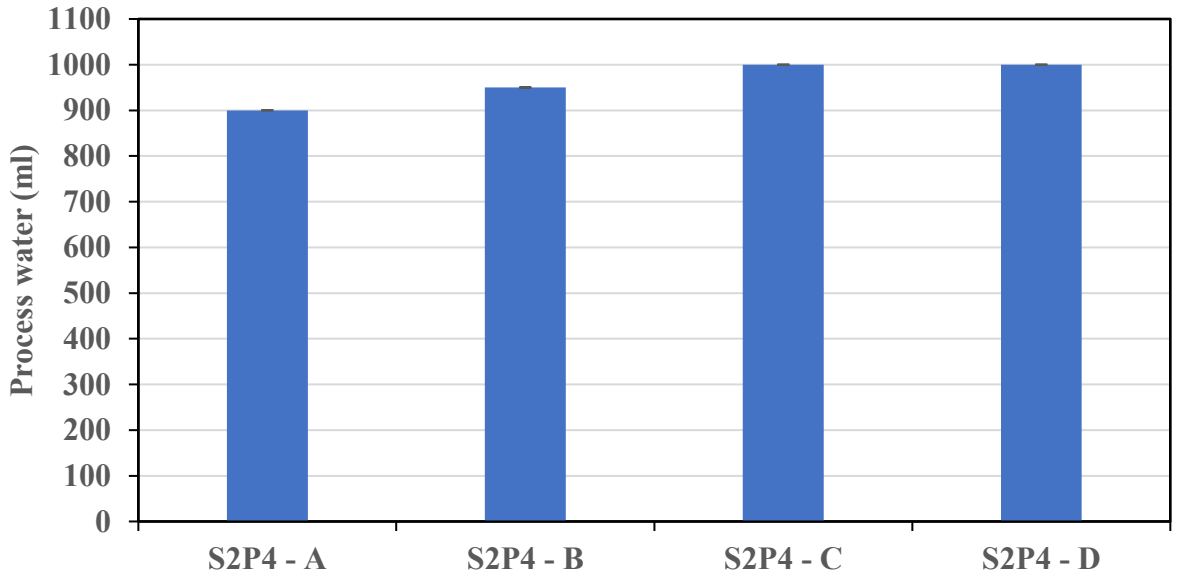


Figure A 2: Process water used by phase 2 fermentations. Legends for size: S2- 500µm-250µm. Legends for protein inclusion: P4 – 3%.

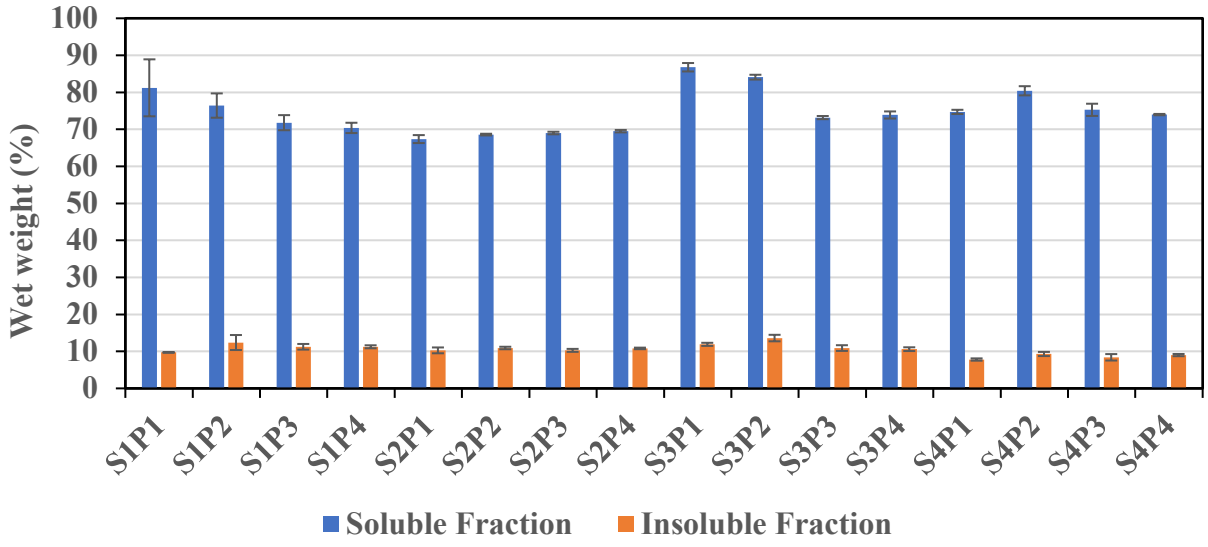


Figure A 3: Mass balance of Phase 1 fermentations - Wet weight, % yield of soluble fraction (protein and Calcium) and insoluble fraction (Chitin). Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

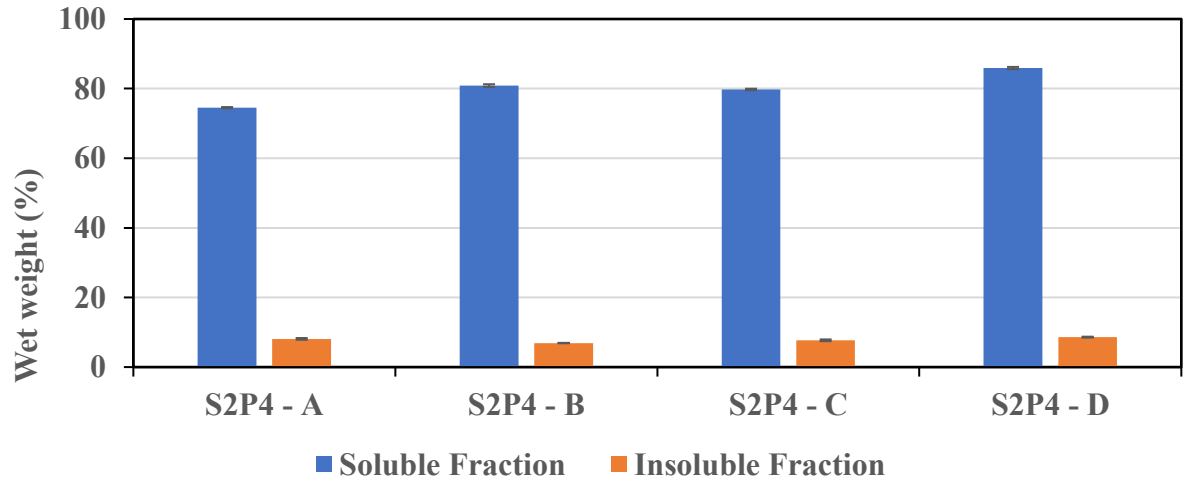


Figure A 4: Mass balance of Phase 2 fermentations - Wet weight, % yield of soluble fraction (protein and Calcium) and insoluble fraction (Chitin). Legends for size: S2- 500µm- 250µm. Legends for protein inclusion: P4 – 3%.

