Multistage Extraction and Purification of Waste *Sargassum natans* to Produce Sodium Alginate: An Optimization Approach

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Abstract

Sargassum in the Caribbean region has affected the livelihood of several coastal communities due to the influx of large quantities of the seaweed in recent times. This article seeks to explore how waste *Sargassum natans* can be utilized to produce sodium alginate. The novelty in this research lies in the optimization process, whereby multistage extraction and precipitation were investigated over commonly used single stage processing, in an effort to maximize both yield and purity. The results showed that a maximum yield of 19% was observed after 1 stage, while the purity was 74% after 4 stages. In addition, optimization of the multistage precipitation process using the Global Optimization Toolbox in MATLAB R2017b provided a novel model which indicated that a compromise between the maximum purity and yield can be obtained at 3 stages; 71 – 74% and 12 – 16% respectively. Furthermore, characterization was done using FTIR and NMR, with results comparable to a commercial sodium alginate brand, giving absorption bands at 1610 cm⁻¹ and 1395 cm⁻¹ and an M/G ratio of 0.51 respectively.

Keywords: Sodium Alginate; *Sargassum natans*; NMR; Multistage Extraction; Multistage Precipitation; Optimization.

1. Introduction

Alginates are polysaccharides that are naturally present in the cell walls of brown seaweeds and are composed of linear binary copolymers of (1→4)-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers (Torres et al., 2007). Alginate exists as a mixed salt of cations that are found in sea water; mainly magnesium, sodium and calcium ions (Larsen, Salem, Sallam, Mishrikey, & Beltagy, 2003), and naturally possesses carboxyl groups in each constituent M and G block residue. The M/G ratio varies depending on the algal species as well as the specific tissues of a given species (Donati et al., 2003). The mannuronic acid forms $\beta(1\rightarrow4)$ linkage, which leads to the M block segments possessing linear and flexible conformations. The guluronic acid has $\alpha(1\rightarrow4)$ linkage that introduces steric hindrance around the carboxyl groups, causing the G block segments to possess folded and rigid structural conformations (Avella et al., 2007).

The most expedient and distinctive property of alginates is their ability to react with divalent metal ions, specifically calcium ions to produce water-insoluble calcium alginate via ionic crosslinking (Grant, Morris, Rees, Smith, & Thom, 1973). The divalent calcium cation, Ca^{2+} , fits into the guluronic acid structure like ‘an egg in an egg-box’, which leads to an increase in the water resistance of calcium alginate films. This increase in water resistance is due to the binding of the alginic acid by forming junction zones (Rhim, 2004). The backbone of the G segments form the water-insoluble calcium alginate due to strong interactions between the divalent cations and the COO^- groups of the base residual of guluronic acid (Avella et al., 2007).

These sodium alginates are widely used as viscosifying/gelling agents and stabilizers in the biomedical, cosmetic, textile, pharmaceutical and food industry due to their rheological properties (Draget, Smidsrød, & Skjåk-Bræk, 2005). In the pharmaceutical field, calcium alginate hydrogels are used as a matrix for drug encapsulation, sustained release of cells/delivery vehicle of drugs, pill disintegration, wound healing and tissue engineering (Lee & Mooney, 2012). More recently, there has been growing attention into the uses of calcium alginate as structural supporting biomaterials for tissue (teeth, bone and cartilage), reconstruction (Chen et al., 2017; Wahl et al., 2015), as taste masking agents (Rajesh, Bhatt, Brahmbhatt, Anand, & Popat, 2015), wound dressings (Carella, Maruccia, Fino, & Onesti, 2013), and as injectable vehicles for topical drug delivery systems (Almeida, Amaral, Lobão, & Lobo, 2014; Barzegari & Saei, 2012). Also, calcium

alginate microparticles are being developed to treat diabetes and Parkinson's disease (Calafiore & Basta, 2014; Hegarty, O'Keeffe, & Sullivan, 2014). Furthermore, new research is underway into the use of calcium alginate-based bio-inks in 3D bio-printing for direct fabrication of artificial living tissue (Axpe & Oyen, 2016).

The procedure to extract sodium alginate from different types of brown seaweeds uses specific methods depending on the growth conditions of the seaweed. Commercial sodium alginate is typically extracted from brown seaweed such as *Laminaria hyperborea*, *Laminaria digitata*, *Ascophyllum nodosum*, *Macrocystis pyrifera* and to a lesser extent from *Laminaria japonica*, *Eclonia maxima* and *Sargassum* sp. (Smidsrød & Skjåk-Bræk, 1990).

The Caribbean has seen a massive influx of *Sargassum* in 2011-2018, which creates a variety of problems for coastal residents as it affects fisher livelihoods; difficulty in launching boats, restricts access to fishery resources, affects tourism and decomposition results in a very foul smell. The *Sargassum* found in Trinidad can be identified as *Sargassum natans* due to its morphological characteristics such as its lanceolate blades with aculeate teeth, lateral smooth branches and air bladders which correlates with previous investigations (Schneider & Searles., 1991; Széchy, Guedes, Baeta-Neves, & Oliveira, 2012).

