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Alginate extraction from *Sargassum* seaweed in the Caribbean region: Optimization using response surface methodology

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ABSTRACT

Sargassum valorization has become increasingly important as the Caribbean region continues to struggle with the massive growth of the seaweed and its damaging effects. Sodium alginate extraction is one method where the seaweed biomass can be utilized to produce a useful biopolymer. However, current processing generally giving low yields of inferior quality, making it unattractive for commercialization. This article seeks to optimize the extraction process using a Box-Behnken Response Surface Design combined with multistage extraction to obtain higher product yield and purity, as well as giving insights, for the first time, into the physiochemical properties of the extracted alginate from *Sargassum* biomass. Optimum conditions were found and confirmed through validation, with a crude yield as high as 28 % after 2 stages and a purity of 92 % for purified alginate samples. Characterization of the bleached alginate through NMR studies validated with FTIR, gave an M/G ratio of 0.45 with a molecular weight of 3.14×10^5 g mol^{-1} and viscosity of 14.10 cP aligned to high gelling capabilities.

1. Introduction

In recent times (2011–2019), there has been massive accumulations of *Sargassum* (brown seaweed) onto the beaches of the Caribbean and Gulf of Mexico (Milledge & Harvey, 2016). This influx has created a variety of problems as it negatively impacts tourism-based economies, aquaculture, and traditional fisheries and fishermen's livelihoods (Smetacek & Zingone, 2013). Furthermore, decomposition releases toxic hydrogen sulphide and ammonia gas from its anoxic interior, which can have detrimental effects on the coastal ecosystems and human health (Resiere et al., 2018).

This seaweed influx has been credited to climate related variations along with warm, nutrient rich (nitrogen and phosphorous) environments (Franks, Johnson, & Ko, 2016). Amaral-Zettler et al. (2016) identified *Sargassum natans (S. natans)* as being the dominant species among the equatorial *Sargassum* influxes onto the Caribbean shores. The *Sargassum* found in Trinidad and Tobago can be identified as *S. natans* due to its morphological characteristics including its lanceolate blades with aculeate teeth, lateral smooth branches and air bladders (Széchy, Guedes, Baeta-Neves, & Oliveira, 2012). Thus, it is vital that this waste *Sargassum* biomass be utilized and its potential maximized and exploited to provide a method for valorization of this natural resource. Commercial brown seaweeds such as Laminaria, *Eclklonia,* and Macrocystis, and to a lesser extent *Sargassum,* contain a valuable hydrocolloid known as sodium alginate and is estimated to be up to 40 % of its dry weight depending on the source (Jung, Lim, Kim, & Park, 2013; Smidsrød & Skjåk-Braek, 1990). However, Sargassum is often used as a last resort commercially since it typically gives low yields (< 19 %) of poor quality alginate (Bertagnolli, Espindola, Kleinubing, Tasic, & da Silva, 2014; Fenoradosoa et al., 2010; Larsen, Salem, Sallam, Mishrikey, & Beltagy, 2003; McHugh, 2003; Torres et al., 2007). Alginates are polysaccharides that are found in the cellular wall matrix of brown seaweeds, and are composed of linear binary copolymers of (1 → 4)-linked β -D-mannuronic acid (M) and $\propto$ -L-guluronic acid (G) monomers (Torres et al., 2007).

These sodium alginates are currently widely used in the biomedical, cosmetic, textile, pharmaceutical and food industry due to their rheological properties (Kurt Ingar Draget, Smidsrød, & Skjåk-Bræk, 2005). However, there is growing interest into the use of alginates as; a cheap protein source (Rajmohan & Bellmer, 2019), alginate ferrogels as smart delivery vehicles for cancer (Kim, Kim, Park, & Lee, 2019), calcium alginate thin films for bioremediation of heavy metal ions (Mohammed et al., 2019), and biomaterials for tissue reconstruction (Chen et al., 2017).

Sodium alginate is traditionally extracted from brown seaweed in 6 steps which involve; formaldehyde pre-treatment of the dried seaweed, acid treatment, alkali extraction, bleaching, precipitation, and drying (McHugh, 1987). The alkaline extraction step is the most important as

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this is responsible for the yield and physiochemical properties (Vauchel et al., 2009), and bleaching is also done in order to improve the color depending on its final use (McHugh, Hernandez-Carmona, Arvizu-Higuera, & Rodriguez, 2001). Therefore, in order to obtain high productivity and commercial applicability, an optimization study on the alkali extraction process is necessary.

Design of Experiments (DoE) can be used for experimental investigations allowing for process modelling and optimization, while minimizing the total number of experimental trials by the simultaneous variation of all key factors (Marchetti, Butté, & Livingston, 2013). Response Surface Methodology (RSM) is a statistical tool that describes a process by evaluation of the interactive effects of independent variables on process parameters (Box & Wilson, 1951). One of these methods is the Box-Behnken Design (BBD) which is efficient and useful in avoiding experiments performed under extreme conditions where unsatisfactory results may occur (Ferreira et al., 2007). BBD has also been successfully used in optimizing the extraction parameters of polysaccharides from different types of biomasses (Ahmad, Alkharfy, Wani, & Raish, 2015; Luo, 2012; Ren et al., 2017).