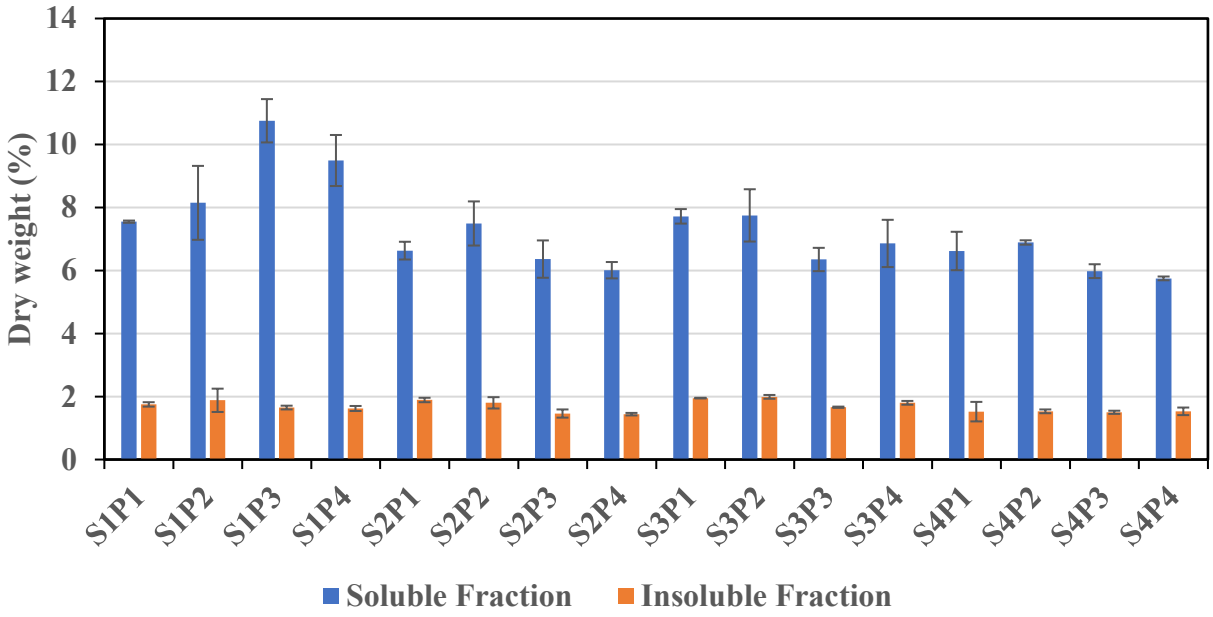


Figure A 5: Mass balance of Phase 1 fermentations - Dry weight, % yield of soluble fraction (protein and Calcium) and insoluble fraction (Chitin). Legends for size: S1 - 2mm - 500µm, S2- 500µm - 250µm, S3 - 250µm - 125µm, S4 - 125µm - 45µm; Legends for protein inclusion P1 – 1%, P2 – 1.5%, P3 – 2%, P4 – 3%.

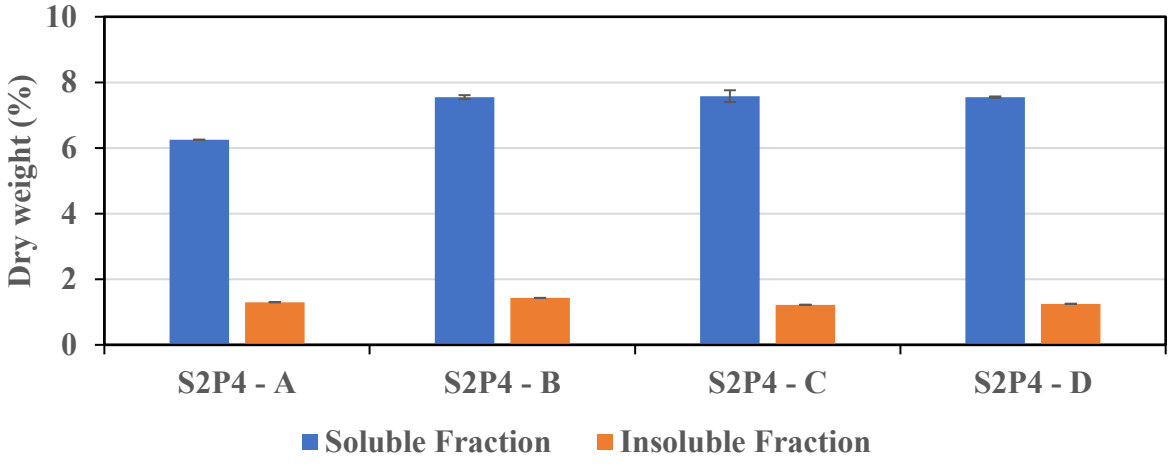


Figure A 6: Mass balance of Phase 2 fermentations - Dry weight, % yield of soluble fraction (protein and Calcium) and insoluble fraction (Chitin). Legends for size: S2- 500µm- 250µm. Legends for protein inclusion: P4 – 3%. 72 - hour staggered addition of inoculum, (S2P4-A), 72 - hour staggered addition of inoculum and carbon source, (S2P4-B), 48 - hour staggered addition of inoculum and carbon source, (S2P4-C), microwave pre-treated, 48 – MAE, hour staggered addition of inoculum and carbon source, (S2P4-D).

Table A1: Mass balance and process water of 5L S2P4 - C Scale-up

5L Scale- up Fermentation (S2P4 - C)				
Process water (L)	Wet Weight (%)		Dry weight (%)	
	Supernatant	Residue	Supernatant	Residue
56	89.90±1.25	10.5±0.07	9.84±0.05	1.39±0.01