Extraction is usually done via acid pretreatment of the seaweed followed by treatment with aqueous alkali solution. The extract is obtained and then precipitated to form alginic acid followed by conversion and purification to form dry powdered sodium alginate. However, in the conventional extraction process, single stage batch extraction and precipitation is carried out. It is anticipated that multistage extraction and precipitation can provide a better yield and a higher quality of sodium alginate. Therefore, in this work we report the use of a multistage extraction and precipitation process of a waste brown seaweed (*Sargassum*) to produce sodium alginate. We also report a novel optimized model for the purification of the sodium alginate extracted. Both the yield and purity of the sodium alginate extracted from *Sargassum natans* are discussed in this work showing that through multistage extraction and precipitation, it is possible to extract high yield and purity sodium alginate.

2. Materials and methods

2.1. Materials

The *Sargassum natans* seaweed was collected from both Manzanilla and Mayaro Bays on the Eastern Atlantic side of the island of Trinidad during the month of May 2017. Formaldehyde (BDH, 36.5 wt. % in water) was used in the seaweed pretreatment process. Acid treatment experiments were performed using different concentrations of sulphuric acid (96.4%) purchased from J.T. Baker (USA). Calcium nitrate solution (1000ppm) used for Atomic Absorption Spectroscopy (AAS) was purchased from Red analytical (USA). For the alkaline extraction process, sodium carbonate (Scharlau, 99.9%) was used. Food grade sodium alginate (purity $\geq$ 96%) purchased from WillPowder (44145S, USA) was used as the analytical standard for High-Performance Liquid Chromatography (HPLC), while commercial grade sodium alginate purchased from Sigma-Aldrich (W201502, USA) was used for comparison studies. The buffer solution used for HPLC was made using phosphoric acid (J.T. Baker, purity $> 86\%$) and sodium hydroxide (J.T Baker, $>99\%$). For the purification process, 50 % (w/v) alcohol (BDH, 94-96% ethanol and methanol) was used. For Nuclear Magnetic Resonance (NMR) analysis, deuterium oxide (Sigma-Aldrich, purity $>99.9\%$), triethylenetetraminehexaacetic acid (TTHA) (Sigma-Aldrich, purity $\geq 98\%$) and sodium deuterioxide (Sigma-Aldrich, purity 99%) were used.

2.2. Extraction process

2.2.1. Seaweed pretreatment

Figure 1 gives the overall extraction process for sodium alginate from *Sargassum natans*. The process begins with seaweed pretreatment whereby bulk impurities such as debris (silt, sand, plastics, and micro-organisms) are removed during continuous washing. After the washing step, the *Sargassum* was preserved in 2% (w/v) formaldehyde solution overnight using the same method according to Fertah, Belfkira, Dahmane, Taourirte, and Brouillette (2017) . Seaweed pretreatment with formaldehyde was essential as the acid pretreatment step alone does not remove all the phenolic compounds. The formaldehyde reacts with the phenolic compounds in the cell walls of the seaweed allowing for enhanced product purity (Fertah, 2017; Wedlock & Fasihuddin, 1990).

After pretreatment, the solution was removed and the *Sargassum* was washed and dried for 48 hours at 40°C.

Once the seaweed was completely dried, it was pulverized in an effort to increase surface area and extraction rates (McHugh, 1987). The pulverized seaweed was sieved and a mean particle diameter of approximately $505 \pm 5 \mu\text{m}$ was calculated and used throughout acid pretreatment and alkaline extraction experiments.

2.2.2. Experimental and analytical methods

For acid pretreatment, alkaline extraction and sodium alginate precipitation samples were carried out in triplicate while two control experiments were used for validation. All experimental errors were analyzed by calculating both the standard deviation and coefficient of variance, while statistical t-tests were used to test significance.

Atomic Absorption Spectroscopy (AAS) was used to quantify the amount of calcium released during acid pretreatment. The conditions for AAS was as follows: calcium line: 422.7nm, slit width: 1.2nm, acetylene/ nitrous oxide flame, 50mm burner (width) operating at a height of 6mm. Calcium standard solutions were made utilizing a 1000 ppm calcium nitrate analytical solution. The calibration curves plotted had an R^2 value of 0.99 within a calibration range of 1-5ppm.

High Performance Liquid Chromatography (HPLC) was used to quantify crude and purified sodium alginate yields. A C18 stationary phase column (150 × 4.6 mm i.d., 3.5 μm) was used. The mobile phase consisted of a buffer solution (0.5mL phosphoric acid) in 1L deionized water at a flow rate of 0.7mL/min at 25°C, adjusted to pH 7.0 with sodium hydroxide (1M). The system was left to equilibrate for 30 minutes prior to sampling. A sample volume of 20 μL was injected, and sodium alginate was eluted at a retention time of 1.50 ± 0.03 minutes using a wavelength of 254nm. Sodium alginate standards were made utilizing a 1g/L food grade alginate solution, and the calibration curves had an R^2 of 0.99 within the calibration range of 0.01-0.03g/L.

