



## Development of high yielding carrageenan extraction method from *Eucheuma Cotonii* using cellulase and *Aspergillus niger*

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### Abstract

Carrageenans are a complex family of water soluble galactans extracted from marine red algae that have several applications such as gelling, thickening and suspending agents in food processing. The yield of carrageenan relies very much on the extraction method. Different carrageenan extraction methods on *Eucheuma cottonii* harvested from Sabah Coast were investigated. Several extraction method i.e. traditional-boiling, fungi-treated and cellulose-treated was studied according to the optimized conditions (pH, temperature and duration) stated by previous studies. The aim of the study is to identify the best extraction method to be used to obtain high yield of carrageenan. The highest yield was obtained through cellulase-treated extraction (45%) followed by traditional boiling (37.5%) and the lowest were observed with fungal treatment (37%).

The viscosity of carrageenan in cellulase-treated method is better than fungi-treatment but still lower than commercial and traditional method. The enzymes produced by *A.niger* might be cutting the long chain into several smaller fragments which makes the carrageenan with low viscosity. Secondly, the extracted carrageenan might not be pure. It may have other cellular impurities which also make it inferior to standard carrageenan. Future study must be focused on incorporating cellulase and fungi fermentation and also on the increase of gel strength.

Keyword: *Aspergillus niger*, Carrageenan, Extraction method, *Eucheuma Cotonii*

## INTRODUCTION

Carrageenans are a complex family of water soluble galactans extracted from marine red algae that have several applications such as gelling, thickening and suspending agents in food processing. Carrageenans have pronounced biological activities or other properties useful in the biomedical field. These polysaccharides are composed of alternating  $\alpha$ -(1-3) and  $\beta$ -(1-4) linked D-galactosyl residues and several types of carrageenan are identified on the basis of the modification of the disaccharide repeating unit by sulphate esters and by the presence of 3,6-anhydrogalactose as 4-linked residues. (Yermak et al., 2006).

Carrageenans exist in three main forms:  $\kappa$ -carrageenan,  $\iota$ -carrageenan and  $\lambda$ -carrageenan. Among these,  $\kappa$ -carrageenan is predominantly obtained by extraction from the tropical seaweed *Kappaphycus alvarezii*, known in the trade as *Eucheuma cottonii* (Rudolp 2000). In this research, we extended our study to the carrageenan extracted from the commercially cultivated seaweed *K. alvarezii* from Sabah. This seaweed is one of the main raw materials for the commercial production of kappa-carrageenan, a widely used gelling sulfated galactan (Bixler, 1996).

In this research, different extraction methods such as the traditional boiling method, commercial enzyme-treated extraction and fungal-treated extraction were systematically varied and the percentage of carrageenan yield was assessed. The purpose of this study was to determine the highest carrageenan yielding extraction method.

## MATERIALS AND METHODS

**Seaweed:** *Kappaphycus alvarezii* (*Eucheuma cottonii*) was collected by hand at the end of November 2008 on the coast of Sabah, Malaysia, which is in a tropical zone. Immediately after sampling, the seaweed was washed several times with clean water in order to remove non-algal materials. The seaweed was then sun-dried, and stored under refrigeration.

**Fungus :** *Aspergillus niger*, a high cellulase producing fungus strain from UPM.

**Carrageenan:** Commercial carrageenan that is used in trades was obtained from University Malaysia Sabah, (UMS). Standard carrageenan Sigma – Aldrich was obtained from local chemical supplier.

**Cellulase :** Cellulase Novozyme NS50013 was obtained from Sigma – Aldrich. This cellulase is produced by fungus, *Trichoderma reesei*.

### Traditional extraction method

Dried seaweeds (20 g) were soaked in 3L water for about 24 h to remove adhering sand, salt and other impurities. The process was repeated twice. The washed seaweeds were cut into fragments approximately 5 mm – 1 cm in length. Extraction (15 g dried algae in 750 ml water) was performed at 70°C for 1 hour (Montalulu et al., 2007). The suspensions were centrifuged (12000 rpm, 4°C, 30 min) and the supernatants designed as the supernatant fraction (S1). One volume of supernatant was poured into two volume of 2-propanol whereby the polysaccharides precipitated as long fibers. The liquors were removed by centrifugation (12000 rpm, 4°C, 30 min) and the samples were designated as the precipitated fraction (S2). The samples were dried under rotary evaporater and recovered in a freeze-dried state. This product was designated as the yield fraction (S3).