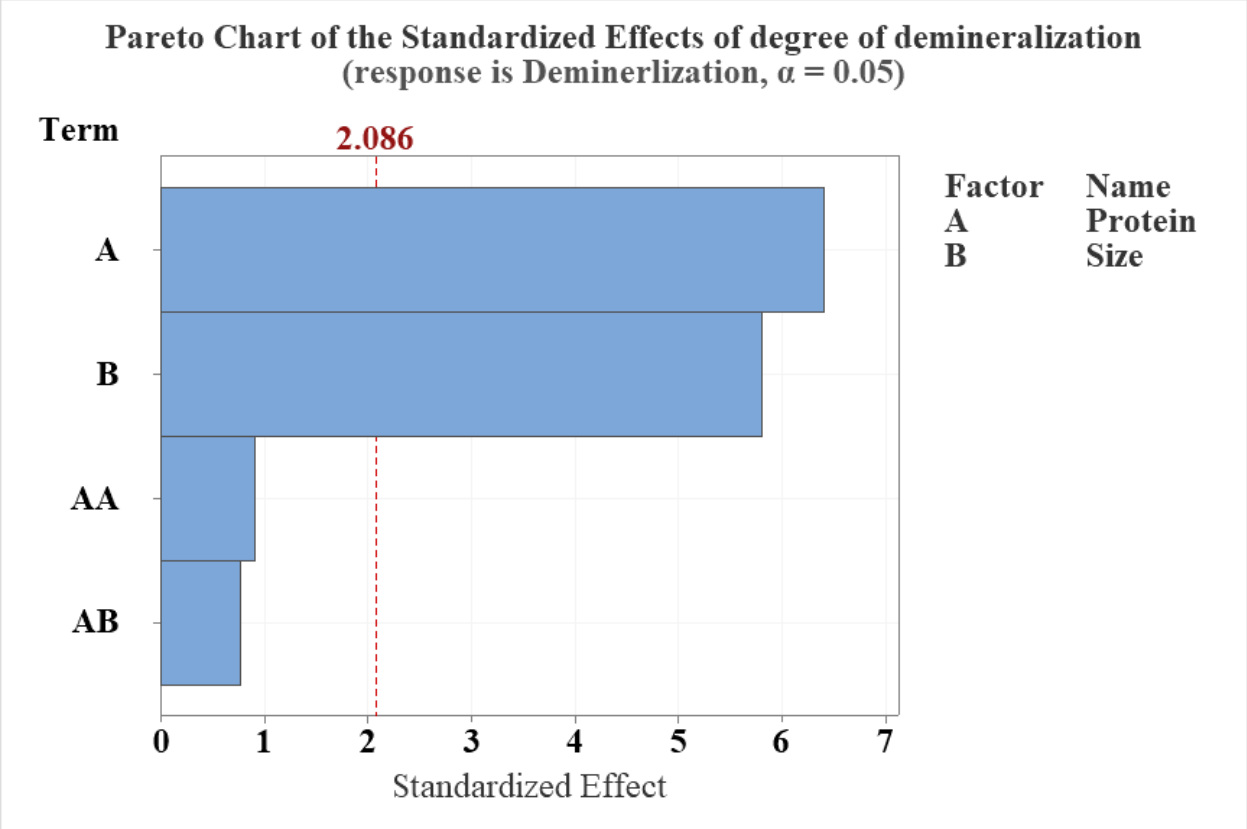
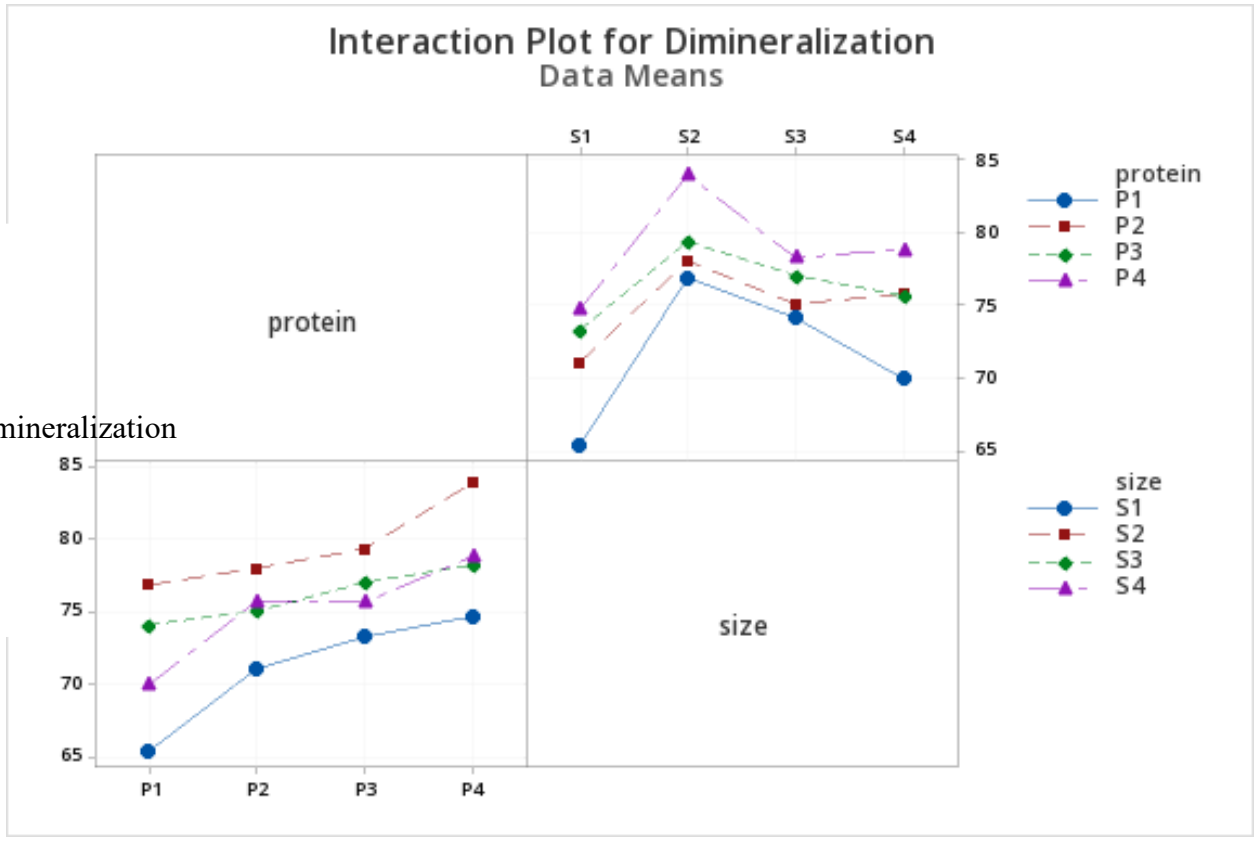


Figure A 7: Pareto chart for shell particle size and protein inclusion in response to degree of demineralization



Degree of demineralization

Figure A 8: Interaction plot for degree of demineralization response with variable shell size and protein inclusion

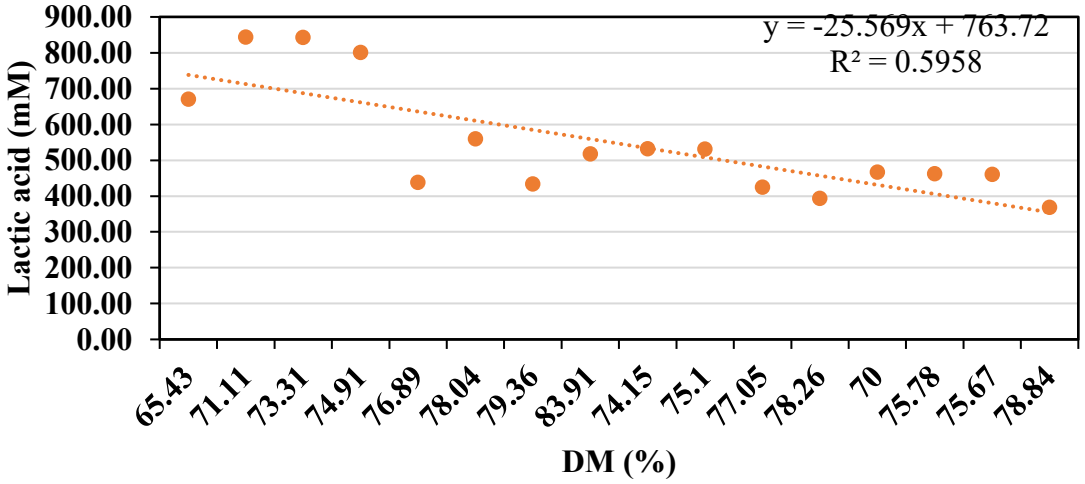


Figure A 9: Correlation between lactic acid and demineralization of phase 1 fermentation