2.2.3. Acid pretreatment

As shown in **Figure 1**, acid pretreatment follows seaweed pretreatment as an important step in the conversion of calcium alginate into alginic acid. In brown seaweeds such as *Sargassum*, alginic

acid is mainly present as the calcium salt of alginic acid although potassium, magnesium and sodium salts may also be present (Bashford, Thomas, & Woodward, 1950). As shown in **Equation 1**, the insoluble calcium alginate, (Ca(Alg)₂), reacts with the sulphuric acid to form alginic acid, (HAlg), and insoluble calcium sulphate, (CaSO₄)

The calcium rate was calculated using the following equation:

$$\text{Calcium Rate} = \frac{\text{mass of Calcium produced (mg)}}{\text{time taken (hr)} \times \text{mass of seaweed used(mg)}} \quad (2)$$

Acid pretreatment of *Sargassum* was performed according to procedures developed by Hernández-carmona, McHugh, Arvizu-Higuera, and Rodríguez-montesinos (1998) and McHugh (1987). In an effort to quantify the efficiency of the acid pretreatment process, the effect of sulphuric acid concentration, volume and temperature on calcium rate was investigated. $1 \pm 0.01\text{g}$ of *Sargassum* was reacted with sulphuric acid within a molar concentration range of 0.1-1M, an excess volume range of 1:5-1:15 (*Sargassum*: H₂SO₄), and over a temperature range of 25-60°C. Temperature was monitored using a water bath (Julabo TW8, Germany). In order to promote mixing, the samples were vortexed for 1 minute and reacted for 1 hour. After the reaction time had elapsed, the samples were filtered using syringe filters (0.45µm), diluted and analyzed for calcium concentration using AAS.

2.2.4. Alkaline extraction

After calcium alginate conversion during acid pretreatment, the insoluble alginic acid formed can be converted into a soluble form as sodium alginate. As shown in **Figure 1**, the alginic acid can be neutralized by the action of sodium carbonate according to **Equation 3** (Hernández-carmona et al., 1998):

The crude yield of sodium alginate can be calculated using **Equation 4**:

$$\text{Yield} = \frac{\text{Concentration of Sodium Alginate Produced (mg/mL)} \times \text{Volume (mL)}}{\text{Mass of Seaweed Used (mg)}} \quad (4)$$

The conditions investigated during alkaline extraction were adapted from many authors (Basha, Rekha, Letensie, & Mensura, 2011; Davis, Ramirez, Mucci, & Larsen, 2004; Fenoradosoa et al., 2010; Hernández-Carmona, McHugh, & López-Gutiérrez, 1999; McHugh, 1987; Torres et al., 2007). Similar to **Section 2.2.3**, in an effort to investigate the efficiency of the extraction process, the effect of sodium carbonate concentration, volume, temperature and reaction time was investigated. Utilizing the best conditions from **Section 2.2.3**, 1 ± 0.01 g of acid treated *Sargassum* was reacted with sodium carbonate within a concentration range of 5-10% (w/v), an excess volume range of 1:5-1:15 (*Sargassum*: Na₂CO₃), over a temperature range of 25-75°C, and reaction times of 0.5-6 hours. After the associated reaction time had elapsed, the samples were centrifuged (Thermo-IEC Centra MP4, USA) at 8000g and filtered. The filtrate was diluted and analyzed for crude sodium alginate using HPLC.

2.3. Multistage alkaline extraction

In an effort to increase the overall crude sodium alginate yield from *Sargassum*, multistage alkaline extraction was carried out utilizing the most efficient conditions from both the acid pretreatment and alkaline extraction steps. Single batch stages were repeated for both steps as identified in **Sections 2.2.3** and **2.2.4**. After centrifugation was carried out at the end of the alkaline extraction step, the residual *Sargassum* was retrieved and extraction was repeated as a second stage. This multistage extraction process was repeated up to 5 stages, and the batch and cumulative crude sodium alginate yields were estimated using HPLC.

2.4. Multistage precipitation process

In an effort to purify the crude sodium alginate extract, precipitation was adapted from the work by McHugh (1987). 0.5M H₂SO₄ was gradually added to the crude extract obtained, after the multistage extraction process was carried out, until a pH of 2 was observed using a pH meter (Thermo Scientific A211, USA). At this point the soluble crude sodium alginate was converted

into insoluble alginic acid. The samples were centrifuged at 8000g for 10 minutes, and the alginic acid was separated. 15mls of alcohol was added to the alginic acid under constant stirring for 2 hours. After 2 hours elapsed, 5% Na₂CO₃ was added to the mixture until the pH increased to 10, forming a thick sodium alginate gel. The samples were centrifuged, and the gel was collected, placed into petri dishes, frozen at -17°C for 24 hours, and freeze dried for 48 hours using an Armfield vacuum freeze dryer (SB4, UK). The purified sodium alginate was then pulverized, and the purity was analyzed using HPLC.

For the multistage precipitation process, this procedure was repeated up to 5 stages by redissolving the purified sodium alginate after each subsequent stage. The true yield was calculated using **Equation 5**:

$$\text{True Yield} = \frac{\text{Mass of Purified sodium alginate}}{\text{Mass of Seaweed Used (mg)}} \quad (5)$$

2.5. Characterization

2.5.1. Fourier transform infrared spectroscopy (FTIR)

IR spectra of the purified alginate samples were obtained using a Perkin Elmer Spectrum 400 FT-IR/ FT-NIR spectrometer with a universal ATR sampling accessory. The spectra were recorded in the range of 650-4000 cm⁻¹ with a 4 cm⁻¹ resolution. The IR-laser wavenumber was set at 15780.00 cm⁻¹, OPD velocity of 0.20 cm/sec and J-stop size of 8.94 mm.

