Multiple-response optimization of the acidic pre-treatment of the brown alga *Sargassum cristaefolium* for the alginate extraction using twin screw extruder

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The first step in the alginate extraction from brown seaweed is the acidic pre-treatment. The acidic pre-treatment affected physical characteristics of brown seaweed in alkaline solution and physicochemical properties of alginate. The aim of this study to investigate the effects of pH and time of the acidic pre-treatment on the multiple response of alginate from brown seaweed *Sargassum cristaefolium* extracted using twin-screw extruder (TSE). Central composite design in Response Surface Method (RSM) was used to find the optimum condition of the acidic pre-treatment based on these responses namely residence time distribution (RTD), yield, intrinsic viscosity, alginate molecule weight. Higher RTD was observed as pH level was approximately neutral, leading to degradation of alginate polymer chain. The results demonstrated that pH and duration of the acidic pre-treatment significantly showed quadratic effects on RTD, intrinsic viscosity, and molecule weight. The optimum condition of the acid pre-treatment was found at pH of 2.90 and time of 61.74 min, resulting in RTD 4.13±0.017 min, yield 35.58±0.23%, intrinsic viscosity 418.77±1.74 ml/g, and molecule weight 198.15±2.47 kDa.

**Keywords:** Twin-screw extruder, acid pre-treatment, alginate, *Sargassum cristaefolium*, response surface methodology

**INTRODUCTION**

Alginate is a polysaccharide isolated from brown seaweed such as *Sargassum cristaefolium*. As a polymer of cell wall component, Alginate is composed of β-(1-4)-D-mannuronic (M) and α-L-guluronic (G) subunits at different proportions and order (Torres et al. 2007; Larsen et al. 2003; Leal et al. 2008; Draget and Taylor, 2011; Fenoradosoa et al. 2010). Ratio of M/G and distribution of G and M subunits remarkably affected characteristics and functional properties of alginate (Davis et al. 2003; Jensen et al. 2012; Lee and Mooney, 2012; Chee et al. 2011; Bertagnolli et al. 2014). It has numerous applications due to its favorable properties such as thickening, thermostable, emulsifying, and gelling agent (Poncelet et al. 1999; Sellimi et al. 2015; Gomez et al. 2009; Rahelivao et al. 2013), and is widely used as food supplement, delivery drug (pharmacy) control, and antitumor (Paula et al. 2007; Moebus et al. 2012; Jensen et al. 2012; Lins et al. 2013).

Alginate extraction using batch process takes time and requires a lot of reactants and solvents
(Torres et al. 2007; Fertah et al. 2014), while extraction using supercritical CO₂, auto-hydrolysis, microwave, and ultrafiltration is expensive and difficult to apply in industrial scale (Balboa et al. 2013; Gonzalez-Lopez et al. 2012; Perez-Lopez et al. 2014; Quitain et al. 2013). Extraction of alginate using twin-screw extruder (TSE) is more effective and applicable for industries due to its continuous process with combination of pressure, shear stress, and temperature in the moving screw (Vauchel et al. 2008; Baron et al. 2010, Hernandez-Carmona et al. 2013). TSE demonstrated an essential role in the transformation of physicochemical materials (Kartika et al. 2010), and was applied for chemical reactor in the extraction of natural resources such as extraction of canola oil and pretreatment of lignocellulose for bioethanol production (Dufaure et al. 1999; Evon et al. 2007; 2013; Zheng and Rehmann, 2014). TSE in the extraction of brown seaweed alginate demonstrated the advantageous properties such as continuous and rapid process, and required low volume of solvents, low waste, and safe operation (Vauchel et al. 2008; Kartika et al. 2008; 2010). Recent studies that involved TSE in the extraction of brown seaweed alginate mostly focused on investigating the effects of screw speed on RTD (Baron et al. 2010). The acidic pre-treatment of brown seaweed is to conversion of alginate salt-including the insoluble calcium and magnesium to alginate acid and this is more easily to extract with sodium carbonate treatment (Hernandez-Carmona et al. 1999). The pH and time level acid pre-treatment affects the physical characteristics of brown seaweed in alkaline solution and physicochemical properties of alginate, and molecule alginate is hydrolyzed at low pH (Lorbeer et al. 2015; Silva et al. 2015). Therefore, a study pertaining the effects of acidic pre-treatment on RTD and physicochemical properties of alginate processed using TSE is required. This study is aimed to evaluate the effects of different pH levels and time of acidic pre-treatment on RTD and physicochemical properties of alginate from brown seaweed Sargassum cristaefolium extracted using twin screw extruder, and to find out optimum condition based on RTD, intrinsic viscosity, yield, and molecular weight.