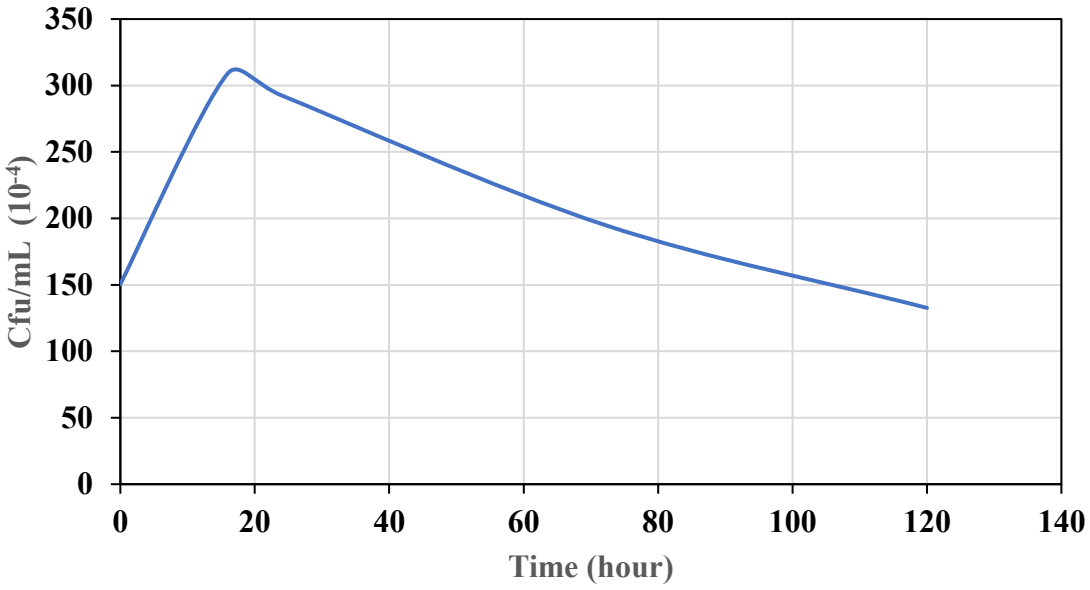


Figure A10: Colony forming units of S2P4. Legends for size: S2- 500µm- 250µm. Legends for protein inclusion: P4 – 3%.

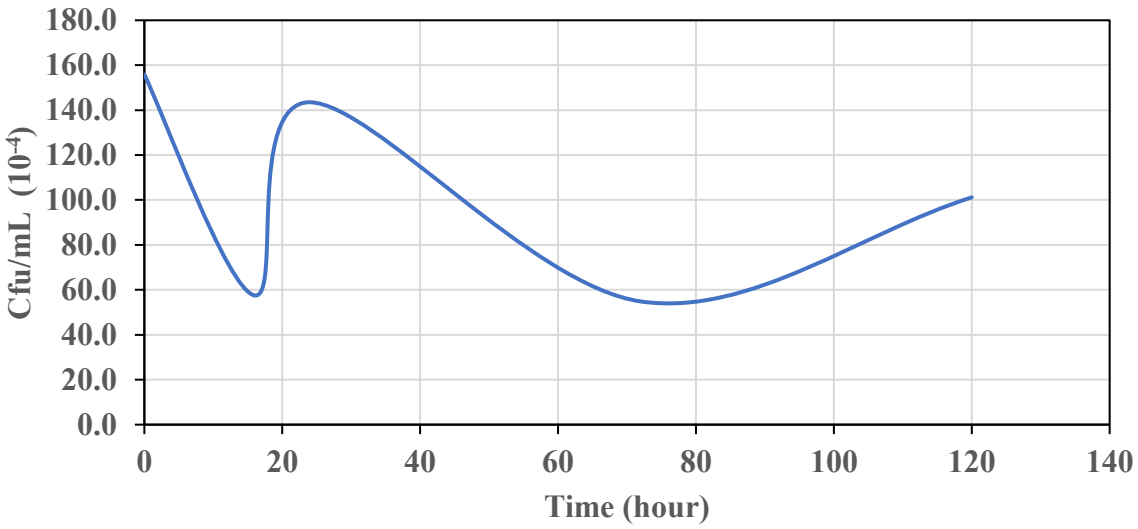
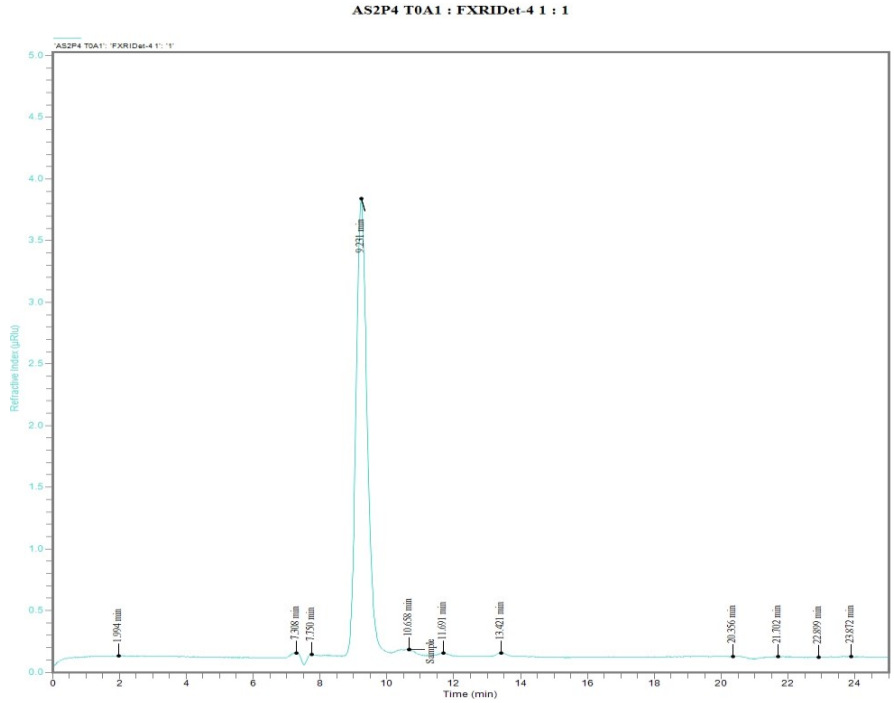


Figure A11: Colony forming units of S4P2. Legends for size: S4- 125µm- 45µm. Legends for protein inclusion: P2 – 1.5%.

a)



b)

