



Extraction assistée par enzyme de phlorotannins provenant d'algues brunes du genre Sargassum et les activités biologiques

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Enzyme-assisted extraction of phlorotannins from *Sargassum* and biological activities



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Doctoral Program of Coastal Resources Managment

Diponegoro University

Semarang

2017

**Extraction assistée par enzyme de phlorotannins
provenant d'algues brunes du genre *Sargassum* et
les activités biologiques**



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Extraction assistée par enzyme de phlorotannins provenant d'algues brunes du genre *Sargassum* et les activités biologiques

Résumé :

En France et en Indonésie, *Sargassum* est une macroalgue abondante mais cette ressource n'est pas encore exploitée. Les composés de l'algue *Sargassum* sont de nature chimique très diverse et certains possèdent des activités biologiques parmi lesquels les phlorotannins. Il est donc intéressant des voies de valorisation dans des domaines tels que l'industrie alimentaire, pharmaceutique, et cosmétique. Par conséquent, la connaissance de la composition biochimique de *Sargassum* qui représente aussi sa valeur nutritionnelle est importante. L'extraction solide-liquide est la technique la plus utilisée pour l'extraction des phlorotannins. L'efficacité de l'extraction est néanmoins limitée par l'accessibilité aux composés, difficulté due à la complexité structurale de la paroi des algues. En conséquence, il est important de considérer la dégradation de la paroi pour favoriser l'extraction des phlorotannins. L'utilisation d'enzymes comme outil technologique est un processus qui pourrait faciliter cette dégradation.

Cette étude a eu trois objectifs principaux; (1) la caractérisation de la composition biochimique de *Sargassum* et l'étude de la variabilité saisonnière, (2) l'extraction solide-liquide des phlorotannins et (3) l'optimisation des conditions d'extraction assistée par enzymes des phlorotannins et l'étude des activités biologiques des extraits produits. Les activités analysées concernent les activités antioxydants (piégeage de radicaux DPPH et pouvoir réducteur FRAP), les activités cosmétiques de blanchiment de la peau (anti-tyrosinase), les activités anti-âge (anti-élastase), les activités antibactériennes, l'inhibition de la formation de biofilm, la mesure de la cytotoxicité et des activités antivirales.

Dans cette étude, les minéraux représentent d'une valeur importance avec une teneur qui atteint 33% suivi par les protéines avec 24% de la matière sèche. Les sucres ne représentent que 13% de la matière sèche. L'étude de la variabilité saisonnière montre que la composition biochimique de *Sargassum*, comme pour la plupart des algues, est fortement affectée par la saison de collecte et semble donc liée au cycle de vie. Ce résultat est également confirmé pour les composés phénoliques.

Cependant des méthodes d'extraction adaptées permettent d'augmenter le rendement. L'extraction solide-liquide conduit à des rendements plus faibles (5 à 24% de la matière sèche de l'algue) comparée à l'extraction solide-liquide assistée par enzyme (26 à 38% de la matière sèche de l'algue). En ce qui concerne la teneur en polyphénols, l'extraction assistée par enzymes permet aussi d'obtenir un meilleur rendement 6% contre 4% de la matière sèche si l'extraction est réalisée sans enzyme.

Après le criblage de l'action de huit enzymes pour extraire les polyphénols de *S. muticum*, Viscozyme, un cellulase, libère une plus grande quantité de composés phénoliques d'après les teneurs déterminées par la méthode de Folin Ciocalteu, résultat confirmé par l'activité anti-radicalaire de l'extrait obtenu

(IC₅₀ 0.6 ± 0.1 mg/mL). Cet extrait montre la plus forte activité anti-biofilm avec 43.7 ± 4.2% d'inhibition de la formation de biofilm de *P. aeruginosa*.

Tous les extraits produits montrent des activités biologiques. L'activité FRAP la plus importante a été montrée par l'extrait produit avec l'enzyme AMG, un exo-amylase, (60.8 ± 1.8 µM of Fe²⁺). L'extrait obtenu avec l'Ultraflo – un xylanase, (24.5 ± 2.6 % of d'inhibition) possède la plus forte activité de blanchiment (anti tyrosinase), tandis que l'extrait de Shearzyme, cellulase et xylanase, (30.9 ± 5.9 % d'inhibition) présente une activité anti-âge plus élevée que les autres extraits.

L'utilisation d'enzymes pour l'extraction des composés phénoliques augmente le rendement d'extraction. De plus, les extraits enzymatiques de *Sargassum* démontrent des activités biologiques intéressantes. Il est important de considérer qu'en utilisant l'extraction assistée par enzyme, d'autres composés que les polyphénols sont co-extraits. En conséquence, il sera intéressant de purifier les extraits pour déterminer la nature des molécules actives. En outre, la caractérisation complète des composés phénoliques de *Sargassum* pourrait aider à mieux comprendre les mécanismes d'action liés aux activités biologiques observées.

Mots-clés : *composition biochimique, variation saisonnières, polyphénols, Sargassum, activités biologiques*

Enzyme-assisted of phlorotannins from *Sargassum* and biological activities

Resume:

In France and Indonesia, *Sargassum* is quite abundant yet this alga has not been optimally exploited. *Sargassum* contains macro and micro molecules and also bioactive compounds, the most known is phlorotannins, showing a wide range of pharmacological properties. Therefore, it is interesting to further valorize this alga for various industrial applications, such as food, pharmacology and cosmetic. Knowledge on biochemical composition of this alga, which also represents its nutritive value, is essential for food industries. Concerning the bioactive compound, solid-liquid extraction is the most commonly used technique in the extraction of phlorotannins from brown algal biomass. However, phlorotannins of brown algae are known to be bound with algal cell wall and the high content of various cell wall polysaccharides in macroalgae limit the active compound accessibility. Thus, these cell walls strongly reduced the extraction efficiency application of conventional extraction. As a consequence, it is important to consider the degradation of algal cell wall as a way to liberate more phlorotannins. Application of enzymes is considered as one process that might facilitate the degradation of algal cell wall leading to the recovery of phlorotannins from brown algae.

This study had three main subjects of interest; they were (1) the characterization of *Sargassum* biochemical composition and its seasonal variation, (2) solid-liquid extraction and (3) enzyme-assisted extraction of *Sargassum* phlorotannins and their bioactivities. The bioactivities analyzed in this study included antioxidant (DPPH radical scavenging and FRAP reducing power), antibacterial, tyrosinase, elastase, biofilm inhibition, cytotoxicity and antiviral activity.

In this study, minerals represented a significant value with a content reaching 33% followed by proteins with 24% of algal dry material. In the contrary, sugars showed only 13% of algal dry material. The study of seasonal variability demonstrated that the biochemical composition of *Sargassum*, as for most algae, is strongly affected by the season and it appears to be related to the life cycle of the algae. Same cases happened with the polyphenol compound of *Sargassum*.

Nevertheless, suitable extraction method is important to choose in order to increase the yield. Solid-liquid extraction yielded lower (5-24% of algal dry material) compared to enzyme-assisted solid-liquid extraction (21-38% of algal dry material). Regarding the polyphenol content, enzyme-assisted extraction also yielded a better yield, 32%, compared to 26% of algal dry material if the extraction is carried out without enzyme.

After the screening of eight enzymes to extract the polyphenols from *S. muticum*, Viscozyme - a cellulase, released a greater quantity of phenolic compounds according to Folin Ciocalteu method, result was also confirmed by the anti-free radical activity of (IC_{50} 0.6 ± 0.1 mg/mL). This extract also managed to

prevent $43.7 \pm 4.2\%$, higher than the other extracts, of biofilm formation generated by *P. aeruginosa*.

Other than antiradical activity, it seems that each enzyme showed different potential. In reducing power, the most important activity was shown by AMG (a α -amylase) extract ($60.8 \pm 1.8 \mu\text{M}$ of Fe^{2+}). Ultraflo (a xylanase) extract had promising potential in inhibiting the tyrosinase at $24.5 \pm 2.6 \%$, while Shearzyme (cellulose and xylanase) extract showed higher inhibition elastase activity than the other extracts, $30.9 \pm 5.9 \%$. Viscozyme extract was able to hamper $43.7 \pm 4.2\%$ of film formation generated by *P. aeruginosa*. However, Shearzyme extracts exhibited strong biofilm inhibition activity against the *E. coli*, $60.3 \pm 10.3\%$ of inhibition.

The application of enzymes in the extraction of the *Sargassum* phenolic compound is quite effective. In addition, *Sargassum* enzyme extracts demonstrate a wide range of bioactivity. It is important to consider that, using enzymes, the phenolic compound is probably not the only compound extracted from the algal matrix by the enzymes. As a result, it will be interesting to continue working on the purified samples pretreated by enzyme-assisted extraction in the future. In addition, the characterization of the *Sargassum* phenolic compound could help to better understand the mechanisms of their bioactivity.

Keywords: *biochemical composition, seasonal variation, polyphenols, Sargassum, biological activities*

Ekstraksi enzimatis senyawa florotanin dari alga coklat *Sargassum* dan aktivitas biologi

Ringkasan :

Di Perancis dan Indonesia, *Sargassum* cukup melimpah namun alga ini belum dimanfaatkan secara optimal. *Sargassum* mengandung makro dan mikromolekul serta senyawa bioaktif, yang paling dikenal adalah florotanin, yang memiliki beragam sifat farmakologi. Oleh karena itu, *Sargassum* menjadi salah satu species alga coklat yang menarik untuk divalorisasi lebih lanjut untuk beragam aplikasi seperti industri makanan, farmakologi, dan kosmetik. Pengetahuan tentang komposisi biokimia dari *Sargassum*, yang juga mewakili kandungan nutrisi, merupakan satu hal yang penting bagi industri makanan. Selanjutnya, aplikasi senyawa bioaktif, ekstraksi padat-cair telah menjadi teknik yang paling umum digunakan dalam ekstraksi florotanin dari biomassa alga coklat. Akan tetapi, florotanin alga coklat dikenal dengan ikatan kompleksnya dengan polisakarida di dinding sel alga dan tingginya kandungan berbagai polisakarida membatasi aksesibilitas senyawa aktif dari matriks dinding sel. Oleh karena itu, keberadaan dinding sel ini mengurangi efisiensi ekstraksi senyawa bioaktif dari alga. Alhasil, penting untuk mempertimbangkan degradasi dinding sel alga sebagai cara untuk mengekstraksi florotanin lebih banyak. Aplikasi enzim dianggap sebagai salah satu proses yang dapat menguraikan dinding sel alga sehingga meningkatkan efisiensi ekstraksi florotanin.

Penelitian ini memiliki tiga fokus utama, yaitu (1) karakterisasi komposisi biokimia *Sargassum* dan variasi musiman, (2) ekstraksi padat-cair dan (3) ekstraksi enzimatis senyawa florotanin *Sargassum* dan bioaktivitas. Bioaktivitas senyawa florotanin yang dianalisis dalam penelitian ini meliputi aktivitas antioksidan (antiradikal DPPH radikal dan FRAP), antibakteri, tirosinase, elastase, penghambatan biofilm, sitotoksitas dan antivirus.

Selain dari polisakarida, tingginya kandungan abu menjadi komposisi dominan kedua di *Sargassum* karena mewakili hingga 35% dari berat kering. Selanjutnya, komposisi biokimia dari *Sargassum* sangat dipengaruhi oleh musim dan berhubungan dengan siklus hidup. Musim mungkin menjadi faktor yang paling mempengaruhi kandungan polifenol dalam *Sargassum*, tetapi metode ekstraksi yang sesuai menentukan efisiensi ekstraksi polifenol dari *Sargassum*.

Ekstraksi padat-cair menghasilkan berat kering lebih rendah (berkisar antara 5-24% dari berat kering) dari ekstraksi enzimatis (21-38% dari berat kering). Kandungan polifenol dari ekstrak enzimatis *Sargassum* memiliki kandungan yang lebih tinggi dari ekstraksi padat-cair.

Berdasarkan evaluasi penapisan enzim untuk mengekstrak polifenol *S. muticum*, di antara delapan enzim, Viscozyme mengekstrak senyawa polifenol lebih banyak dan mempunyai aktivitas antiradikal yang kuat. Hal ini mungkin berkaitan dengan kondisi optimum yang digunakan selama proses ekstraksi, yaitu 50 ° C selama 3 H. Oleh karena senyawa polifenol bersifat sensitif terhadap panas dan mudah teroksidasi oleh paparan cahaya, sangat disarankan untuk menerapkan suhu yang sedang antara 40 sampai 60 ° C. Selain itu, ekstrak enzimatis dari

polifenol dari *Sargassum* dianggap memiliki farmakologis menjanjikan, nutraceutical, dan sifat cosmeceutical.

Dalam studi ini, kandungan mineral dalam *Sargassum* mencapai 33% diikuti oleh protein dengan 24% dari total berat kering alga. Sebaliknya, kandungan gula menunjukkan hanya 13% dari total berat kering alga. Studi tentang variabilitas musiman menunjukkan bahwa komposisi biokimia dari *Sargassum* sangat dipengaruhi oleh musim dan berkaitan dengan siklus hidup dari alga, sama halnya dengan kandungan polifenol pada *Sargassum*.

Namun demikian, metode ekstraksi yang cocok penting untuk dipertimbangkan dalam rangka meningkatkan efisiensi ekstraksi. Ekstraksi padat-cair menghasilkan lebih rendah (5-24% dari bahan kering alga) berat kering dibandingkan dengan ekstraksi enzimatik (26-38% dari bahan kering alga). Mengenai kandungan polifenol, ekstraksi enzimatik juga mengekstrak lebih banyak, 6%, dibandingkan dengan 4% dari total berat ekstrak jika ekstraksi dilakukan tanpa enzim.

Setelah skrining dengan menggunakan delapan enzim untuk mengekstrak polifenol dari *S. muticum*, Viscozyme - karbohidrase, membebaskan lebih banyak senyawa polifenol, dan menunjukkan aktivitas antiradikal bebas yang kuat (IC_{50} 0.6 ± 0.1 mg/mL). Ekstrak ini juga berhasil mencegah $43.7 \pm 4.2\%$, lebih tinggi dari ekstrak lainnya, pembentukan biofilm yang dihasilkan oleh *P. aeruginosa*. Ekstrak AMG mempunyai aktifitas reduksi besi atau FRAP pada $60.8 \pm 1.8 \mu M$ dari Fe^{2+} . Ekstrak Ultraflo memiliki potensi yang menjanjikan dalam menghambat tirosinase di $24.5 \pm 2.6\%$, sedangkan ekstrak Shearzyme menunjukkan aktivitas antielastase yang lebih tinggi dari ekstrak lainnya, $30.9 \pm 5.9\%$.

Aplikasi enzim dalam ekstraksi senyawa polifenol *Sargassum* cukup efektif. Selain itu, ekstrak enzimatik dari *Sargassum* menunjukkan bioaktivitas yang sangat luas. Namun demikian, sangatlah penting untuk diêrhatkan bahwa, dengan menggunakan enzim, senyawa polifenol mungkin bukan satu-satunya senyawa yang diekstrak dari matriks alga. Oleh karena itu, sangat disarankan untuk melanjutkan penelitian dengan menggunakan sampel yang dimurnikan. Selain itu, karakterisasi senyawa polifenol *Sargassum* bisa membantu untuk lebih memahami mekanisme bioaktivitas dari senyawa tersebut.

Kata kunci: komposisi biokimia, variasi musiman, polifenol, *Sargassum*, aktivitas biologi

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Introduction

Brown algae (Phaeophyceae) consist of 2038 species from 20 order in which order of Fucales contributes 597 species. *Sargassum*, known as gulf-weed or sea holly belonging to family Sargassaceae, contains 353 species that have been accepted taxonomically (Guiry & Guiry 2015). *Sargassum* species are spread throughout the tropical and subtropical regions attached on the substrates with its holdfast. This species is characterized by its short stipe differentiating to numerous branches that have many leaf-like laterals and spherical vesicles as tool for flotation. Due to the presence of this floating vesicle, *Sargassum* often detaches from the substrates and forms vast pelagic mats as massive floating biomass as in the Sargasso Sea (Mattoo & Payri 2011). In Indonesia, there are 58 species recorded by Atmadja and Prud'homme van Reine (2014). To the extent of our knowledge, *S. muticum* is the sole *Sargassum* species found in France. Moreover, this is not the native species from this region as it was imported unintentionally in European waters in 1970s (Josefsson & Jansson 2011). *Sargassum* has been acknowledged for its massive influx of biomass along the coast (Milledge & Harvey 2016).

The interesting fact of *Sargassum* species is that communities in some regions, for example in China and Japan, have noticed the important role of this species in pharmacology and food industry. It has been mentioned that *Sargassum*, particularly *S. fulvellum* (Turner) C. Agardh, *S. henslowianum* C. Agardh, *S. thunbergii* (Mertens ex Roth) Kuntze, and *S. horneri* (Turner) C. Agardh, has been part of Chinese traditional medicine from the ancient times and has been formulated in classical Chinese prescription. Meanwhile in Japan, *S. fusiforme* becomes a popular side dish boiled with water and served with the soy sauce (Liu et al. 2012). Supposedly, the finding of ancient manuscript concerning medicinal uses of *Sargassum* has drawn the attention and made this species compelling for further study.

Recognition of *Sargassum* pharmacological properties with wide range of potential derived from its extracts or isolated pure components have unveiled the remarkable prospect of this species for modern medicine. Moreover, the high content of polysaccharides in *Sargassum* cell wall highlights even more the potential application of this species for hydrocolloids and biomaterial industries (Mirshafiey & Rehm 2009; Rhein-Knudsen et al. 2015). For this reason, *Sargassum* has been considered as sustainable resources with appealing bioactivities related to its primary and secondary metabolites (Liu et al. 2012; Yende et al. 2014). Previous studies have reported that *Sargassum* produces diverse metabolite of structural class such as terpenoids, polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, plastoquinones, steroids, glycerides etc. (Yende et al. 2014).

Among the arsenal metabolite compound synthesized by *Sargassum*, polyphenols are one of the most extensively studied as promising bioactive compounds with potential health benefits. Polyphenols in brown algae are mostly phlorotannins, an oligomer of phloroglucinol (1,3,5-trihydroxybenzene). Phlorotannins have been recognized as the most significant group of biologically active substances determining the pharmacological value of algae (Kim & Himaya 2011). The potential health benefits of phlorotannins comprise antioxidant, antibacterial, anti-HIV, anticancer, anti-arthritis activities and enzyme inhibitory effects (Li et al. 2011).

The successful of phlorotannins extraction is determined by the appropriate selection of extraction method. Rate and yield of extraction are two important factors contributing to the economic feasibility of an extraction process. There are several techniques of extraction available for obtaining polyphenol compounds from brown algae biomass, *i.e.* extraction with water, acid and alkaline hydrolysis, alternative and conventional solvent extraction (CSE) (Michael and Chojnacka, 2014). Up until now, the CSE consisting of liquid-liquid (L/L), solid-liquid (S/L) and Soxhlet apparatus (Michael and Chojnacka, 2014) extraction is the most commonly used technique in the extraction of phenolic

compound of algal biomass. Type of solvents, solid/liquid ratio, temperature, time, and agitation are mentioned as crucial factors to increase efficiency of extraction by improving the solubility and mass transfer (Puri et al. 2012; Li et al. 2017). Nevertheless, this extraction technique is often characterized by applications of organic solvents in high volume that are often toxic, long extraction time, low extraction yield of bioactive and present low selectivity (Ibanez et al. 2012). Phlorotannins are easily oxidized due to light and heat-sensitive. As complex polysaccharides linkages constitute the brown algal cell wall in high content, solid-liquid extraction can be a long and exhausting process. It could lead to the degradation of phlorotannins during the process resulting low yield of target compound (Wijesinghe & Jeon 2012; Franco et al. 2008). Considering that phlorotannins are cell-wall-bound compounds, they cannot be easily extracted using typical extraction methods with aqueous solvents. Thus, it limits the accessibility of solvents to target compound. In other word, the complexity of polysaccharides in algal cell becomes the main physical barriers causing the decrease of extraction efficiency. Therefore, degradation of algal cell wall has been considered as important step so that the active compounds become more accessible (Wijesinghe & Jeon 2012). To disrupt the integrity of algal cell wall, enzymes are required as they can hydrolyze the components of cell wall thereby increasing the permeability of cell wall that can result higher extraction yields (Puri et al. 2012). A successful use of this technique to enhance the recovery of phenolics compounds from several terrestrial plants has been reported. This enzyme-assistant extraction method on macroalgae has also been reported in several studies (Wijesinghe & Jeon 2012).

Sargassum might have promising potential as sustainable resources for diverse applications. In order to announce algae for human consumption either directly as vegetables or integrated in foods, the details of their nutritional compositions as well as variations among species are required to acknowledge (Gomez-Ordenez *et al.*, 2011). However, not all algae have been thoroughly studied related to their nutritional values and one of them is *Sargassum*. In

addition, to our knowledge, there have not been any studies that compare between solid-liquid and enzyme-assisted extraction of *Sargassum* polyphenols, in this case phlorotannins. Moreover, characterization of *Sargassum* phlorotannins extracted with enzymes is still scarce.

Objectives

This work is part of a search for active molecules within species of Indonesian and French Sargassaceae (Phaeophyceae, Fucales) especially through phlorotannins. Furthermore, this work is conducted within joint supervisor framework between two universities, Université Bretagne-Sud (UBS) in France and Diponegoro University (UNDIP) in Indonesia.

In summary, the objectives of this study will be:

1. To evaluate the biochemical composition of different species of *Sargassum* and its seasonal variation of represented by French *S. muticum*,
2. To determine the phenolic compound of different species of *Sargassum* extracted with different type of solvents in the S/L extraction technique followed by its seasonal profile in the French *S. muticum*,
3. To evaluate and to compare the efficiency and the biological activity of *Sargassum* extracted with two different techniques, i.e. the S/L and enzyme-assisted extraction,
4. To evaluate the biological activity of the fractions of *Sargassum* enzyme-assisted extract.

Phase I

In this phase, we studied both the biochemical composition of different species of *Sargassum* and its seasonal variation represented by one species of *Sargassum*, i.e. the French *S. muticum*. The biochemical compositions that will be evaluated are protein, neutral sugar, sulfates groups, polyphenol, uronic acid, nitrogen, and ash.

Later, the seasonal phenolic content of *S. muticum* S/L extract will also be analyzed as well as its seasonal profile. For the S/L extraction, four different solvents (water, methanol, acetone and methanol 50%) were applied during the extraction of phenolic extraction.

Phase II

In this phase, *Sargassum* polyphenol was extracted using solid-liquid extraction in four different solvents, namely water, methanol, methanol 50%, and ethanol 75%. Later, the antibacterial and antioxidant activity represented by DPPH radical scavenging activity and reducing power capacity of *Sargassum* polyphenol were evaluated.

Phase III

In this phase, focus of our work will be more about the biological activity of *Sargassum* enzyme-assisted extracts both the crude and fractionated samples. Therefore, in this part, the biological activity analysis will be extended and be more focused to the potential of this extracts for cosmeceutical purpose.

The biological activities that were evaluated were the antibacterial, antioxidant represented by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity and FRAP (Ferric Reducing Ability of Plasma), inhibition of tyrosinase and elastase as well as antiviral activity and cytotoxicity based upon cell viability of Vero Cell/HSV-1 and fibroblast model.

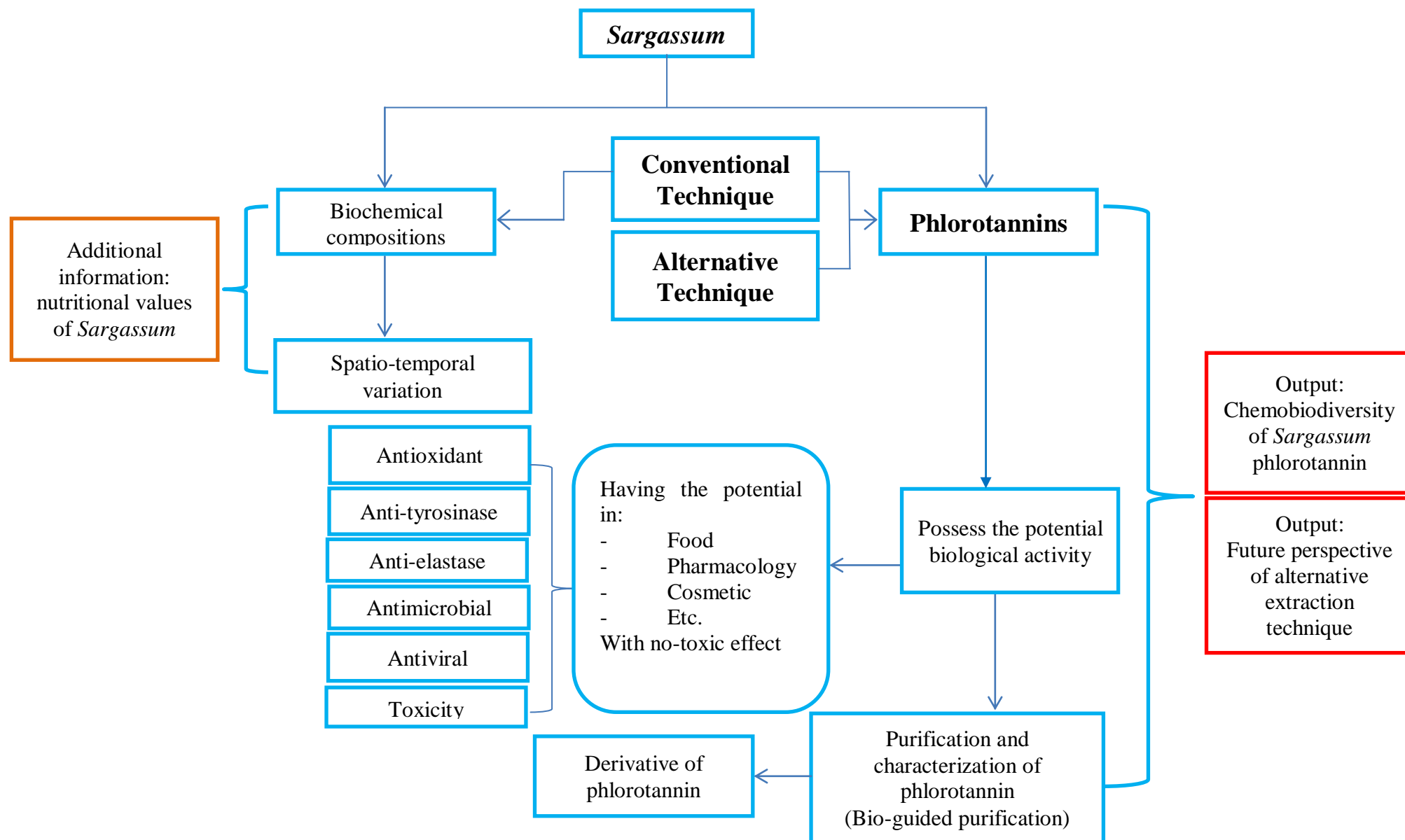


Fig 1. Summary of research conceptual framework

Chapitre 1 Literature Review

1.1 General introduction

Definition of algae classes is very complex and many other criteria are involved, such as the nature and locations of the reserve glucanes, the nature of their cell walls, the number of periplastidial membranes, the shape of mitochondrial crystal, the presence and ultra structure of flagella, the type of mitosis, the mode of nutrition, reproduction and habitat (de Reviers, 2002)). In the biological sense of the word, the word algae (algae) is used to designate a large, varied, and heterogeneous group of organisms that, at present, do not have a clear cut, formal taxonomic status since it keep on up-dating and is under constant and rapid revision at all levels (Barsanti and Gualtieri, 2006; Mouritsen *et al.*, 2013).