2.5.2. Nuclear magnetic resonance (NMR)

The samples for NMR analysis was done according to ASTM F2259 – 10 (2012). Firstly the alginate was depolymerized via acid hydrolysis. 100 mLs of 0.1% (w/v) alginate solution was prepared and the pH was adjusted to 5.6 using 0.1 M hydrochloric acid. The alginate solution was placed in a water bath at 100°C for 1 hour. The pH was re-adjusted to 3.8 with 0.1 M hydrochloric acid and replaced into the water bath at 100°C for 30 minutes. The pH was then neutralized to 7 with 0.1 M sodium hydroxide and the sample was freeze-dried overnight. The alginate sample was then dissolved in 5mL of 99.9% D₂O and freeze-dried again. 10-12 mg of the sample was then

dissolved in 1 mL of 99.9% D₂O. 0.7 mL of the alginate was added to a NMR tube along with 40 µL of 0.15 M TTHA (triethylenetetraminehexaacetic acid). The pH of the TTHA was adjusted to 5.5 using sodium deuteroxide (NaOD). The TTHA was used as a chelator to minimize traces of divalent cations interacting with the alginate. The NMR spectra were recorded on a NMR spectrometer (Bruker AVANCE™ 600 MHz) operating at 80°C with the sample spinning at 20 Hz using a standard one-dimensional pulse program, a frequency of 600 MHz, a spectral window of 6kHz with a pulse angle of 90°, 2 seconds relaxation delay and acquisition time of 5.453 seconds collecting 65536 data points. 64 scans were accumulated for signal averaging. The chemical shifts of the anomeric proton signals were done according to ASTM-F2259-10 method with A (guluronic acid anomeric proton) at around 5.2 ppm; B1 (H-5 proton of the central guluronic acid residue in a GGM triad) at 4.78 ppm ; B2 (H-5 proton of the central guluronic acid residue in a MGM triad) at 4.75 ppm ; B3 (anomeric proton of the mannuronic acid residue neighboring a mannuronic acid) at 4.71 ppm ; B4 (anomeric proton of the mannuronic acid residue neighboring a guluronic acid) at 4.68 ppm and C (guluronic acid proton 5) at 4.48 ppm.

3. Results and discussion

3.1. Fourier transform infrared spectroscopy (FT-IR)

The small amounts of polyphenolic residues present in alginates make them strongly fluorescent (Fertah, 2017). The spectrum produced (**Figure 2**) gave a strong and sharp band at 1610 cm⁻¹ as this represents the strong carboxylate asymmetric stretching vibration. The absorption band at 1395 cm⁻¹ shows the weaker COO symmetric stretching vibration of the carboxylate group. The characteristic peaks obtained in this study correlates closely with previous studies (Bi, Mahmood, Arman, Taj, & Iqbal, 2007; Fenoradosoa et al., 2010; Fertah, 2017; Latifi, Sadegh Nejad, & Babavalian, 2015; Papageorgiou et al., 2010; Rhein-Knudsen, Ale, Ajalloueian, & Meyer, 2017b) giving strong bands at around 1600 and 1400 cm⁻¹ indicative of the asymmetric and symmetric vibrations. The presence of these carboxylate groups further show that the alginate was extracted at alkaline pH in the form of a sodium alginate salt (Rhein-Knudsen et al., 2017b). Furthermore, the spectra for sodium alginate produced from the *Sargassum* was similar to the sodium alginate

from the commercial brand, and resembles the finger print region (1600-1400 cm^{-1}). Hence, this further confirms the presence of sodium alginate in the extracted samples.

3.2. Nuclear magnetic resonance (NMR)

Figure 3(A) shows the NMR spectra of the sodium alginate sample extracted from the *Sargassum natans* with the peaks representing the anomeric and other protons at varying carbon positions on the uronic acid sequence. The assignment of the peaks obtained in the spectra are slightly different from those in the ASTM standard used. These differences in the chemical shifts obtained in the alginate extract with the ASTM standard F2259 – 10 (2012) can be attributed to the presence of trace elements of metal ions in the alginate extracted and differences in the chemical composition and properties such as molecular weight. The presence of the metal ions (calcium) causes line broadening and selective loss of signal intensity.

However, from **Figure 3(B)**, the chemical shifts of the signature protons for the alginate extracted from *Sargassum natans* and commercial brand Sigma overlay precisely, confirming that the polysaccharide extracted from the *Sargassum natans* was in fact alginate.

The block structure and M/G ratio was calculated according to the calculation proposed in the ASTM standard F2259 – 10 (2012). The M/G ratio (0.51) of the alginate extracted in this study correlates closely to the M/G ratio of the alginate extracted from *Sargassum natans*, 0.47 (Rhein-Knudsen et al., 2017b). The M/G ratio of this sodium alginate from *Sargassum natans* can be elucidated to lie within the range of the *Sargassum* genus from **Table 1** having enriched guluronic acid content. The commercial brand Sigma, has a high M/G ratio compared to *Sargassum* and this was not unexpected as commercial brands extracted from *Ascophyllum* and *Laminaria* range from 1.2 to 2.2 (Grasdalen, 1983). The uronic acid sequence highly influences the ability of the alginate to form gels (Penman & Sanderson, 1972). M-blocks show flexible and linear conformations while G blocks have rigid structural conformations giving rise to a distinct stiffness of the molecular chains (Avella et al., 2007). Gel formation occurs mainly as a result of the formation of enriched GG junction zones. Alginates that possess a low M/G ratio give hard and brittle gels while those with a high M/G ratio form soft and elastic gels (Penman & Sanderson, 1972). Hence this extracted alginate possesses a high guluronic acid content of 66% (standard deviation $\pm 1\%$) and will most likely form strong gels.