There are a few papers that have investigated the effects of alkaline extraction on alginate yield from *Macrocytis pyrifera* (Hernandez-Carmona, McHugh, & López-Gutiérrez, 1999), *S. muticum* (Mazumder et al., 2016) and *S. latifolium* (Fawzy, Gomaa, Hifney, & Abdel-Gawad, 2017), however, these contain no information with regards to the bleaching effects and purity of the alginate extracted. Hence, in this work we report on the use of a novel BBD design combined with multistage extraction to further optimize extraction through the investigation of the linear and interactive effects of different alkali extraction parameters. These parameters include extraction temperature, alkali concentration, and excess volume of alkali: dried seaweed ratio and extraction time. Among these parameters, extraction temperature and extraction time are known to increase mass transfer; thus, higher extraction times and temperatures promote a higher extraction efficiency. However, higher temperatures can decrease molecular weight and viscosity which negatively impact alginate gelling ability, and as such, 80 °C was considered the upper limit for temperature for our experimental design supported by past research (Hernandez-Carmona et al., 1999). For strong gelling, it has been postulated that molecular weight needs to be within a narrow window of 320,000–340,000 g/mol (Draget, Simensen, Onsøyen, & Smidsrød, 1993). Thus, this study builds on our previous research (Mohammed et al., 2018) and aims to obtain yields and purities greater than 17 % and 67 %, respectively, utilizing the new methods and conditions proposed in this study. Furthermore, this study gives insight into bleaching effects, and physiochemical properties including molecular weight, viscosity, purity and M/G ratio from the *S. natans* alginate that has never been reported before. This provides key information as to how this waste equatorial *Sargassum* can be utilized and valorized as a potential new source of alginate for future use.

2. Materials and method

Proximate analysis was carried out on the raw seaweed according to AOAC (1990) methods. Protein content was determined using the micro-Kjeldahl method by converting the nitrogen content (6.25 x N) adapted from Marinho-Soriano, Fonseca, Carneiro, and Moreira (2006), while lipid analysis was carried out using solvent extraction with petroleum ether (SER 148, Italy). The ash content was obtained using a muffle furnace (Box furnace, Germany) at 550 °C for 5 h. Moisture content was determined by heating the seaweed in an oven (Precision Thelco, USA) at 135 °C until a constant weight was obtained. Carbohydrate content was estimated by difference.

Guided by our previous work on the extraction of sodium alginate (Mohammed et al., 2018), process parameters as well as ranges were established and optimized in this study. The extraction process was governed by 6 steps, namely; initial pretreatment with formaldehyde, acid treatment with sulphuric acid, alkali extraction, bleaching, precipitation, and drying. Alkali extraction conditions included varying temperature (22 − 80 °C), alkali concentration (1–10 % (w/v)) Na_2CO_3, alkali volume to dried seaweed ratio; 5 − 15 mL Na_2CO_3: 1 g dried seaweed and extraction time (0.5 − 6 hours). BBD, with four independent variables stated above, at three levels (high, low and middle) was chosen to investigate the effect of the extraction treatments on the crude extraction yield- with the data generated used for model fitting. Details of the coded and uncoded factors and responses can be found in Table 1.

Table 1
Coded and un-coded Box-Behnken design (BBD) and response.

	Independent variables				Response
Run	A: Temperature (°C)	B: Concentration (% w/v)	C: Excess volume (mL)	D:Time (hours)	Crude yield (%)
1	0(51)	0(5.5)	0(10)	0(3.25)	6.12
2	0(51)	1(10)	0(10)	−1(0.5)	3.28
3	0(51)	−1(1)	0(10)	1(6)	4.12
4	1(80)	1(10)	0(10)	0(3.25)	12.32
5	−1(22)	1(10)	0(10)	0(3.25)	4.72
6	0(51)	0(5.5)	0(10)	0(3.25)	9.14
7	0(51)	0(5.5)	1(15)	−1(0.5)	5.95
8	0(51)	1(10)	0(10)	1(6)	4.89
9	1(80)	0(5.5)	0(10)	1(6)	21.74
10	0(51)	0(5.5)	−1(5)	−1(0.5)	3.76
11	0(51)	−1(1)	1(15)	0(3.25)	4.31
12	0(51)	0(5.5)	0(10)	0(3.25)	7.21
13	1(80)	−1(-1(1))	0(10)	0(3.25)	12.49
14	1(80)	0(5.5)	−1(5)	0(3.25)	11.13
15	1(80)	0(5.5)	1(15)	0(3.25)	14.15
16	0(51)	−1(1)	0(10)	−1(0.5)	2.97
17	0(51)	0(5.5)	0(10)	0(3.25)	7.02
18	0(51)	0(5.5)	−1(5)	1(6)	5.94
19	0(51)	−1(1)	−1(5)	0(3.25)	0.49
20	−1(22)	−1(1)	0(10)	0(3.25)	0.58
21	0(51)	0(5.5)	1(15)	1(6)	4.74
22	0(51)	0(5.5)	0(10)	0(3.25)	6.85
23	−1(22)	0(5.5)	0(10)	−1(0.5)	3.34
24	1(80)	0(5.5)	0(10)	−1(0.5)	13.59
25	−1(22)	0(5.5)	−1(5)	0(3.25)	1.40
26	0(51)	1(10)	−1(5)	0(3.25)	6.85
27	−1(22)	0(5.5)	1(15)	0(3.25)	3.64
28	0(51)	1(10)	1(15)	0(3.25)	2.54
29	−1(22)	0(5.5)	0(10)	1(6)	1.66

()- Values inside the brackets indicate the actual value used within the experimental methodology.