Algae might resemble to plant yet they are more likely to be saltwater dwelling, simple, multicellular, macro thallic organisms that fall into the rather outdated general category of 'plants'. They are the oldest members of the plant kingdom, extending back to many hundreds of millions years. They are typically plant without leaves and stems as well as root system to take in water or nutrients (like the terrestrial plant) as their cells are in direct contact with the surrounding water from which they derive their nourishment. They do not form embryos as their reproductive structures consist of cells that are potentially fertile and lack of sterile cells covering or protecting them. For the multicellular algae, they are generally attached with so-called *holdfast* serving as anchorage functions (Mouritsen *et al.*, 2013).

Most macroalgae come from either brown algae (phylum Ocrophyta, class Phaeophyceae) or red algae (phylum Rhodophyta). A few marine macroalgae occur in the phylum Charophyta since about 600 large green algal taxa are in the phylum Chlorophyta (Dawes, 2016). Macroalgae in these four groups have more fundamental differences regarding cytological, chemical and life histories, between one another than with the vascular plants (Dawes, 1998). Dring (1982)

listed 900 species of green algae, 997 brown algae, and 2540 red algae worldwide. Since then, field, culture, and molecular studies have identified many new and cryptic species and taxonomic relationships. These advancements in phycology have resulted in major changes in the classification of algae (Schneider and Wynne, 2007, 2013; Wynne and Schneider, 2010). Thus the number of seaweed species has increased. Guiry and Guiry (2015) listed 4422 species in the Charophyta and 6055 in the Chlorophyta, 2041 species in the class Phaeophyceae, and 7107 species in the Rhodophyta. In W.R. Taylor's 1957 flora *Seaweeds of the Northeastern Coast of North America*, he listed 170 genera (37 green, 57 brown, 76 red algae) and 401 species (95 green, 142 brown, and 164 red algae), while Mathieson and Dawes (2016) have described 248 genera (57 green, 89 brown, 102 red) and 535 species (146 green, 180 brown, and 209 red taxa) for the same geography (Dawes, 2016).

For centuries, coastal communities have harvested a wide variety of marine macroalgae within the entire algal group, *i.e.* brown, green and red algae. At first, they were frequently used for domestic human consumption; later, industrial uses were discovered. Macroalgae is a lucrative business, driven by economic rather than environmental conditions (Delaney *et al.*, 2016). Along with the growth of human population, the need to look for alternatives natural resources raises. Moreover, the scarcity of land for cultivation, infertility of soil, limitation of various crops, requirement for more fodder, requisition of specific compounds for life-saving medicines, and other specialized requirements have compelled mankind to search more into the depth of the ocean (Murty and Banerjee, 2012). Aside from consumptive forms, macroalgae also include in the non-consumptive forms as medicine, fertilizer and animal feed, inputs into industrial process and for other purposes such as building materials (Delaney *et al.*, 2016).

1.2 Brown algae (Phaeophyceae)

The kingdom Chromista has over 21,500 taxa and includes the phyla Dinophyta (Dinoflagellates), and the Ochrophyta. The latter phylum contains 16 classes and

over 3700 species including the brown algae in the class Phaeophyceae (Guiry and Guiry, 2015). Based on nucleotide sequences, it appears that the Phaeophyceae diverged from the diatom phylum Bacillariophyta that is also in the kingdom Chromista around 200 million years ago (Hori and Osawa, 1987). The majority of species in the Ochrophyta occur in the classes Chrysophyceae (613 species that range from unicellular to filamentous freshwater taxa), Xanthophyceae (559 species including the coenocytic marine genus *Vaucheria*), and the Phaeophyceae (with about 265 genera and 2040 species; (Guiry and Guiry, 2015)). Over 95% of the brown algae are marine (Wang *et al.*, 2013).

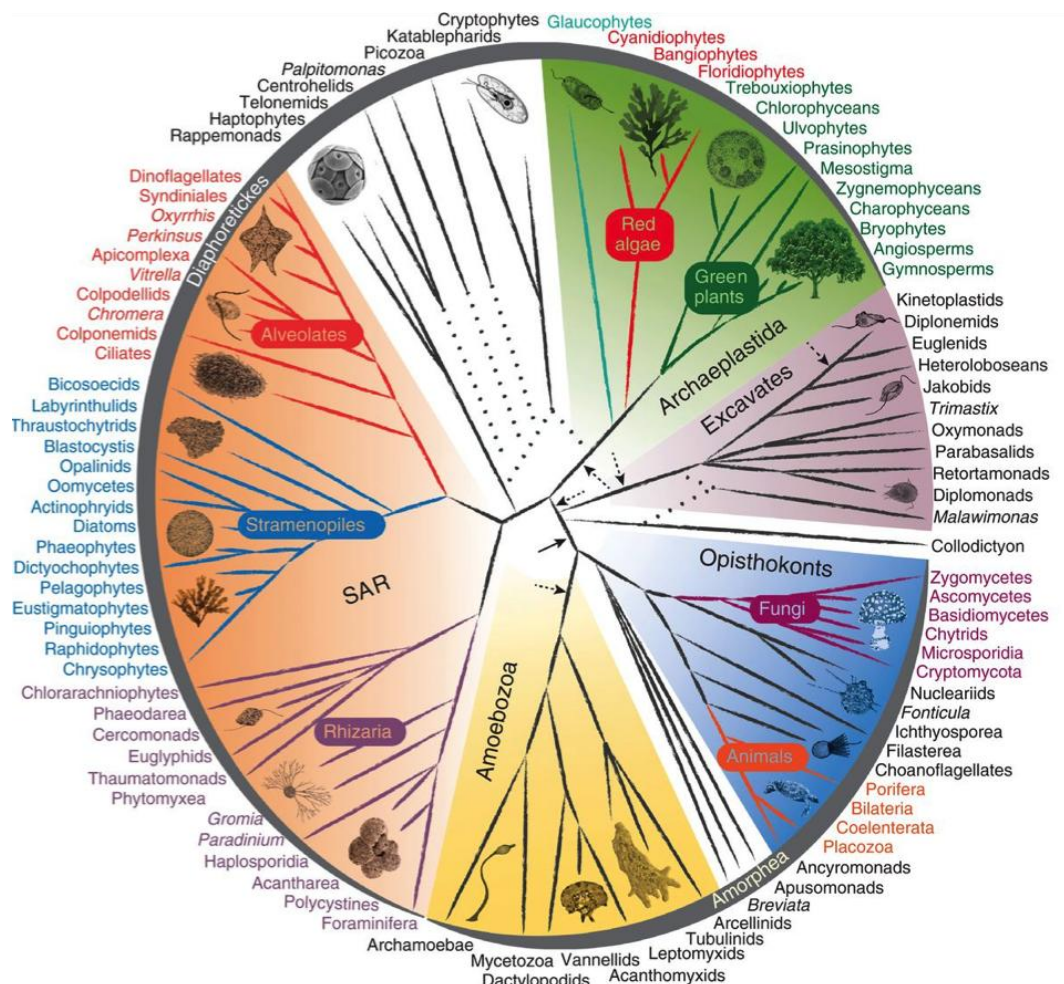


Fig 1-1 Phylogenetic tree showing the position of Phaeophyceae within the Eucaryotic group (Burki, 2014).

Fig 1-1 illustrates the phylogenetic tree showing the position of Phaeophyceae within the eukaryotic group based on Burki (2005). As presented in this figure, brown algae are included in the SAR group – an acronym of its constituents, *i.e.* stramenopiles, alveolates, and rhizaria, particularly in stramenopiles (also known as heterokonts). This super-group, most recently recognized, embraces a very large of diversity of protists, including diatoms, multicellular algae and heterotrophic, often parasitic species such as oomycetes and *Blastocystis* (Burki, 2014).

In addition to Fig 1-1, Fig 1-2 illustrates the phylogenetic tree of eukaryotic based on the major component of cell wall. Cesa* represents members of the cellulose synthase family whose proteins assemble into rosette terminal complexes, Cesa is the ancestral form of cellulose synthases, and CslA/C is a single gene that is most similar to the land plant CslA and CslC gene families. The arrows indicate the origin of the plastids: PE (solid arrow), primary endosymbiosis; SE1 (dotted arrow), secondary endosymbiosis scenario 1 in which the Alveolata, Cryptophyceae, Haptophyta, and Stramenopiles originate from a common ancestor, which acquired its plastids by a secondary endosymbiosis event with a red alga (Cavalier-Smith, 1999), which Rhizaria subsequently lost and the Chlorarchniophyta regained (Keeling, 2010); SE2 (dashed arrows), secondary endosymbiosis scenario 2, separate acquisitions of rhodobionts (Baurain *et al.*, 2010).

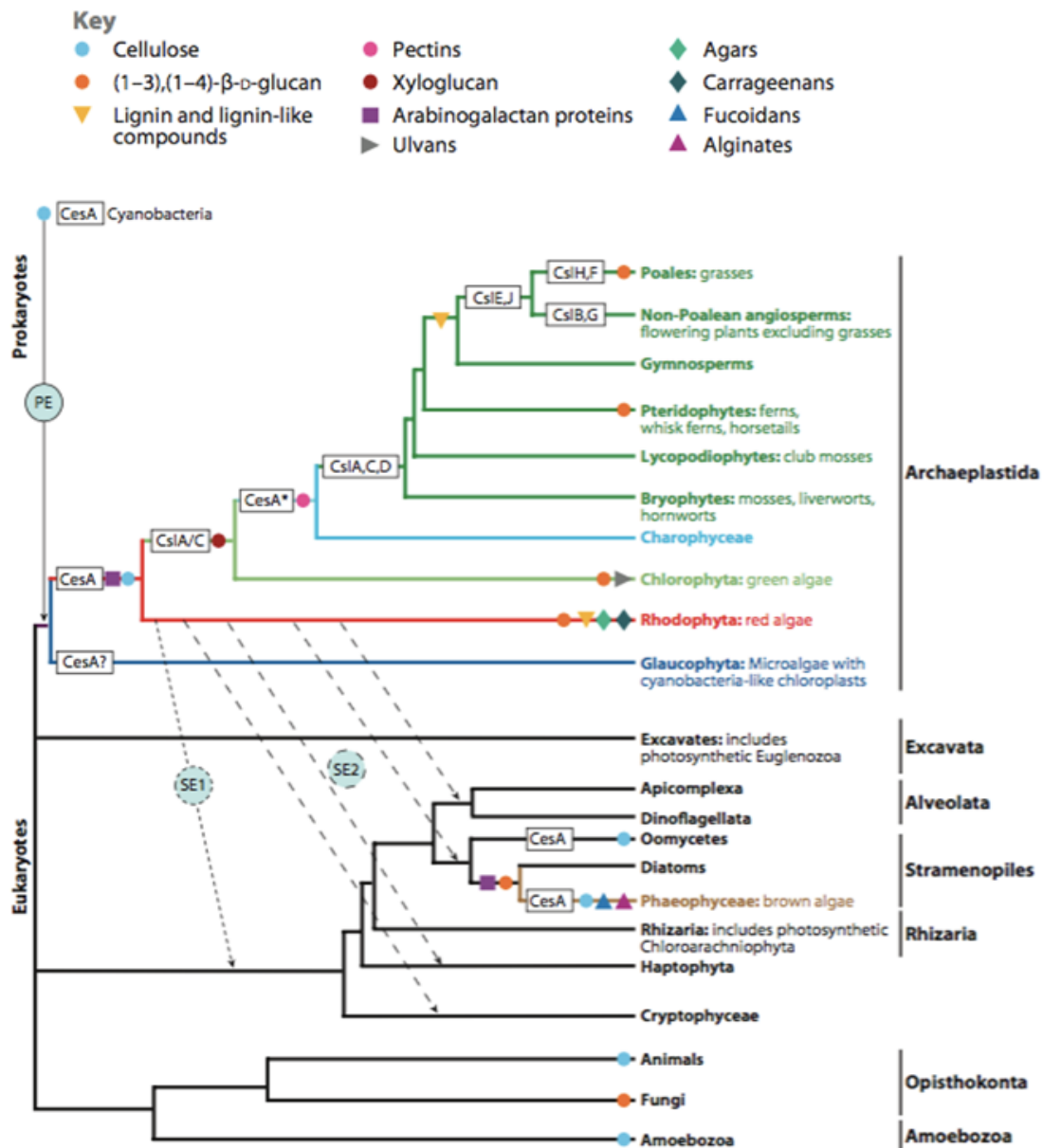


Fig 1-2 Simplified phylogenetic tree representing the major cell wall component (Popper *et al.*, 2011).

Members of brown algae are multicellular, and their primary photosynthetic pigments are chlorophylls 'a' and 'c' but derive their brownish colour characteristic from a mixture of xanthophyll pigments (β -carotene, neofucoxanthin, and other carotenoids), of which fucoxanthin is the most important (Dring, 1982; Dawes, 2016). Primary reserve of brown algae is chysolaminaran, a β -1,3-linked glucan occurring in solution in special vacuoles as well as mannitol and lipid. Cell wall of brown algae have a felt-like weft of

cellulose microfibrils stiffened by calcium alginate, which is a commercially important phycocolloid (Dawes, 1998). In general, brown algal life histories are either haplodiplontic or diplontic with morphological appearance ranging from filamentous thalli (*Ectocarpus fasciculatus* Harvey) to large complex with blades, stipes and floats as with *Sargassum filipendula* C. Agardh. Most of larger algae, belonging to the 17 orders in the class Phaeophyceae, occur in the orders Desmarestiales, Dictyotales, Fucales, and Laminariales. Brown algae are most inhabited the cold (arctic to boreal) to temperate waters, while most species of Dictyoles and Sporochnales are mainly found in subtropical or tropical waters. Many brown algae are lithophytes and grow attached to hard substrate (Dawes, 2016), and some of them, such as members of the order Fucales (the rockweeds), are commonly found along rocky seashores (Venugopal, 2011). The major brown algae, which include such genera as *Macrocystis*, *Laminaria*, *Sargassum*, *Pterygophora*, and *Nereocystis* grow upward like trees and spread their blades at the surface of the water, where they obtain the maximum amount of light. The Pacific coast of both North and South America is dominated by *Macrocystis*, whereas *Laminaria* is dominant in Atlantic waters and in Japan (Venugopal, 2011).

Several methods of reproduction occur in algal reproduction, they are vegetative by the division of a single cell or fragmentation of a colony, asexual by the production of motile spore, or sexual by the union of gametes. Vegetative and asexual modes allow stability of an adapted genotype within a species from a generation to the next. Sexual mode involves plasmogamy (union of cells), karyogamy (union of nuclei), chromosome/gene association, and meiosis, resulting in genetic recombination. In this mode, variation is less yet possible as it is mostly because of the waste of gametes that fail to mate (Barsanti and Gualtieri, 2006).

In Fucales, the reproduction occurs by releasing the haploid gametes and the biological life cycle is monogenetic diplophase. Adult individuals are monoic or dioecious; they are meiosporophytes ($2n$ chromosomes), producing gametes by

chromatic reduction. Gametes are produced in conceptacles. The conceptacles are grouped into receptacles at apexes of fertile lateral in the Genera of *Cystoseira* and *Bifurcaria*, at the base of pleuridia along the axis in the Genera *Turbinaria* and along the fertile laterals in the Genera of *Sargassum* (Le Lann, 2009).

The plant body of *Sargassum* is a diploid sporophyte, reproducing both by vegetative and sexual methods. It does not multiply asexually by means of spores. Instead it reproduces by vegetative method and the formation of sex organs. Fragmentation is the only known method of vegetative reproduction in this alga. Their vegetative reproduction involves fragmentation. Each fragment grows into new plant. The separation commonly occurs accidentally or by death and decay of older parts separating the younger parts of the branches. In the Sargasso Sea, huge masses of floating *Sargassum* are formed by continuous vegetative reproduction. Their conceptacles can be seen occasionally, however they are functionless since the maintenance of the population is entirely by vegetative means (Graham and Wilcox, 2000). The sexual reproduction is oogamous and involves the fusion of motile sperm or male gamete with a non-motile ovum or female gamete. They are respectively borne inside antheridia and oogonia. They are also termed as microsprangia and megasporangia. The spores germinate inside the sporangia to produce gametes. The sex organs are produced inside special cavity called conceptacles. Conceptacles are found inside small finger-like branchlets called receptacles and usually bear only one type of sex organs (Ogawa, 1976; Critchley *et al.*, 1991).

A large number of antheridia develop in each male conceptacle. They are borne on the lower branches of paraphyses. The first antheridium is borne at the tip of a 3-celled filament which arises from a cell of the fertile layer. The basal cell remains at the level of the fertile layer. The middle one is known as stalk cell. The upper or antheridial cell develops into an antheridium. Antheridium is ovoid in outline. It has a thick wall which becomes mucilaginous towards maturity. Internally the antheridium contains initially a single diploid nucleus and a few chromatophores. The diploid nucleus divides meiotically and then mitotically to

form 64 haploid nuclei. The chromatophores also divide. They ultimately get placed near the nuclei in one to one ratio. The cytoplasm cleaves and the contents of the antheridium get divided into 64 haploid protoplasts. Each such protoplast transforms itself into a single sperm or antherozoid. Thus 64 sperm develop inside an antheridium (Ogawa, 1976; Critchley *et al.*, 1991).

The antherozoids are then expelled into the medium while the oosphere, after rupture of the oogone wall, remains on the surface of the design. The antherozoids move towards the oosphere by chemotaxis, attracted by fucoserratene, pheromone excreted by the oospheres (De Reviers 2003a). After gametes melt, zygotes form on the surface of receptacles (Yoshida, 1983). The zygotes germinate to give seedlings bearing 8 to 16 rhizoids, which once released, sediment rapidly and attach to a hard substrate, usually near the parental thallus (Stiger and Payri 1999).

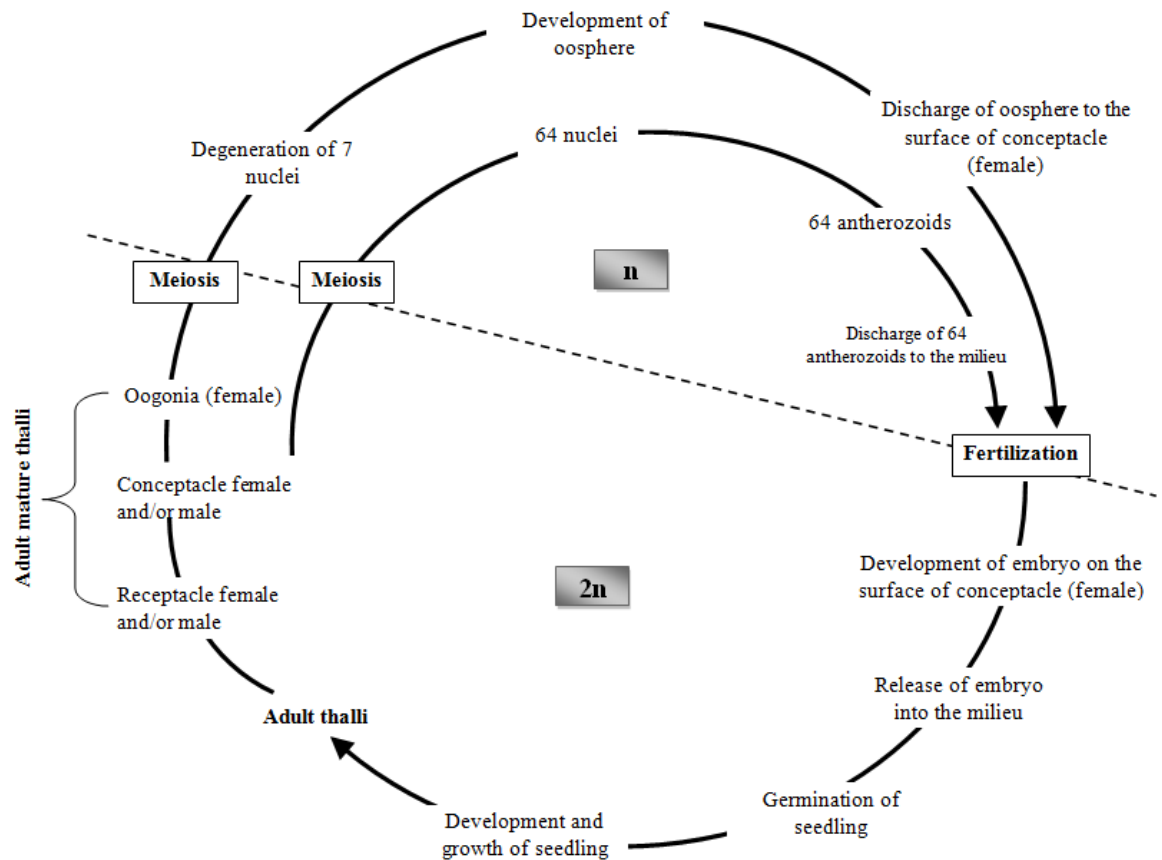


Fig 1-3 Life cycle of Sargassaceae according to Stiger (1997).

1.2.1 Brown algae *Sargassum*

Classification of brown algae *Sargassum* according to Lee (2008) and Guiry and Guiry (2015) is:

Phylum: Ochrophyta

Class: Phaeophyceae

Order: Fucales

Family: Sargassaceae

Genus: *Sargassum*

The thallus of *Sargassum* is differentiated into root-like form called holdfast; their main cylindrical axis into stem-like form called stipe and their strand into leaf-like form called blade (Fig 1-4). In addition, they have air bladder in every corner of their thallus. These bladders are responsible for their floating ability. As a result, they are considered as a disturbing community especially around the harbor and coastal. Furthermore, by having bladders, *Sargassum* is well known by its fast and long-distant distribution. Other factors that influence their distribution are their fast growth rate, first year fertility and monoecious reproductions – eggs and sperms come from the same gametophyte. Main axis of *Sargassum* is perennial and slow growth rate; therefor their main lateral can grow annually (Graham and Wilcox, 2000).

Both the growth and the distribution of *Sargassum* are influenced by their surroundings, which is their environmental. Each environmental factor gives different effect to different species of *Sargassum* especially related to their metabolism. There are several environmental factors considered as the most influenced variables based on previous researches. The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, salinity, temperature, and turbulence. The most optimal parameters as well as the tolerated ranges are species-specific and the various factors may be inter-independent and a parameter optimal for one set condition is not necessarily optimal for another (Barsanti and Gualtieri, 2006).