MATERIALS AND METHODS

Materials and Reagents
Sargassum cristaefolium brown seaweed was obtained from Poteran Island, Sumenep, Madura, in April 2016. Fresh brown seaweed was washed using tap water and immediately transported to laboratory (within 24 h). The seaweed was washed and submersed in 0.1% KOH for 1 h, and re-washed to remove residue. The seaweed was sun dried, grounded, and sieved at 60 mesh (Subaryono, 2010). The seaweed was submerged in 0.1% formaldehyde overnight, and then washed, dried at 50°C for 6 h in cabinet dryer (Wedlock and Fasihuddin, 1990; Hernandez-Carmona et al. 1999). All chemicals including KOH, formaldehyde, hydrochloric acid (HCl) 35%, ethanol 96%, Na₂CO₃ were technical grade, and reagent for analysis were analytical grade.

Twin-screw extruder
The alginate extraction was performed using intermeshing co-rotating twin-screw extruder (Berto Industry BEX-DS-2256), with capacity of 7 kg/h. Three thermocouples were set to produce heat. The barrel temperature was monitored in control panel, and the high temperature was reduced by using air compressor, diameter of die was 8 mm. The extruder was operated at screw speed of 0-180 rpm, feed screw speed of 0-35 rpm. Barrel and screw profile of twin-screw extruder Berto Industry BEX-DS-2256 were exhibited in Figure 1.

Extraction of sodium alginate
Pre-treatment of brown seaweed
Brown seaweed was dissolved in 0.03 M HCl according to the treatment (pH 1-5) for 30-90 min with ratio of 1:20 (b/v), depended on the treatment, and gently stirred at speed of 500 rpm. The brown seaweed was rinsed by distilled water to eliminate acid excess, and the remaining water was removed using pressure machine (Hernandez-Carmona et al. 1999).

Alginate extraction using twin-screw extruder
Pre-treated brown seaweed (at pH 1-5 for 30-90 min) was gradually added with Na₂CO₃ solution (2.25%) with ratio of 1:3 (b/v), stirred and transferred into extruder hopper. Extrusion experiment for alginate extraction was operated at feed screw speed of 30 rpm, screw speed of 75 rpm, temperature of 60°C. Brown seaweed was
moved along the rotating screws and then was released in the opening die. The extrudate was then dissolved in Na$_2$CO$_3$ 2.25% with ratio of 1:10 (b/v), stirred. Alginate filtrate was centrifuged at 5000 rpm for 10 min to obtain supernatant. The filtrate was added with ethanol 96% with ratio of 1:2 (v/v), kept for 1 h and filtered. Alginate was washed (twice) using ethanol 70% and 96%, filtered and pressed. The alginate obtained was oven-dried at 45°C for 24 h, crushed and sieved at 60 mesh.

**RSM experimental design**

The acidic treatment was optimized using RSM central composite design consisting of two variables pH ($x_1$) and time acid pre-treatment ($x_2$). The design resulted in 13 combinations randomly ordered with 5 replications at center point (run 9-13) (Montgomery, 2005), as presented in Table 1. Based on experimental data, the second order was used as follow:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ij} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j$$  \hspace{1cm} (1)

where $Y$ is response; $\beta_0$ is intercept coefficient; $\beta_i$, $\beta_{ij}$ are regression coefficients for linear, quadratic, and interaction; $x_i$, $x_j$ are independent variables of pH and time acid pre-treatment.