3.3. Effect of H₂SO₄ concentration and excess volume on calcium rate

The acid pre-treatment process is very important since it converts all the insoluble salts that remain in the algal matter after washing, into alginic acid which is converted to sodium alginate upon alkaline treatment (A. Haug, 1964). Insight into acid pretreatment conditions have not been looked at in any precise or scientific nature in previous studies (Davis et al., 2004; Fenoradosoa et al., 2010; Larsen et al., 2003; McHugh, 1987; Rhein-Knudsen et al., 2017a; Torres et al., 2007). Furthermore, calcium rate gives a measure of the extent of alginic acid conversion and has not been reported by any previous research. Acid pre-treatment is typically done with a dilute mineral acid usually sulphuric acid or hydrochloric acid (A. Haug, 1964; McHugh, 1987). In this study, sulphuric acid was chosen due to its lower pK_a (-10) compared to hydrochloric acid (-7) (Lide, 2005). Hence, sulphuric acid would provide higher alginic acid conversion.

Figure 4(A) shows the effect of increased molar concentration on the total amount of calcium released (**Equation 2**). It can be shown from **Figure 4(A)** that as the molar concentration of the sulphuric acid increases from 0.1 M to 0.5 M, there was a general increase in the calcium rate with a maximum rate of 0.26 mg calcium/(mg.seaweed.h) being recorded. However, it was observed that as the molar concentration increased above 0.5M, the calcium rate decreased to 0.17 mg calcium/(mg.seaweed.h). Sulphuric acid is a strong acid and in aqueous solution fully dissociates into [H⁺] and [SO₄²⁻]. High acid concentration can lead to degradation of the cell walls within the *Sargassum* structure. These cell walls are comprised of glucans which are polysaccharides composed of glucose, and at low pH these glucans can be preferentially hydrolyzed (Yanagisawa, Kawai, & Murata, 2013). As such, as the pH decreases, the hydrolysis reaction competes with the calcium conversion process. Hence, it can be elucidated that as the molar concentration of the acid increases, the rate of hydrolysis increases and as such a decrease in the calcium rate was observed. Although the effects of acid hydrolysis were observed at 1M, the overall calcium rate was 1.4 times higher than that at 0.1M, showing that the calcium conversion process favors a higher acid concentration.

Figure 4(B), which shows that as the acid volume increased, the calcium rate increased, following the same general trend as **Figure 4(A)**. These results can be explained as a larger volume of acid

added to the seaweed results in more $[H^+]$ ions being readily available, increasing the calcium ions exchanged and the amount of alginic acid formed.

3.4. Effect of temperature on calcium rate

The effect of temperature on calcium rate is an important parameter that has not been investigated fully in previous work done on acid pretreatment. 0.5M sulphuric acid at an excess volume of 1:15, were used to conduct experiments to analyze the temperature for optimum Ca^{2+} ions liberation. **Figure 4(C)** relates the relationship between the calcium rate and the temperatures tested (25°C, 30°C, 40°C, 50°C, and $60 \pm 0.1^\circ C$). It was observed that as the temperature increases, the calcium rate also increases, with a maximum temperature recorded at 40°C. An increase in temperature allows for increased $[H^+]$ ions and therefore increased calcium rate. Similar acid pretreatment results have been reported by (McHugh, 1987) as degradation of the alginate occurs with most species of seaweed at temperatures beyond 50°C.

The results suggest that 40°C was the optimum temperature for acid treatment at 0.5M sulphuric acid using 1:15 excess volume. A Student's T-test ($p = 0.05$) was also done in order to assess whether the averages at 50°C and 60°C were significantly different from 40°C. The results showed that they were insignificant and therefore confirmed that 40°C was the optimum temperature.

3.5. Effect of Na_2CO_3 concentration and excess volume on sodium alginate yield

Previous research into alkaline extraction (Basha et al., 2011; Davis et al., 2004; Fenoradosoa et al., 2010; Hernández-Carmona et al., 1999; McHugh, 1987; Torres et al., 2007) have been carried out but no specific operating ranges have been given.. Thus, it was important that conditions be investigated to understand its effect on sodium alginate yield, and to find the desired conditions that maximizes the sodium alginate yield obtained from *Sargassum natans*.

From **Figure 5(A)**, it was observed that crude sodium alginate yield (**Equation 4**) decreased by more than 50% as the alkaline concentration increased above 5%. The 5% Na_2CO_3 concentration gave the highest yield of crude sodium alginate with a value of 6.08% whereas 10% Na_2CO_3 gave the lowest yield at a value of 1.65%. A Student's T-test ($p = 0.05$) was performed for both 1g: 10mL and 1g: 15mL at 7% sodium carbonate. The results indicated that they were both statistically

significant. Another Student's t-test ($p = 0.05$) was done for all 3 excess volumes at 10% sodium carbonate. However, the results showed that they were not statistically significant.