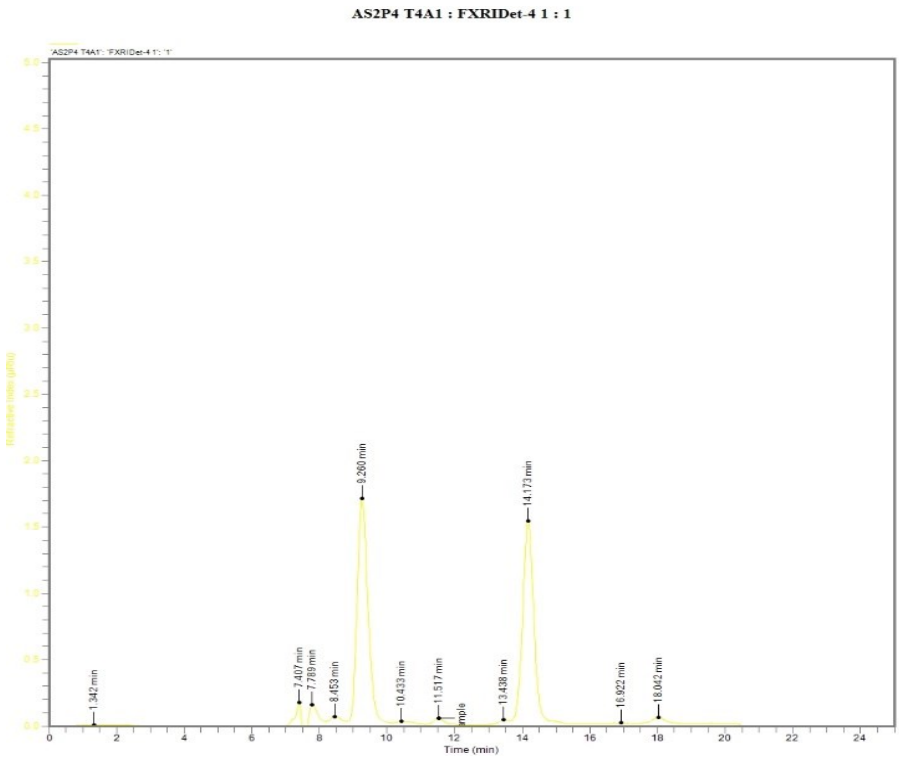
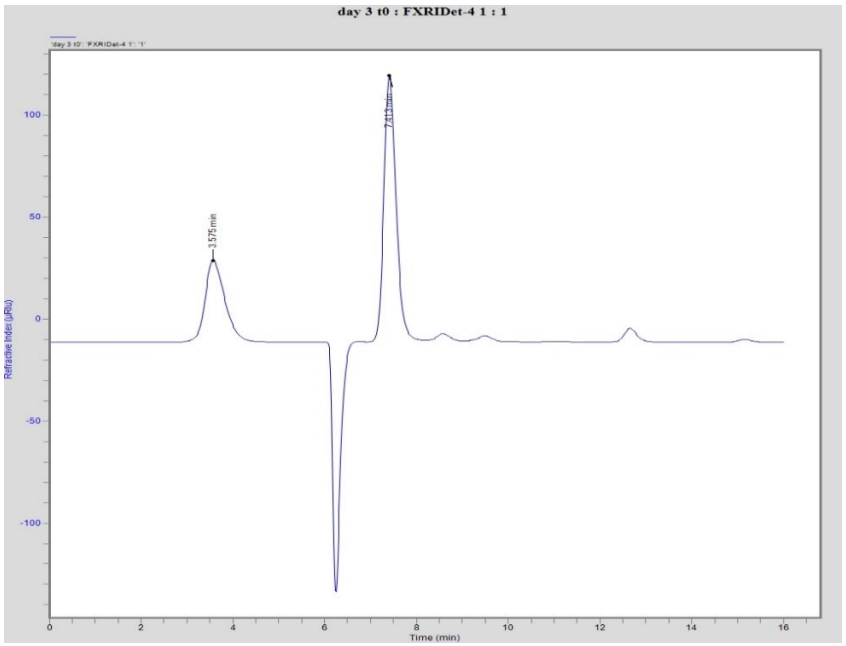


Figure A 12: S2P4 lactose and lactic acid chromatogram at a) 0 hours b) 120 hours.). Legends for size: S2- 500µm- 250µm.
Legends for protein inclusion: P4 – 3%.

a)



b)

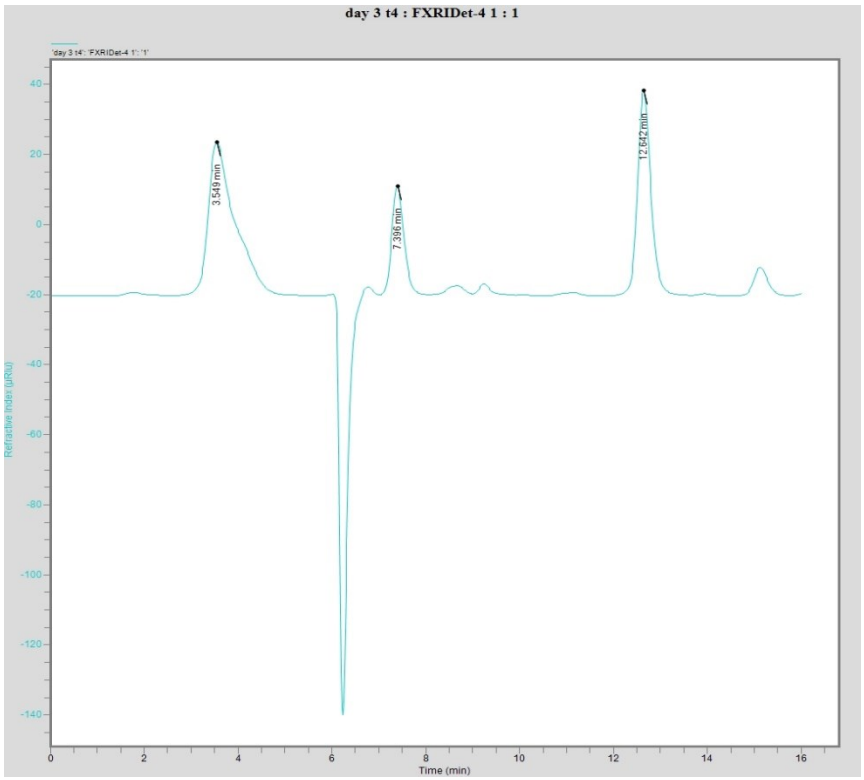
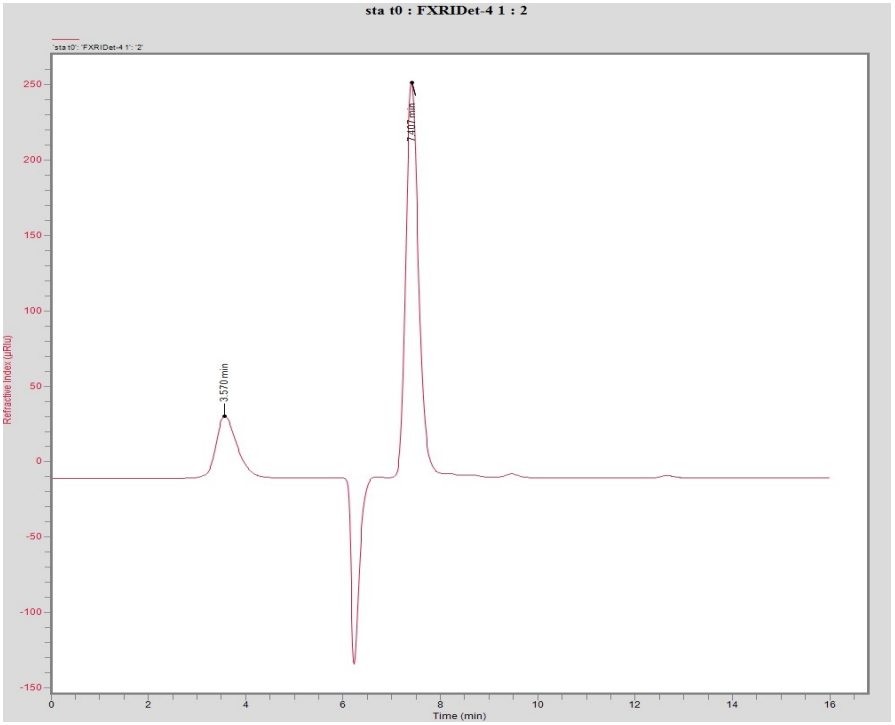


Figure A 13: S2P4-A lactose and lactic acid chromatogram at a) 0 hours b) 120 hours. 72 - hour staggered addition of inoculum, (S2P4-A)

a)



b)