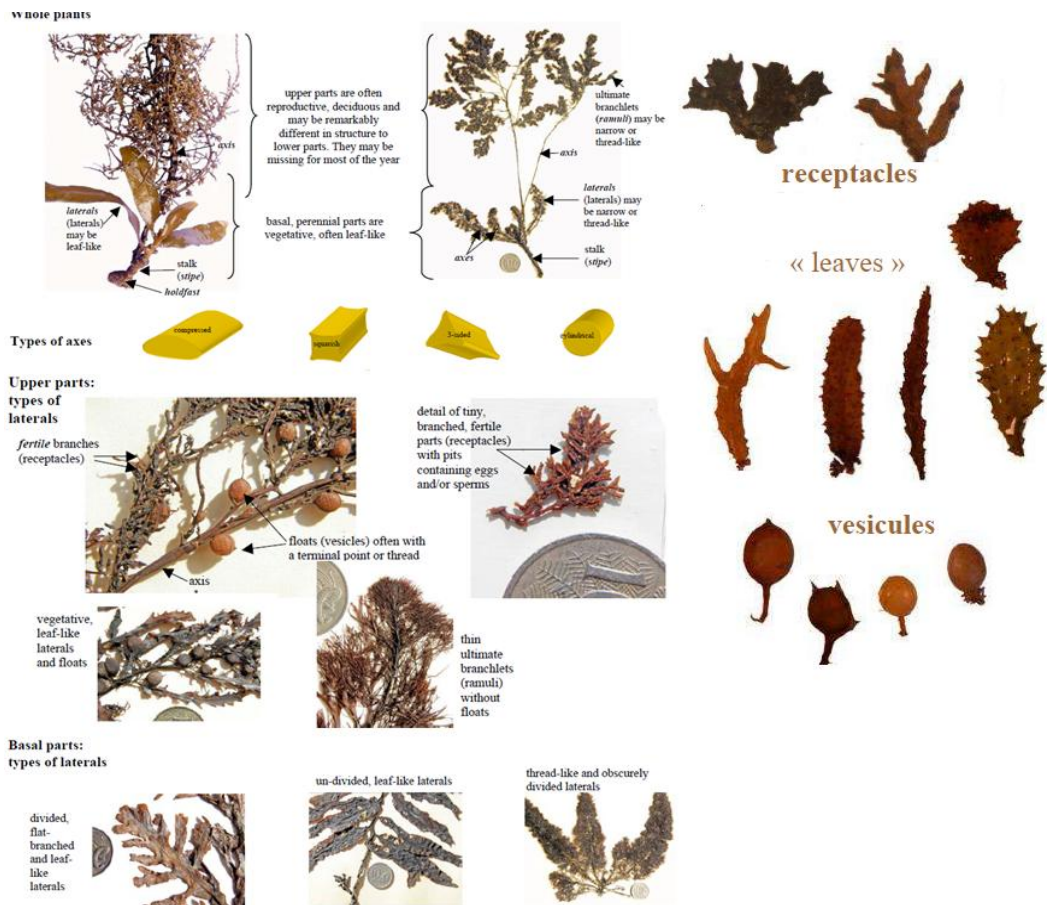


Fig 1-4 General morphology of *Sargassum* (Baldock, 2009; Mattio *et al.*, 2009).

1.2.1.1 *S. aquifolium*

Taxonomic synonym of *S. aquifolium* is *S. echinocarpum* (Bold and Wynne, 1985). In general, the morphology of this alga is similar with other *Sargassum* species. The difference lays on its flattened branches, with lance-like leaves, vesicles which are mucronate with winged pedicles (Low and Chou, 2013).



Fig 1-5 *S. aquifloium* (pers. documentation).

1.2.1.2 *S. ilicifolium*

S. ilicifolium is generally brown or dark green. Oogonia and antheridia occur in conceptacles embedded in receptacles on special branches (10). Their extensions are modified pedicles on which berry like bladders are borne. Linear-lanceolate fronds possess a dentate sinuate margin, distinct midrib and small numerous irregularly placed cryptostomata. The shapes of their vesicles are globular often without prolongation at the top. Then shapes of their receptacles are cylindrical, filiform and irregularly ramified (Sumithra and Arunachalam, 2014).



Fig 1-6 *S. ilicifolium* (pers. documentation).

1.2.1.3 *S. muticum*

S. muticum brown color varies from dark brown to pale, yellowish brown depending on season and growing conditions. They have regularly alternating lateral shoots or branches on a central perennial stem. They attach to substrate with their disc-shaped holdfast. They have numerous small air bladders of 2-3 mm round lying on the stems. These bladders help them to stand upright in the water or float if there is any part detached from the basal.

S. muticum has a 75 – 120 cm long frond, but it reaches 1.5 – 2 m long in Swedish waters, 6 – 7 m in French waters and up to 8.5 m in Norwegian waters. Their lateral branches detach in summer or autumn, leaving a short perennial basal stem to over winter (Wallentinus, 1999). During the summer, their receptacles develop in the areas where the annual shoot attaches to the stem but may also sit on top of the branch.



Fig 1-7 *S. muticum* Yendo (Fensholt) (pers. documentation).

Sheltered hard-bottoms are their habitat preferences. However, they may also attach to hard substrate on soft-bottom, such as stone or shells. *S. muticum* tolerates water currents but they are not fond to direct wave exposure. They are often found in shallow waters, in lower intertidal and upper sub tidal zones. *S. muticum* stands for a wide range of abiotic conditions and is thus considered highly invasive ((Wallentinus, 1999)). Their optimal salinity level for growth is 34 ‰. They need a temperature of more than 8 °C at least four month to reproduce (Steen, 2004) (Steen and Rueness, 2004).

1.2.1.4 *S. polycystum*

The morphology of *S. polycystum* is not different from other Phaeophyceae. They have a cylindrical small spiny thallus with a disc-shaped holdfast and air-bladders in every corner. Typical characteristics of this species are their small, pointed and jagged blades. *S. polycystum* can be found at the tide pool area, the reef flats, the reef slope, the ridge and the lagoon. They grow mostly in the tropical region with temperature ranged from 27.5 – 29.3 °C and water salinity 32 – 33.5 ‰. In general, *Sargassum* needs of light intensity is higher compared to the red algae (Kadi, 2005). They need 6500 – 7000 lux of light intensity (Boney, 1965). Consequently, they inhabit mostly along the coast with 0.5 – 5 m.



Fig 1-8 *S. polycystum* (pers. documentation).

1.3 Abiotic factors

1.3.1 Temperature

The ideal temperature for algae varies depends on where they live. In general, for algae inhabit the polar zone they can tolerate temperature $< 10^{\circ}\text{C}$, the temperate zone between $10 - 25^{\circ}\text{C}$ and tropical $> 20^{\circ}\text{C}$. Temperate algae can tolerate cold water at least down to -1.5°C (seawater freezing point) (Lobban and Harrison, 1997).

Temperatures have fundamental effects on the chemical-reaction rates related to the metabolic rates of algae. The effects of a given temperature change will not be same on all metabolic processes, because of differing temperatures sensitivities of enzymes and the influences of other factors, including light, pH, and nutrients (Lobban and Harrison, 1997).

Temperatures optima vary among species and among strains as well as between heteromorphic life-history stages. Changes with the age of the thallus have also been noted. Norton (1977) has suggested that *S. muticum* shows a high growth rate at high temperatures and it is what makes them so invasive in relatively warm waters.

S. muticum optima temperature for maximum growth is 25 °C. He also reported that the temperature range of *S. muticum* germlings is able to tolerate a temperature between 10-30 °C. This species is able to tolerate an annual temperature range spanning about 10 °C to 15 °C (Hales and Fletcher, 1989). *S. polycystum* is intolerant to seawater temperature > 30 °C, thus they have a lower optimum growth temperature between 20-25 °C and tolerance limit between 25-30 °C (Hwang *et al.*, 2004). Rao *et al.* (2002) reported that their rapid increase in growth and maximum development were shown during the low period of low temperature (22 to 28 °C). For *S. ilicifolium*, its highest growth rate was recorded during the cold season at mean temperature 28 °C to 30 °C. In regions where maximum temperatures are lower, *S. ilicifolium* maintains relatively high biomass all through biomass through the year (Ateweberhan *et al.*, 2005), for example at the Philippines, with the mean annual temperature 29.1 °C to 32.7 °C (Hurtado and Ragaza, 1999). *S. aquifolium* has an optimum temperature for its growth at 23 – 30 °C with low tolerant limit between 19 – 23 °C. Its germlings attained maximum growth at 35 °C and its maximum growth at 35 °C (Hamza *et al.*, 2015).

In Indonesia, as tropical characterized with its two season, i.e dry and rainy season, there are not much researches related specifically to the seasonal variation or effects of temperature towards the growth rate of *Sargassum*. Apparently, the optimum temperature ranges from 27.25 °C to 29.30 °C (Kadi, 2005).

1.3.2 Salinity

Algae are found mostly in the spray zone (supralittoral zone) are subjected to the open air for certain period of time frequently experience salinity fluctuation. Evaporation causes an increase in the salinity. The most important effects of salinity are the osmotic consequences of the movements of water molecules along water-potential gradients and the flow of ions along electrochemical gradients. These processes take place simultaneously and both are regulated in part by the

semipermeable membranes that surround cells, chloroplasts, mitochondria and vacuoles.

In the brown algae, changes of salinity as well as temperature and irradiance might induce the phenolic production (Ragan and Glombitza, 1986). Connan and Stengel (Connan and Stengel, 2011) have demonstrated that the reduction of salinity decreases the phenolic content and increase in phenolic exudation in the *Fucus vesiculosus* and *Ascophyllum nodosum*. The increase of phenolic exudation was assumed to be a mechanism that prevents the copper from entering and damaging the photosynthesis apparatus of Algae. Thus, this mechanism gave the ability of algae to tolerate high copper contamination in the surrounding – also known as a detoxification mechanism (Connan and Stengel, 2011).

Algae are extremely tolerant to changes in salinity. One of them is *S. muticum* who has been well known by their tolerance towards salinity ranges, i.e between 25 to 34‰. Pacific Northwestern *S. muticum* can tolerate different range of salinities, which is 24 to 25.5 ‰, 27 to 35 ‰ and 21 to 30 ‰ (J. and Loo, 1990; Clemence, 2008; Josefsson and Jansson, 2011). In Indo-Pacific Ocean, *Sargassum* tolerates the change of salinity range between 32 – 33.5 ‰ (Kadi, 2005).

1.3.3 Nutrients

The nutrient availability will affect algae's growth rate. C, H, O, P, Mg, Cu, Mn, Zn and Mo are considered to be required by all algae (O'Kelley, 1974; DeBoer and Ryther, 1977). Among these possible limiting elements, nitrogen appears to be the most frequently limit the growth of algae (Topinka and Robbins, 1976), followed by phosphorus as the second most limiting nutrient (Manley and North, 1984). Nitrate, nitrite, ammonium and phosphate concentrations vary from 0 to 30, 1, 3, and 2 μM , respectively, for most pristine temperature areas (Lobban and Harrison, 1997). The concentrations of these elements vary depends on the

physical and biological parameter of the surrounding environment where the algae live.

1.3.4 Light

Light intensity plays important role since it is the source of energy during photosynthetic reactions in algae. However, the requirements greatly vary depends on the depth and the algae density. If the light is too high, it might lead to photo-inhibition.

Light intensity also affects the production of phenolic contents found in the brown algae. *Fucus spiralis*, *F. vesiculosus* and *A. nodosum* that grow under high daily irradiation inhabited the intertidal and eulittoral zone had a higher phenolic content than algae grow in the low intertidal or sublittoral zone (Abdala-Diaz *et al.*, 2006).

Most often employed light intensities range between 100 and 200 $\mu\text{E sec}^{-1}$, which corresponds to about 5 to 10 % of full daylight (2000 $\mu\text{E m}^{-2} \text{sec}^{-1}$) (Barsanti and Gualtieri, 2006). Kadi (2005) also reported that *Sargassum* needed light intensity ranged between 6000 – 7500 lux equivalents to 100 – 200 $\mu\text{E m}^{-2} \text{sec}^{-1}$. Nevertheless, the optimum light intensity for maximum growth required by marine algae is species-specific. For example, *S. muticum*, their maximum germling growth was recorded at high light intensity (200 $\mu\text{E m}^{-2} \text{sec}^{-1}$).

1.3.5 Wave and currents

These two physical factors are an incredible source of energy to the coastal environment. It can contribute to ten times more energy than does the solar radiation. This tremendous energy input explains the high productivity of coastal macroalgal communities. In other words, this energy gives a positive effect on algae. By constantly moving the frond of algae, waves and currents reduce the shading and bring a constant resupply of inorganic nutrients to the algae. These

factors also reduce the nutrient depletion by reducing the thickness of the boundary layer of unmoving water associated with algae surfaces. In addition, waves and current can remove sessile organism that compete with algae for attachment in the intertidal zone (Lobban and Harrison, 1997).

Along with wave and currents, tidal pattern also affects the algae especially to the structural communities. However, tidal patterns based on the observation by Pavia and Brock (Pavia and Brock, 2000) seems to regulate the phenolic content in the tissue of *A. nodosum*. Greater phenolic concentration was found during the low tides than during immersion. In addition, this variation also correlated to the air temperature (case for *P. canaliculata*) and to the tidal regime as in the case of *A. nodosum* (Connan, 2004).

1.3 Biotic factor

Impact of herbivores on macroalgae have been extensively studied and considered as one of crucial factor affecting the metabolic system of algae. It is believed that herbivores of macroalgae have a number of counter adaptations, including feeding specialization and sequestration of defenses. Such adaptation represents potential steps toward a top-down control of macroalgal communities (Pereira and da Gama, 2008).

To counter attack the herbivores, algae employ a number of chemical, morphological, and structural defense mechanisms. These defense mechanisms vary according to spatial and temporal patterns of both seaweed and their associated herbivores. Moreover, such mechanisms are metabolically costly for algae. Previous study on the grazing of *F. vesiculosus* by isopod showed a temporally variable defense pattern that improve the algae's costs/benefit ratio since the deterrence of herbivore involves redistribution of metabolism from growth and primary towards defense (Flöthe *et al.*, 2014). Grazing activity caused by fish, crabs, sea urchins, gastropods, amphipods, and isopods on algae as their primary nutrient source, might result in the alteration of algal communities. If the grazing activity is so intense, it can destroy a kelp forest occupying several square

kilometers. Biomass, coverage, and composition of *Sargassum* sp in central Japan was altered by the presence of sea urchin *Diadema setosum* (Ishikawa and Kurashima, 2016).

Other than grazing, microbial colonization and epibiosis on algae also contribute to algal community (Pereira and da Gama, 2008). As marine macroalgae are continuously immersed in seawater they are regularly colonized by many microbial populations forming surface biofilm which exert significant physiological health pressures by changing the hydrodynamics, increasing resistance to heat transfer, and enhancing subsequent colonization by other fouling organisms and corrosion and destruction of underlying surfaces leading to death of the host (Stern *et al.*, 1996). The encrusting bryozoan *M. membranacea* is an invasive species along the Atlantic coast of Nova Scotia, to which a disruption in sea urchin-kelp dynamics has been attributed (Scheibling *et al.*, 1999). By overgrowing kelp fronds, it causes thallus fragmentation and loss, decimating kelp beds.

Algal defense mechanisms have evolved a variety of strategies including: (1) temporal and spatial escape, (2) chemical defense, (3) morphological or structural defense, and (4) tolerance (Pereira and da Gama, 2008). Herbivore pressures fluctuate as a function of available habitats and seasonal patterns. Abundant populations of unpalatable seaweeds coexist with attractive, palatable seaweeds; fish (as example) rapidly consume the slow growing *Hypnea musciformis* when cultured in isolation, but when the unpalatable brown alga *Sargassum* grows adjacent to *H. musciformis* it grows well (Hay, 1986). Another interesting defense performed by macroalgae is the production of secondary metabolites such as aromatic compounds, terpenes, and many more, as a mechanism to deter epibionts and herbivores (Kubanek *et al.*, 2004; Wiencke *et al.*, 2007; Blunt *et al.*, 2012). In the case of brown algae, they are well known for its polyphenols (phlorotannins) productions (Jormalainen *et al.*, 2003).

1.4 Biochemical composition of *Sargassum*

Algae have been subjected to many researches these past years and some of them have been introduced and used in food, cosmeceutical and pharmaceutical industries. Biochemical constitutions of algae have been widely conducted worldwide. Polysaccharides, proteins, minerals, vitamins, dietary fiber, and lipids have been reported frequently (Peng *et al.*, 2013). These constituents of algae, including major nutrients, like proteins and minerals, and micronutrients, like lipids and vitamins, provide high nutritional values that are beneficial for human (Marinho-Soriano *et al.*, 2006; Patarra *et al.*, 2011). Because of their high nutritional value, algae are traditionally consumed as food or as herbal medicine. Furthermore, they are also used as fodder, fertilizer, fungicides, herbicides, dietary supplements, and as a resource of phycocolloids such as agar, alginate, and carrageenan for various industrial applications (Peña-Rodríguez *et al.*, 2011; Kiliç *et al.*, 2013).

Biochemical composition of algae varies, depending on species, time of collection, geographic habitat and on many external conditions, like water temperature, light intensity, and nutrient concentration in water (Marinho-Soriano *et al.*, 2006; Sudatti *et al.*, 2011). Additionally, chemical composition of algae demonstrates great differences of concentration of nutritional factors such as proteins, lipids, minerals, or dietary fiber within diverse genera of algae (Marinho-Soriano *et al.*, 2006; Leal *et al.*, 2008; Stengel *et al.*, 2011).

1.5.1 Cell wall and sugars

Algae are widely known with their large amount of polysaccharides as constituting their cell wall and reserved as their storage. In general, algal cell wall is distinguished as skeletal and amorphous matrix. The skeletal phase is mainly composed of cellulose in the form of crystalline microfibrils. As for the amorphous matrix phase, separates the cell wall from the cells and serves as cement, mostly contains a network of anionic polysaccharides called phycocolloids (Jouanneau, 2015).

Brown algal cell walls (Fig 1-9) are generally composed of two layers with cellulose making up the main structural skeleton (Kloareg and Quatrano, 1988) and its content ranges from 5.7 to 14 % (Kloareg and Quatrano, 1988; Park *et al.*, 2000). Amorphous component of brown algal cell wall is made up of alginic acid and fucoidan (Lee, 2008), whereas their mucilage and cuticle polysaccharides are mainly composed of fucoidans, alginate and laminarin with total ratio 3:1:1 (Balboa *et al.*, 2013). Alginate generally makes up to 10-30% of the algae dry weight (Park *et al.*, 2000) as for fucoidan, their content range from 1-25% (Obluchinsjaya *et al.*, 2002; Kusaykin *et al.*, 2008). Beside structural polysaccharides, algae also contain storage polysaccharides, notably laminaran and mannitol (Stiger-Pouvreau *et al.*, 2016).

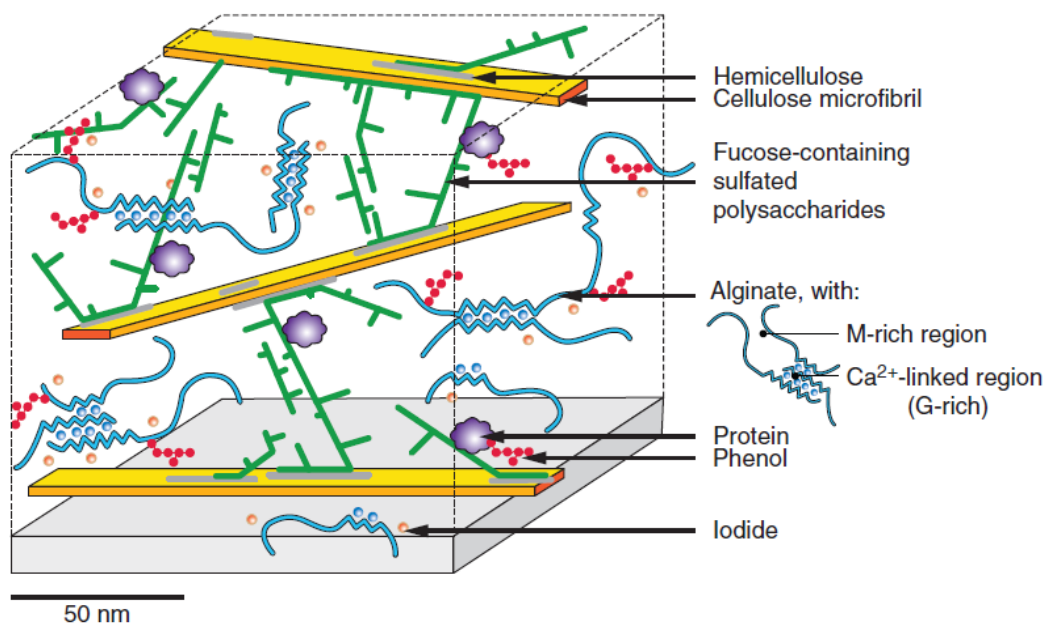


Fig 1-9 Cell wall model of brown algae from the order Fucales (Deniaud-Bouet *et al.*, 2014).

Table 1-1 Content of total polysaccharides in some species of Algae (% of dry weight) (^aAhmad *et al.*, (2012); ^bGomez-Ordenez *et al.*, (2010); ^cHaque *et al.*, (Haque *et al.*, 2009); ^dMarinho-Soriano *et al.* (2006); ^ePatarra *et al.*, (2011); ^fBurtin, (2003a).

Genera	Total polysaccharides (% of dry weight)
<i>Fucus</i>	Chapitre 2 63.88 ^e
<i>Padina</i>	Chapitre 3 85 ^a
<i>Sargassum</i>	Chapitre 4 83 ^a ; 47.43 ^c ; 68 ^d
<i>Turbinaria</i>	Chapitre 5 83 ^a
<i>Laminaria</i>	Chapitre 6 30.23 ^c ; 37.3 ^f

Figure 1-9 presents the cell wall of brown algae proposed by Deniaud-Bouet (2014). Cellulose microfibrils are sparse having a ribbon-shaped like form. Alginates and fucose-containing sulfated polysaccharides form the greater part of cell wall polymers, the latter acting as cross-linkers between cellulose microfibrils. Phenols are likely associated with alginates and proteins. Large amounts of iodide are also present in the wall (Deniaud-Bouet *et al.*, 2014).

1.5.1.1 Crystalline microfibrils form

The skeletal phase is mainly composed of cellulose in the form of crystalline microfibrils, which can be replaced by xylan in the green and red algae, in addition to mannan in the green algae. Brown algal embedding matrix is predominantly alginic acid or alginate (salt of alginic acid) with smaller amounts of sulfated polysaccharide (fucoidan). In red algae, this matrix contains sulfated galactans, *i.e.* carrageenan, agar, porphyran (Stiger-Pouvreau *et al.*, 2016).

Algal cellulose is rarely a pure β -1,4 glucan ; more frequently it contains sugar than glucose, commonly xylose (Mackie and Preston, 1974). Algal cellulose is homopolysaccharides ; whereas their hemicellulose is heteropolysaccharides whose constituents are hexoses, pentoses, and uronic acids. Cellulose belongs to polymers that are very difficult to dissolve and this is determined by compact packing – mainly crystalline, with single amorphous region – of cellulose chains in microfibrils. Adjacent chains are linked by multiple hydrogen bonds instead of covalents. This linkage is the reason why cellulose is insoluble in water, dilute

acid and alkalies, and even in organic solvents (Tarchevsky and Marchenko, 1991).

Unlike cellulose, hemicelluloses are more easily soluble to water due to the looser packing of component molecules; hence they are more accessible to solvents. Hydrolysis of hemicelluloses into sugars components is usually carried out with 2 M hydrochloric acid at 100 °C. Hemicelluloses can be subdivided into several groups on the basis of monosaccharide: 1) arabinans ; 2) xylans (homo- and heteropolysaccharides including arabinoxylans, glucuronoxylans, arabinoglucuronoxylans, galactose, arabinose, rhamnose, glucuronic and galacturonic acids, etc.) ; 3) glucans (homopolysaccharides including β -1,3-D-glucan callose, and heteropolysaccharides such as arabinoglucans) ; 4) galactans (arabinogalactans) ; 5) mannans (glucomannans, galactomannans, and glucogalactomannans ; 6) fructosans and glucofructosans ; 7) galacturonans, and 8) mannuronans (Tarchevsky and Marchenko, 1991).

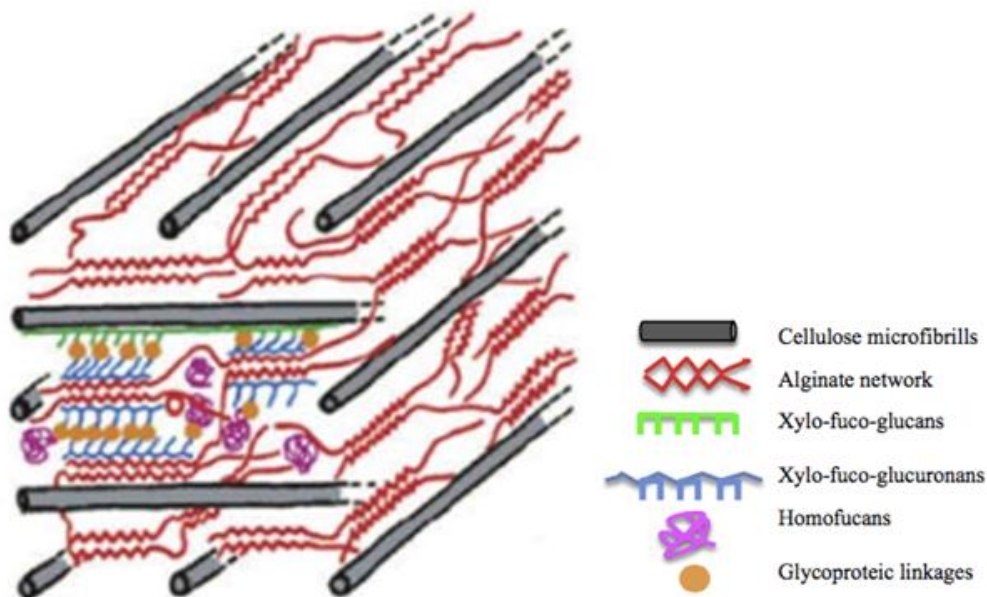


Fig 1-10 Detail of brown algal cell wall composition (Michel *et al.*, 2010).

1.5.2.1 Charged matricial form

As for the amorphous matrix phase, separating the cell wall from the cells and serving as cement, mostly contains a network of anionic polysaccharides called phycocolloides (Jouanneau, 2015).