The accuracy of polynomial model was analyzed using Software Design-Expert version 7 to obtain correlation coefficient (R) and determination coefficient ($R^2$) of observe variables (RTD, yield, intrinsic viscosity, and molecule weight). The significance of R and $R^2$ was statistically evaluated using F-test.

**Determination of residence time distribution**

Residence time distribution (RTD) of brown seaweed was time at which the material injected until the material released in the opening die.

**Determination of Yield**

Yield was determined as ratio of the dry weight alginate extracted to dry weight of brown alga used for extraction, then multiplied by 100%.

**Determination of intrinsic viscosity**

Alginate viscosity was assessed at 25 °C using viscometer glass with capillary diameter of 0.56 mm. To prepare alginate solution, alginate (30 mg) was dissolved in 10 ml of distilled water, stirred for 3 h at 25 °C (Chee et al. 2011). The different concentrations (0.05-0.3 g/dL) of alginate were then made. (t) was relatively measured to ($t_0$). Intrinsic viscosity was determined by extrapolating of $\eta_{sp}/c$ concentration to zero.

Relative viscosity,

$$\eta = \frac{t}{t_0}$$  \hspace{1cm} (2)

Specific viscosity,

$$\eta_{sp} = \eta^{-1}$$  \hspace{1cm} (3)

Reduction viscosity,

$$\frac{\eta_{sp}}{c} = \frac{\eta}{c}$$  \hspace{1cm} (4)

Intrinsic viscosity,

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c}$$  \hspace{1cm} (5)

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**Table 1. Experimental design and responses**

<table>
<thead>
<tr>
<th>No</th>
<th>Actual variable</th>
<th>Code variable</th>
<th>Pre-treatment</th>
<th>Response</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>pH</td>
<td>Time(min)</td>
<td>$x_1$</td>
<td>$x_2$</td>
</tr>
<tr>
<td>1</td>
<td>3.00</td>
<td>60.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>30.00</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>0.17</td>
<td>60.00</td>
<td>-1.414</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5.00</td>
<td>90.00</td>
<td>+1</td>
<td>+1</td>
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<tr>
<td>5</td>
<td>3.00</td>
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</tr>
<tr>
<td>6</td>
<td>5.83</td>
<td>60.00</td>
<td>+1.414</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1.00</td>
<td>90.00</td>
<td>-1</td>
<td>+1</td>
</tr>
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<td>8</td>
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<td>30.00</td>
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<td>-1</td>
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<tr>
<td>9</td>
<td>3.00</td>
<td>17.57</td>
<td>0</td>
<td>-1.414</td>
</tr>
<tr>
<td>10</td>
<td>3.00</td>
<td>60.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>3.00</td>
<td>102.43</td>
<td>0</td>
<td>+1.414</td>
</tr>
<tr>
<td>12</td>
<td>3.00</td>
<td>60.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>3.00</td>
<td>60.00</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Determination of molecular weight**

Determination of alginate molecular weight was based on correlation of average intrinsic viscosity and molecular weight, determined by using formulation of Mark-Houwink (Eq. 6), where $k = 0.023$ dL/g and $a = 0.984$ proposed by Clementi et al. (1998) quoted by Fertah et al. (2014), Chee et al. (2011) and Torres et al. (2007) to empirically relate $[\eta]$ and the weight-average molar mass ($M_w$).

$$[\eta]=kM_w^a$$  \hspace{1cm} (6)

The $[\eta]$ is intrinsic viscosity (dL/g), and $M_w$ is molecular weight (kDa).