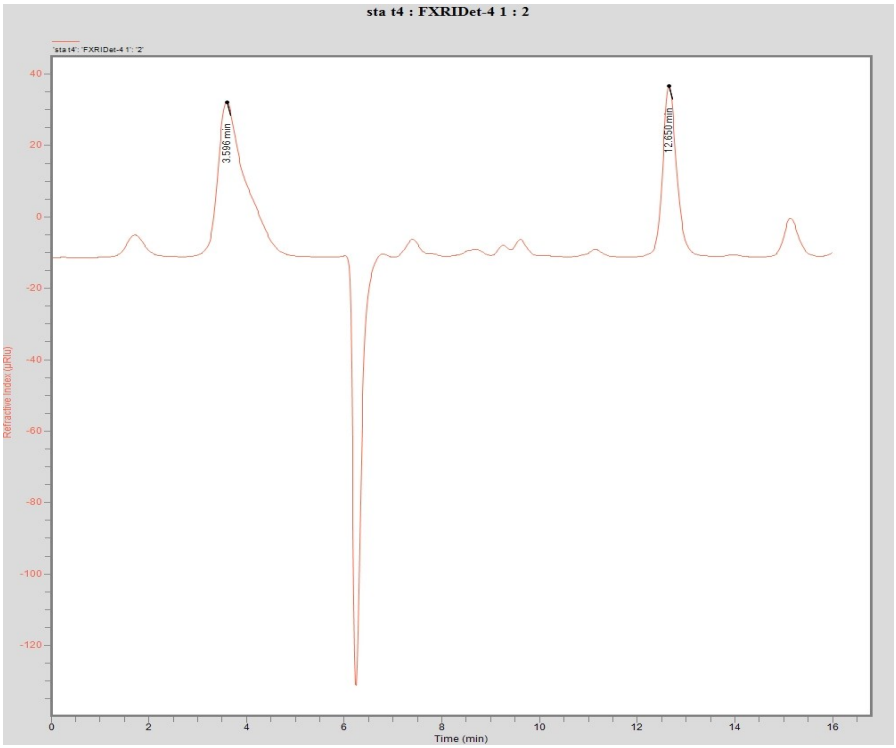
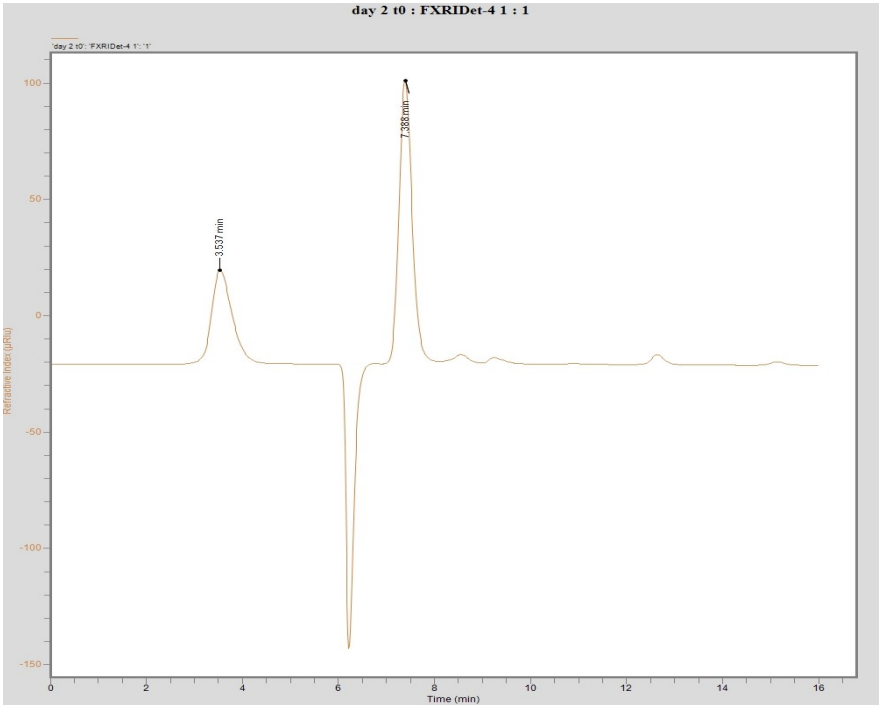


Figure A 14: S2P4-B lactose and lactic acid chromatogram at a) 0 hours b) 120 hours. 72 - hour staggered addition of inoculum and carbon source, (S2P4-B)

a)



b)

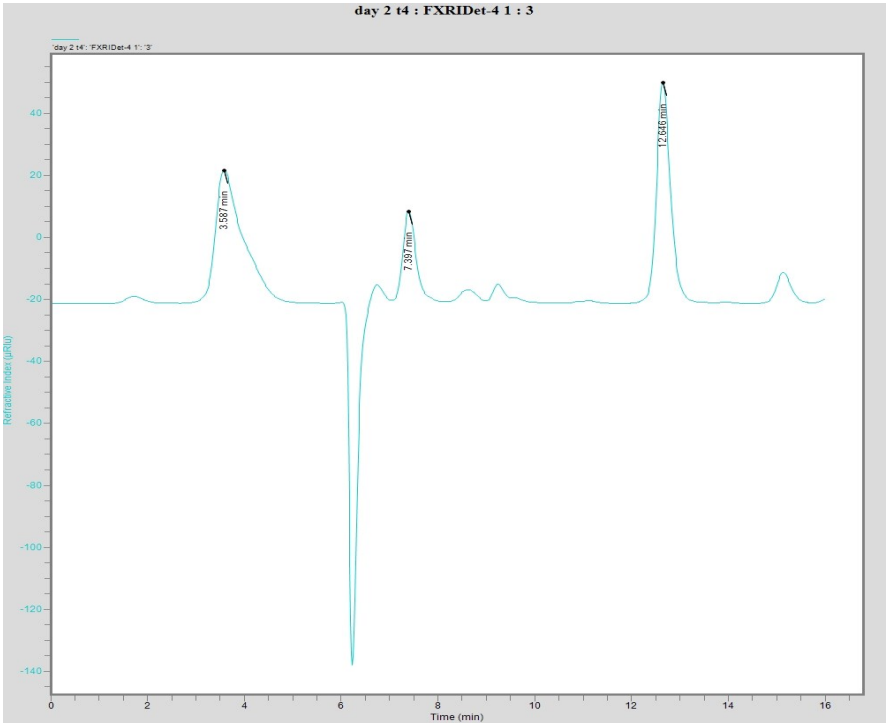
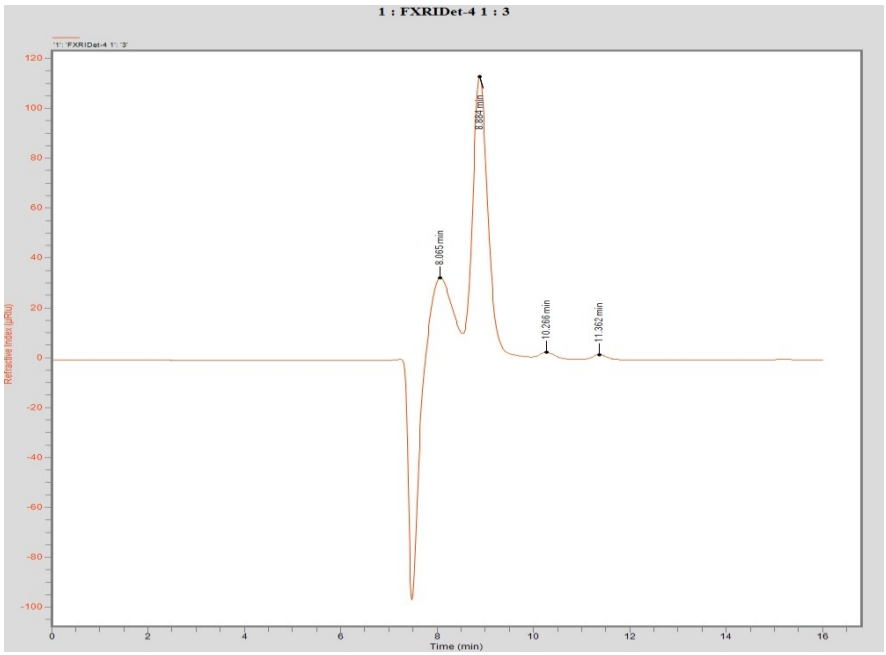