Alginic acid consists of (1-4) linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues (Figure 15). In the mixed salts form – known as alginates – they contain several cations such as Na^+ , K^+ , Mg^{2+} and Ca^{2+} (Percival and McDowell, 1967). Brown macroalgae contain alginates ranging up to 40% of the dry weight (Stiger-Pouvreau *et al.*, 2016).

To isolate alginic acids, the algal biomass is pretreated with dilute acid to remove polyvalent cations, and the polymer is extracted in the form of readily soluble sodium salt by treatment with sodium carbonate solution at elevated temperature. Alginic acids are precipitated after acidification of the extract, whereas sodium alginate may be precipitated with ethanol; alternatively, insoluble calcium alginate may be obtained by addition of calcium chloride ((Haug, 1962; Hernandez-Carmona *et al.*, 1999a, 1999b). Alginic acids isolated from various sources may differ in the M/G ratio depends on the species, the part of the thallus, their surrounding conditions and the harvest period (Haug *et al.*, 1974; Apoya *et al.*, 2002; Chandia *et al.*, 2004; Skriptsova *et al.*, 2004). Moreover, the molecules may differ in block composition, since uronic acid residues are usually arranged in two homopolymeric blocks, poly-M and poly-G, divided by approximately alternating sequences, poly-MG. Such a blockwise distribution of monomeric units is the result of specific biosynthetic pathway, where the formation of linear polymannuronan precursors is followed by epimerization of some β -D-mannuronic acid residues at C5 under the action of mannuronan-C(5)-epimerase, giving rise to the appearance of α -L-guluronic acid residues, single or contiguous, within the polymeric molecule (Skjak-Braek, 1992; Ertesvag *et al.*, 1996).

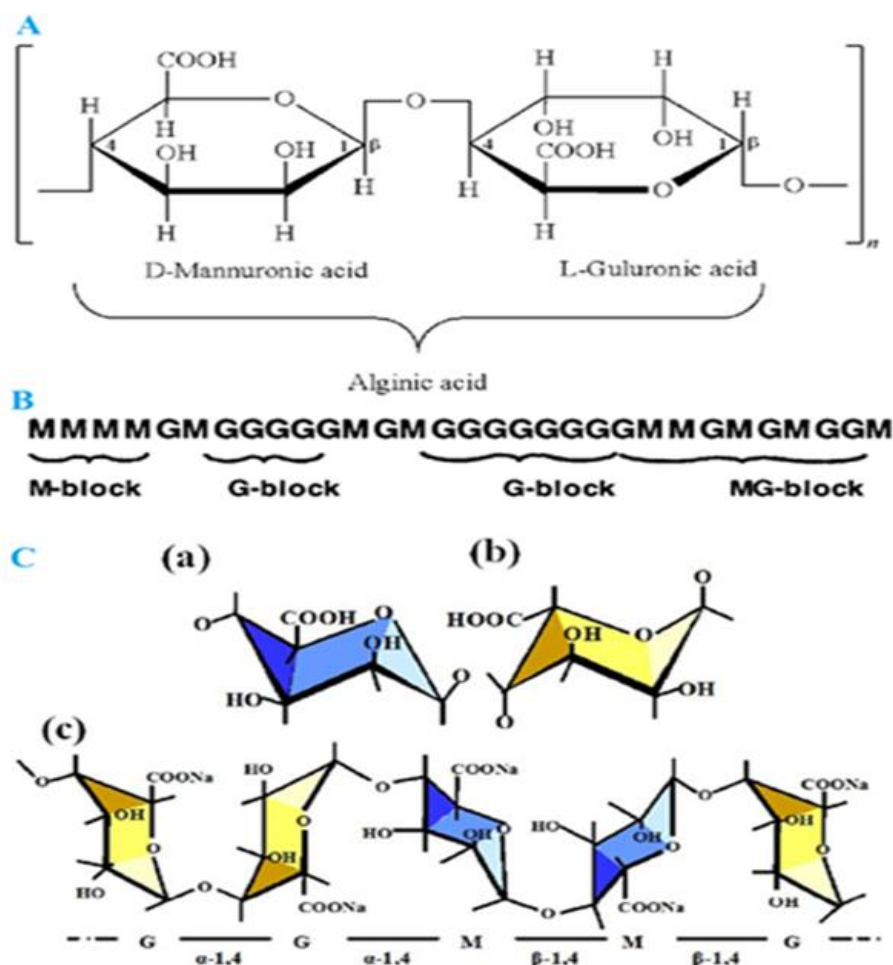


Fig 1-11 Chemical structure of alginates of brown algae; (A) Alginic acid; (B) Distributions of blocks; (C) Ring conformation (Draget *et al.*, 2005; Kashima and Imai, 2012).

Physical properties of alginate including solubility depend on the M/G ratio and the molecular mass ranging from 150 to 1700 kDa depending on the source. Mass molecular of *Padina gymnospora* alginate was about 5000 kDa (Salgado *et al.*, 2007). Molecular weight of alginate extracted from brown algae *Costaria costata* ranged from 394 to 714 kDa (Imbs *et al.*, 2009). Molecular weight distribution of alginate can have implications for the uses of alginate itself. Low molecular weight containing only short G-blocks may not take part in gel-network formation; as a consequent, it do not contribute to the gel strength (Draget *et al.*, 2005). Therefore, to have great gel strength, an alga should have

more G block units than M blocks. Alginates provide flexibility and strength for algal thallus (Milani and Maleki, 2012);

Various industries related to food processing, pharmaceuticals, feed and cosmetics use alginic acid extracted from *Laminaria hyperborean*, *L. digitata*, *L. japonica*, *Ascophyllum nodosum*, *Macrocystis pyrifera*, *Sargassum*, *Saccharina* and *Undaria* (Jayasankar, 1993; Sartori, 1997; Lee and Mooney, 2012). It has been reported that alginic acid leads to a decrease in the concentration of cholesterol, exerts an anti-hypertension effect, can prevent absorption of toxic chemical substances, and plays a major role as dietary fiber for the maintenance of animal and human health (Murata and Nakazoe, 2001; Nishide and Uchida, 2003; Kim and Lee, 2008). These dietary polysaccharides are not found in any land plants (Balboa *et al.*, 2013). Alginates have been considered only as thickening, gel forming, and stabilizing agents, however, recent pharmacological research discovered that alginates isolated from *Sargassum* demonstrated anticancer (*in vivo*), antiviral (*in vitro*), and hypolipidemic (*in vivo*) activity (Mao *et al.*, 2004; de Sousa *et al.*, 2007; Sinha *et al.*, 2010).

Fucoidans or fucans or fucanoids are complex sulfated polysaccharides primarily composed of sulphated L-fucose (Holdt and Kraan, 2011; Balboa *et al.*, 2013) with small proportions of galactose, mannose, xylose, glucose, rhamnose and uronic acid (Pomin and Mourau, 2008; Usov and Bilan, 2009). Figure 1-12 presents the general structure of fucoidan and proposed fucoidan structure isolated from one of *Sargassum* species, *S. myriocystum*.

Molecular weight of fucoidan varies according to species, geographic habitat, season, and even extraction method. Therefore, the exact molecular weight of fucoidan is under debates (Kim, 2012). *Sargassum* sp. extracted by water had molecular weight of fucoidan at 57.7 and 2.7 kDa, while HCl extract of this species had molecular weight at 46.6 and 3.38 kDa (Junaidi, 2013). Regarding the extraction method, fucoidans are easily extracted from the algal cell wall with hot water or acid solution. Mannitol, alginic acid, and laminaran are often

occurred in fucoidans extraction, thus, ethanol precipitation is employed to isolate the fucoidans from brown algae. In addition, elevated temperature of more than 70°C is frequently selected as the extraction condition. Even though, the ambient or slightly elevated temperature is still served as a preferred step for isolating fucoidans prior to any fractionation and purification process (Ale and Meyer, 2013).

Fucoidans account for 10-20% of dry weight and are not found in other divisions of algae or in terrestrial plants. They are very soluble and do not develop highly viscous solutions (Balboa *et al.*, 2013). The chemical composition of fucoidan depends on the algal species, part of the thallus and the harvesting period (Skriptsova *et al.*, 2010). Table 1-2 describes the chemical composition of fucoidan in several species of brown algae.

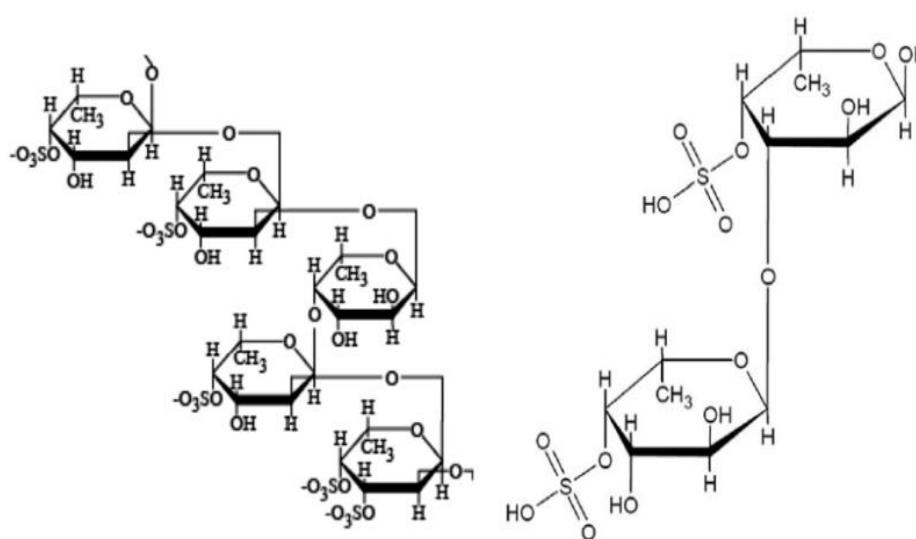


Fig 1-12 Fucoidan structure – a branched sulfated polymer with L-fucose building blocks (Left) Probable structure of fucoidan from *S. myriocystum* as proposed by Badrinathan *et al.* (2012).

Many previous studies have revealed the wide range potential showed by fucoidans especially related their bioactivity. They are known to show anti-tumor (Maruyama *et al.*, 2006; Alekseyenko *et al.*, 2007), anticoagulant (De Zoysa *et al.*, 2007; Rodrigues *et al.*, 2011), anti-inflammatory, antithrombotic (Cumashi *et al.*, 2007; Patel, 2012; Dore *et al.*, 2013), antioxidant (Yuan *et al.*, 2005; Lim *et al.*, 2014; Wang *et al.*, 2015), antiviral (Bouhlal *et al.*, 2011; Elizondo-Gonzalez *et al.*,

2012; Wang *et al.*, 2012; Rabanal *et al.*, 2014) and anticancer properties (Villarreal-Gómez *et al.*, 2010; Fedorov *et al.*, 2013; Marudhupandi *et al.*, 2014; Wang *et al.*, 2015).

Table 1-2 Chemical composition of fucoidan from several brown algae.

Brown Algae	Chemical Composition	References
<i>Fucus vesiculus</i>	Fucose, sulfate	Black <i>et al.</i> (1952), Nishino <i>et al.</i> (1994)
<i>F. evanescens</i>	Fucose/sulfate/acetate (1/1.23/0.36)	Bilan <i>et al.</i> , (2002)
	Fucose, xylose, galactose, uronic acid and sulfate	Cumashi <i>et al.</i> , (2007)
<i>F. distichus</i>	Fucose/sulfate/acetate (1/1.21/0.08)	Bilan <i>et al.</i> , (2004)
	Fucose, xylose, galactose, uronic acid and sulfate	Cumashi <i>et al.</i> , (2007)
<i>F. serratus</i>	Fucose/sulfate/acetate (1/1.12)	Bilan <i>et al.</i> , (2006)
<i>Lessonia vadosa</i>	Fucose/sulfate (1/1.12)	Chandi <i>et al.</i> , (2008)
<i>Macrocystis pyrifera</i>	Fucose/galactose (18/1), sulfate	Black <i>et al.</i> , (1952)
<i>Turbinaria ornate</i>	Fucose/galactose (3/1), sulfate	Than <i>et al.</i> , (Thanh <i>et al.</i> , 2013)
<i>Undaria pinnatifida</i> (Mekabu)	Fucose/galactose (1/1.1), sulfate	Lee <i>et al.</i> , (2004)
<i>Ascophyllum nodosum</i>	Fucose (49%), xylose (10%), GlcA (11%), sulfate	Percival <i>et al.</i> , (1968)
<i>Himanthalia lorea</i> and <i>Bifurcaria bifurcata</i>	Fucose, xylose, GlcA, sulfate	Mian <i>et al.</i> (1973)
<i>Padina pavonia</i>	Fucose, xylose, mannose, glucose, galactose, sulfate	Hussein <i>et al.</i> , (1980a, 1980b)
<i>Laminaria angustata</i>	Fucose/galactose/sulfate (9/1/9)	Kitamura <i>et al.</i> , (2014)
<i>Ecklonia kurome</i>	Fucose, galactose, mannose, xylose, GlcA, sulfate	Nishino <i>et al.</i> , (1994)
<i>Sargassum stenophyllum</i>	Fucose, galactose, mannose, GlcA, glucose, xylose, sulfate	Duarte <i>et al.</i> , (2001)
<i>S. muticum</i>	Glucose, xylose, galactose, mannose, fucose	Balboa <i>et al.</i> , (2013)
<i>S. polycycstum</i>	Glucose, xylose, rhamnose, fucose, galactose, mannose	Sugiono <i>et al.</i> , (Sugiono <i>et al.</i> , 2014)
<i>S. wightii</i>	Fucose, sulfate	Marudhupandi and Kumar, (2013)

Marinova – a Tasmanian company – has managed to isolate fucoidans from a range of species and provide characterized fractions for either investigational research or as ingredients for nutraceutical and cosmetic applications. They have successfully isolated and supplied commercial volumes of

fucoidan extracted from *Undaria*, *Lessonia*, *Macrocystis*, *Cladosiphon*, *Durvillea*, *Laminaria*, *Ecklonia*, *Fucus*, *Sargassum*, *Ascophyllum* and *Alaria* with Halal- and Kosher-certified (Holdt and Kraan, 2011).

1.5.3 Storage metabolites of brown algae

Laminaran, also called laminarin, is carbohydrate reserve of brown algae located in plastids of each cells. Amount of laminaran varies up to 34% of dry weight; it is preferably present in algae of the order Laminariales. Nevertheless, this carbohydrate reserve was also discovered in *S. fusiforme* and *S. trichophyllum* in low amounts, less than 1% (Rioux and Turgeon, 2015).

Laminaran is a polymer made of glucose units, varying in structure and composition that depends on the species. It is basically composed of chains of 1,3- β -D-glucose units (Holdt and Kraan, 2011; Rioux and Turgeon, 2015), a linear polysaccharide made up of 25-50 glucose units. The terminal reducing end of laminaran is diverse, corresponding to a glucose residue in G-type laminarins and to a mannitol residue in M-type laminarins. This diversification generates two forms of solubility: soluble and insoluble, for each type of laminaran. First characteristic of laminaran is a complete solubility in cold water, while second is only soluble in hot-water (Ruperez *et al.*, 2002). Laminarans have an average molecular weight of 5 kDa. The molecular weight of soluble laminaran, extracted from *L. digitata*, is about 5.3 kDa, while the insoluble form of laminaran, obtained from *Laminaria hyperborea*, is about 3.5 kDa (Chapman and Chapman, 1980).

Other carbohydrate reserve in brown algae aside from laminaran is mannitol, a six-carbon alcohol. Mannitol represents up to 30% of algal dry weight and it is accumulated mainly in *Laminaria* and *Saccharina* species (Holdt and Kraan, 2011). In *A. nodosum* and *Fucus* species, mannitol was produced with content lower than 16% of dry weight. Meanwhile, mannitol content in *S. pacificum* (formerly known as *S. mangarevense*) was 14% of dry weight as reported by Zubia *et al.* (Zubia *et al.*, 2008). As reviewed by same authors, the mannitol content of *Sargassum* ranged from 1% to 12% of dry weight.

Laminaran and mannitol content vary considerably according to algal species, age and part of the thallus, and seasons. Meanwhile, the extraction of these reserve polysaccharides is usually with acid solution (Rioux and Turgeon, 2015). Laminaran has been reported to possess various biological activities. Laminarin of *A. nodosum* and *L. hyperborean* demonstrated an antioxidant and antibacterial activities (Kadam *et al.*, 2015c). Antiviral, anticoagulant, and antiproliferative activities were also reported from the laminaran (Hoffman *et al.*, 1995; Miao *et al.*, 1999; Devillé *et al.*, 2007). As for mannitol, the bioactivity reported was its efficacy in protecting the photosynthetic apparatus from low-salinity damage (Lobban and Harrison, 1997).

1.5.2 Proteins

Proteins of marine algae have attracted less attention and their structure and biological properties are not as widely documented as their polysaccharides (Chojnacka *et al.*, 2012). However, recently, they have gained increasing attention due to their functional properties and bioactivity that is potential for further application (Michalak and Chojnacka, 2015).

The content of brown algae protein is generally low ranging between 6-13% of dry weight compared to the green (15-25%) and red algae (15-30%) (Kim, 2012; Balboa *et al.*, 2013). However, their content in different species is dependent on season and is linked to their surrounding variables including nutrient supply. They are present in a diverse range of forms and cellular locations, i.e as a component of cell wall, enzymes and bound to pigments and carbohydrates (Stengel *et al.*, 2011). Table 3 displays protein content of algae from different groups.

Table 1-3 Protein content (% of dry weight (w/w)) of algae according to Harnedy and Fitzgerald (2011) (modified).

Algae	Protein content (% of dry weight)	Reference
<i>Undaria pinnatifida</i>	11-24	Fleurence, (2004)
<i>Laminaria digitata</i>	15.90	Marsham <i>et al.</i> , (2007)
<i>L. saccharina</i>	6-11	Morrissey <i>et al.</i> , (2001)
<i>Fucus vesiculosus</i>	5-10	Morrissey <i>et al.</i> , (2001)
<i>F. serratus</i>	17.4	Marsham <i>et al.</i> , (2007)
<i>Ascophyllum nodosum</i>	5-12	Morrissey <i>et al.</i> , (2001)
	3-15	Fleurence, (2004)
<i>Himanthalia elongata</i>	6-11	Morrissey <i>et al.</i> , (2001)
<i>Sargassum variegatum</i>	3.0	Ambreen <i>et al.</i> , (Ambreen <i>et al.</i> , 2012)
<i>S. corrifolium</i>	16.07	Haque <i>et al.</i> , (Haque <i>et al.</i> , 2009)
<i>S. filipendula</i>	5.6	
<i>S. vulgare</i>	13.6	Marinho-Soriano <i>et al.</i> , (2006)

1.5.3 Lipids

Brown algae contain relatively lower contents of lipids having less than 5% of dry weight. Quantitative analysis revealed that total lipid content of a major brown algae family, Sargassaceae, was higher in subarctic zones (approximately 5% of dry weight) than tropical zones (0.9-1.8% of dry weight). In spite of their low total lipid content, brown algae are rich source of polyunsaturated fatty acids (PUFAs) (van Ginneken *et al.*, 2011). Fatty acids are the main constituents of fat raw material in living organisms distinguished into saturated fatty acids (SAFA) and unsaturated fatty acids. The unsaturated fatty acids are later known as two major groups, namely monounsaturated fatty acids (MUFAs) and PUFAs (Kulikova and Khotimchenko, 2000). PUFAs generally contain high levels of omega-3 (ω 3) and omega-6 (ω 6) (Holdt and Kraan, 2011). *Sargassum* is rich in fatty acids with 20 carbon atoms, such as eicosapentaenoic acid (EPA, ω 3 C20:5) and arachidonic acid (AA, ω 6 C20:4). Certain species shows different content of these fatty acids. As an example, *S. kjellmanianum* is a rich source of ω 6; whereas *S. thunbergii* has high content of ω 3 (Burtin, 2003b).

Table 1-4 Total lipid content in some brown algae.

Algae	Total lipid content (% of dry weight)	Reference
<i>U. pinnatifida</i>	5	(Billakanti <i>et al.</i> , 2013)
<i>S. miyabei</i>	0.7	(Kulikova and Khotimchenko, 2000)
<i>S. horneri</i>	0.82-0.96	(Hossain <i>et al.</i> , 2003)
<i>Alaria esculenta</i>	1.3-1.5	
<i>L. digitata</i>	0.8-1.1	
<i>L. hyperbora</i>	1.1-1.4	(Maehre <i>et al.</i> , 2014)
<i>F. vesiculosus</i>	2.6-3.5	
<i>Pelvetia canaliculata</i>	3.7-5.8	
<i>A. nodosum</i>	4.5	
<i>F. serratus</i>	3.7	(van Ginneken <i>et al.</i> , 2011)
<i>S. natans</i>	1.0	

1.5.4 Minerals

Marine algae have the capacity to absorb inorganic substances from their surroundings, because of this capacity, they are acknowledged as a significant source of essential minerals (Na, K, Ca, and Mg) and trace elements (Fe, Zn, Mn, and Cu) (Misurcova, 2012; Peng *et al.*, 2015). Algae's absorption capacity is regarded with the presence of polysaccharides in algal cell wall. Different type of polysaccharides also becomes the reason why various group of algae demonstrate differ ability to uptake minerals. Capacity of brown algae to absorb metal is higher than green and red algae due to the high presence as a result of high presence of alginic acid, alginate and salt of alginic acids. These polysaccharides of brown algae have strong ion-exchange properties. Moreover, cellulose and carboxyl groups coexisted in brown algal cell wall contribute to the accumulation of metals as well (Hu *et al.*, 1996). It is highly possible that contents of accumulated minerals in marine algae are higher than those of in the environment by several orders of magnitude (Peng *et al.*, 2015).

Minerals content in algae vary, depending on diverse genera of algae, season, and geographic location. Brown algae, particularly from the genera *Laminaria*, are a great source of iodine. The highest concentration of iodine is in *L. japonica*, 5.6 and 3.0 mg/kg from all investigated algae (Misurcova, 2012).

Table 1-5 Mineral contents in some brown algae (Fajardo *et al.*, 1998; Kolb *et al.*, 2004; Santoso *et al.*, 2006; Manivannan *et al.*, 2008; Misurcova *et al.*, 2009; Misurcova, 2012).

Algae	Ca	Mg	P	Na	Fe	K
<i>L. japonica</i>	8.8 g/kg	5.5 g/kg	3.0 g/kg		73.8 mg/kg	
<i>U. pinnatifida</i>	9.5 g/kg	4.1 g/kg	4.5 g/kg	36.1 mg/kg	133 mg/kg	71.2 g/kg
<i>S. polycystum</i>	18.7 g/kg	5.7 g/kg			277 mg/kg	
<i>P. australis</i>	28.3 g/kg	4.0 g/kg			446 mg/kg	

1.5.5 Polyphenols

Phlorotannins, are structural classes of polyketides found exclusively in brown algae and classified into six group based upon variations in their assemblage from the polymerization of phloroglucinol units (Arnold and Targett, 2000, 2002). Stored within cells in vessels called physodes, phlorotannins have been reported from almost all brown algae orders. According to Vo *et al.* (2012), phlorotannins are classified in three primary groups based on their inter-phloroglucinol linkages. They are fucols, phlorethols and fucophlorethols.

Monomeric unit of phlorotannins, *i.e.* phloroglucinol, is proposed to be formed via the acetate-melanote (polyketide) pathway. Two molecules of acetyl coenzyme A are converted into malonyl co-enzyme A through the addition of carbon dioxide. A polyketomethylene chain is formed by three malonyl CoA units, and the polyketide species thus generated undergoes a Claisen-type cyclization reaction to yield a hexacyclic ring system. This hexacyclic ring, a triketide, undergoes tautomerisation to give phloroglucinol which is thermodynamically more stable (Fig 1-13). This whole sequence of reactions is carried out by an enzyme complex that converts acetyl CoA and malonyl CoA into the final product without giving any intermediates. This enzyme complex is known to combine the polyketide synthase and polyketide cyclase activities (Jihane Achkar *et al.*, 2005). Study on *Ectocarpus siliculosus* reveals that type III polyketide synthase (PKS III) catalyzes major step in the biosynthetic pathway of phlorotannins (Meslet-Cladière *et al.*, 2013).

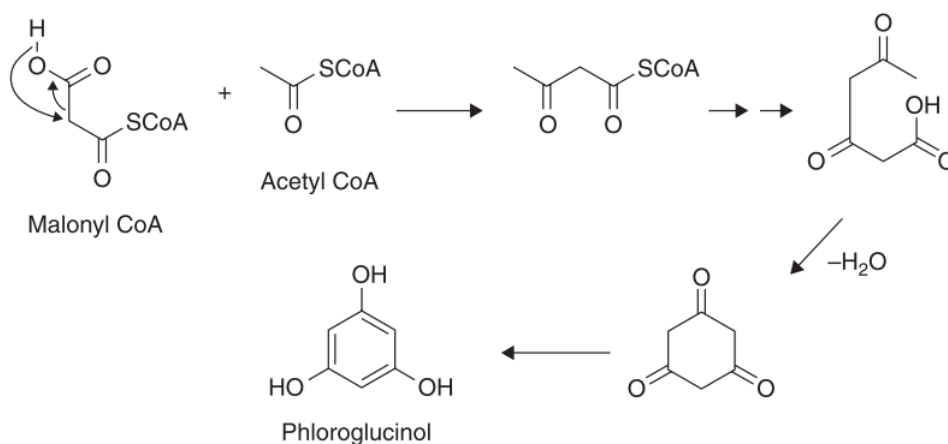


Fig 1-13 Biosynthetic pathway of phlorotannins.