**RESULTS AND DISCUSSION**

**Residence time distribution**

The results showed that pH and time of the acid pre-treatment significantly affected RTD. Lower pH and longer acid-treatment time resulted in lower RTD (Figure 2). This condition of lower pH may induce enhancement of Ca/H ion exchange, which more easily reacts and dissolves in sodium carbonate solution to form sodium alginate. The Ca/H ion exchange follows the first order, and its rate shows proportional correlation with pre-treatment time and acid concentration (Myklestad, 1968). The physical characteristic of brown seaweed in sodium carbonate solution at ratio of 1:3 (b/v) smoother with reduced pH level and increased duration acid pre-treatment. This phenomenon is due to the formation of porous cell wall and remove phenolic compounds because of acid pre-treatment of brown seaweed (Bertagnolli et al. 2014). This acid pre-treatment also could promote of Ca/H ions exchange, conversion of alginate salts to algic acid was higher as acid concentration and pre-treatment time increase. The algic acid was more extractable with sodium carbonate solution compare alginlate salt (calcium and magnesium alginate) (Gomez et al. 2009; Hanh et al. 2011). Hernandez-Carmona et al. (1999) and Silva et al. (2015) reported that exchange of Ca/H ions was higher in pre-extraction in 0.1 M HCl than that pre-extraction at pH 4 or without pre-extraction treatment, alginate extractability was improve with more acid conditions.

**Yield**

Treatments of pH and acid pre-treatment time remarkably influenced extractability of alginate, higher yield was obtained at lower pH and longer pre-treatment time (Figure 2). The yield was higher at condition of pH 3 and acid pre-treatment time of 60 min, but lower at condition of pH 5 and duration acid pre-treatment in the range of 30-90 min. The widely reported that pH and duration acid treatment quadratic effect on the extractability of alginate, the extractability of alginate was increase with more acid conditions and longer duration acid treatment (Lorbeer et al. 2015). This presumably reflects conversion of insoluble alginate, calcium, magnesium to more soluble and extractable alginate using sodium carbonate. Ca/H ion exchange was relatively higher with increase in acid concentration and acid pre-treatment time. Higher acid concentration and longer acid pre-treatment effectively reduced phenolic components which could promote alkaline extraction, decreased phenolic compounds associated with alginates and inhibited alginate extraction (Gonzalez-Lopez et al. 2012; Deniaud-Bouet et al. 2014). Alginate yield using acid-treatment at pH 2 was higher than that of at pH 5 or without acid treatment (water) (Lorbeer et al. 2015; Rahelivao et al. 2013; Jayasangkar, 1993). Pre-treatment which was performed at too low pH and excessive time led to degradation of alginate polymers (Gomez et al. 2009; Hernandez-Carmona et al. 1999; Silva et al. 2015).

**Intrinsic viscosity**

Experimental data show that intrinsic viscosity was higher at pH 3 and acid pre-treatment time of 60 min, but lower at pH 5 and duration time of 30-90 min. The highest intrinsic viscosity (438.27 ml/g) was obtained at pH 3 and 60 min, while the lowest intrinsic viscosity (136 ml/g) was found at pH 5 and 90 min. Intrinsic viscosity obtained in this research was relatively similar to that reported by Rahelivao et al. (2013), Fenorodosa et al. (2010), Torres et al. (2009), and higher than result reported by Mahmoud and Siddique (2010), Fertah et al. (2014) and Sellimi et al. (2015).

Treatment of pH and time acid pre-treatment exhibited quadratic effect on intrinsic viscosity of alginate extracted by twin-screw extruder (Figure 2). Intrinsic viscosity was positively correlated at pre-treatment condition of pH 3 and 60 min, decreased at acid pre-treatment condition of pH 1 and 5 for the range of 30-90 min. This is because the degradation of the polymer chain is getting worse at the pre-treatment of pH 1 and 5. Degradation of alginate molecules resulted from reaction of β-elimination in 4-O-glycosidic bonds.
Tabel 2. The quality of fit of the second-order models.