The optimum extraction conditions were obtained using Derringer's desirability function and verified, while statistical analysis was carried out using Analysis of Variance (ANOVA). The regression coefficients of the linear, quadratic and interactive terms of the model and their effects were generated; significance was evaluated by the F-test at a significance level of $p \leq 0.05$, CI 95 %. The precision of the model developed was validated using the coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}) and predicted coefficient of determination (R^2_{pre}).

After identifying optimum conditions using model validation, multistage extraction was carried out followed by bleaching, precipitation and freeze-drying. Multistage extraction was carried out as the maximum amount of alginate cannot be obtained via a single stage, and based on previous research conducted, 2 stages were found to be ideal in promoting greater extraction efficiency (Mohammed et al., 2018). Multistage extraction involves reacting the residual *Sargassum* biomass carried over from the previous extraction stage, with fresh alkali. Bleaching was done on a volume ratio of 1:50 (sodium hypochlorite: alginate) and the hypochlorite was added slowly for 20 min prior to precipitation according to the method developed by (Mohammed et al., 2018). Frozen samples were then placed in a freeze dryer (Armfield

SB4) for 48 h and pulverized into a powder.

High Performance Liquid Chromatography (HPLC) was used to quantify the crude sodium alginate yields and purity of the extracted alginate; a C18 stationary phase column (150 × 4.6 mm i.d., 3.5 μm) was used. The mobile phase consisted of a buffer solution (0.5 mL phosphoric acid) in 1 L deionized water at a flow rate of 0.7 mL/min at 25 °C, adjusted to pH 7.0 with 1 M sodium hydroxide adapted from Awad and Aboul-Enein (2012). A sample volume of 20 μL was injected, and alginate was eluted at a retention time of 1.50 ± 0.03 min using a wavelength of 254 nm. Alginate standards were made utilizing a 1 g/L analytical sodium alginate solution, and the calibration curve gave a R^2 of 0.99 within the calibration range of 0.05 − 0.25 g/L. The crude yield of sodium alginate after extraction was calculated using the following equation:

$$\text{Crude yield} = \frac{\text{Concentration of sodium alginate produced}\left(\frac{mg}{ml}\right) \times \text{Volume } (mL)}{\text{Mass of Seaweed}\,(mg)} \quad (1)$$

After bleaching and precipitation to produce pure alginate, the purified yield of sodium alginate produced was calculated as follows:

$$\text{Purified Yield}\,(\%) = \frac{\text{Mass of purified alginate}\,(g)}{\text{Mass of seaweed used}\,(g)} \times 100 \quad (2)$$

Gel Permeation Chromatography (GPC), Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared Spectroscopy (FTIR) were carried out on precipitated purified alginate samples as crude samples were impure and non-distinguishable in terms of characterization when comparing to analytical standards. The molecular weight distribution of the alginates were determined via GPC. 0.1 % (w/v) alginate samples were chromatographed on a GPC column (Agilent PL Rapide Aqua- H, 150 mm x 7.5 mm, particle size 8 μm) with an RI detector (Agilent Technologies, G1362A). The column was eluted at 25 °C with a mobile phase of 0.1 M $NaNO_3$. 100 ml of the alginate sample was injected and the flow rate adjusted to 1 ml/min. The methodology using Polyethylene oxide (PEO) standards (100 −1000 kDa, Sigma Aldrich, Switzerland) was adapted from Kapishon, Whitney, Champagne, Cunningham, and Neufeld (2015)) owing to its water solubility and polymeric similarity. A calibration curve of Ln MW (molecular weight), y, against retention time (x) was obtained giving an R^2 of 0.97 for the PEO standards. The molecular weights of the samples were estimated using the respective calibration curve (y = −2.11 x + 15.29).

NMR analysis was carried out according to ASTM F2259−10 (2012). A 0.1 % (w/v) solution of purified alginate was prepared and D_2O was used as the solvent. The NMR spectra were recorded using a NMR spectrometer (Bruker AVANCE TM 600 MHz) operating at 80°C with the sample spinning at 20 Hz with a standard one-dimensional pulse program, at a frequency of 600 MHz, a spectral window of 6 kHz with a pulse angle of 90°, 2 s relaxation delay and acquisition time of 5.453 s. 64 scans were accumulated for signal averaging. The chemical shifts of the anomeric proton signals, block structure and M/G ratio calculation were estimated according to ASTM-F2259-10 method.

FTIR spectra of purified alginate samples were obtained using a Shimadzu IRAffinity-1S spectrometer. The spectra were recorded in the range of 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1}. The IR-laser wavenumber was set at 15802.00 cm^{-1} and a scan speed of 2.8 mm/sec. Further analysis was done evaluating the performance of bleaching on purified alginate appearance using color measurements as well as its effects on viscosity, molecular weight and monomeric M/G ratio. A detailed analysis of the methodology along with relevant equations used can be found in the Supplementary Data Section 1.