Figure A 15: S2P4-C lactose and lactic acid chromatogram at a) 0 hours b) 120 hours. 48 - hour staggered addition of inoculum and carbon source, (S2P4-C)

a)



b)

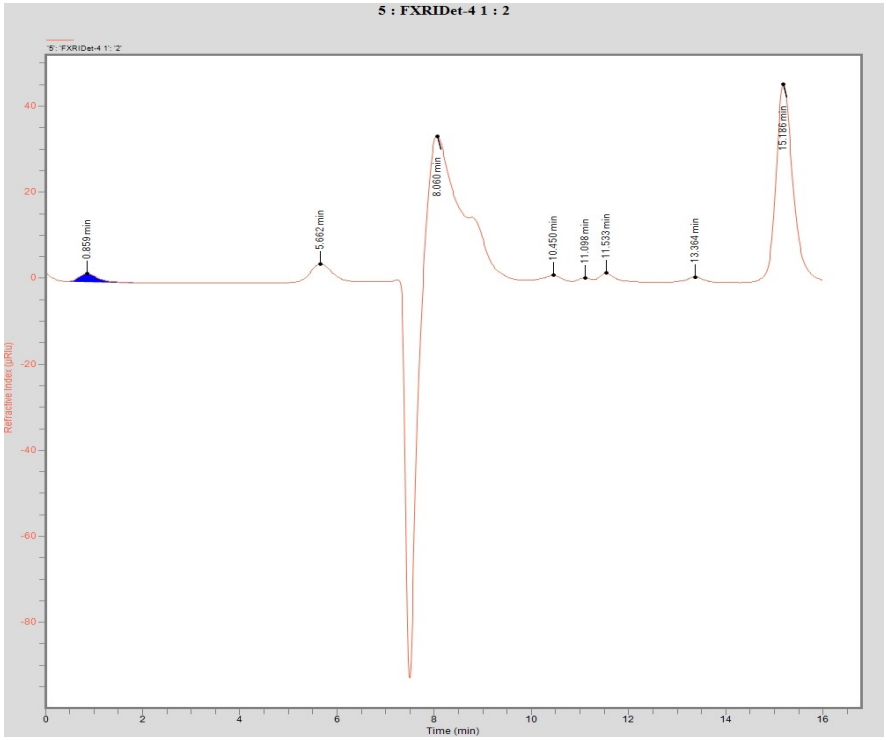
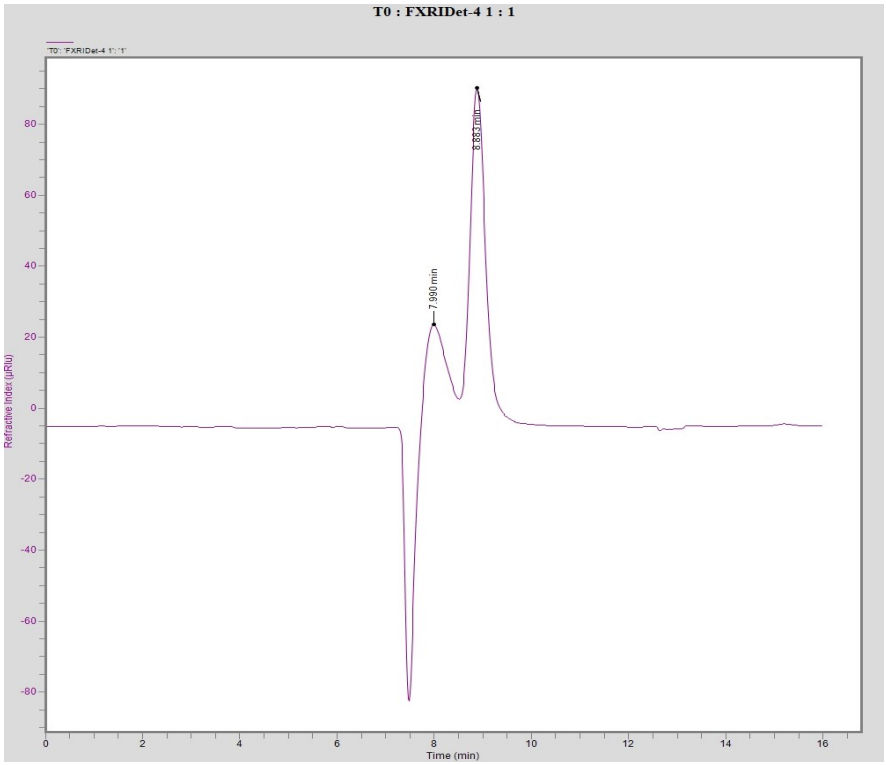


Figure A 16: S2P4-D lactose and lactic acid chromatogram at a) 0 hours b) 120 hours. 48 – MAE, hour staggered addition of inoculum and carbon source, (S2P4-D).

a)



b)

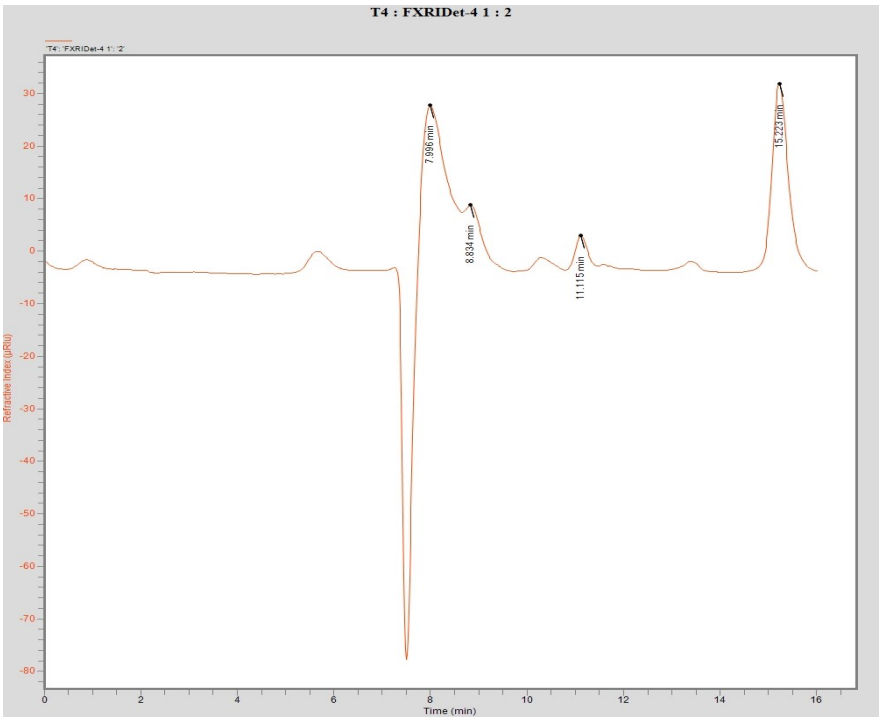


Figure A 17: 5L Scale-up S2P4-C lactose and lactic acid chromatogram at a) 0 hours b) 120 hours. 48 - hour staggered addition of inoculum and carbon source, (S2P4-C)