<table>
<thead>
<tr>
<th>Function</th>
<th>Model significance (Ps)</th>
<th>Lack of fit ($P_L$)</th>
<th>Corelation coefficient ($R^2$)</th>
<th>Coefficient of variance (C.V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residence time distribution</td>
<td>0.0005</td>
<td>0.0558</td>
<td>0.9335</td>
<td>1.60</td>
</tr>
<tr>
<td>Yield</td>
<td>0.0059</td>
<td>0.1271</td>
<td>0.8656</td>
<td>4.28</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
<td>&lt; 0.0001</td>
<td>0.3367</td>
<td>0.9854</td>
<td>6.22</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>&lt; 0.0001</td>
<td>0.3367</td>
<td>0.9915</td>
<td>6.22</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
<td>&lt; 0.0001</td>
<td>0.3367</td>
<td>0.9854</td>
<td>6.22</td>
</tr>
</tbody>
</table>

Table 3. Components and optimized response, goal, limits, and importance in the optimization stages of the formula

<table>
<thead>
<tr>
<th>Response component</th>
<th>Target</th>
<th>Lower limit</th>
<th>Upper limit</th>
<th>importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>In range</td>
<td>1</td>
<td>5</td>
<td>3(+++)</td>
</tr>
<tr>
<td>Waktu (menit)</td>
<td>In range</td>
<td>30</td>
<td>90</td>
<td>3(+++)</td>
</tr>
<tr>
<td>Residence time distribution (menit)</td>
<td>In range</td>
<td>4</td>
<td>4.58</td>
<td>3(+++)</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>Maximum</td>
<td>27.35</td>
<td>36.18</td>
<td>5(++++++)</td>
</tr>
<tr>
<td>Intrinsic viscosity (ml/g)</td>
<td>Maximum</td>
<td>139.95</td>
<td>438.27</td>
<td>5(++++++)</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>maximum</td>
<td>65.05</td>
<td>207.53</td>
<td>5(++++++)</td>
</tr>
</tbody>
</table>

Figure 1. Schematic modular barrel and screw profile of corotative twin screw extruder Berto Industry BEX-DS-2256 (serial number: BC-0405-054-08-004). TC= groove transfer direct pitch element (TC1=300 mm, TC2=220 mm, TC3=140 mm, TC4= 120 mm), TM= groove mixing pitch element (80 mm), Total long element=800 mm
Sugiono et al., Multiple-response optimization of the acidic

Figure 2. Response surface (left) and contour plot (right) of experimental responses as a result of different pH levels and acid pre-treatment time
which formed deoxyhexopyranuronic, and hydrolytic reaction was catalyzed by proton from glycuronan chain, as well as auto-oxidation of reduction components (Smidsrod et al. 1969; Haug et al. 1967). Hernandez-Carmona et al. (1999) found that acidity level in pre-treatment stage negatively correlated with alginate viscosity, the viscosity rapidly decreased in acid treatment at 0.1 M HCl. The acid pre-treatment at around neutral condition ineffective reduction of phenol, low Ca/H ion exchange, higher residence time distribution, and stronger specific mechanical energy effect, leading to breakdown of alginate polymer main chains (Wedlock and Fasihuddin, 1990; Kartika et al. 2010; Baron et al. 2010). Alginate viscosity with acid pre-treatment was higher than that of without acid pre-treatment (Jayasankar, 1993).

**Alginate molecular weight**

Experimental data demonstrated that the highest molecular weight was obtained at pH 3 and 60 min, while the lowest one was found at pH 5 and 90 min. Statistical analysis showed that acid pre-treatment at pH 1-5 and 30-90 min significantly affected molecular weight of alginate (P<0.05). Alginate molecular weight was positively increased until pH 3 and duration of 60 min, and decreased at pH 1 and 5 (Figure 2). This is associated with degradation of alginate polymer chains induced by β-elimination in acid pre-treatment stage at low pH level (Gomez et al. 2009; Hernandez-Carmona et al. 1999). In addition, it also might be affected by auto-oxidation of phenolic compounds, which possibly promoted formation of peroxide hydrogen in acid pre-treatment near neutral pH level. Reducing components by peroxide hydrogen yielded radical hydroxyl that could break alginate molecules.