3. Results and discussion

3.1. Statistical analysis

Following the benchmarks described in Section 1- Supplementary Data for statistical significance and model validation, the empirical relationship between the crude extraction yield and the independent variables was fitted by a second order polynomial equation as this presents the basis for response surface designs giving the best approximation, accounting for curvature in the real response function (Carley, Kamneva, & Reminga, 2004). The final equation obtained via backward elimination of insignificant factors ($p > 0.05$) in terms of coded factors was given as:

$$Sqrt\,(\text{Crude alginate yield}\,\%) = +2.61 + 1.11A + 0.26B + 0.15C + 0.12D - 0.36AB + 0.38AD - 0.60BC + 0.32A^2 - 0.54B^2 - 0.36C^2 \quad (3)$$

The yield has both linear and quadratic effects by the four process variables. The quadratic effects suggest that there is curvature in the response function due to interaction between process variables, and will allow for an accurate approximation of the optimum to the true response. From Eq. (3) and the coefficient for each parameter, it was found that the greatest positive linear impact on the extraction yield was extraction temperature (A), the greatest negative interactive effect was the mutual interaction between alkali concentration and excess volume of alkali: dried seaweed ratio (BC), and the greatest positive interactive effect was the interaction between temperature and extraction time (AD). The experimental data was analyzed by ANOVA giving the level of significance of each regression coefficient evaluated using their respective p values -these results are presented in Table 2.

Two linear coefficients (A and B), three quadratic coefficients (A^2, B^2 and C^2) and all three interactive coefficients (AB, AD and BC) were significant. Linear coefficients C and D were found to be statistically insignificant, and it can be postulated that these linear effects had no effect on the alginate yield. However, considering their interactions with other process conditions, synergistic effects (AD) and antagonistic effects (BC) were observed. The model gave a high f-value of 36.70, indicating the model's significance at a low probability value ($p < 0.0001$). A large f-value signifies that the variation in the response can be explained by the regression model. The Lack of Fit f-value of

Table 2
Analysis of Variance (ANOVA) for the Response Surface Model.

Source	Sum of squares	Degree of freedom	Mean square	F value	p value
Model	22.45	10	2.24	36.79	< 0.0001
A	14.85	1	14.85	243.43	< 0.0001
B	0.80	1	0.80	13.07	0.0020
C	0.25	1	0.25	4.14	0.0569[a]
D	0.16	1	0.16	2.61	0.1238[a]
AB	0.52	1	0.52	8.48	0.0093
AD	0.58	1	0.58	9.43	0.0066
BC	1.43	1	1.43	23.49	0.0001
A^2	0.70	1	0.70	11.53	0.0032
B^2	1.94	1	1.94	31.83	< 0.0001
C^2	0.87	1	0.87	14.24	0.0014
Residual	1.10	18	0.061		
Lack of fit	0.93	14	0.067	1.61	0.3443[a]
Pure error	0.17	4	0.041		
Cor Total	23.55	28			
Adeq. prec.	25.465				
R^2	0.9534				
R^2_{adj}	0.9274				
R^2_{pre}	0.8689				

[a] Not significant (P > 0.05), A- Temperature, B- Alkali concentration, C- Excess volume of alkali: dried seaweed ratio, D- Extraction time.

1.61 with an associated p value of 0.3443 implied that the Lack of fit was insignificant relative to the pure error ($p > 0.05$), and further diagnosis of residuals showed no abnormality. This indicated that there was a high degree of validity in using a second order polynomial fit, and that it was statistically sound.

Additionally, the goodness of fit was determined by the coefficient of determination, R^2, which was found to be 0.9534. This meant that 95.34 % of the total variation in the results was attributed to the variables investigated, and only 4.66 % cannot be explained by the model. An R^2 value higher than 0.90 suggests that the empirical model fits the actual data significantly (Lawson & Erjavec, 2016). The values of the adjusted determination coefficient R_{adj}^2 (0.9274) was also high indicating that the experimental and predicted values possess a high degree of correlation. In addition, the difference between the R_{pred}^2 (0.8689) and R_{adj}^2 (0.9274) was less than 0.2, indicating that there is reasonable agreement with the model (Anderson & Whitcomb, 2015). Furthermore, adequate precision measures the signal to noise ratio and ratios greater than 4 are desirable and indicate adequate model discrimination. The adequate precision was found to be > 25 indicating that the model is significant. As a result, the developed models can be used to navigate the design space.

3.2. Influence of process variables on crude alginate yield

The influence of process variables in the form of linear and binary interactions greatly influence the maximization of the response. The 2-D contour plots, presented in Fig. 1A-C was utilized to showcase the nature and effects of the extraction process variables on extraction yield. The plots illustrate the effects of any two process variables while the others are kept as constant (at zero level in terms of coded factors) indicating the location of the highest yields in the region of experimentation. From the ANOVA analysis for linear effects, temperature (A) was found to be the highest statistically significant positive extraction factor ($p < 0.0001$) followed by alkaline concentration (B) ($p = 0.002$). Excess volume of alkali: seaweed (C) demonstrated a weak effect on its own ($p = 0.0569$) with noticeable stronger effects ($p = 0.001$) through interactions with concentration (B) as shown in Fig. 1C.

However, extraction temperature (A) and alkaline concentration (B) exhibited a very strong mutual interaction (Fig. 1A) at $p = 0.0093$, where the yield was seen to increase with an increase in alkali concentration up to 6% (w/v) beyond which there was an observed decline. Furthermore, it was noted that the highest extraction yield obtained through this AB interaction was at the maximum temperature of 80 °C. These findings correlate with those of Mazumder et al. (2016), where alginate yields increased linearly with alkali concentrations, but were seen to decline at higher alkali percentages- with a maximum yield observed at the highest temperature reported at 92 °C. Thus, it can be observed that both extraction temperature and alkali concentration play a key role in the extraction of alginate.