There is specificity within genera as seen with the fucols in *Fucus* and eckols in *Ecklona*. Fucols (Geiselman and McConnell, 1981), phlorethols, fucophlorethols, fuhalols, isofuhalols (Grosse-Damhues and Glombitza, 1984) and eckols differ in the number of the hydroxyl groups present and in their bond linkages. Among these structures, the fucols are attached by ether bonds. The phloroglucinol units are often esterified or acylated, and can dimerize or polymerize into larger units. These compounds also exhibit a broad range of size, thought to polymerize as they age and thereby grow larger. They are typically 10 – 100 kDa, although their range spans from as small as 126 Da to as large as 650 kDa (Boettcher and Targett, 1993; Targett and Arnold, 2001). Figure 1-14 illustrates the chemical structure of main groups of phlorotannins and Table 1-6 presents a summary of several brown algae derived-phlorotannins.

Ecklonia cava, *E. stolonifera*, *E. kurome*, *Eisenia bicyclis*, *Ishige ookamurae*, *Sargassum thunbergii*, *Hizikia fusiformis*, *Undaria pinnatifida* and *Laminaria japonica* are some marine brown algae that have been most reported for their phlorotannins as well as its health beneficial biological activities (Li *et al.* 2011). From *Ecklonia cava*, several studies managed to isolate their different type of phlorotannins, i.e. phloroglucinol, eckol, fucodiphloroethol G, phlorofucofuroeckol A, 7-phloroeckol, dieckol, and 6,6'-Bieckol (Jung *et al.*, 2006; Yoon *et al.*, 2009b; Wijesekara *et al.*, 2010; Li *et al.*, 2011a).

Phlorofucofuroeckol A was also found in *E. stolinifera* and *E. kurome* (Nagayama *et al.*, 2003; Yoon *et al.*, 2009b). *E. stolinifera* also contained triphloroethol B and 2-phloroeckol as reported by Nagayama *et al.* (Nagayama *et al.*, 2003). 6,6'-Bieckol was also isolated from *Ishige okamurae* (Zou *et al.*, 2008; Yoon *et al.*, 2009a). Eckol, phlorofucofuroeckol B, 6,8'-Bieckol, and 8,8'-Bieckol were successfully isolated from the *Eisenia bicyclis*. Ferreres *et al.* (2012) found 22 different phlorotannins belonging to eckol and fucophlorethol main groups from *Cystoseira nodicaulis*, *C. tamariscifolia*, *C. usneoides* and *Fucus spiralis*.

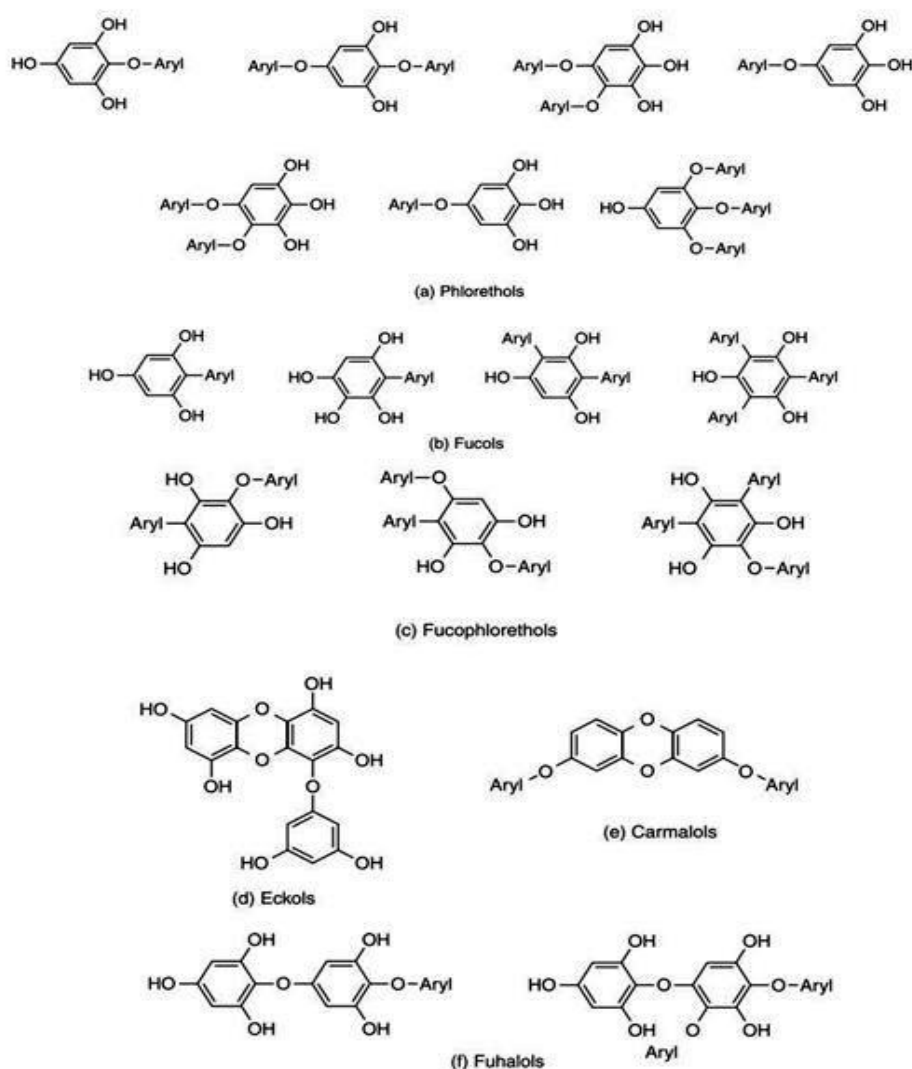


Fig 1-14 Chemical structure of main groups of phlorotannins characterized from several brown algae; (a) phlorethols, (b) fucols, (c) fucophlorethols, (d) eckols, (e) carmalols, and (f) fuhalols.

Table 1-6 Main group and sub-group of phlorotannins families in different species of brown algae.

Main group of phlorotannins	Sub-group	Species	Interphloroglucinol linkages and Molecular size	Reference
Fucols	Tetrafucol-A and B	<i>Fucus vesiculosus</i> L	With only phenyl linkages	Glombitza <i>et al.</i> , (1975); Truus <i>et al.</i> , (2004)
Phlorethol	Triphlorethol C and tetraphlorethols A & B Tetraphlprethol C	<i>Laminaria ochroleuca</i> <i>Ecklonia maxima</i> (Osbeck) Papenfuss	With only aryether bonds -	Koch <i>et al.</i> , (1980) Glombitza and Vogel (1985)
	Pentaphlorethol B and Hexaphlorethol A	<i>Cystophora retroflexa</i> (Labillardiere) J. Argadh	-	Sailler <i>et al.</i> , (1999)
Fuhalols	Isotrifuhalol Hydroxytrifuhalol B, Hydroxypentafulhalol A, hydroxyheptafulhalol B, Hydroxynonafulhalol A	<i>Chorda filum</i> <i>Sargassum spinuligerium</i>	Phlorethol with additional hydroxyl group - -	Grosse- Damhues <i>et al.</i> , (1984) Glombitza <i>et al.</i> , (1997a); Glombitza and Keusgen, (1995); Keusgen <i>et al.</i> , (1997) Glombitza and Shmidt, (1999)
Eckols	Trimers eckol and Dioxinodehydroeckol	<i>Ecklonia maxima</i> & <i>Esenia arborea</i> Areschoug	Phlorethols includes 1,4-dibenzodioxin system -	Glombitza and Vogel, (1985); Glombitza and Gerstberger (1985)

Table 1-6 Main group and sub-group of phlorotannins families in different species of brown algae (Continued).

Main group of phlorotannins	Sub-group	Species	Interphloroglucinol linkages and Molecular size	Reference
Fucophlorethols	Eckol, Phlorofucoeckol-A, Dieckol, 6,6'-bieckol and 8,8'-bieckol	<i>Eisenia bicyclis</i>	-	Kim <i>et al.</i> , (Kim <i>et al.</i> , 2013) ; Fuji <i>et al.</i> (2013)
	Tetramers 2-phloroeckol and 7-phloroeckol	<i>Eisenia bicyclis</i> (Kjellman) Setchell, <i>E. kurome</i> Okamura and <i>E. stolonifera</i> Okamura	-	Shibata <i>et al</i> (2004, 2008); Kim <i>et al.</i> , (2011); Kita <i>et al.</i> , (1990); Nagayama <i>et al.</i> , (Nagayama <i>et al.</i> , 2003); Kang <i>et al.</i> , (Kang <i>et al.</i> , 2012) ; Kim <i>et al.</i> , (2005) ; Goo <i>et al.</i> , (2010)
	Diphlorethohydroxycarmalol	<i>Carpophyllum maschalocarpum</i>		Li and Glombitza (1991)
	Triphlorethohydroxycarmalol	<i>Ishige okamurae</i> Yendo		Zou <i>et al.</i> , (2008) ; Yoon <i>et al.</i> , (2009a)
			With aryether and phenyl linkages	
	Fucophlorethols	<i>Himanthalia elongate</i> (Linnaeus)	~ 994 Da	Glombitza and Grosse , (1985)
	Quaterfucononaphlorethol	<i>Carpophyllum maschalocarpum</i> (Turner)	1.738 Da	Li and Glombitza (1991)
	Fucophlorethols	<i>Sargassum spinuligerum</i> and <i>Cystophora torulosa</i> (R. Brown)	-	Glombitza <i>et al.</i> , (1997a)
	Fucophlorethols-B	<i>Laminaria ochroleuca</i>		Koch <i>et al.</i> , (1980);
	Fucodiphlorethol-C and G	<i>Eisenia bicyclis</i> , <i>Ecklonia cava</i>	-	Martinez and Castaneda, (2013); Ham <i>et al.</i> , (2007);

Table 1-6 Main group and sub-group of phlorotannins families in different species of brown algae (Continued).

Main group of phlorotannins	Sub-group	Species	Interphloroglucinol linkages and Molecular size	Reference
	Trifucodiphlorethol A	<i>Fucus vesiculosus</i>		Parys <i>et al.</i> , (2010)
	Bisfucotriphlorethol	A, <i>Cystophora torulosa</i>	-	Glombitza et al. (1997b)
	Bisfucoetraphlorethol	A,		
	Bisfucopentahlorethol	A,		
	Hydroxyfucodiphlorethol	B,		
	Hydroxyfucotriphlorethol	B,		
	Bisfucotriphlorethol,			
	Hydroxybisfucopentaphlorethol			
	A and Fucodifucotetraphlorethol			

1.5.5.1 Role of phlorotannins in brown algae

Phlorotannins are commonly characterized as chemical protective defense, which also in the case of this algae, exhibit primary functions for example in growth and the development of the cell wall (Schoenwaelder and Wiencke, 2000). Koivikko (2008) stated that the links between cell wall (alginic acid) and phlorotannins are the ester bond and the hemiacetal bond, both of which are the covalent bonds. Therefore, it makes them hard to degrade.

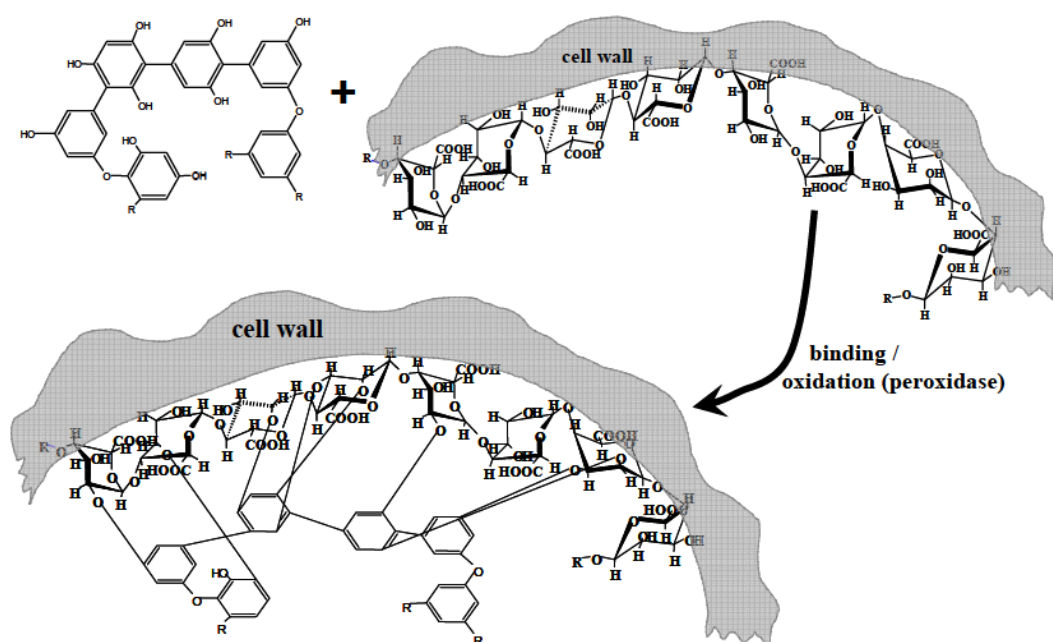


Fig 1-15 Illustration of phlorotannin polymer covalent bond at the alginic acid cell wall of brown algae as proposed by Koivikko (2008).

Apparently, phlorotannins also play another role in the defense mechanisms of Algae towards any stress or changing in their surroundings, thus they are also known as stress compound (Stengel *et al.*, 2011). Phlorotannins are involved in a protective mechanism for examples against grazing (Koivikko *et al.*, 2005; Coleman *et al.*, 2007; Ank *et al.*, 2013), attachment and settlement of bacteria or other epibiosis effect (Mokrini *et al.*, 2008; Ank *et al.*, 2013), ultraviolet (UV) radiation (Macaya *et al.*, 2005; de la Coba *et al.*, 2009), metal

contamination (Connan and Stengel, 2011; Warneke and Long, 2015).

1.5.5.2 Phlorotannins content in brown algae

The total content of phlorotannins in brown algae ranges from < 1 – 14% of dry weight and has been shown to depend on environmental factors such as salinity, light and nutrient availability (Hemmi *et al.*, 2005; Jormalainen and Honkanen, 2008; Holdt and Kraan, 2011; Le Lann *et al.*, 2012). Variation of total phenolic content seems to occur among local populations of algae (Pavia *et al.*, 1999; Hemmi and Jormalainen, 2004; Koivikko *et al.*, 2008). Such variation can be explained by genetic differentiation among populations and/or response to local changes of environmental factors (Koivikko *et al.*, 2008).

Spatial variation in levels of brown algae phenolic compounds has been examined across very large spatial scales, such as between hemispheres and between temperate and tropical habitats (Stiger *et al.*, 2004; Le Lann *et al.*, 2012)). Variation in phenolic levels has also been examined across regional scales (tens to hundreds of km) for several species of Australasian brown algae (Steinberg, 1989), *Lobophora variegata* in Caribbean and Atlantic waters (Arnold *et al.*, 1995), *Fucus vesiculosus* in Atlantic waters (Targett *et al.*, 1992), and *Ascophyllum nodosum* in Europe (Pavia and ÅBerg, 1996). Steinberg (1989) reported that phenolic levels of Australasian kelps and rockweeds often differed significantly among sites, but within a species the highest phenolic concentration was generally less than twice the lowest concentration. Table 1-7 presents the seasonal variation of brown algal phlorotannins. In addition, Table 1-8 summarizes the total phenolic content with other biochemical composition from several species of brown algae.

Table 1-7 Polyphenol contents variation in different species of brown algae.

Species	Total Phenolic Content	Type of variation	References
<i>Cystoseira tamariscifolia</i>	~ 2 % in winter ~ 8 % in summer	Seasonal	Abdala-Diaz <i>et al.</i> , 2006
<i>Stypopodium zonale</i>	~ 0.5% (without herbivore) ~ 0.7 % (with herbivore) ~ 0.6 % (low epibiosis) ~ 0.6% (medium epibiosis) ~ 0.4% (high epibiosis)	Simulated herbivory Epibiosis effects	Ank <i>et al.</i> , 2013
<i>Fucus serratus</i>	4.27 % in lower intertidal zone	Interspecific	Connan <i>et al.</i> , 2004
<i>Ascophyllum nodosum</i>	~ 5.8 % in lower intertidal zone		
<i>Bifurcaria bifurcate</i>	3.73 % in lower intertidal zone		
<i>Himanthalia elongate</i>	2.17 % in lower intertidal zone		
<i>Laminaria digitata</i>	0.13 % in subtidal zone		
<i>F. spiralis</i>	3.88 % in upper intertidal zone	Seasonal	Connan <i>et al.</i> , 2004
<i>F. vesiculosus</i>	~ 5.8 % in upper intertidal zone		
<i>Pelvetia canaliculata</i>	3.39 % in upper intertidal zone		
<i>L. digitata</i>	0.09 % in summer 0.19 % in winter		
<i>F. vesiculosus</i>	7.84 % in spring ~ 7% in summer		
<i>H. elongate</i>	0.49 % in winter 4.84 % in summer	Geographical location with depth and reef morphology	Le Lann <i>et al.</i> , 2012
<i>P. canaliculata</i>	3.72 % in summer ~ 3 % in winter		
<i>A. nodosum</i>	7.18 % in summer ~ 6% in winter		
<i>Sargassum cristaefolium</i>	0.08 %		
<i>S. aquifolium</i>	0.10-0.71 %		
<i>S. polycystum</i>	0.09-0.74%		

Table 1-8 Total phenolic content and biochemical composition from several brown algae (expressed in % of dry material).

Species	Phenol	Carbohydrates	Protein	Ash	Reference
<i>S. fusiforme</i>	0.01	-	8.85	0.33	Shafay, (2014)
		17-69	11.6	19.77-26	Pereire, 2012
<i>S. ilicifolium</i>	1.93	38.72	38.72		Ganapathi <i>et al.</i> , 2013
<i>S. polycystum</i>	0.23	34.93	7.78	21.87	Ahmad <i>et al.</i> , 2012
	-	36.59	3.07	38.64	Triwisari, 2010
<i>S. vulgare</i>	0.38	93.34	5.85	0.19	Shafay, 2014
<i>S. wightii</i>	2.17	-	6.40	-	Seenivasan <i>et al.</i> , 2012
<i>Turbinaria ornate</i>	7-8 x 10 ⁻³	14	15	-	Phartiban <i>et al.</i> 2013
<i>Dictyota dichotoma</i>	0.01–0.02	10	10		
<i>T. conoides</i>	0.34	41.03	7.40	21.37	Ahmad <i>et al.</i> , 2012
<i>Hormophy sacuneiformis</i>	0.15	40.57	6.42	26.81	Ahmad <i>et al.</i> , 2012
<i>Padina gymnospora</i>	0.25	26.86	5.93	45.04	Ahmad <i>et al.</i> , 2012
	32.3	-	10.5	-	Rameshkumar <i>et al.</i> , 2012
<i>Cystoseira barbata</i>	1.05	-	5.60	14.24	Frikha <i>et al.</i> , 2012

1.5 Extraction of algal bioactive compound

Samples preparation is used to increase the efficiency of an analysis, to eliminate or reduce potential interference, to enhance the sensitivity of the analysis by increasing the concentration of the compound of interest in the assay mixture and to transform it into a more suitable form that is easily separated, detected and/or quantified. The sample produced in this step should have a high concentration of target compound free of interfering compounds from the matrix. One of the key procedures in this step is the extraction of the target compound (Palma *et al.*, 2013).

Extraction is a preliminary treatment of the analytic protocol in which a compound or group of compounds is preferentially transferred from a matrix into a different phase. The objective is making the sample available for introduction into analytic instruments either for qualitative or quantitative analysis. However, the difference between this two analyses is in qualitative the achievement of the exhaustive extractions and complete compound stability are ignored. In other word, they are not regarded as important. In the contrary, when dealing with quantitative analysis, it is highly important to achieve the complete extraction of the target compounds while preserving their original profile (Palma *et al.*, 2013).

The principal of the extraction process are related to several important properties, i.e. (1) high yield, (2) high selectivity/purity with low amount of interfering or undesirable co-extracted compounds, (3) high sensitivity allows for different quantification techniques producing a high slope in the calibration curve, (4) low limit of detection thus components in the extracts obtained can be detected at low levels (Palma *et al.*, 2013).

In general, extraction is a mass transfer process of one or more components from one phase to another one. In the most case of natural products, the samples used in the extraction process are solid materials, although in some cases liquids samples are also used. The extraction solvent is usually a liquid, but it can also be a solid or a supercritical fluid (Palma *et al.*, 2013). In understanding

the basic mechanism of extraction in any different techniques of extraction existed, both the target compounds and the extraction solvent applied must be considered. In addition, it is crucial to know the properties of the solvent medium in which the samples is to be dissolved. As a general rule, non-polar solutes are dissolved by non-polar solvents while polar solutes are dissolved by polar solvents. The interactions between solute and solvent are determined by the vapor pressure of the solute, the solubility of the solute in the solvents, the hydrophobicity, and the acid/base properties of both solute and solvents ((Palma *et al.*, 2013).

The basic mechanism of extraction as mentioned above is schematically presented in figure with following steps (Palma *et al.*, 2013):

1. The solvent is transferred from the fluid phase to the solid surface and pervades it,
2. The solvent penetrate into the solid matrix by molecular diffusion,
3. The soluble material is solubilized by desorption from the matrix into the extraction solvent – the breakage of chemical bonds may be required for desorption of the target analytes from solid matrix,
4. The solution containing the solutes returns to the surface of the solid by molecular diffusion,
5. The solution is transferred from the solid surface to the bulk fluid by natural or forced convection.

The method of extraction applied to particular solid matrix depends on the raw material to be processed and on the final product of interest. Some types of extraction techniques have existed. The most commonly used conventional techniques are soaking extraction, Soxhlet extraction and distillation. Each one of them presents different advantages and disadvantages, thus there is no single and

standard extraction technique for extracting the bioactive compounds from natural products (Palma *et al.*, 2013).

Besides the technique, the efficiency of extraction is also function of the process conditions. Certain external factors affect the concentration of the desired compounds in the extract such as solvent type, temperature, solvent to feed ratio, contact time, and particle size (Palma *et al.*, 2013). Therefore, the best extraction method with optimized conditions should be performed for preparing each particular product. Nevertheless, Cowan (1999) stated that the extraction efficiency of any conventional method mainly depends on the choice of the solvents. The polarity of the target compound is the most important factor for solvents choice. Azmir *et al.* (Azmir *et al.*, 2013) added that molecular affinity between solvents and solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility should also be considered in selection of solvent for bioactive compound extraction.

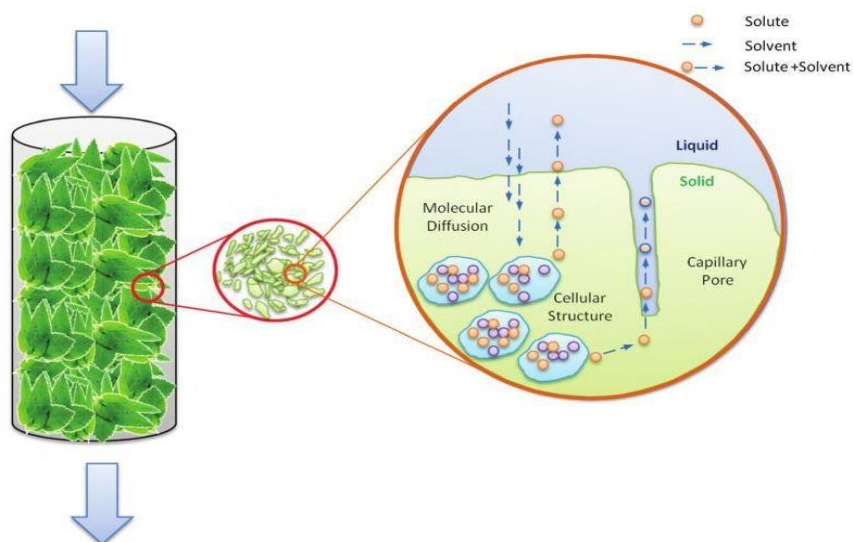


Fig 1-16 Basic mechanism of extraction based on Palma *et al.* (2013).

1.6.1 Algal model

Most of published studies concerning the extraction of marine algae natural product involved the conventional extraction method, i.e. solvents extraction

consisting of liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and extraction in Soxhlet apparatus (Michalak and Chojnacka, 2014). The main principal of this method is to extract the targeted compound by using the appropriate solvents. Under this circumstance, the extraction yield varies from 8% to 30% of algal dry material (Robic *et al.*, 2009). This is due to the presence of various polysaccharides in large quantities in their cell wall and some compounds attached to the cell-wall (cell-wall-bound compounds). Thus, it strongly reduces the efficiency of extraction. In addition, some organic solvents used for the extraction are often toxic and they result a great amount of waste after the process has finished.

Extraction of bioactive compounds is mostly performed by conventional extraction, such as solid-liquid extraction. Recently, focus is given to the production of algal extract without using the toxic organic solvents or destructive extraction conditions that could deteriorate biologically active compound from algal cells. A trend involving safer or even less solvents, more efficient, higher extraction yields compared to the conventional method and perhaps if possible with shorter time and lower cost. In order to be able to develop the application of marine natural products derived from marine algae in the future, an environmentally friendly and sustainable trend should be followed.