### Fungal treated extraction

The basal medium (without carbon source) for cellulase production had the following composition (Table 1.1). The fungi were fermented in 500 ml Erlenmeyer flasks containing 200 ml of the basal medium supplemented with 15 g seaweed blend for 7 days at 30 °C. Each flask was inoculated with 2 ml spore suspension ( $10^4$  spores  $\text{ml}^{-1}$ ) from 7 days-old *Aspergillus niger* stock culture.

Table 1: Basal Medium (without carbon source)

Material	Mass
NaNO <sub>3</sub>	2.0 g L <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	1.0 g L <sup>-1</sup>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g L <sup>-1</sup>
FeSO <sub>4</sub>	10.0 mg L <sup>-1</sup>

(Preliminary study: Fungi were grown in 500 ml Erlenmeyer flask containing 200 ml Potato Dextrose Broth (PDB). Incubation was done in a shaker incubator for 14 days. After 14 days, it was supplemented with 15 g seaweed, (*E.cottonii*) and let to ferment for 7 days at 200 rpm in 35°C)

The suspensions were centrifuged (12000 rpm, 4°C, 30 min) and the supernatants designed as the supernatant fraction (S1). One volume of supernatant was poured into two volume of 2-propanol whereby the polysaccharides precipitated as long fibers. The liquors were removed

by centrifugation (12000 rpm, 4°C, 30 min) and the samples were designated as the precipitated fraction (S2). The samples were dried in oven at 40°C and recovered in a freeze-dried state. This product was designated as the yield fraction (S3).

### **Enzyme (cellulase) treated extraction**

Optimization of carrageenan extraction from seaweed *kappaphycus alvarezii* using cellulase method was modified from the method used by Patindol et al (2007) to extract oligosaccharide from rice bran using cellulase enzyme.

20g of grinded seaweed is mixed into 200ml distilled water. 0.1g of cellulase is added into the mixture and boiled in water bath with shaker at 50°C for 1 hour. The suspensions were then centrifuged (5000 rpm, 4°C, 15 min) and the supernatants designed as the supernatant fraction (S1). One volume of supernatant is poured into two volume of 2-propanol whereby the polysaccharides precipitated as long fibers. The liquors were removed by centrifugation (12000 rpm, 4°C, 30 min) and the samples were designated as the precipitated fraction (S2). The samples were dried under rotary evaporator and recovered in a freeze-dried state. Weight of the dried carrageenan sample is recorded. Freeze dried sample (S3) is grinded into powdered form and stored in a sealed bottle for further analysis.

After the preliminary study using cellulase, respond surface model (RSM) is used to optimize the extraction yield.

## RESULT AND DISCUSSION

Carrageenans are phycocolloids derived from galactan polysaccharide. The major polysaccharide constituent of the *Eucheuma cottonii* (red seaweed) cell walls are the carrageenan. The amount present varies with ecological factors such as light, nutrient, wave exposure and temperature. Since carrageenan is bound to the cell walls, degrading the seaweed cell wall can lead to high carrageenan yield.

The traditional method focuses on the osmosis capability of the seaweed. When seaweeds were exposed to water, it will naturally uptake water and releases some chemical. The identity of the substance is still unknown. Preliminary studies done with the water used to soak the seaweed showed the absence of polysaccharide.

Heating up the seaweed with water, forces the polysaccharide (carrageenan) to be extracted out of the seaweed. This method will produce native carrageenan, which will be free of chemicals. However, the extraction will be lower compared to alkali treatment.

Alkali will perform two function: firstly it promotes swelling and maceration of the weed to aid in bringing the carrageenan into solution, while, secondly, when employed at sufficient high concentration, it effects cleavage of 6-sulfate groups from the carrageenan to generate 3,6-anhydro-D-galactose residues in the polysaccharide chain (Stanly, 1987).

This alkaline treatment removes colouring matter and some proteins and makes the carrageenan more easily extractable. Some alkaline elimination of 6-sulfate may also occur during this treatment. The natural precursors of kappa-carrageenan ( $\mu$ ) are non-gelling carrageenans due to irregularities in the 6-sulfate ester groups on some D-galactose. Most of these 6-sulfate units convert to the corresponding 3,6-anhydro-D-galactose during alkaline industrial carrageenan extraction, imparting a higher degree of regularity to the molecule (Freile-Pelegrin, 2006). This will increase the gel strength of the carrageenan.