Extraction time (D) as a linear term exhibited a poor effect ($p = 0.1238$), but its interaction with temperature (A) had a high level of significance ($p = 0.0066$) indicating a strong synergistic effect. From Eq. (3) it was observed that extraction temperature and time possessed the only significant positive interactive effect, and Fig. 1B further illustrates this strong interaction giving the highest yield (20.35 %). These yields were the highest observed for all conditions considered, proving that optimal conditions enabled greater extraction efficiency (Fawzy et al., 2017). It can be postulated that this high temperature facilitates the penetration of the alkali solution into the cell walls and causes the algal tissues to become soft, thereby increasing the solubility and diffusion of the alginate into the alkali. This finding is consistent with high temperature extraction (80 − 100 °C) for brown seaweeds (Basha, Rekha, Letensie, & Mensura, 2011; Fenoradosoa et al., 2010; Hernandez-Carmona et al., 1999). The long extraction time of 6 h allows longer exposure of the seaweed to the alkali resulting in enhanced penetration, and this long extraction time agrees with the optimum of 5 h for the extraction of alginate from *S. vulgare* (Torres et al., 2007) and *S. flipendula* (Bertagnolli et al., 2014). Thus, extraction temperature and time are critical parameters in the solid-liquid extraction process.

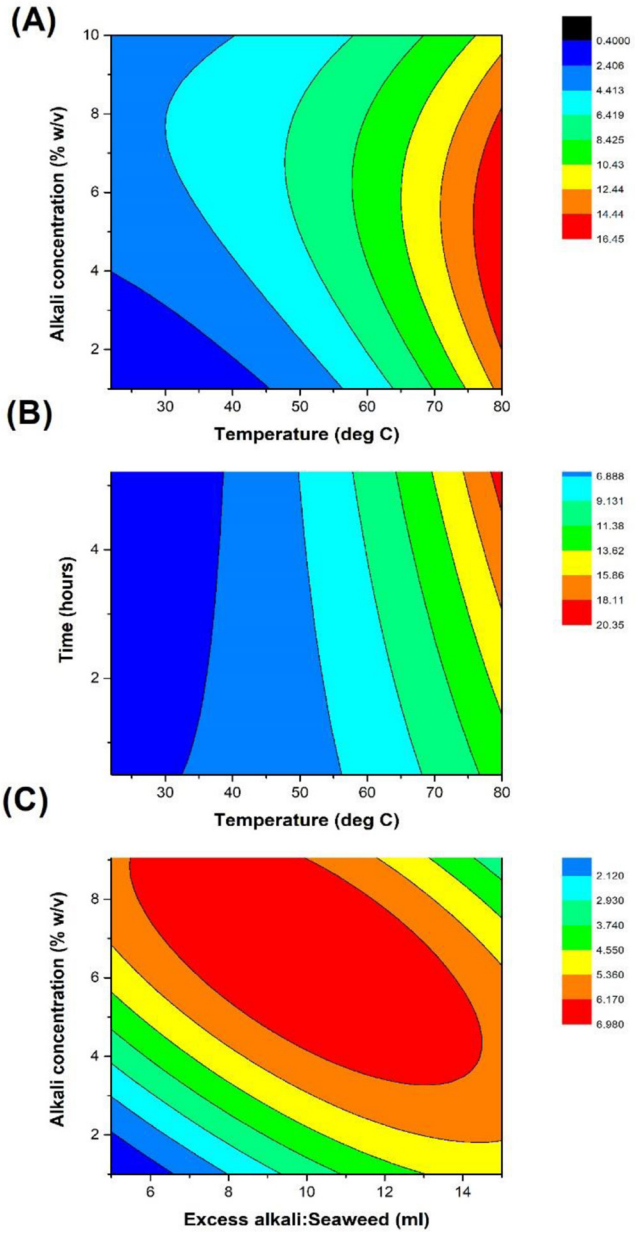

Fig. 1. 2D distribution of the response (extraction yield of alginate) as a function of process variables. A) alkali concentration and temperature, B) extraction time and temperature and, C) alkali concentration and excess alkali: dried seaweed. Colors simulate crude yield responses from blue (lowest) to red (highest). Ranges are A) 0.40–16.45, B) 6.89–20.35 and C) 2.12–6.98. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

From Fig. 1C, it can be seen that the mutual interaction between the excess volume and concentration (BC) is the best at the center of the plots, and that there exists an optimum where a compromise is favored at around 6% (w/v) and 10:1 excess volume. At high concentrations, alkaline degradation of the alginate can occur resulting in the formation of monocarboxylic and dicarboxylic acids, and also β-elimination of the glycoside bonds (Moen, Horn, & Østgaard, 1997). Therefore, the lower yields detected can be attributed to this degradation process. Past research carried out on alkaline extraction of brown seaweed generally consider low concentrations, in the range of 1–5 % (w/v), (Davis,

Ramirez, Mucci, & Larsen, 2004; Fenoradosoa et al., 2010; McHugh, 1987). A combination of 25:1 and 2% (w/v) (Basha et al., 2011) and 32:1 and 2% (w/v) (Fenoradosoa et al., 2010) excess volume: seaweed ratio and alkaline concentration, respectively, have been reported for different *Sargassum* genus giving yields of 21 % and 10 %. These excess volumes reported are larger in comparison to the volumes used in this study, but ultimately still produce a high yield. Thus, the conditions proposed here demonstrate the added economic profitability in using DoE, as utilizing lower volumes will result in net lower operational costs, and can provide benchmarks for commercial applicability of alginate extraction within the Caribbean region.