There are different methods of extraction that have been applied to obtain bioactive compounds of algae, like polysaccharides, polyphenols, proteins, lipids and pigments (Azmir *et al.*, 2013; Kadam *et al.*, 2015a). Figure 1-17 illustrates different methods of extraction that have been used to extract bioactive compounds in algae. Table 1-9 summarizes the application of different extraction methods to obtain bioactive compounds from algal material. The expected outputs of these new techniques of extraction are higher extraction yields, specificity and efficiency of bioactivity by preserving the original efficacy of active compounds, mild reactive conditions, shorter time, less solvents, better products and lower cost (Tanniou *et al.*, 2013a).

Various type of extraction methods used to attain bioactive compounds in algae (Jeon *et al.*, 2012; Kadam *et al.*, 2013; Michalak and Chojnacka, 2014).

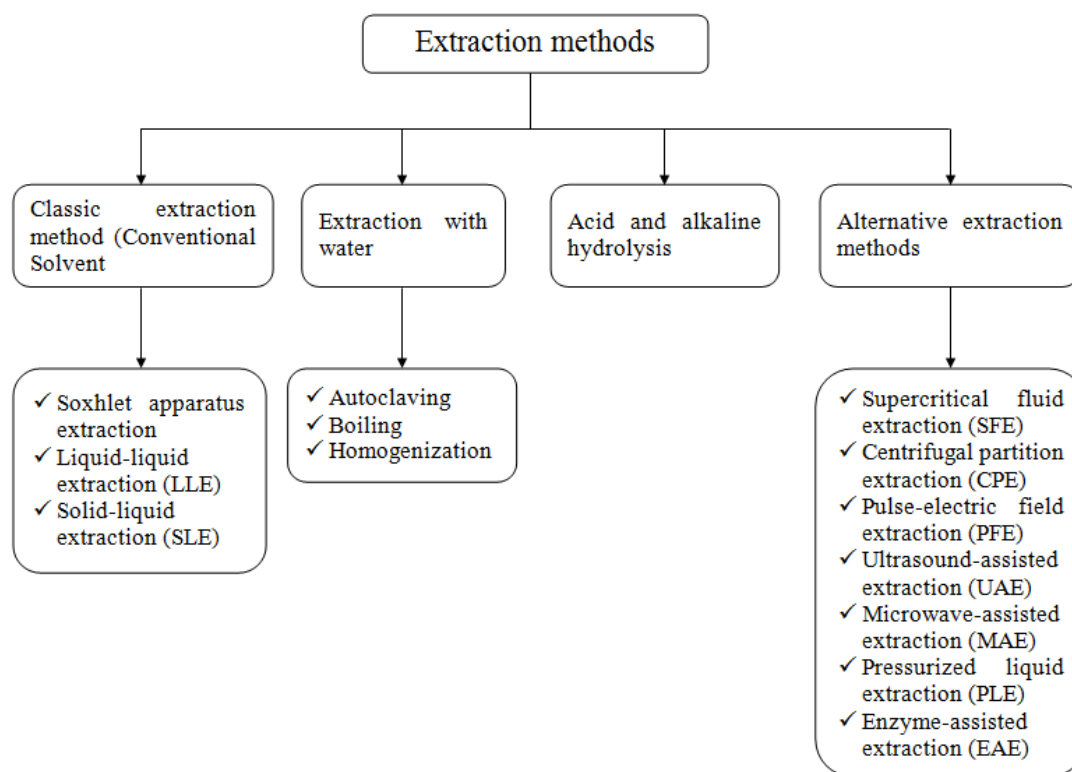


Fig 1-17 Various type of extraction methods used to attain bioactive compounds in algae (Jeon *et al.*, 2012; Kadam *et al.*, 2013; Michalak and Chojnacka, 2014).

Table 1-9 Examples of different extraction methods applied in the search of algal bioactive compounds.

Algae	Extraction method		Bioactive compound	Content	Bioactivity	References
<i>S. muticum</i>		• SLE	Polyphenol	• 18.5%	Antioxidant	(Tanniou <i>et al.</i> , 2013a)
		• CPE		• 10%		
		• PLE		• 22%		
		• SC-CO ₂		• 3%		
<i>Stypocaulon scoparium</i>		SLE	Polyphenol	123-328 mg GAE/100g	Antioxidant	(Lopez <i>et al.</i> , 2011)
<i>Dictyopteris polypodioides</i>	Acid and alkaline hydrolysis		• Alginate • Mannuronan	• 0.92 and 1.25 g • 0.75 and 1.15 g	-	(Karaki <i>et al.</i> , 2013)
<i>S. filipendula</i>	Alkaline hydrolysis		Sulfated polysaccharide	41-66%	Antioxidant	(Costa <i>et al.</i> , 2011)
<i>Gracilaria tenuistipitata</i>	Water extraction		Polyphenols Flavonoid Ascorbic acid	98.9 µg GAE/mg 22.6 µg QE/mg 1.6 µg AA/mg	Antioxidant	(Yang <i>et al.</i> , 2012)
<i>Halimeda monile</i>	Water extraction		Polyphenols Carotenoids Terpenoids	179.5 µg/g 42.2 µg/g -	Antioxidant	(Batista-Gonzalez <i>et al.</i> , 2012)
Micro-macroalgae	and SC-CO ₂		Neutral lipids Phospholipids Glycolipids Liposoluble pigments	-	-	(Crampon <i>et al.</i> , 2011)
<i>E. cava</i>	EAE		Polyphenol	901 µg/100 ml	Antioxidant and antiproliferative	(Athukorala <i>et al.</i> , 2006)

Table 1-9 Examples of different extraction methods applied in the search of algal bioactive compounds (continue).

Algae	Extraction method	Bioactive compound	Content	Bioactivity	References
<i>S. muticum</i>	EAE PLE	Polyphenol	6.1 – 10 mg GAE/g 21.8-39.2 mg	Antioxidant	(Sanchez-Camargo <i>et al.</i> , 2015)
<i>A. nodosum</i>	UAE	Polyphenol Fucose Uronic acid	143.1 mg GAE/g 87.1 mg/g 128.5 mg/g	-	(Kadam <i>et al.</i> , 2015b)
<i>S. muticum</i>	EAE	Polyphenol Sulfated sugar Nitrogen	200.5-300.5 µg/g 5.9-9.7 mg/g 22.1-29.6 mg/g	Antioxidant Prebiotic a-glucosidase inhibitory	(Rodrigues <i>et al.</i> , 2015)
	UAE	Polyphenol Sulfated sugar Nitrogen	2.35 µg/g 10.1 mg/g 24.4 mg/g		
	Hot water extraction	Polyphenol Sulfated sugar Nitrogen	275.8 µg/g 9.5 mg/g 24.6 mg/g		
	EAE	Polyphenol Sulfated sugar Nitrogen	106.0-123.1 µg/g 38.6-59.8 mg/g 11.7-19.4 mg/g		
<i>Osmundea pinnatifida</i>	UAE	Polyphenol Sulfated sugar Nitrogen	103.7 µg/g 42.8 mg/g 10.3mg/g	Antioxidant Prebiotic a-glucosidase inhibitory	
	Hot water extraction	Polyphenol Sulfated sugar Nitrogen	118.6 µg/g 41.8 mg/g 12.7 mg/g		

Table 1-9 Examples of different extraction methods applied in the search of algal bioactive compounds (continued).

Algae	Extraction method	Bioactive compound	Content	Bioactivity	References
<i>F. vesiculosus</i>	MAE	Fucoidan		-	(Rodriguez-Jasso <i>et al.</i> , 2011)
		• Total	• 3.6-		
		• sugar	• 5.6%		
<i>Undaria pinnatifida</i>	Sonication MAE	• Sulfates	• 6.1-		(Punín Crespo <i>et al.</i> , 2006)
			• 8.3%		
		Hydrocarbons	40.4-93.7% 34.6-90.5%	-	

1.6.2 Solid-liquid extraction (SLE) of polyphenols

Extraction is a technique to separate compounds based on their solubility. It is a preliminary treatment of the analytic protocol in which a compound or group of compounds is preferentially transferred from a matrix into a different phase. S/L extraction is concerned with the extraction of a soluble constituent from a solid by means of solvents. If the solute is uniformly dispersed in the solid, the material near the surface will be dissolved first, leaving a porous structure in the solid residue. The solvent will then have to penetrate this outer layer before it can reach further solute, and the process might become progressively more difficult and the extraction rate will fall. This is due to the chemical structure of the solid material that some of them might have a complex bond. Thus, it makes them hard to penetrate. Generally, the process can be considered in three parts: first the change of phase solute as it dissolves in the solvent, secondly its diffusion through the solvent in the pores of the solid to the outside of the particle, and last, the transfer of the solute from the solution in contact with the particles to the main bulk of the solution (Richardson *et al.*, 2002).

Since algae become important sources of remarkable bioactive compounds, these compounds have been extracted from various genera of algae. Polyphenols from brown algae are one of bioactive compounds that have been extensively studied for their outstanding antioxidant activity. Extraction of polyphenols is usually influenced by their chemical nature, extraction method employed, sample particle size, storage time and conditions and presence of the interfering substances (Naczki and Shahidi, 2004). Polyphenols extraction from brown algal material are mostly used solid-liquid extraction and preferable solvents applied are the aqueous ethanol or methanol solutions (Stiger-Pouvreau *et al.*, 2014). As previously described (see 1.2.4.6), polyphenol in brown algae coexists as complexes with polysaccharides and proteins in algal cell wall. In addition, as secondary metabolite, polyphenols are not the only compounds produced by algae as defense system (Macaya *et al.*, 2005, Jormalainen and Honkanen, 2008). Therefore, polyphenol extracts from plant material are always a mixture of

different classes of polyphenols or non-polyphenols that are soluble in the solvent system used. Additional steps may be required to remove the unwanted polyphenols and non-polyphenols constituents, solid-phase extraction techniques and fractionation based on acidity are commonly used to eliminate the unwanted constituents (Onofrejová *et al.*, 2010). It is also important to consider the temperature applied in extracting polyphenols as they are sensitive to high temperature. In most studies, 40 °C has been proposed as the most optimal and favorable condition for polyphenol extraction (Gironi and Piemonte, 2011; Tanniou *et al.*, 2013a; Hong *et al.*, 2014; Ismail and Abdullah, 2015).

Solubility of polyphenols is defined by the polarity of solvents, degree of polymerization of polyphenol, and interaction of polyphenols with other constituents and formation of insoluble complexes (Naczki and Shahidi, 2004). Table 1-10 shows some example of solvents that have been used to obtain polyphenol from brown algae.

Polyphenol compounds in brown algae, also known as phlorotannins, are considered as a pool composed by different nature and/or polarities since they are oligomers and polymers of phloroglucinol. SLE has been selected to extract polyphenol based on the solubility preference of phlorotannins towards the organic solvents. Therefore, different solvents might extract different group of phlorotannins. If the organic solvent is favorable for polyphenols, it is highly possible that the polyphenols can be extracted in large quantities. As for productivity in SLE of phlorotannins, polar solvents extract more polyphenols. In other word, the efficiency of extraction often decreases if the solvents are not appropriate. Aside from this, other disadvantage of SLE is time-consuming, and often uses large volumes of toxic organic solvents. In addition, the complex linkage of brown algal cell wall between polysaccharides, protein and phlorotannins becomes another hindrance in SLE. Thus, the efficiency of extraction is not as optimal as expected since SLE could not break the complex linkage of constituents in algal cell wall. However, it is likely possible that even the appropriate solvent has been chosen for phlorotannins extraction, other

bioactive compounds with same preference as phlorotannins might come along. As a result, it becomes a contaminant in the extractants and creates interference in the bioactivity. For this reason, it is also crucial to consider the suitable isolation method for phlorotannins.

Table 1-10 Application of polyphenol extraction using different organic solvents and their bioactivity potential in brown algae from previous studies.

Species	Solvents	Bioactivity Potential	References
<i>S. muticum</i>	Ethyl-acetate; Ethanol; Acetone; Methanol; Water	Antioxidant	Tanniou <i>et al.</i> , (2013b, 2014)
<i>S. filipendula</i> , <i>S. duplicatum</i> , <i>S. crassifolium</i> , <i>S. binderi</i> , <i>Padina sp</i>	<i>S.</i> Ethanol, Ethyl acetate, n-Hexane	Antioxidant	Bambang <i>et al.</i> , (2013)
<i>S. polycystum</i> , <i>S. duplicatum</i> , <i>S. echinocarpum</i> <i>S. longifolium</i>	<i>S.</i> Hexane, Ethyl acetate, Methanol Dimethyls sulfoxide (DMSO), benzene, acetic acid, hexane, chloroform, diethyl ether	Antibacterial and antifouling Antimicrobial	Widowati <i>et al.</i> , (2014) Ponnanikajamdeen <i>et al.</i> , (2014)
<i>Ecklonia cava</i>	Hexane, diethyl ether, acetone, ethyl acetate, acetonitrile, methanol and water	Antibacterial	Choi <i>et al.</i> , (2014)
<i>Eisenia bicyclis</i>	Methanol, hexane, dichloromethane, ethyl acetate, n-buthanol, water	Antibacterial	Lee <i>et al.</i> , (2014)
<i>Fucus vesiculosus</i> , <i>Ascophyllum nodosum</i> , <i>F. serratus</i>	Ethanol (61.7 %)	Antioxidant	Keyrouz <i>et al.</i> , (2011)
<i>Cystoseira trinodis</i>	Dichloromethane	Antioxidant	Sathya <i>et al.</i> , (Sathya <i>et al.</i> , 2013)
<i>F. vesiculosus</i>	Ethanol (80%)	Antioxidant	Wang <i>et al.</i> , 2012

1.6.3 Enzyme-assisted extraction (EAE) of polyphenols

Marine algae are well-known by their chemically complex and heterogeneous structure, i.e. sulfated and branched polysaccharides associated with proteins and various bound ions including calcium and potassium (Jeon *et al.*, 2012; Wijesinghe and Jeon, 2012a). This cell wall structure is even more complex when it comes to the family of brown algae as they have a strong interaction between protein and phlorotannin (Stern *et al.*, 1996; Koivikko, 2008). This physical barrier has limited the efficiency of solid-liquid extraction technique of biologically active compounds from the marine algae (Wijesinghe and Jeon, 2012a). Thus, it is highly recommended to degrade this complex cell wall yet in the same time it does not have the negative side effect to the target compound during the process. Enzymatic degradation of cell wall polymers as proposed in the enzyme-assisted extraction has drawn many attention and become a more attractive alternative to chemical and mechanical processes (Wijesinghe and Jeon, 2012a). Table 1-11 displays the main differences between conventional and enzyme-assisted extraction.

Table 1-11 Differences between the conventional extraction technique and enzyme-assisted extraction.

Method of extraction	Advantage	Disadvantage	References
Conventional Extraction	Easy application for industrial scale	Longer extraction time Requirement of costly and high purity solvent Low extraction selectivity Thermo labile compounds Toxic solvents are often used Low bioactive yield	Michalak and ChojnackA (2014); Azmir <i>et al.</i> (2013)
Enzyme-assisted Extraction	High bioactive yield Facilitate the solubility of bioactive compounds Food grade enzymes are applicable Nontoxic, ecofriendly Preparation simplicity Moderate conditions Applicable for a large-scale operation	Not yet applicable in the industrial-scale because of the enzyme cost	Kadam <i>et al.</i> (2013); Puri <i>et al.</i> (2012); Wijesinghe and Jeon (2012a); Jeon and Wijesinghe (2011); Sander <i>et al.</i> (2009); Athukorala <i>et al.</i> (2006)

Applying the cell wall degradation enzymes has received many attentions. This application is considered as a promising technique in hydrolyzing the cell wall and the bound phytochemicals to enhance the release of that high-value compound. EAE has been introduced for the past few years as one of the environmentally friendly technique of extraction. This technique is performed under moderate conditions which protect the biologically active compounds from degradation. Carbohydrases and proteases have been found to break down the cell wall of marine algae and release the desired bioactive compound. Some of the commonly used enzymes are Viscozymes, Celluclast, Termamyl, Ultraflo, carragenanase, agarase, xylanase, Kojizyme, Neutrase, Alcalase and Umamizyme (Ahn *et al.*, 2004; Park *et al.*, 2004; Heo *et al.*, 2005; Wijesinghe and Jeon, 2012a). A list of some examples of enzymes as well as their optimal conditions used in EAE of bioactive compounds from brown algae is presented in Table 1-12.

Table 1-12 Summary of the most used enzymes in enzyme-assisted extraction of algal bioactive compound, particularly phlorotannins (Kim *et al.*, 2011; Wijesinghe and Jeon, 2012b).

Enzymes	Temperature (°C)	pH	Enzyme composition
Viscozyme	50	4,5	Arabanase, cellulase, β -glucanase, hemicellulase and xylanase
Celluclast	50	4,5	Group of enzymes catalyzing the breakdown of cellulose into glucose, cellobiose, and higher glucose polymer
Termamyl	60	6,0	Heat-stable α -amylase
Ultraflo	60	7,0	Heat-stable multiactive β -glucanase
Neutrase	50	6,0	Endoprotease
Flavourzyme	50	7,0	Endoprotease and exopeptidase activities
Alcalase	50	8,0	α -endoprotease

Enzyme-assisted extracts appeared to have a higher antioxidant activity compared to those obtained using conventional extraction methods (Park *et al.*, 2004). Prior to dimethyl ether extraction, enzymatic extracts of *Undaria pinnatifida* enhanced the yields of total lipids by 15-20% and fucoxanthin by 50%

(Billakanti *et al.*, 2013). EAE is also well suited to the extraction of phlorotannins and other phenolic compounds from marine algae (Kim *et al.*, 2006b; Siriwardhana *et al.*, 2008; Wang *et al.*, 2010), as it assists in breaking the complex bonding between polyphenol and proteins. Further, this technique can be applied for large-scale operations as a promising, an environmentally friendly and a feasible alternative to traditional extraction method for algal bioactive (Michalak and Chojnacka, 2014; Kadam *et al.*, 2015c). Besides algae, the recovery of polyphenols from the terrestrial plant has been reported, they are citrus peel (Li *et al.*, 2006), grape skin (Pinelo *et al.*, 2006), grape seed (Passos *et al.*, 2009), pomegranate (Mushtaq *et al.*, 2015), unripe apples (Zheng *et al.*, 2008), black currant (Landbo and Meyer, 2004) and cauliflower (Huynh *et al.*, 2014).

Degradation of algal cell wall has been considered as an important preliminary step for bioactive compounds extraction, including polyphenols. By breaking the complex linkage of polysaccharides in algal cell wall, it allows releasing the polyphenols and makes them more accessible to extract. Regarding the yield of extraction, this method results higher yield of extraction since the EAE is a key to “open a door”, in this case the complex algal cell wall, and to liberate almost everything that is “kept inside the door”, in other words phlorotannins. In order to make this method more efficient, it is strongly suggested to determine the suitable enzyme for brown algal cell wall.

Table 1-13 Application of enzyme-assisted extraction in brown algae phlorotannins and their bioactivity potential.

Algae	Enzymes	Activities	References
<i>Ecklonia cava</i>	AMG	Antiproliferative & Antioxidant	Athukorala <i>et al.</i> (2006)
<i>E. cava</i> , <i>Ishige okamure</i> , <i>S. fulvellum</i> , <i>S. horneri</i> , <i>S. coreanum</i> , <i>S. thunbergii</i> , <i>Scytosiphon lomentaria</i>	Viscozyme, Celluclast, Termamyl, Ultraflo, Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase	Antioxidant	Heo <i>et al.</i> (2003)
<i>S. muticum</i>	Alcalase and Viscozyme	Antioxidant	Sanchez-Camargo <i>et al.</i> (2015)
<i>E. cava</i>	Viscozyme, Celluclast, Termamyl, Ultraflo, Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase	Hydrogen-peroxide scavenging	(Kim <i>et al.</i> , 2006a)
<i>E. radiata</i>	Viscozyme, Celluclast, Ultraflo, Alcalase, Neutrase, Flavourzyme	Antioxidant	(Charoensiddhi <i>et al.</i> , 2014)

1.6 Upgrading of *Sargassum* – bioactive compounds or extracts

Sargassum exists in large number of species with more than 350 species (Guiry and Guiry, 2015) growing mostly anchored to the bottom with its holdfast with thalli floating in water column. Its fast growth supported by its vegetative reproduction and floating ability has made *Sargassum* a floating jungle in the ocean causing golden tides. As it gets washed away on to the shore in substantial amounts, *Sargassum* forms a mini-mountain piling up along the coast. Later, *Sargassum* creates a putrid sulfurous odor if its massive influx of biomass is left to decompose on the beach. Inundation of *Sargassum* on the coast occurs naturally since it relates to *Sargassum* life cycle. Nevertheless, the existence of *Sargassum* in massive amount can be disturbing for coastal communities. As example in the Caribbean, it spent at least \$120 million to clean up the massive influx of *Sargassum* (Milledge and Harvey, 2016). Instead of spending the amount for merely cleaning them from the coast, it might be better to valorize

Sargassum since many studies has revealed a vast array of potential in particularly for foods, pharmacology, and cosmetics industries (Holdt and Kraan, 2011; Rebours *et al.*, 2014). Pharmacological properties of *Sargassum* crude extracts or isolated pure compounds have been widely recognized including antibacterial, antifungal, antiviral, anticoagulant, antioxidant, antimelanogenic, anti-inflammatory, hypoglycemic, hepatoprotective and neuroprotective activities. This suggests that *Sargassum* is a rich source of health and promoting agents. Prior to these exceptional findings, *Sargassum* has been traditionally used as medicine in the ancient times in China to treat goitre. *Sargassum* has been mentioned as “Hai Zao” and it is believed that *Sargassum* could soften hard lumps, dispel nodes, eliminate phlegm and induce urination in humans (Liu *et al.*, 2012).

In traditional medicine of China, *Sargassum* has been formulated in more than 226 prescriptions to treat various diseases. *S. pallidum* (Turner) C. Agardh and *S. fusiforme* (Harvey) Setchell have been listed as treatments for arteriosclerosis, skin diseases, high blood pressure, hepatolienomegaly, neurosis, angina pectoris, chronic bronchitis and esophagitis. *S. fulvellum* (Turner), *S. henslowianum* C. Agardh, *S. thunbergii* (Mertens ex Roth) Kuntze, *S. horneri* (Turner) C. Agardh, commonly named as “Hai Qian” are another *Sargassum* species that are popular in traditional medicines and food ingredients in the south east region of China. *Sargassum* was also used exclusively for medicinal purpose at ancient times in Monte Verde, South America (Dillehay *et al.*, 2008). *Sargassum vulgare* C. Agardh, and *Sargassum thunbergii* Yendo from Japan have been or are used as antihelmintics (Chapman and Chapman, 1980; Kang *et al.*, 2008). Beside medicines, *Sargassum* is also benefit in environmental application, for example U.S. has made a patent regarding the application of *S. natans* in sequestering gold from water (Kuyucak and Volesky, 1988). In food industry Japan, *S. fusiforme* also known as Hijiki is served as food, named as “vegetable for longevity”. It is a side dish prepared by soaking and boiling the dried algae with water and served with soy sauce (Liu *et al.*, 2012). *S. polycystum*, has been

eaten as sea vegetables in Asian countries such as Malaysia and Vietnam (Hong *et al.*, 2007; Matanjun *et al.*, 2009).

Records about traditional use of *Sargassum* in medicine have made this genus interesting to explore. Thus, *Sargassum* has been the most extensively as alternative natural resources with economic value. Identified bioactive meroterpenoids such as 2-methyl-6-(3-methyl-7oxo-2,5-octadienyl)-1,4-benzoquinone (Horie *et al.*, 2008) and sargafuran (Kamei *et al.*, 2009) with significant antibacterial activity ($2\text{ }\mu\text{g/mL} < \text{MIC} < 15\text{ }\mu\text{g/mL}$) could provide scientific evidence for the traditional therapeutic claims of *Sargassum*. The pharmacological activity of *Sargassum* extracts and research of its bioactive constituents provide scientific evidence that underpins the traditional therapeutical claims made for *Sargassum* such as furuncle and abscesses (Liu *et al.*, 2012).

The iodine in *Sargassum* was believed to play an important role in therapeutical function. However, the role of iodine may have been overestimated and other bioactive metabolites may have made more significant contributions toward *Sargassum*'s therapeutic claims (Liu *et al.*, 2012). Research suggests that *Sargassum* may play a role as an immunomodulator (Yende *et al.*, 2014). Antimalarial activity has been found *Sargassum natans* (Linnaeus) Gaillon (Orhan *et al.*, 2006). *S. hemiphyllum* lipophilic fraction of the dichloromethane/methanol extract exerted potent in vitro antiplasmodial activity ($\text{IC}_{50} = 2.8\text{ }\mu\text{g/mL}$; (Lategan *et al.*, 2009)). Samee *et al.* (2009) showed that semipurified hydroethanolic extracts from *Sargassum* growing in Pakistan or China had an antihyaluronidase activity proportional to the phlorotannin contents of the ethyl acetate fractions. In addition, the IC_{50} of the extract from *Sargassum tennerimum* was found to be similar to that of catechin. *Sargassum* in India is mostly exploited for its alginates production in hydrocolloid industries either for local or for export (Subba Rao and Mantri, 2006).

Table 1-14 List of important bioactive compounds from *Sargassum* species that have been extensively studied.