Alkaline degradation at high concentrations results in the formation of monocarboxylic and dicarboxylic acids (Niemelä & Sjöström, 1985), and hence can explain the decrease in the yields of sodium alginate extracted from *Sargassum natans* observed at 7% and 10% Na_2CO_3 . The pH of the aqueous solutions of 7% and 10% Na_2CO_3 are of moderate alkalinity ($\text{pH} > 10$) (Kroschwitz & Seidel, 2004) and thus promotes degradation of the alginate significantly (A. Haug, 1964; Moen, 1997). Furthermore, alkalis can react with alginates causing β -elimination of the glycoside bond which reduces the yield of the intact polysaccharide. Also, effects of this degradation process can be more pronounced at $\text{pH} > 10$ (Arne Haug, Larsen, & Smidsron 1963).

Figure 5(B) shows the general trend that as the alkali volume increased, crude sodium alginate yield increased, with the highest yields being reported with the highest volumes used. Similar to the acid pretreatment results observed in **Section 3.3**, an increase in the volume of alkali added increases the neutralization effect that occurs between alginic acid and Na_2CO_3 , and also allows for better solubilisation of the extracted crude sodium alginate. Hence, a greater yield of extraction would be obtained. Using alkali volumes of 1:10 and 1:15 at concentrations of 7% and 10% Na_2CO_3 gave low yields further indicating that pH has a pronounced effect on the conversion of the alginic acid. Hence, from the results shown in **Figures 5(A) and (B)** a low concentration of Na_2CO_3 was favorable for the alkaline extraction process.

3.6. Effect of temperature and reaction time on sodium alginate yield

From **Figure 5(C)**, it was observed that the crude sodium alginate yield increased over the temperature range of 25°C to 65°C (7 % to 15 %) utilizing a concentration of 5% Na_2CO_3 , at an excess of 1:15, and an extraction time of 2 hours. Furthermore, a maximum yield was found at 65°C and this was recorded as the optimum temperature. These results correlated well with Torres et al. (2007) who reported an optimum temperature at 60°C in the extraction of sodium alginate from *Sargassum vulgare*. However, there was an observed decrease in the yield between 65°C and 75°C which can be attributed to the higher temperatures leading to the breakdown of uronic acid chains and consequently lower viscosities for the sodium alginate. Whistler and BeMiller (1959) reported that alginates are readily degraded by alkaline solutions at high temperatures resulting in

the formation of new dicarboxylic saccharinates. It could be quite possible that this degradation reaction explains the decrease in sodium alginate yield at 75°C.

Reaction time plays an integral role in the alkaline extraction and there has been no thorough analysis done to investigate how sodium alginate yields from *Sargassum* trend over a range of reaction times. **Figure 5(D)** shows the crude sodium alginate yield obtained when the seaweed was reacted over different time periods at the optimized conditions (65°C, 1:15 excess volume, 5% Na₂CO₃). A Student's T-test ($p = 0.05$) was carried out on the data above (**Figure 5 D**) and it was found that for all reaction times investigated, the yields reported were not significantly different. The reasoning behind this could be that the extraction equilibria associated with the sodium alginate conversion reaction occurs rapidly during the first hour of extraction, and hence, the maximum amount of sodium alginate liberated was accounted for within this particular time frame. During a reaction, the mass transfer resistance is often reduced allowing for rapid equilibrium (Klaewkla, Arend, & Hölderich, 2011). From **Figure 5(D)**, the highest crude yield reported was 15% after one extraction stage. It can be elucidated from the results, that increased reaction time does not affect the overall crude sodium alginate obtained from *Sargassum natans*.

3.7. Effect of multistage extraction and multistage precipitation on sodium alginate yield and purity

Conventional extraction and precipitation of sodium alginate from seaweed is usually based on single stage batch processing. Previous research (**Table 2**) has reported results based on one purification stage with very little analysis on purity. The objective of the extraction process was to recover as much alginate as possible so as to obtain a high yield. In order for complete extraction to occur, a concentration gradient must be established between the concentration of solute at the surface of the solid particles and that in the bulk solution.

In single stage extraction, as the phases approach equilibrium, the gradient ceases and hence, the overall rate of extraction decreases (Brennan, Grandison, & Lewis, 2006). After one stage, the seaweed may still contain a significant amount of alginate and can be recovered via further extraction of the solute with fresh solvent. The rate of diffusion determines the length of time needed to achieve equilibrium between phases (Toledo, 1991). In this solid-liquid extraction, the sodium carbonate must diffuse into the cell walls of the seaweed in order for neutralization to

occurs, and the alginate must diffuse out of the ‘carbonate-saturated seaweed’ into the bulk solution. However, this cannot be achieved fully using single stage extraction and hence the need for multistage extraction (Berk, 2009).

Multistage extraction and precipitation of sodium alginate from *Sargassum natans* has never been explored as a process for maximizing yield and purity. This study demonstrates the optimum number of extraction and purification stages to maximize both yield and purity of the sodium alginate at the optimum conditions reported previously; 5% Na₂CO₃, 1:15 excess volume, at a temperature of 65°C and a reaction time of 2 hours.

From **Table 2**, it was found that after one extraction stage, the crude sodium alginate yield obtained was 15%. After the first extraction stage, it was assumed that the seaweed residue still contains crude sodium alginate that can be extracted. Upon a second extraction stage, it was found that the residual alginate found accounted for a crude yield of 2%. By reacting the residue with fresh solvent, a new concentration gradient can be established allowing for increased extraction and an overall cumulative yield of 17%. These findings correlate well with past research utilizing multistage extraction techniques such as; in the extraction of polyphenols from olive leaves, the extraction yield increased to more than 50% after 3 stages compared to single stage extraction (Stamatopoulos, Chatzilazarou, & Katsoyannos, 2014). Furthermore, in the extraction of protein from *Jatropha curcas* seeds, the recovery increased from 35% to 73% after 4 stages (Lestari, Mulder, & Sanders, 2010).