3.3. Optimization and validation of model results

The desirability function is a useful tool in finding the optimum combination of process conditions that maximizes the response based on the data generated and model predicted. The optimum process conditions obtained from Derringer's desirability function analysis was an extraction temperature of 80 °C, alkaline concentration of 3.75 (w/v) %, excess volume of alkali: seaweed ratio of 1:12.63, and an extraction time of 6 h. Under these conditions, the maximum crude alginate yield predicted was 21.21 % giving a desirability of 0.922, where a value of 1 represents full desirability (Derringer & Suich, 1980). Furthermore, upon using these optimum conditions, an experimental extraction yield of 20.76 ± 0.73 % (n = 3) was obtained providing excellent agreement with the predicted value with an experimental error of 2.1 %. Thus, the model utilizing BBD with the aid of the desirability function, was adequate in predicting the optimum conditions.

3.4. Effect of BBD and multistage extraction on yield

Multistage extraction, using 2 stages, at the optimum conditions (80 °C, alkali concentration of 3.75 (w/v) %, excess volume of alkali: dried seaweed ratio of 1:12.63, and an extraction time of 6 h) stipulated by the BBD, was considered to maximize the overall extraction yield. Based on the BBD with increased desirability, these new optimized conditions were applied, giving superior results when compared to our previous work (Mohammed et al., 2018) – a 38 % rise for the first stage (from 15 % to 20.76 ± 0.73 %), and 270 % for the second stage (from 2% to 7.40 ± 0.24 %), giving an overall increase in crude extraction yield of 66 % (cumulative yield increase from 17 % to 28.16 ± 0.97 %). Therefore, a more complete and enhanced extraction was obtained via optimization combined with multistage extraction.

The crude yields of alginate extracted from different *Sargassum* sources in comparison to the commercial brown seaweeds are given in Table 4. The yield reported in this study (28 %) is amongst the highest reported for the most studied *Sargassum* sources from different shorelines globally (4–26 %, Table 4). Thus, this yield of 28 % is critically important in providing a foundation for which the waste *Sargassum* biomass can be valorized. Variations can be attributed to several factors, such as; differences in extraction conditions, inter-species and geographic differences, seasonal variation as well as age and type of tissue used (Saraswathi, Babu, & Rengasamy, 2003). Furthermore, this yield is comparable to certain commercial sources such as *S. longicruris, M. pyrifera, L. hyperborea and S. lattisima* (Table 4). In addition, the industrial specifications for sodium alginate yield should not be less than 18 % based on the European Food Safety Authority (EFSA et al., 2017), and thus the conditions reported in this study fully meet the requirements for a commercial alginate process using *Sargassum*.

3.5. Assessment of bleached and unbleached alginate

Bleaching is an essential step in the extraction process as the final color of the extracted alginate is brown and deemed not aesthetically pleasing. However, bleaching has its disadvantages as it greatly influences the rheological properties of the alginate including its molecular weight, viscosity and M/G ratio. Bleaching is a critical part of the extraction process which determines its utilization and suitability for specific commercial applications.

Table 3
Physiochemical properties of bleached and unbleached purified alginate.

Property	Type of alginate			
	Unbleached	Bleached	Food grade	(Mohammed et al., 2018)
Purified Yield (%)	24.37 ± 0.74	24.33 ± 0.12	–	17
Purity (%)	92.21 ± 0.76	91.19 ± 0.62	99	67
Whiteness Index (WI)	50.56	75.54	88.79	–
Viscosity (cP)	18.00	14.10	–	–
Molecular weight (x10^5 g mol^{-1})	3.20	3.14	5.41	3.46

(-) Indicates data not available.

A Student's T-test (p = 0.05) was performed on the yield and purity with indicated that bleaching had no significant effect on the extraction process- similar to the work done by Istini, Ohno, and Kusunose (1994). Therefore, the removal of impurities was facilitated within the precipitation step, showing that this step is crucial in the extraction of high purity alginate as the overall purified yield decreases (from 28 % to 24 %) as shown in Table 3. However, bleaching significantly increased the Whiteness Index of the alginate compared to the unbleached sample, representing a 49 % increase; similar to the findings of McHugh et al. (2001). Upon bleaching, the viscosity and molecular weight decreased as shown in Table 3. The viscosity loss correlates well with those found in the literature (Mao, Zhang, Sun, & Ren, 2011; McHugh et al., 2001; Vauchel, Arhaliass, Legrand, Kaas, & Baron, 2008) and this can be attributed to the degradation of the osidic linkages in MG and MM blocks which are more sensitive to degradation than GG blocks within the alginate.

Furthermore, on examining data from our past research (Mohammed et al., 2018) conducted at 65 °C, a decrease in molecular weight (by 26,000 g/mol) was observed compared to our current optimized methodology. This evidence shows that higher extraction temperatures can lead to depolymerization and the breakdown of uronic acid chains. However, it should be noted that the molecular weight is still within the range for strong gel formation and as such, 80 °C remains as an upper temperature bound and a suitable compromise for high yields with relatively good product integrity.

3.6. Proximate analysis of the raw seaweed

The chemical composition of the raw seaweed is given as follows; protein 4.7 ± 0.23 %, lipid 0.01 ± 0.00 %, ash 13.43 ± 0.01 %, moisture 18.9 ± 0.05 and carbohydrate (inclusive of fiber) of 63.1 ± 0.06 %. This carbohydrate fraction correlates very closely to that of *S. natans* and *S. fluitans* (Oyesiku & Egunyomi, 2014) where a value of 63.45 % carbohydrate content (inclusive of fibre) was observed. The crude yield of the sodium alginate (28 %) extracted in this study represents 44 % of the total carbohydrates present in the seaweed. This confirms that the main carbohydrate constituent present in the cell walls of the seaweed was sodium alginate, which correlates well with characterization research conducted on algal material (Stiger, Bourgougnon, & Deslandes, 2016) (Fig. 2).