Bioactive compound	Reference
Polysaccharides	(Josephine <i>et al.</i> , 2008; Sinha <i>et al.</i> , 2010; Ji <i>et al.</i> , 2011; Lee <i>et al.</i> , 2011; Zhang <i>et al.</i> , 2012; Dore <i>et al.</i> , 2013; Yende <i>et al.</i> , 2014)
Polyphenols	(Glombitza <i>et al.</i> , 1997a; Keusgen and Glombitza, 1997; Stiger <i>et al.</i> , 2004; Santoso <i>et al.</i> , 2005; Chandini <i>et al.</i> , 2008; Ghereib, 2010; Budhiyanti <i>et al.</i> , 2012; Tanniou <i>et al.</i> , 2013a; Hardouin <i>et al.</i> , 2013; Widowati <i>et al.</i> , 2014; Perez-Lopez <i>et al.</i> , 2014; Montero <i>et al.</i> , 2015; Samudra <i>et al.</i> , 2015)
Lipids and fatty acids	(Bhaskar <i>et al.</i> , 2004; Bazes <i>et al.</i> , 2009; Wiraswati and Warganegara, 2011; Gerasimenko <i>et al.</i> , 2014; Balboa <i>et al.</i> , 2016)
Pigments	(Heo and Jeon, 2009; Resita <i>et al.</i> , 2010; Pangestuti and Kim, 2011; D'Orazio <i>et al.</i> , 2012; Heo <i>et al.</i> , 2012; Hu <i>et al.</i> , 2015; Sivagnanam <i>et al.</i> , 2015)
Proteins and amino acids	(Marín <i>et al.</i> , 2009; Peng <i>et al.</i> , 2013; Polo <i>et al.</i> , 2015)
Minerals	(Oyesiku and Egunyomi, 2011; Kalaivanan and Venkatesalu, 2012a; Michalak and Chojnacka, 2013)

Among the bioactive compound contained in marine algae, polysaccharides and polyphenols are the two most studied especially related to their biological activity. In this study, phlorotannin, a phloroglucinol derivatives from the brown algae functioning both as a primary and secondary metabolite compound will be the main subject. Brown Algae are one of marine algae group well-known for their strong antioxidant property shown by this compound. However, other studies have also stated that this compound has a vast array of potential as antimicrobial, anticancer, antihypertensive, antidiabetic, antiallergic, etc (Vadlapudi, 2012; Wijesinghe and Jeon, 2012; Thomas and Kim, 2011; Wijesekara *et al.*, 2010).

The function of phlorotannins as stress compound has brought another

interesting point of view particularly related to the search of marine natural product. Allegedly, their ability to defense themselves against diverse environmental stress makes them to have number biological activities that can be further prepared as basic materials for the pharmaceutical and cosmeceutical industries. Further, these biological activities have been widely studied and one of the most outstanding activities possessed by phlorotannin is its antioxidant activity. It has been proven that brown algae phlorotannin has demonstrated as one of the strongest and important antioxidant activity (Ahn *et al.*, 2004; Shibata *et al.*, 2008; Girija *et al.*, 2013).

Strong correlation between the total phenolic content and antioxidant activity in brown Algae has been reported by many previous studies (Wang *et al.*, 2009; Rajauria *et al.*, 2010; Eom *et al.*, 2011; Namvar *et al.*, 2013). Other studies have revealed that phlorotannins demonstrated a great potential against 1,1-diphenyl 1,2-picrylhydrazyl (DPPH), hydroxyl, superoxide and peroxy radicals (Li and Glombitza, 1991; Nakai *et al.*, 2006; Santoso *et al.*, 2012; Tierney *et al.*, 2013). In general, the antioxidant properties work by inhibiting the initiation or propagation reactions by inactivating or scavenging free radicals. Therefore, it restrains or inhibits completely the lipid oxidation (Gupta and Abu-Ghannam, 2013). The phenol rings play their role as electron traps. Thus, they are responsible for the multifunctional antioxidant properties like the scavenging of hydroxyl radicals, peroxy radicals or superoxides. Further, their metal chelating potency appeared to be dependent upon their unique phenolic structure and the number as well as location of the hydroxyl groups (Santoso *et al.*, 2004). The chemical structure of algae phlorotannin is constituted up to eight interconnected rings (Wang *et al.*, 2008). This factor explains why phlorotannins perform more potent antioxidant properties compared to the terrestrial polyphenols (Gupta and Abu-Ghannam, 2013) and make them as an interesting compound to further develop.

Apparently, phlorotannins do not only perform strong antioxidant but also

antimicrobial property (Eom *et al.*, 2013, 2012; Lopes *et al.*, 2012; Choi *et al.*, 2010; Lee *et al.*, 2010; Eom *et al.*, 2008; Lee *et al.*, 2008; Plouguerné *et al.*, 2008; Lin *et al.*, 2006; Ahn *et al.*, 2004). Wang *et al.* (2009) reported the sensitivity of *Eschericia coli* to brown algae *Ascophyllum nodosum* phlorotannins and the terrestrial tannins. Their results demonstrated that the inhibition of *E. coli* growth of phlorotannins was greater than the hydrolysable and condensed tannins isolated from *Rhus semialata* and *Schinopsis balansaei* – both are terrestrial plants. The oxidation of tannins and the liberation of hydrogen peroxide become the factors that are responsible for antimicrobial properties of phenolic compounds as mentioned by Smith *et al.* (2003). A different source of phenolic produces different amounts of hydroxide peroxide under aerobic conditions. The highest production is associated with phenolic that are rich in hydroxyl groups (Akagawa *et al.*, 2003). In addition, the number of hydroxyl groups available to form hydrogen bonds with protein lead to the increase of tannins affinity to protein (Mueller and Harvey, 2006). Easily oxidized upon exposure to air and contain more hydroxyl groups are two characteristics shown by phlorotannins. These factors account for their greater antibacterial activity (Wang *et al.*, 2009). Furthermore, the degree of polymerization of phloroglucinol also contributes to this outstanding antibacterial property (Nagayama *et al.*, 2002) that does not exist in the terrestrial tannins (Wang *et al.*, 2009). Despite the differences shown by the phlorotannins and terrestrial tannins, they seem to share similarity in the mechanisms of antimicrobial activity. Those mechanisms are inhibitions of extracellular bacterial enzymes, deprivation of substrate required for growth or direct inhibition of oxidative phosphorylation (Wang *et al.*, 2009).

Besides those two potential, phlorotannins also perform other biological activity such as anti-proliferative and antitumor (Nwosu *et al.*, 2011; Yang *et al.*, 2010; Imbs *et al.*, 2008); antidiabetic (Nwosu *et al.*, 2011; Jung *et al.*, 2008); α -Glucosidase and α -Amylase inhibition (Lee *et al.*, 2008), angiotensin-I-converting enzyme (ACE I) inhibition (Jung *et al.*, 2006; Athukorala and Jeon, 2005), anti-inflammatory (Kim *et al.*, 2014; Kim *et al.*, 2010); matrix metalloproteinases

(MMPs) inhibition (Kim *et al.*, 2006), hyaluronidase inhibition (Ferrerres *et al.*, 2012; Shibata *et al.*, 2002), tyrosinase inhibition (Yoon *et al.*, 2009; Heo *et al.*, 2009; Kang *et al.*, 2004), phoroprotective (Cha *et al.*, 2012; Le Lann *et al.*, 2008; Heo *et al.*, 2009) and antiallergic (Lee *et al.*, 2008; Sugiura *et al.*, 2007).

Phlorotannin has attracted many attentions as it has a promising potential for further application in the pharmaceutical, cosmeceutical and food industries. One genus that has been well and most studied related to their phlorotannin is the *Ecklonia*, Genera of kelp belonging to the family Lessoniaceae. The phlorotannin from this genus, particularly from *E. cava*, *E. kurome*, *E. maxima*, has been successfully purified and characterized, for example phloroglucinol, eckol, dieckol, fucodiphloroethol, phlorofucofuroeckol etc. (Mwangi *et al.*, 2013; Yotsu-Yamashita *et al.*, 2013; Wijesikara *et al.*, 2010; Koivikko, 2008). Besides this Genera, the characterization of phlorotannin from other Genera of Phaeophyceae has also been successfully reported such as the *Cystoseira abies-marina* (Montero *et al.*, 2014), *C. tamariscifolia* (Hudson) Papenfuss, *C. nodicaulis* (Withering) M. Roberts, *C. usneoides* Linnaeus and *Fucus spiralis* Linnaeus from the Fucales (Ferrerres *et al.*, 2012) and *Eisini bicyclis* (Kjellman) Setchell from the Eisenia (Fujii *et al.*, 2013).

Phlorotannins of *Sargassum* have also revealed their wide range of pharmacological properties. Many studies have revealed the promising potential of phlorotannins derived from this genus. *S. hystrix* (Harnita *et al.*, 2013; Budhiyanti *et al.*, 2011), *S. filipindeula*, *S. duplicatum*, *S. crassifolium*, *S. binderi* (Bambang *et al.*, 2013), *S. polycystum* (Chan *et al.*, 2011), *S. myriocystum*, *S. plagiophyllum*, *S. ilicifolium* (Ganapathi *et al.*, 2013), *S. muticum* (Namvar *et al.*, 2013; Tanniou *et al.*, 2012), *S. boveanum* (Zahra *et al.*, 2007) are some examples of *Sargassum* species that have been reported for their potential antioxidant properties of their phenolic compounds. However, only few studies have reported the characterization of phlorotannin from this genus, i.e fuhalols, deshydroxyfuhalols and fucophloroethols from *S. spinuligerum* (Glombitza *et al.*, 1997; Glombitza and Keusgen, 1995), bifuhalol from *S. ringgoldianum* (Nakai *et*

al., 2006), 2-(4-(3,5(dihydroxyphenoxy)-3,5-dihydroxyphenoxy) benzene-1,3,5-triol (DDBT) from *S. patens* (Kawamura-Konishi *et al.*, 2012).

1.7 Production and potential application of brown algae

Most of algal cultivation activities come from the red algae, Rhodophyceae. Major red algae species that are cultivated include *Kappaphycus* and *Euchema*, (primary raw materials for carrageenan), followed by *Gracilaria* (raw materials for agar) and *Poryphyra* (nori, for direct human consumption). Figure 1-19 presents the division of algal industrial application and most of algal dry materials are used for direct human consumption known as sea vegetables. Second place of algal application is for hydrocolloid and last is for other applications, like medical applications, fertilizer, animal feeds, and other biotechnological applications. In 2011, 83% algal production was designated for human consumption (Craigie, 2011). Afterwards, processed food industry, the primary market for algal hydrocolloids continue to grow rapidly. Hydrocolloids products from algae, i.e. agar, alginate and carrageenan, serve as texturing agents and stabilizers (Bixler and Porse, 2011).

The brown algae processing industry in Europe is divided into two main categories, (1) alginic acid production and (2) products for agriculture such as fertilizer and animal feeds (Frangoudes *et al.*, 2012). Colloids have been applied to multiple industrial applications, such as food processing, cosmetics and pharmaceuticals. Alginate, carrageenan and agar are widely known for their gelling, stabilizing and thickening properties. These products have been commercialized as additives with an average 1 million tons of wet algae are used every year in order to extract these three compounds. In France itself, there are nearly 90 companies that process or sell algae-based products (Mesnildrey *et al.*, 2012). Hydrocolloid extraction and commercialization have contributed 75% of algae-based products. Meanwhile, for direct consummation of fresh or dried algae, algae production only contributes 1% with 19 species authorized for general consumption. As for the focus of French stakeholders interested in algae-

based products, the majority of cultivated algae is used for material purposes, for example in the specialty of chemical (cosmeceutical, nutraceuticals and pharmaceuticals) (Brinker and Sternberg, 2014).

In European level, France is in the second position after Norway based on their algae production in 2012 with 57 % and 14 % respectively. Approximately, 95 000 tons of fresh algae were collected. Every year, France produces between 40.000 and 70.000 tons of fresh algae (Kerlero de Rosbo and Bernard, 2014). Brown algae contributed 95 % of the production mainly from the genus *Laminaria* – with total 75% consisted of *L. digitata* (60%) and *L. hyperborea* (15%) (BDI, 2012; Mesnildrey *et al.*, 2012).

The distinguished annual production of algae in France is due to the fact that their production still depends on the natural stock. Furthermore, as a four season country, natural biomass of algae is extremely seasonal-dependent. Aquaculture production only contributes 50 tons of algae with two principal species cultivated, i.e. *Undaria pinnatifida* (Wakamé) and *S. latisima* (Kombu) (Kerlero de Rosbo and Bernard, 2014). They are mainly harvested from the coast of Brittany, particularly in the Finistère and Côtes d'Armor (Kerlero de Rosbo and Bernard, 2014). Figure 1-17 describes the percentage of algae applications in France dominating by the food-processing industry, chemistry and microbiology as the main markets.

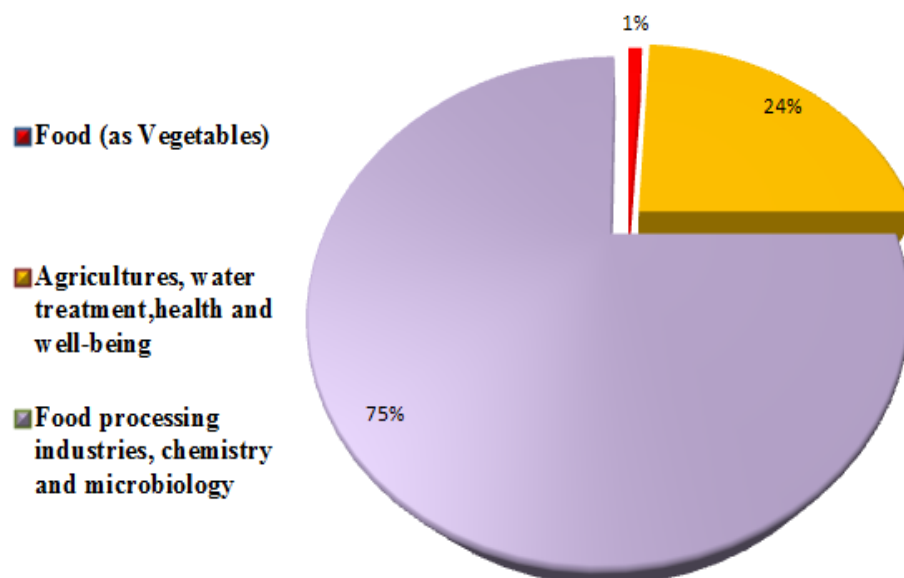


Fig 1-18 Classification of algae based on their utilization in France (French production and imports) according to CEVA (2009)(Mesnildrey *et al.*, 2012)

There are 24 algae authorized for human consumption in France, in which 6 of them are particularly present in the littoral zone; kombu, wakame, nori, dulse, sea lettuce and bean (Figure 1-18). Table 1-12 displays the lists of marine algae that are allowed for human consumption (CEVA, 2014).







Kombu (<i>Saccharina latissima</i>)	Wakamé (<i>Undaria pinnatifida</i>)	Nori (<i>Porphyra sp.</i>)	Dulse (<i>Palmaria palmata</i>)	Laitue de mer (<i>Ulva sp.</i>)	Haricot de mer (<i>Himanthalia elongata</i>)
					

Fig 1-19 Macroalgae mainly consumed in France (CEVA, 2014).

Table 1-15 Macroalgae that are allowed for human consumption (CEVA, 2014).

Species	Common name
Brown algae	
<i>A. nodosum</i>	-
<i>F. vesiculosus</i> , <i>F. serratus</i>	-
<i>Himanthalia elongate</i>	“Haricot de mer” (bean)
<i>U. pinnatifida</i>	Wakame
<i>L. digitata</i>	Kombu
<i>L. saccharina</i>	Kombu royal
<i>L. japonica</i>	Kombu
<i>Alaria esculenta</i>	Atlantic wakame
Red algae	
<i>Palmaria palmate</i>	Dulce
<i>Porphyra umbilicalis</i>	Nori
<i>P. tenera</i>	Nori
<i>P. dioica</i>	Nori
<i>P. purpurea</i>	Nori
<i>P. laciniata</i>	Nori
<i>P. leucostica</i>	Nori
<i>Chondrus crispus</i>	Pioca
<i>G. verrucosa</i>	Ogonori
<i>Lithothamnium calcareum</i>	Maerl
Green algae	
<i>Ulva</i> sp.	“Laitue de mer” (sea lettuce)
<i>Enteromorpha</i> sp.	Aonori

In 2009, Indonesia was the second world producer contributing 16% of algae production, second consumer (13%) and first exporter of macro-algae by 32% of flow rate. 87% of its market was concentrated in the production of *Eucheima* spp. In contrary with China and Japan, and similar to France, the Indonesian production of algae contributes the market with their colloids, particularly carrageenan used as stabilizer, emulsifier and gelling agents for food-processing industries (Kerlero de Rosbo and Bernard, 2014).

Algae production is mostly concentrated on Sulawesi, producing almost 65% of the total production volume. Most of the productions come from the exports of dry materials and the rest from the hydrocolloid production, especially carrageenan and agar-agar from the red algae. Neish (2012) stated that red algal galactan algae (RAGS) from the genus *Kappahycus/ Eucheima* and *Gracilaria* are the most cultivated algae in Indonesia. In 2010, the yields of carragenophytes

reached 9.000 tons with different quality levels; 9% was refined and 21% was semi-refined for non-food purposes (Neish, 2012).

Basically, there seven main species that are commercially harvested, namely *K. striatus*, *E. spinosum*, *K. alvarezzi*, *Gracilaria sp.*, *Sargassum sp.*, *Gellidium sp.*, and *Caulerpa sp.* Predominant Indonesian algae crops are *K. alvarezzi* – commercial name “cottonii”, and *K. striatus* – commercial name “sacol”. About 50% of *Kappaphycus* crops are exported to China; and 20% are processed by domestic processors and the rest is exported to Philippines and other countries (Neish *et al.*, 2015). *K. alvarezzi*, *E. spinosum* and *Gracilaria sp.* are known to produce kappa- and iota- carrageenan, and agar. Accordingly, algal production in Indonesia is most likely designated for hydrocolloid production. For carrageenan, there are two types of products according to the production process, *i.e.* refined and semi-refined carrageenans, known as RC and SRC, respectively.

Almost similar to France’s case, *Sargassum* species in Indonesia is also in the state of being underexploited. For local anglers, the application of *Sargassum* is still for animal’s food (fodder) (pers. comm.) despite of the fact that alginates from brown algae are important substances for food, beverages, medical/pharmaceuticals, and non-food industries (Wouthuyzen *et al.*, 2016). Previous studies have revealed that *Sargassum* species extracts or compounds from Indonesia have shown some pharmacological potential. As an example, alginate of *Sargassum sp.* in Indonesia has been acknowledged to enhance immune system in catfish *Clarias sp* (Isnansetyo *et al.*, 2014).

French and Indonesian *Sargassum* have been long considered as a potential natural resource with economic value. *Sargassum* is normally proposed as alginates sources for hydrocolloids industry as texturing agents. There have been many studies carried out regarding the prospect of *Sargassum* extracts in food, pharmacological and cosmetics industries beside the hydrocolloid industry. Results reveal the hidden yet remarkable pharmacological properties of *Sargassum* as it contains extremely active compounds that are possible for further

formulation. Moreover, this species might also be suggested as proteins, vitamins and minerals source for human consumptions. As can be seen from Table 1-12, *Sargassum* has not been included as one of macroalgae authorized for human consumption in France, though *Sargassum* has been consumed in certain countries such as in Japan. In order to authorize this species as a ‘safely-consumed’ product, it is highly essential to perform a series analysis required according to the regulations existed in each designated countries. Thus, *Sargassum* can be included as one of the authorized marine resources for commercial interest.

Others have started considering exploiting the massive biomass of *Sargassum*. For example, Japan has recognized *Sargassum* as source of sustainable bioenergy. Lipids and starch from *Sargassum* have been proven to be exploitable for bioenergy. Exploitation of *Sargassum* biomass is considered one of the best options for increasing renewable energy due to its massive influx along the coast. Bioethanol production from seaweed in Japan, entitled the “Ocean Sunrise Project,” aims to produce seaweed-based bioethanol by farming and harvesting *S. horneri*, utilizing 4.47 million km² (the sixth largest in the world) of unused areas of the Exclusive Economic Zone (EEZ) and maritime belts of Japan (Aizawa *et al.*, 2007). As *Sargassum* contains important proteins, fiber, macro- and microminerals (especially in potassium, nitrates, calcium, iron and PUFAs), this species has been targeted as alternative source of animal feeds, fertilizer and biostimulants. Dry material of *Sargassum* has been integrated as supplements in animal feeds. It is believed that this species could improve the growth rate, survival rate and immune system of cultivated animals as reported in shrimp *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, and rainbow trout *Oncorhynchus mykiss* (Huynh *et al.*, 2011; Hafezieh *et al.*, 2014; Sudaryono *et al.*, 2015; Zamannejad *et al.*, 2016). As fertilizer, algae have been applied to soils as manure and as conditioning agents (Hong *et al.*, 2007). Extract of *Sargassum* also stimulates the germination, improve the growth and biochemical constituents and enhance the immunity of certain plants, like lentil (*Vigna mungo*), mung bean (*Vigna radiata*), mustard (*Brassica juncea* L.), garden cress (*Lepidium sativum*),

and tomato (*Solanum lycopersicum* L.) (Kavipriya *et al.*, 2011; Kalaivanan and Venkatesalu, 2012b; Hernández-Herrera *et al.*, 2014; Siregar *et al.*, 2014; Godlewska *et al.*, 2016). Sargasso Sea Alliance has revealed that there are about 90 patents referencing *Sargassum*. Various industrial, medical and nutritional uses are proposed including applications focused on inhibiting HIV infection, as an antibiotic, antifungal and antifouling substance, biofuel, fertilizer and animal feeds (Laffoley *et al.*, 2011).

Table 1-16 Several studies on *Sargassum* bioactive compounds with pharmacological potential.

Species	Location	Solvent	Result	Reference
<i>S. fillipendulla</i>	Pulau Talango, Kab. Sumenep Madura	methanol, acetone, hexane, ethyl acetate	Antioxidant activity and preservation for fish	(Khotimah <i>et al.</i> , 2013)
<i>S. duplicatum</i>	-	Ethanol, kloroform, ethyl acetate	alternative therapy for IBD treatment	(Aulanni'am <i>et al.</i> , 2011)
<i>S. echinocarpum</i>	Talango Island, Madura	Ethanol, methanol, acetone	Antioxidant activity	(Firdaus <i>et al.</i> , 2012)
<i>S. duplicatum</i>	Pantai Raca, Nusa Kambangan	Ethanol, methanol, hexane, ethyl acetate	Inhibition of manoldehide and peroxidase	(Septiana and Asnani, 2013)
<i>S. crassifolium</i>	Pulau Sumba	Methanol, hexane, dichlorometana, water	Antioxidant activity	(Supriyono, 2007)
<i>S. hystrix</i>	South Coast of Yogyakarta	Methanol, chloroform, deionized water, ethyl acetate	Candidate for photoprotection of oxidative damage to the DNA	(Harnita <i>et al.</i> , 2013)

Table 1-16 Several studies on *Sargassum* bioactive compounds with pharmacological potential (continue).

Species	Location	Solvent	Bioactivity	Reference
<i>S. crassifolium</i> , <i>S. polycystum</i> , <i>S. echinocarpum</i>	Craggy Coast of Gunung Kidul, Yogyakarta	alginate extraction	Potential to produce alginate	(Mushollaeni and S, 2012)
<i>S. echinocarpum</i>	Pari Island	alginate extraction	Potential to produce alginate as the viscosity reach the SIGMA's standar	(Rasyid, 2010)
<i>S. polycystum</i> , <i>S. duplicatum</i> , <i>S. echinocarpum</i>	Teluk Awur, Jepara	Hexane, Ethyl acetate, Methanol	Antibacterial activity	(Widowati <i>et al.</i> , 2014)
<i>S. muticum</i>	Grand Dellec, Plouzané	chloroform	Antibacterial, antifungal and anti-microalgal	(Plouguerne <i>et al.</i> , 2010)
	Cote-d'armor, Brittany	Alginate extraction and biomethane	Methane production	(Jard <i>et al.</i> , 2013)
	Grand Dellec, Plouzané	Enzymatic hydrolysis	Anti-herpetic activity	(Hardouin <i>et al.</i> , 2013)
	Northern and southwestern of Brittany	Methanol:water (50%; v/v)	Antioxidant activity	(Connan <i>et al.</i> , 2006)
	Locmariaquer	Ethanol:water and ethanol:dichloromethane	Antifouling activity	(Bazes <i>et al.</i> , 2009)

Chapter 2 Material and Methods

2.1 Sampling Sites and abiotic factors

In this work, two locations, France and Indonesia, were chosen in order to compare the biochemical composition of *Sargassum* sp. extracts along with their biological activities. Illustration of sampling location is described in Figure 2-1.



Figure 2-1 Overall view of sampling sites (Above) Saint Gildas de Rhuys, Morbihan, France; (Below) Java Island, Indonesia.

2.1.1 Overview of studied location in France

The region of Brittany is located in the temperate climatic zone of the northern hemisphere bordered with the English Channel – known as La Manche (in French) – on the north and Atlantic Ocean on the south and west. This region is between two marine eco-regions; they are the Celtic Sea and Atlantic Continental Shelf of South Europe (Spalding *et al.*, 2007). As a consequence, this difference leads to a distinguished environmental conditions and fauna indicated between the coastal of north and south Brittany (Ehrhold *et al.*, 2006).

The coastal border of south Brittany is located on the north continental shelf of Gascogne Gulf. It extends 150 km from east and west, between Guérande peninsula and the point of Raz bordered with a series of islands on the south (Houat, Hoëdic, Belle-Île, Groix and the archipelago of Glénans). The size of this area represents around 400.000 ha. The eastern side of South Brittany coast is bordered with the estuarine of Vilaine and Loire. From east to west, the zone includes four big bays, which are Vilaine, Quiberon, Concarneau and Audierne (Dutertre *et al.*, 2014).