Besides the pros of alkali treatment, the operation inevitably involves some degradation of polysaccharide due to the rigors (heat, alkalinity) of processing (Stanly, 1987). Higher concentration of sodium hydroxide could promote depolymerisation of the carrageenan.

The primary focus of this research is the fungal treated and cellulase treated carrageenan extraction method. Fungus, *Aspergillus niger*, produces a cell wall degrading enzyme, cellulase. This enzyme was used to breakdown the seaweeds cell wall, and free the carrageenan attached in between the cell wall. The difference between these methods is the direct introduction of enzyme in the enzyme hydrolyzes, which will have specific activity on the seaweed cell wall and will only attack cellulose. Theoretically, this method was supposed to produce higher carrageenan yield.

The extracted carrageenan yield percentage is shown in table 2.

Table 2: Carrageenan extraction method and the percentage of yield.

<b>Extraction Method</b>	<b>Sample weight (g)</b>	<b>S3 (g)</b>	<b>Yield (%)</b>
Traditional	10	3.75	37.5%
Fungal	10	3.7	37.0%
Cellulase-treated	10	4.5	45.0%

### Fungal-treated extraction

Fungal treated extraction has produced the lowest yield (figure 1) compared to cellulase-treated extraction but at the same par with traditional extraction. Dry cell weight (Figure 2) of the biomass have shown a good cell growth which proves that, *Aspergillus niger* is able to hydrolyze the seaweed cell and extract out the carrageenan and other cellular compounds. This is also proved by the cellulase activity test by DNS method (Figure 2).

Figure 1: *Aspergillus niger* treated carrageenan extraction yield

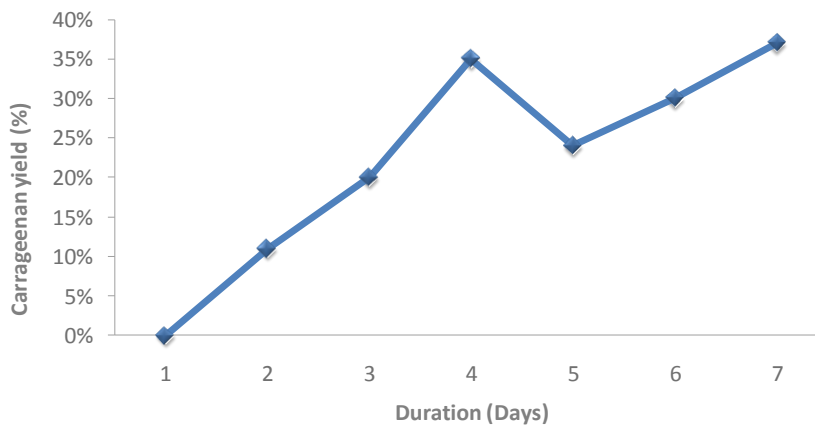
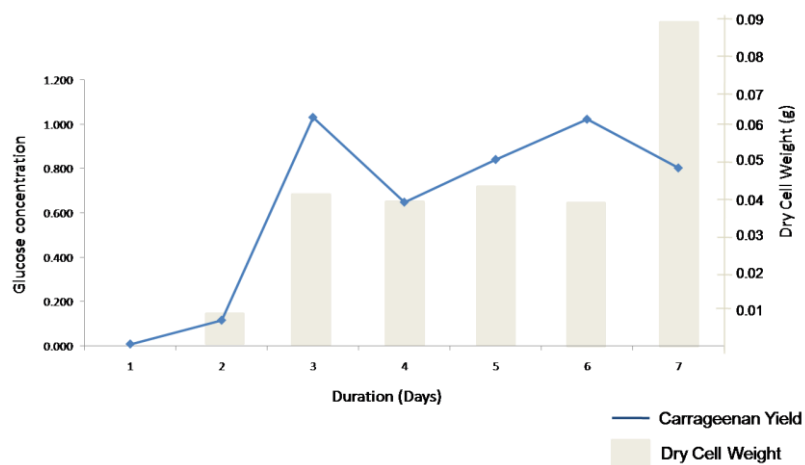