However, it was observed that beyond 2 stages the crude yield was very low (< 1%) and it was no longer feasible to carry out extraction. This can be explained whereby the net molar transport stops when equilibrium was reached between the phases. As a result the chemical potential of the extractable solute was the same in all phases (Berk, 2009). Hence, after two extraction stages, equilibrium was attained and all the available alginate within the seaweed was recovered. Thus, it can be shown that multistage extraction can in fact be used to maximize the crude yield of sodium alginate from *Sargassum*.

In an effort to improve the purity of the sodium alginate extracted from *Sargassum*, multistage precipitation was carried out utilizing the crude extract obtained from 2 extraction stages. The freeze-dried sodium alginate obtained after the first purification stage was re-dissolved, and the precipitation process was repeated for each subsequent stage up to 5 stages.

After the first precipitation stage, the true yield and purity was found to be 19 % and 67% respectively. From **Table 2**, it can be shown that as the number of precipitation stages increases, the yield decreases while the purity increases. From stage to stage, the equilibrium changes and this can result in lower yields observed in each subsequent stage. The reducing yields can be attributed to imperfect separation of the alginate from the impurities which may result in the discarding of fractions that are deemed insufficiently pure. Also, as more impurities are removed, according to **Equation 5**, the true yield can be expected to decrease. However, as the number stages increases, the impurity removal rate increases thus allowing for enhanced sodium alginate purity.

The true yield of sodium alginate reported in this study correlates well with that of other *Sargassum* sources (**Table 2**). Hence, alginate yields obtained in this study can be postulated to lie within the range for the *Sargassum* genus. It should be noted that the true yield found in this study was lower than the alginate extracted from other commercial algal species such as *Ascophyllum nodosum*, *Lessonia nigrescens*, *Macrocytis pyrifera*, *Laminaria spp*, *Durvillea willana* and *Marginariella boryana*, which reports yields of up to 61% of their dry weight (Draget et al., 2005; Panikkar & Brasch, 1996; Rinaudo, 2007). However, the true yield value of 19% was not surprising as McHugh (2003) states that *Sargassum* usually gives sodium alginate that has borderline quality and of a low yield. Nevertheless, through multistage precipitation, the purity was relatively high and found to increase from 67% to 74%.

Also, it can be elucidated that the differences in the yields reported in literature can be due to the fact that the extraction process was carried out using different *Sargassum* species. Additionally, the extraction yield can be affected by the period and place of collection of the seaweed. Furthermore, the low yields reported in literature could have occurred as the extraction for that particular species may not have been carried out at the ideal conditions. Therefore, it can be elucidated a more complete extraction of sodium alginate can be attained using multistage extraction and multistage precipitation rather than single stage extraction and precipitation.

3.8. Data optimization of multistage precipitation

Optimization has never been used as a method for improving the sodium alginate yield and purity from *Sargassum natans*. Process optimization is an important tool that can be used to maximize efficiency, by setting specified parameters without violating constraints.

Figure 6(A) shows the relationship between purity and yield between subsequent stage precipitations illustrated in **Table 2**. The equations for the purity and yield are as follows:

$$P = -0.80032N^2 + 6.4042N + 61.28534 \quad (6)$$

$$Y = -2.975N + 21.497 \quad (7)$$

Where P – Purity (%), Y – Yield (%), and N – Number of stages.

By maximizing **Equation 6** as shown in the **Equation 8**, the maximum purity was found to be 74% at four stages with a corresponding yield of 10%.

$$\frac{dP}{dN} = -0.80032(2)N + 6.4042 = 0 \quad (8)$$

The graph in **Figure 6 (A)** clearly shows that a compromise between maximum purity and yield can be found at $2 \leq N \leq 3$. To obtain a better understanding of the model and estimate of the number of stages required for high purity and yield, multi-objective optimization was used. In this multi-objective optimization problem, the objectives are to maximize the functions for purity and yield, and therefore determine the optimum number of stages required.

The ‘gamultiobj’ function in the Global Optimization Toolbox in MATLAB R2017b was utilized. In MATLAB, the objective functions were set up to minimize – P and – Y within the constraint of $1 \leq N \leq 5$. It should be noted that the ‘gamultiobj’ function only considers minimization problems and hence, the equivalent was to minimize – P and – Y.

The MATLAB model predicts several conditions for which the optimized objective functions fit, and the results are graphically shown in **Figure 6(B)**. It is evident that high purity and high yield occurs at Stage 3 and this is in accordance with the estimated number of stages from **Figure 6(A)**. If the optimum number of stages are used, the purity and yield should lie within the ranges of 71 – 74% and 12 – 16% respectively.

5. Conclusions

This study provides optimized process conditions for the multistage sodium alginate extraction and purification process, and provides a novel optimized model for maximizing purity and yield of extracted sodium alginate from *Sargassum natans*.