3.7. NMR analysis

Alginate composition in terms of its guluronic and mannuronic acid residues (M and G blocks) is an essential property of the alginate, and greatly influences its gelling ability. NMR spectroscopy is the main technique used to investigate this uronic acid sequence. Fig. 3 shows

Table 4
Uronic acid sequence for purified alginate samples and sodium alginate yields for crude extracts compared to previous literature.

Species	$^{1}F_G$	$^{2}F_M$	$^{3}F_{MM}$	$^{4}F_{GG}$	$^{5}F_{GM}$	$^{6}M/G$ Ratio	Crude Yield (%)	Reference
S. natans (unbleached)	0.67	0.33	0.24	0.59	0.09	0.49	–	This study
S. natans (bleached)	0.69	0.31	0.23	0.61	0.08	0.45	28	This study
Commercial alginate	0.46	0.54	0.36	0.27	0.18	1.19	–	This study
S. natans	0.66	0.34	0.62	0.32	0.04	0.51	17	(Mohammed et al., 2018)
S. natans	0.68	0.32	0.61	0.25	0.07	0.47	23	(Rhein-Knudsen et al., 2017)
S. fluitans	0.64	0.36	0.55	0.28	0.08	0.57	21	(Davis et al., 2004)
S. oligocystum	0.62	0.38	0.31	0.55	0.14	0.62	19	(Davis et al., 2004)
S. thunbergii	0.66	0.34	0.17	0.48	0.34	0.53	13	(Davis et al., 2004)
S. vulgare	0.56	0.44	0.55	0.43	0.01	1.27	17	(Torres et al., 2007)
S. subrepandum	–	–	–	–	–	–	21	(Basha et al., 2011)
S. turbinaroides	0.52	0.48	0.36	0.39	0.25	0.94	10	(Fenoradosoa et al., 2010)
S. latifolium	0.55	0.45	0.41	0.51	0.08	0.82	4	(Larsen et al., 2003)
S. muticum	0.49	0.51	0.17	0.15	0.34	1.04	26	(El Atouani et al., 2016)
Commercial brown seaweeds								
A. nodosum	0.54	0.46	0.28	0.36	0.18	0.85	32	(Rioux, Turgeon, & Beaulieu, 2007)
S. longicruris	0.59	0.41	0.07	0.25	0.34	0.70	24	(Rioux et al., 2007)
M. pyrifera	0.37	0.63	0.42	0.16	0.21	1.70	26 – 38	(Panikkar & Brasch, 1996)
D. antartica	0.20	0.80	0.64	0.16	0.03	4.00	37 – 52	(Panikkar & Brasch, 1996)
L. digitata	–	–	–	–	–	–	31 – 37	(Schiener, Black, Stanley, & Green, 2015)
L. hyperborea	–	–	–	–	–	–	21 – 33	(Schiener et al., 2015)
S. lattisima	–	–	–	–	–	–	25 – 31	(Schiener et al., 2015)

$^{1}F_G$ = G/(M + G), $^{2}F_M$ = M/(M + G), $^{3}F_{MM}$ = MM/(M + G), $^{4}F_{GG}$ = GG/(M + G), $^{5}F_{GM}$ = MGG/(M + G) and $^{6}M/G = (1\text{-}F_G)/F_G$. (-) Indicates that no data is given.

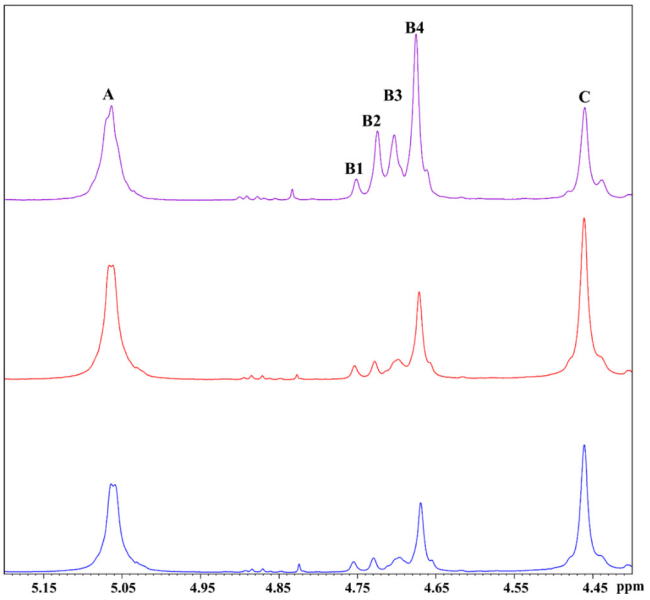

Fig. 2. NMR spectra of purified alginate showing monads, diads and triads. Purple represents food grade alginate, red represents unbleached extracted alginate, and blue represents bleached extracted alginate. The assignments of the signals are A (G); B1 (GGM); B2 (MGM); B3 (MG); B4 (MM) and C (GG). _ Indicates where the anomeric proton exists. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