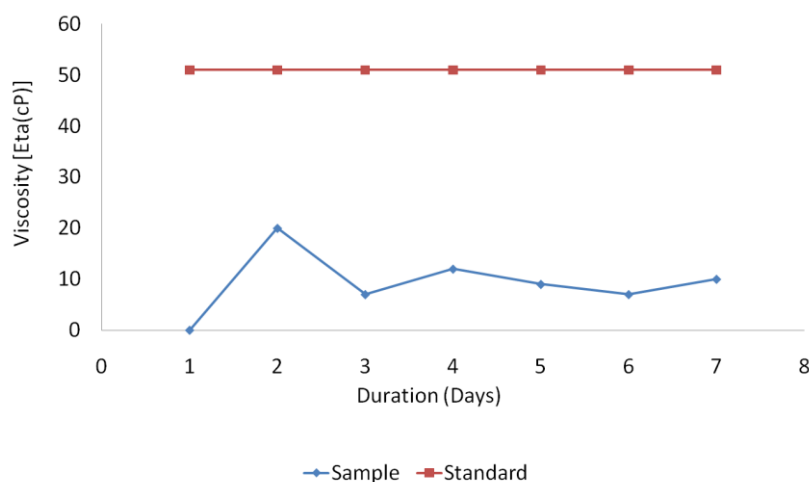
Figure 2: Cellulase activity and dry cell weight of *Aspergillus niger*



However the viscosity of fungal treated method was very low if compared to a standard carrageenan (Figure 3). This maybe explained by the long chain structure of carrageenan. Carrageenan is a polysaccharide which has a long repeating carbon chain with attached sulfate. Standard carrageenan may have a longer chain which explains the higher viscosity.

Another factor will be the purity of extracted carrageenan. The hydrolysis of seaweed may have extracted other cellular impurities which would have attached to carrageenan. The impurities couldn't be separated through alcohol precipitation and centrifugation. Other technique must be deduced to produce high carrageenan extract.

Figure 3: Viscosity of carrageenan extract from 7 day fermentation



### Enzyme (cellulase) treated extraction

#### The ash, water content and water activity for *Eucheuma cottonii* were

The traditional carrageenan yield was 37.5% of dry weight. An increase in yield by 16.7% was observed after cellulase treatment. The viscosity of carrageenan extracted by cellulase treatment is much lower than the traditional extraction. This may due to the impurities in the extraction whereby alcohol precipitation and centrifugation would not yield pure carrageenan. A suitable method is being studied to obtain pure carrageenan.

Respond surface model (RSM) have produced an experimental design where the result is shown below (table 3). Based from table 3, extraction yield have given the highest value of 60.2% at the temperature of 50 °C for 3 hours with 2% w/w cellulase enzyen. This shows that the optimum extraction temperature of cellulase is between 45-50 °C. Other than that, carrageenan can only be extracted at higher temperature when the cell walls are being ruptured.

Table 3: Effect of factors such as Enzyme Concentration, time, and temperature on the carrageenan extraction yield

Experiment	Independent Variables			Responding Variable
	Temperature (°C)	Time (Hours)	Enzyme Conc. (%)	Extraction yield (%)
1	40	2	1	29.4
2	30	1	2	0
3	50	1	0	40.1
4	30	3	0	0
5	30	2	1	0
6	40	1	1	23.3
7	40	2	1	29.2
8	40	2	1	31.2
9	50	3	2	60.2
10	30	1	0	0
11	50	3	0	42.6
12	40	2	1	28.4
13	30	3	2	0
14	50	1	2	45.3
15	50	2	1	44.1
16	40	2	2	27.5
17	40	3	1	32.5
18	40	2	1	30.3
19	40	2	0	27.8
20	40	2	1	29

### CONCLUSION

Based from the results, cellulase extraction has produced the higher carrageenan yield (45%) compared to fungus treated extraction (37%). Direct introduction of cellulase have proved specific activity on the cell walls of *Eucheuma cottonii* and not the product, carrageenan unlike fungus treatment which hydrolyses carrageenan as carbon source. However, the advantage of using fungus will be cost effectiveness, where it can self produce the enzymes.



Both extraction methods have impurity problems whereby a new and improved purifying method must be deduced.

Further studies must be focusing on the incorporation of the benefits from two methods i.e fermentation of *Aspergillus niger* to produce cellulase and than use the produced cellulase to extract carrageenan from seaweed *Eucheuma cottonii*.

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