For acid pretreatment a maximum calcium rate of 0.26 mg calcium/(mg.seaweed.h) was found utilizing a concentration of 0.5M H₂SO₄, a 1:15 excess volume, and at a temperature of 40°C. Also, for alkaline extraction a maximum crude yield of 15% was reported using a concentration of 5% Na₂CO₃, a 1:15 excess volume, at a temperature of 65°C, and a reaction time of 2 hours. Furthermore, it was found that at high acid and alkali concentrations, degradation occurred resulting in low alginic acid conversion and low crude yields of sodium alginate respectively.

Multistage extraction proved that 2 stages were needed in order to maximize the cumulative crude yield of sodium alginate to 17%, while multistage precipitation showed that a true yield and purity of 19% and 67% respectively can be obtained after one stage. Optimization of the multistage precipitation process was investigated, and the results showed that a compromise between maximum sodium alginate yield and purity can be obtained after 3 stages; 12-16% and 71-74% respectively. Furthermore, characterization of the sodium alginate from *Sargassum natans* was carried out using FTIR with characteristic peaks obtained confirming the presence of sodium alginate, giving the carboxylate asymmetric stretching vibration at 1610 cm⁻¹ and symmetric vibration at 1395 cm⁻¹. Also NMR characterization gave insight into the uronic acid structure of the extracted sodium alginate, giving an M/G ratio of 0.51 with a high guluronic acid content of 66%.

Hence, the results of this study provides a novel optimized process that can be applied to other alginate systems (brown seaweeds). This new process can be fundamentally important as alginates are becoming increasingly attractive within the healthcare, pharmaceutical, environmental and food industries, where an optimization system can lower costs and provide a higher product quality and yield. Furthermore, this study provides the foundation for future work into the fabrication of films from sodium alginate extracted from *Sargassum natans*, for use as an alternative to conventional plastics for packaging and encapsulation.

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Table 1: Uronic acid sequence and M/G ratio of sodium alginate samples

	F_G	F_M	F_{GG}	F_{MM}	F_{GM}	F_{MG}	M//G Ratio	Reference
<i>S. natans</i>	0.66	0.34	0.62	0.32	0.04	0.04	0.51	This study
<i>Sigma</i>	0.44	0.56	0.33	0.46	0.10	0.10	1.29	This study
<i>S. fluitans</i>	0.64	0.36	0.55	0.28	0.08	0.08	0.57	(Davis et al., 2004)
<i>S. oligocystum</i>	0.67	0.33	0.58	0.24	0.09	0.09	0.49	(Davis et al., 2004)
<i>S. dentifolium</i>	0.66	0.34	0.55	0.23	0.11	0.11	0.52	(Larsen et al., 2003)
<i>S. natans</i>	0.68	0.32	0.61	0.25	0.07	0.07	0.47	(Rhein-Knudsen, Ale, Ajalloueian, & Meyer, 2017a)
<i>S. thumbergii</i>	0.66	0.34	0.48	0.17	0.17	0.17	0.53	(Davis, Llanes, Volesky, & Mucci, 2003)

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Table 2: Sodium alginate yield and purity after multistage extraction and precipitation

Stage	Multistage Extraction		Multistage Precipitation	
	Crude Yield (%)	Cumulative Yield (%)	True Yield (%)	Purity (%)
1	15	15	19	67
2	2	17	15	71
3	<1	17	13	73
4	<1	17	10	74
5	<1	17	7	73
<i>Sargassum</i> Species		True Yield (%)	Reference	
<i>S. subrepandum</i>		21	(Basha et al., 2011)	
<i>S. turbinarioides</i>		10	(Fenoradosoa et al., 2010)	
<i>S. fluitans</i>		17.7	(Davis et al., 2004)	
<i>S. oligocystum</i>		18.6	(Davis et al., 2004)	
<i>S. latifolium</i>		4.3	(Larsen et al., 2003)	
<i>S. asperifolium</i>		12.1	(Larsen et al., 2003)	
<i>S. dentifolium</i>		3.3	(Larsen et al., 2003)	

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Figure 1: Overall process for extraction of sodium alginate from *Sargassum natans*.

674

675 **Figure 2: FTIR spectra of sodium alginate from extracted from *Sargassum natans* and that**
676 **of the commercial brand Sigma.**

677

678 **Figure 3: (A) NMR spectra of alginate extracted from *Sargassum natans* showing monads,**
 679 **diads and triads. (B) Comparison of NMR spectra of alginate extracted from *Sargassum***
 680 ***natans* (---) and commercial brand Sigma (---). A (G); B1 (GGM); B2 (MGM); B3 (MG);**
 681 **B4 (MM) and C (GG).**

Figure 4: Calcium rate [mg. Calcium Produced/ (h.mg seaweed)] as a function of (A) Acid concentration [M] at 25°C . (B) Excess volumes [mL] at 25°C. (C) Temperature [°C] using 0.5M H₂SO₄ at 1:15 excess.

Figure 5: Yields of sodium alginate as a function of (A) Na_2CO_3 concentration [%] for 2 hours at 25°C. (B) At excess volume [mL] for 2 hours at 25°C. (C) Temperature [°C] for 2 hours, using 5% Na_2CO_3 and 1:15 excess volume. (D) Reaction time [Hours] using 5% Na_2CO_3 and 1:15 excess volume at 65°C.

Figure 6: (A) Purity and yield of alginate extracted for increasing stages. (B) MATLAB optimization model for purity and yield of alginate extracted for increasing stages.