Carbon Balance of 5L Scale-up Fermentation:

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow 4\ C_3H_6O_3$$

Presence of lactose: 288.9mM

Actual presence of lactic acid: 865.92mM

(theoretical lactic acid presence: 1155.6mM)

Only 75% of lactic acid is produced

Lactose added: 500 gram

Molecular weight of Lactose: 342.3 g/mol

Molecular weight of Lactic acid: 90.08 g/mol

500/342.3 = 1.46

Since only 75% of lactic acid is produced,

375/70.08 = 4.16

L.H.S - 12 (no. of carbon)*12 (atomic weight of Carbon)*1.46 = **210.24 of C**

R.H.S - 12 (atomic weight of Carbon)*3(no. of carbon)*4.16 = **149.87 of C**

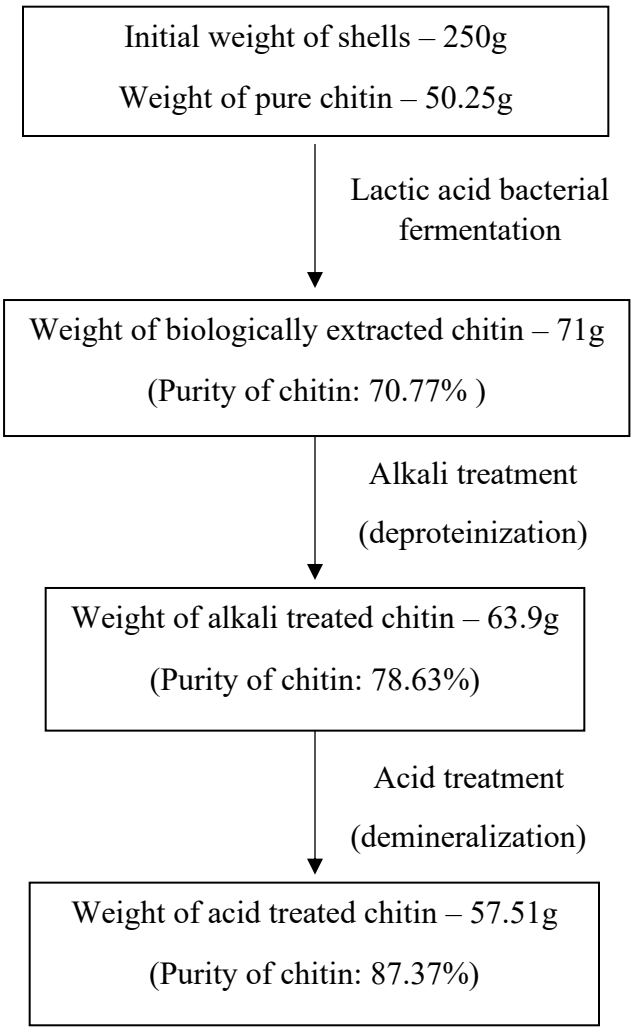


Figure A 18: Purification of biologically extracted chitin by alkali and acid treatment

The above process is used to determine the purity of chitin extracted using biological process and to determine the purity of chemically treated extracted chitin. The weight of pure chitin is determined by the composition of shell particle size 500µm-250µm (Chitin) as shown in table 4.1 from the total shell load of 5L fermentation. The purity of the biologically, deproteinized and demineralized chitin are found using Eq., A1.

$$\text{Chitin purity} = (\text{weight of pure chitin} / \text{weight of extracted chitin}) \times 100 \quad \text{Eq., A1}$$

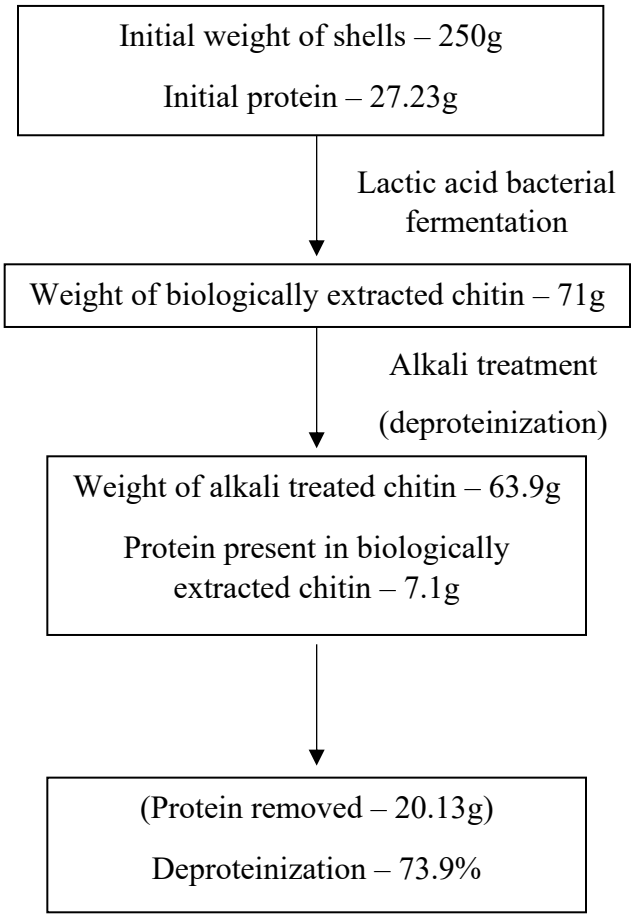


Figure A 19: Calculation of degree of deproteinization using chemical treatment.

In literatures, the degree of deproteinization is calculated using the theoretical percent of nitrogen in acetylated chitin (6.9) and the theoretical percent of nitrogen in proteins (6.25) (Steeves, 2019; Wei Xu *et al.,* 2020). The theoretical value may vary within organism and thus it does not give a conclusive degree of deproteinization.

To overcome the problem, the above process can be used to determine the degree of deproteinization using chemical treatment. The biologically extracted chitin is treated with alkali to remove the remaining protein present in residual chitin. The initial protein is calculated by the proximate composition of shell particle size 500µm-250µm (protein) as shown in table 4.1 from the total shell load of 5L fermentation. The weight loss after the chemical treatment is the weight

of protein present in the biologically extracted chitin. Thus, the protein removed can be calculated from the initial protein and protein removed from biologically extracted chitin. The degree of deproteinization is calculated by using the Equation A2,

$$\text{Degree of deproteinization} = (\text{Protein removed} / \text{Initial protein}) \times 100 \qquad \text{Eq., A2}$$