**Figure 3. Desirability value of optimum condition predicted by Design Expert version 7**

![Design-Expert® Software Desirability](image)

**Design Points**

<table>
<thead>
<tr>
<th>A: pH</th>
<th>B: Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>3.00</td>
<td>4.00</td>
</tr>
<tr>
<td>5.00</td>
<td>6.00</td>
</tr>
</tbody>
</table>

**Prediction 0.921**
Prediction model and statistical analysis
RSM was used to optimize effects of acid pretreatments on alginate extracted by twin-screw extruder. The various pH levels (1, 3, 5) and pretreatment time (30, 60, 90 min) are variables for central composite design (CCD). The second-order polynomial models RTD, yield, intrinsic viscosity, and molecule weight (code variables) are presented below:

\[ y = +4.11 + 0.16 x_1 - 0.077x_2 + 0.11x_1^2 - 0.073x_2^2 + 0.15x_1x_2 \quad R^2=0.9335 \]
\[ y = +34.81 - 0.42x_1 + 0.98x_2 - 2.62x_1^2 - 2.11x_2^2 + 1.12x_1x_2 \quad R^2=0.8617 \]
\[ y = +425.46 -9.15x_1 + 2.24x_2 -130.86 x_1 - 129.18 x_2^2 -12.58x_1x_2 \quad R^2=0.9914 \]
\[ y = +310.89 - 6.70x_1 +1.65x_2 -97.35 x_1^2 -96.12x_2^2 -9.21x_1x_2 \quad R^2=0.9915 \]

Polynomial models of second order are evaluated according to significance, lack of fit, correlation coefficient \( R^2 \) and coefficient of variance (C.V.), as observed in Table 2. The proper prediction models have significance \( P<0.05 \), \( R^2 \geq 0.8 \), lack of fit \( >1 \), coefficient of variance (C.V.) \( \leq 10\% \) (Montgomery, 2005). Based on these parameters, second order polynomial models meet the criteria, thus they are acceptable for predicting optimal response.

Multiple-response optimization and verification
After the mathematical models are determined, optimization is carried out to obtain the best desirability, considering target and importance of responses (Table 3). The optimum condition for extraction of brown seaweed alginate was obtained at pH 2.90 and acid treatment time of 61.74 min. At this condition, the prediction of response value was RTD 4.096 min, yield 34.87 %, intrinsic viscosity 425.327 ml/g, molecular weight 201.333 kDa, which resulted in desirability value of 0.921 (Figure 3). Desirability value most equal to 1 indicated that optimum point predicted by Design Expert had high validity (Ale et al. 2012).

The prediction of optimum condition (pH 2.90 and time 61.74 min) was the verified using 3-replicated experiments, which resulting in RTD 4.13±0.017 min, yield 35.58±0.23 %, intrinsic viscosity 418.77±1.74 ml/g, and molecule weight 198.15±2.47 kDa. The verification demonstrated 95% PI low and 95% PI high, indicating that the verification results in attaining maximum yield, intrinsic viscosity, and molecular weight were consistent and valid (Qiao et al. 2009; Sugiono et al. 2014). The optimum condition of the acid pretreatment of brown seaweed in this research agreement with the findings of Rahelivao et al. (2013), but slightly lower than the pH of 3.2 suggested by Lorbeer et al. (2015) and the pH of 4 suggested by Gomez et al. (2009). Multiple-response alginate at the optimal condition was higher than result reported by Vauchel et al. (2008).

CONCLUSION
Different pH levels and acid pre-treatment time significantly altered residence time and quality of brown seaweed \textit{Sargassum cristaefolium} alginate extracted using twin-screw extruder. The optimum acid pre-treatment condition was achieved at pH 2.90 and time 61.80 min, resulting in RTD 4.13±0.017 min, yield 35.58±0.23 %, intrinsic viscosity 418.77±1.74 ml/g, and molecular weight 198.15±2.47 kDa.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest".

ACKNOWLEGEMENT
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AUTHOR CONTRIBUTIONS
This manuscript is part of the PhD Dissertation (Agricultural Product Technology) of first author advised by all co-author. Sugiono was responsible for conception, design, analysis and interpretation of data, drafting and revising and give final approval of the version to be submitted. Masruri, Teti Estiasih and Simon Bambang Widjanarko were responsible for supervise for conception, design, analysis and interpretation of data, and processing of the manuscript.
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