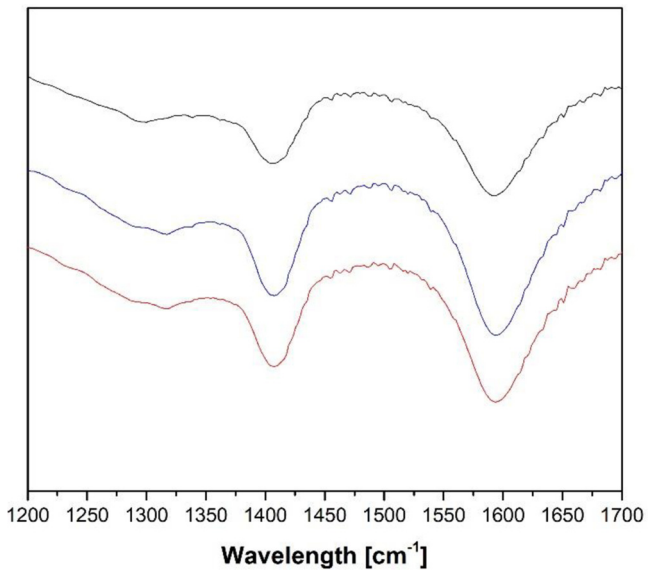

Fig. 3. FTIR spectra of sodium alginate samples. Black represents food grade alginate, blue represents bleached extracted alginate, and red represents unbleached extracted alginate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the NMR spectra of the alginate samples with the peaks representing the anomeric and other protons at the different carbon positions on the uronic acid sequence. The chemical shifts of the signature protons for the *S. natans* alginate (bleached and unbleached) and the commercial food grade alginate correlate well, further confirming the nature of the extracted product. The block structure and M/G ratio was determined using ASTM standard F2259 – 10 (2012) and presented in Table 4.

The M/G ratio found for the alginate extracted from *S. natans* in this study (0.45) is in agreement with the ratio found by Rhein-Knudsen, Ale, Ajalloueian, and Meyer (2017) (0.47) and fits within the range for the *Sargassum* genus (Table 4). The small dissimilarities may be due to differences between species, geographical location as well as extraction methodology. The M/G ratio in this study compared to our past study (Mohammed et al., 2018) showed a decrease, and this can be postulated to be as a result of the longer extraction times and higher extraction temperature leading to a breakdown of the uronic acid sequence. A higher M/G ratio was found for the commercial alginate compared to *Sargassum,* and this can be attributed to the fact that commercial alginates are extracted from commercial brown seaweeds which range from 0.7 to 4.0 (Table 4).

Comparing the bleached and unbleached extracted alginate, it was found that bleaching resulted in a decrease in the M/G ratio. This can be attributed to higher oxidation of the osidic linkages in MG and MM blocks over GG blocks (Andriamanantoanina & Rinaudo, 2010). Thus,

bleaching of the alginate will favor stronger gel formation due to the concentration of G blocks increasing within the polymer matrix as it is known that gel formation depends strongly on the presence of GG blocks (Haug & Larsen, 1962).

3.8. FTIR analysis

The spectra produced gave strong and sharp bands at 1595 cm^{-1} and 1406 cm^{-1} which can be attributed to the strong carboxylate (COO) asymmetric stretching vibration, and the weaker carboxylate symmetric vibration of the mannuronate and guluronic moieties, respectively, found within the alginate polymeric matrix. The characteristic peaks obtained in this study correlate closely with previous studies (Fenoradosoa et al., 2010; Fertah, 2017; Latifi, Sadegh Nejad, & Babavalian, 2015; Mohammed et al., 2018; Rhein-Knudsen, Ale, Ajallouеian, & Meyer, 2017) confirming the presence of sodium alginate.

Overall, our results align well with the literature (Fawzy et al., 2017; Fertah, 2017; Mohammed et al., 2018; Torres et al., 2007) in supporting extraction time and temperature as compatible indicators promoting extraction yield- and for the first time demonstrating their validated compatibility through BBD. While it is clear from our results (Fig. 1B), that extending the design space to higher temperature and extraction time can increase our observed alginate yields (Chee, Wong, & Wong, 2011; Fawzy et al., 2017), a compromise must be accepted to ensure product integrity is maintained. Thus, based on our current design space, our research illustrates the impact of experimental design and optimization in promoting productivity and extraction efficiency.

4. Conclusions

In this paper we present, for the first time, optimized conditions for the extraction of sodium alginate from *Sargassum* biomass. Optimum conditions identified were 12.63 mL of 3.75 % (w/v) Na_2CO_3 for 6 h at 80 °C producing a yield of 20.76 ± 0.73 %- agreeing closely with the predicted value of 21.21 % after one extraction stage. A cumulative yield of 28.16 ± 0.97 % was achieved after two stages, and was amongst the highest yields reported for the *Sargassum* genus. Additionally, amidst all parameters examined, extraction temperature and extraction time highly influenced the extraction yield having the greatest positive effects. Upon precipitation, the purified yield attained was 24.37 ± 0.74 % with a purity of 92.21 ± 0.76 %. Bleaching of the alginate had no significant effect on the yield and purity, but improved whiteness and decreased molecular weight and viscosity, while characterization using NMR and FTIR identified extracts as sodium alginate with a M/G ratio of 0.45 thus, increasing its potential to form strong gels.

CRediT authorship contribution statement

Akeem Mohammed: Methodology, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Arianne Rivers:** Data curation, Investigation. **David. C. Stuckey:** Validation, Writing - review & editing. **Keeran Ward:** Supervision, Project administration, Validation, Formal analysis, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.116419.

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