Morbihan is one of the French departments within South Brittany, situated on the northwest of France with 6.823 km² of total area. This department is surrounded by others departments namely, Finistère, Côtes-d'Armor, Ille-et-Vilaine and Loire-Atlantique with Atlantic Ocean on the south-west. Morbihan Gulf, an inland sea with 11.500 ha area widespread with a number of small islands, spreads to the north of Quiberon Bay. This area has a total size of 117.000 ha corresponding to “Mor-Braz” (Dutertre *et al.*, 2014).

Sargassum muticum (Yendo) Fensholt was collected monthly from February 2014 – February 2016 from the St. Gildas de Rhuys (47° 30' 03" N 2° 50' 12" W), one of the commune within the Morbihan department of South Brittany, France. Based on their position, the hydrology characteristic of this area

is influenced by the Biscay Bay characteristic. Biscay Bay region ranges from the West Brittany (48°N) to the Strait of Gibraltar (36 °N). The continental shelf located in this Bay is about 140 km wide and it becomes narrower to the south with about 50 km off the southern France. Sediments covering the continental margin mainly consist of thick turbidity sheet-fan deposits. In the south-western Brittany, the substrate is known as Grande Vasière – large muddy area.

S. muticum was hand-sampled by cutting the basal part of the thalli near the holdfast. They were rinsed with seawater after sampling and transported into the laboratory in closed boxes with no excess seawater. The collected samples were then rinsed thoroughly using tap water to remove the epiphytes and residues attached to samples. *S. muticum* was then grinded with Jupiter Universal (Wernau, Germany) using a 3 mm perforated grill. The grinded seaweeds were then lyophilized to obtain a dry material for further analysis and experiments.

In the north-east of Biscay Bay, mainly in summer, upwelling events occur off Southern Brittany and the Landes coastline and may induce low-salinity (Koutsikopoulos and Le Cann, 1996; Puillat *et al.*, 2006). Temperature in this region varies between 7 and 15 °C in the sea surface and between 5.5 and 7 °C in the deep waters. The average salinity is about 35 ‰ or higher in both surface and deep water.

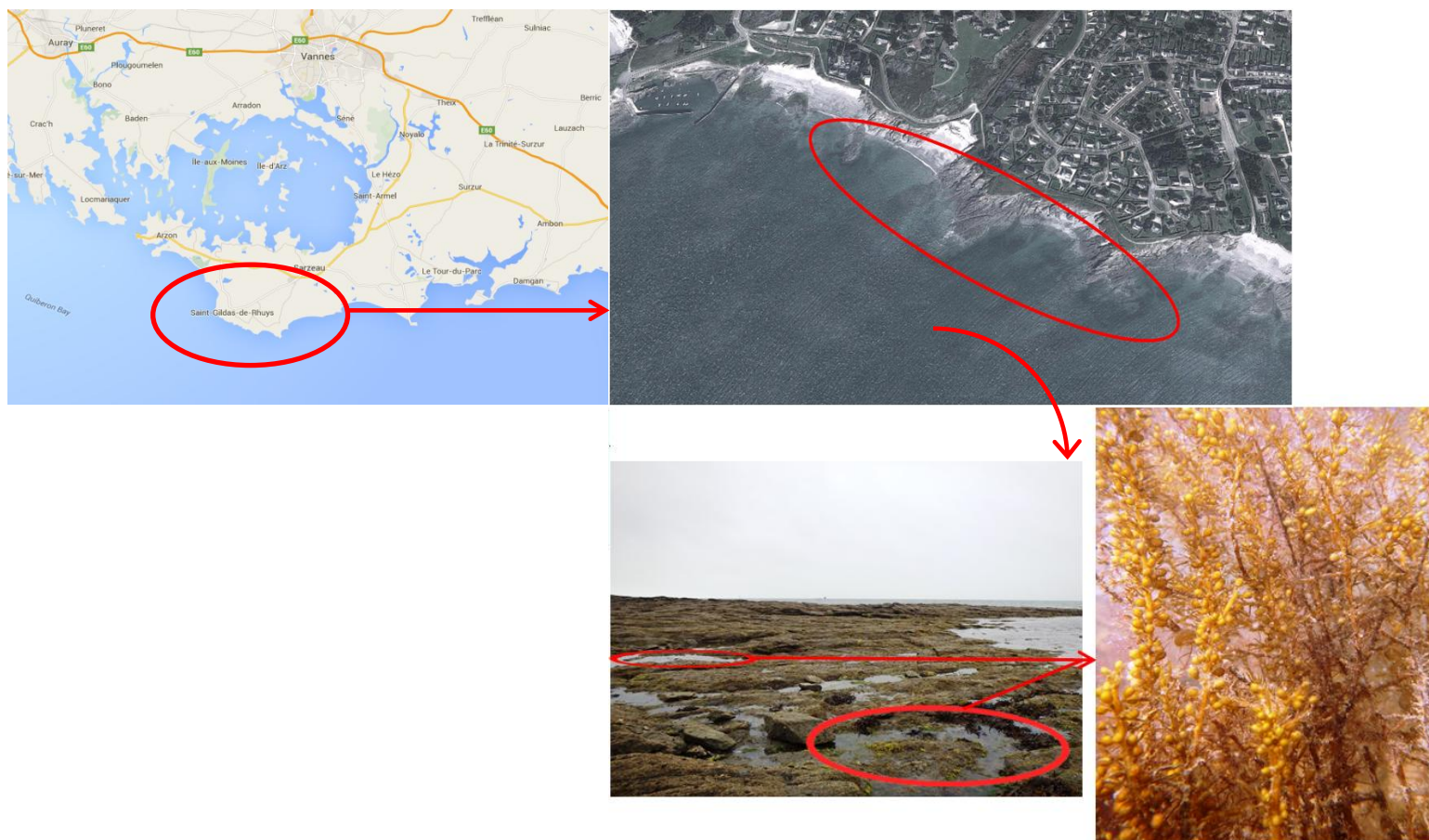


Figure 2-2 Overall illustration of sampling sites at St. Gildas de Rhuys, Vannes, France.

2.1.2 Overview of studied location in Indonesia

The second location represented the tropical region is located in one of the main island in Indonesia, which is at Jepara, Central Java. Jepara is one of a city in the north of Central Java with 82.73 km² of coastal zone with total area covers 100.413,189 ha, located between 5°43'20,67" - 6°47'25,83" South and 110°9'48,02" – 110°58'37,40" East. This region extends up to the north where the Karimunjawa Islands is bordered by the Java Sea on the north and west side.

Jepara has a tropical climate with average air temperature between 21 and 32 °C with maximum precipitation (600 mm) occurring during the rainy season between January and March usually followed by the highest high tide. During this season, strong waves might reach 5 m high with wind speed 12 – 16 knots. The driest season happens on August with only 20 mm precipitation. Most of the substrate found in this region is fringing reef with white sands.

There were two different sampling sites, *i.e.* Panjang Island (06°34'30" South and 110°37'30" East) and Telukawur (6,1268° South 110,4° East) with 30 minutes away between these two sites. Both of them are located at Jepara, Java Central. *S. polycystum* was collected from Panjang Island, Jepara in October 2013 then *S. aquifolium* and *S. ilicifolium* were collected from Telukawur, Jepara in April 2014. All seaweeds were hand-sampled by cutting the basal part of the thalli near the holdfast. They were rinsed with seawater after sampling and transported into the laboratory in closed boxes with no excess seawater. The collected samples were then rinsed thoroughly using tap water to remove the epiphytes and residues attached to samples. *S. aquifolium*, *S. ilicifolium* and *S. polycystum* were air-dried and chopped to pieces and grinded using multiuse blender.

The water quality of Telukawur where *S. aquifolium* and *S. ilicifolium* were collected, and Panjang Island in Jepara, Indonesia where *S. polycystum* was sampled, was measured *in-situ* for its salinity, temperature, pH, and dissolved oxygen, and *ex-situ* for its nitrate and phosphate content. Details on water quality are presented in the

table 2-1.

Table 2-1 Water quality of sampling sites based on *in-situ* measurements.

Water Quality	Telukawur	Panjang Island
Salinity (‰)	27	30.3
Temperature (°C)	28.2	31.6
pH	7.7	8.13
Dissolved oxygen (DO/ mg/l)	6.3	8.09
Phosphate (mg/l)	0.1	n.d
Nitrate (mg/l)	1.1	n.d

n.d: not determined



Figure 2-3 Overall view of sampling sites at Jepara, Central Java, Indonesia, (Left) Panjang Island, (Right) Telukawur.

2.2 Preparation of algal material

2.2.1 Species, sites and period of collection

Algal species used in this study was *S. aquifolium*, *S. ilicifolium*, *S. polycystum*, and *S. muticum*. Table 2.2 presents the detail of *Sargassum* species used in this study collected from different sites in different period.

Table 2-2 List of *Sargassum* species, location and period of collection from this study.

Species	Location	Region	Period of collection
<i>S. muticum</i>	Saint Gildas de Rhuys, Morbihan	Temperate (France)	February 2014 – February 2016
<i>S. aquifolium</i> <i>S. ilicifolium</i>	Telukawur, Jepara	Tropical (Indonesia)	April 2014
<i>S. polycystum</i>	Panjang Island, Jepara	Tropical (Indonesia)	October 2013

2.2.2 Preparation of the samples, drying and grinding

Thallus of French *Sargassum*, *S. muticum*, were cut by scissor, approximately 5 cm from the holdfast. Algal material was quickly cleaned with seawater and was kept in closed box to keep them away from direct contact with sunlight. Further, algal material was washed with tap water from the remaining sand and epiphytes and was grinded with a Jupiter's Universal grinder (Wernau, Germany) using a 3-mm perforated grill. Grinded algal material was lyophilized, and the dry algal materials were stored in the dark for further analysis.

Indonesian *Sargassum*, i.e. *S. aquifolium*, *S. ilicifolium* and *S. polycystum*, was collected by cutting the thallus as well, approximately 5 cm from the holdfast. Algal material was quickly cleaned with seawater and was kept in closed box to keep them away from direct contact with sunlight. Further, algal material was washed with tap water from the remaining sand and epiphytes. Indonesian *Sargassum*, was naturally dried for five to seven days. To maintain the quality of the target compound, it is crucial to prevent any direct contact with sunlight. Thus, algal material was dried under the shade. After the drying, they were cut to small

pieces with scissor and were grinded by using multi-use home blender.

2.2.3 Determination of the dried algal material

Fresh algal material of 3 g was weighed and placed in glass tube and dried in 100 °C until constant weight of the sample was attained (Gupta *et al.*, 2011). The dry algal material was determined by following formula:

$$DW (\%) = \frac{W_f}{W_i} \times 100$$

D = dry weight of algae express in %

W_f = final weight of algae (g)

W_i = initial weight of algae (g)

2.3 Biochemical composition of algal material

2.3.1 Acid hydrolysis

Ten mg of dry material were introduced to 5 mL of HCl 1 M in different vials. All prepared samples were then put in the heating block of 100 °C for 2 hours with agitation every 30 minutes to optimize the process. Samples were left in the box of ice for 5 minutes. In order to complete the samples concentration to 1 mg/mL, 5 mL NaOH 1 M were added to each different vials. All tubes were kept in 4 °C before further analysis and the designated analysis were protein, sugar, sulfates groups, uronic acid, nitrogen, ash, and polyphenol content analysis.

2.3.2 Water hydrolysis

Ten mg of dry material were introduced to 5 mL of H₂O in different vials. All prepared samples were then put in the heating block of 100 °C for 2 hours with agitation every 30 minutes to optimize the process. Samples were left in the box of ice for 5 minutes. In order to complete the samples concentration to 1 mg/mL,

5 mL H₂O were added to each different vial. All tubes were kept in 4 °C before further analysis and the designated analysis were protein, sugar, sulfates groups, uronic acid, nitrogen, ash, and polyphenol content analysis.

2.3.3 Protein content analysis

Dosage of total protein is using the BSA (Bovine Serum Albumin) method based on the reaction of Biuret reagent which results in the reduction of Cu²⁺ ion to Cu⁺ by the protein in the alkaline medium. Twenty-five µL of standards (Bovine Serum Albumin) or samples was introduced into the micro plate well then added with 200 µL of reactive A + B (Pierce BCA protein assay kit, Thermo Scientific, 23227). Bovine Serum Albumin was prepared in different concentration ranged from 0 – 100 µg/mL. The micro plate was then incubated for 1 hour in 37 °C. Optical density was then measured at 562 nm. All samples were made in triplicate. Content of protein was calculated using the following formula:

$$\% = \left[\frac{(OD - b)}{a} \right] \times D \times \frac{1}{1000} \times 10$$

With OD is the optical density of the designated analysis b is the intercept of the calibration curve, a is the slope of the calibration curve, D is 1/factor of dilution, 1/1000 is the conversion of mg from µg, 10 is from 1 mg samples and conversion to % (Smith *et al.*, 1985; Kessler and Fanestil, 1986; Wiechelman *et al.*, 1988; Brown and Jeffrey, 1992).

2.3.4 Sugar content analysis

The principle of this analysis is based on the formation of degradation products of sugars by the action of a strong acid, derivatives of furfural. These chromogenic products are then condensed with Polyphenols to obtain a chromophore. Anhydrous glucose served as standard for the calibration curve prepared in

different concentration ranged from 0 – 100 µg/mL.

One mL of samples (dilution 1/10) or standard was put in the tubes then added with 50 µL of phenol 75 % and followed by agitation by using the vortex. 2.5 mL of sulfuric acid was added and put them in the cold water of 20 °C for 10 minutes. After 10 minutes, all samples were agitated and placed in the water bath of 30 °C for 10 minutes. Optical density is read against the blank at 490 nm. All samples were made in triplicate (Dubois *et al.*, 1956).

$$\% = \left[(OD - b) / a \right] \times D \times 1/1000 \times 10$$

With OD is the optical density of the designated analysis b is the intercept of the calibration curve, a is the slope of the calibration curve, D is 1/factor of dilution, 1/1000 is the conversion of mg from µg, 10 is from 1 mg samples and conversion to % (Smith *et al.*, 1985; Kessler and Fanestil, 1986; Wiechelman *et al.*, 1988; Brown and Jeffrey, 1992).

2.3.5 Sulfates groups content analysis

This assay is based on the colorimetric method by Jacques *et al.* (1986) (Guillaume, 2010) which reacts specifically with sulfate groups linked to the polysaccharide backbone. This reaction is based on the formation of a complex between sulphate groups and the 3- amino-7-(dimethylamino) phenothizin-5-ium (Azure A, Sigma-Aldrich A6270) to give a purple. 17 % of dextran sulfate in concentration ranging from 0 – 100 µg/mL served as standard. 200 µl of samples or standard was added with 2 mL of Azure A (10 mg/L) and the optical density against the blank was read at 535 nm. All samples were made in triplicate.

$$\% = \left[(OD - b) / a \right] \times D \times 1/1000 \times 10$$

With OD is the optical density of the designated analysis b is the intercept of the calibration curve, a is the slope of the calibration curve, D is 1/factor of dilution, 1/1000 is the conversion of mg from μg , 10 is from 1 mg samples and conversion to % (Smith *et al.*, 1985; Kessler and Fanestil, 1986; Wiechelman *et al.*, 1988; Brown and Jeffrey, 1992).

2.3.6 Uronic acid content analysis

Uronic acid could react to the presence of meta-hydroxydiphenyl (MHDP) and this reaction creates a pink coloration. To enhance the coloration, tetraborate is added and the addition of sulfamic acid minimizes the interferences of neutral sugars detection in the presence of sulfuric acid (Blumenkrantz and Asboe-Hansen, 1973).

Diluted samples solution or standard of 400 μL was added with 40 μL of sulfamic acid 4 M. Prior to the introduction of 2.4 mL of sodium tetraborate 75 mM, the solution was agitated and re-agitated afterward. All samples were incubated for 20 minutes at 80 °C then put in the ice for 5 minutes. Then, 80 μL of MHDP 0.15 % was then added and the optical density was read at 525 nm. Glucuronic acid with concentrations ranged from 0 – 400 $\mu\text{g/mL}$ served as standards for the calibration curve (Guillaume, 2010).

$$\% = \left[(OD - b) / a \right] \times D \times 1/1000 \times 10$$

With OD is the optical density of the designated analysis b is the intercept of the calibration curve, a is the slope of the calibration curve, D is 1/factor of dilution, 1/1000 is the conversion of mg from μg , 10 is from 1 mg samples and conversion to % (Smith *et al.*, 1985; Kessler and Fanestil, 1986; Wiechelman *et al.*, 1988; Brown and Jeffrey, 1992).

2.3.7 Nitrogen content analysis

By using the Kjeldahl method, there are three main steps to follow. First is the digestion, i.e. the decomposition of nitrogen in organic samples by utilizing a concentrated acid solution. This is accomplished by boiling the samples in concentrated acid in order to obtain ammonium sulfate solution as a final product. Second step is distillation – adding excess base to the acid digestion mixture to convert NH_4^+ to NH_3 followed by boiling and condensation of the NH_3 gas in a receiving solution. Last step is the titration, i.e. to quantify the amount of ammonia in the receiving solution (Bradstreet, 1965).

One hundred mg of dry material and 2.5 g of catalyst were introduced in to the glass tube. The catalyst is composed of 100 g potassium sulfate, 20 g of copper sulfate pentahydrated and 2 g of selenium. Later, 15 mL of sulfuric acid (96 – 97%) was added along with several glass beads. All the glass tubes specialized for the nitrogen analysis were put in the mineralizer support at 350 °C for 30 min. BSA (Bovine Serum Albumin) was used as control. All samples and controls were analyzed in triplicate.

After 30 min of mineralizing process, the samples were then distilled by introducing 50 mL of boric acid, concentration 40 g/L, in to the distiller. The distiller was set up before running the analysis with following conditions: 60 mL of concentrated sodium hydroxide (30%) in 5 min (time of analysis). 0.5 mol/L of sulfuric acid was prepared for the titration. The acid was introduced slowly while in to the samples that have been distilled (the distillate) added with a few drops of Taschiro indicator while mixing them on the magnetic stirrer. During the addition of acid sulfuric onto the distillate, the changing color of distillate was observed and the volume of acid needed to cause the color to change was noted. As the molarity of acid sulfuric is changeable depends on the samples.

2.3.8 Ash content analysis

Ash is the general term used to describe the inorganic matter in a fuel. In biomass fuels, the ash content may originate from the biomass itself, e.g. materials that the plant absorbed from the water during its growth. In food analysis, ash content represents the total mineral content. Ashing is the first step in preparing a food sample for specific elemental analysis. Since certain foods are high in particular minerals, ash becomes important (Marshall, 2010).

The principle is to incinerate materials at high temperature (525 °C or higher). 100 mg of dry material was put in the oven of 100 °C for 30 minutes then burnt on the Bunsen flame for 10 minutes. Later, the samples were kept in the incinerator of 585 °C for 3 hours. Prior to measure their final weight, all samples were put in the desiccators for 1 hour.

$$\% = \frac{M_f}{M_i} \times 100$$

M_f = final mass of algae (g)

M_i = initial mass of algae (g)

2.3.9 Polyphenols content analysis

Folin-Ciocalteu is a commonly used method for measuring the polyphenols content. This assay is based on the reduction-oxidation (redox) reactions, which are usually considered to be relatively stoichiometric and on the redox potential of the polyphenols hydroxyl group (Hagerman and Butler, 1989).

20µl of samples (0.25 mg/mL) or standard (Phloroglucinol) was added with 130 µl H₂O was then introduced to the samples or standard solution followed by 40 µl of Folin-Ciocalteu. In the end, 40µl mL of Na₂CO₃ 200 g/L was added. The reaction was then incubated in 70 °C for 10 min and put in the

box of ice to stop the reaction for 5 min. Then the optical density was read against the blank prepared at 620 nm. Phloroglucinol was used as a standard for the calibration curve prepared in different concentrations ranged from 0 – 100 µg/mL (Le Lann *et al.*, 2008).

$$\% = \left[(OD - b) / a \right] \times D \times 1/1000 \times 10$$

With OD is the optical density of the designated analysis b is the intercept of the calibration curve, a is the slope of the calibration curve, D is 1/factor of dilution, 1/1000 is the conversion of mg from µg, 10 is from 1 mg samples and conversion to % (Smith *et al.*, 1985; Kessler and Fanestil, 1986; Wiechelman *et al.*, 1988; Brown and Jeffrey, 1992).

2.4 Extraction phenolic compounds

2.4.1 Solid-liquid extraction (SLE)

There were four different solvents chosen for the polyphenols extraction, there were water, methanol 50 % (v/v), pure methanol and ethanol 75 % (v/v). Later, 40 °C and 3 h were chosen as temperature and time of extraction, it was based on the study performed by Tanniou (2014).

Twenty-five g of dry algal material was diluted in 300 mL of solvents. The filtered samples were then evaporated until dryness, then, the dried extract obtained was then added by 20 mL of H₂O in order to get the crude extract. Finally, the raw extracts were frozen and lyophilized for further analysis, *i.e.* polyphenols content, bioactivity analysis and chromatographic analysis.

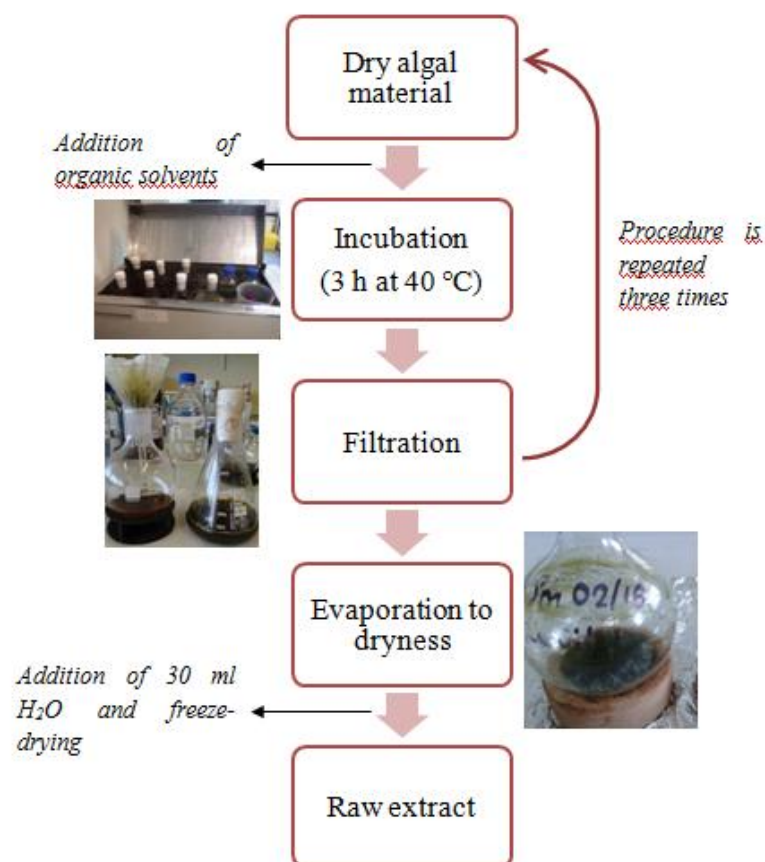


Fig 2-4 Solid-liquid extraction of *Sargassum* polyphenols.

2.4.2 Enzyme-assisted extraction (EAE)

In this study, 19.5 g of dry algal material was diluted in 300 mL of water, then 2.5 % (of dry algal material) of commercial enzyme (two protease and six carbohydrase) was added. Sample solutions were incubated for 3 hours in 50 °C. In advance of filtration, all samples were denatured for 20 minutes in 90 °C. The soluble samples were then frozen and lyophilized for further analysis. As for the insoluble samples (residues), they were stored in – 20 °C. The eight enzymes used were Neutrase, Ultraflo, Alcalase, AMG, Shearzyme, Termamyl, Viscozyme and Celluclast. Method was based on Heo *et al.* (2003) and Hardouin *et al.* (2014). Table 2-3 shows details of the commercial enzymes used in the EAE screening. Aqueous extraction served as control, *i.e.* extraction without enzyme. Figure 2-4 shows the screening of *Sargassum* enzyme-assisted extraction of

polyphenol compounds.

Table 2-3 Commercial enzymes applied for the EAE and their optimal conditions based on Kadam *et al.* (2013) and Wijesinghe and Jeon (2012).

Enzymes	Type of enzymes	pH (optimal)	T °C (optimal)
Neutrase	Protease	5.5-7.5	50
Ultraflo	Carbohydrase	7.0	60
Alcalase	Protease	6.5-8.5	50
AMG	Carbohydrase	4.5	60
Shearzyme	Carbohydrase	6.8-7.2	50
Termamyl	Carbohydrase (endoamylase)	6.0	60
Viscozyme	Carbohydrase	3.3-5.5	50
Celluclast	Carbohydrase	4.5	50

For optimizing the conditions of phlorotannin extraction, several different parameters were chosen, i.e. temperature and concentration of enzyme applied. Based on the first screening of EAE by using eight commercial enzymes, enzyme that extracted the highest polyphenol was chosen for optimization. In addition, another enzyme was introduced in this part of work, which is the Protamex as a protease. Protamex is known as enzyme that works optimal at 40 °C, which is also favorable for polyphenol extraction (Park *et al.*, 2005).

The extraction procedure was almost the same as previously described (Heo *et al.*, 2003; Hardouin *et al.*, 2013). The difference lied in the temperature and the concentration of enzyme. Instead of 50°C, 40°C was applied. Then, as for the enzymes concentration, 5% and 10% were determined in order to comprehend whether or not the increase of enzyme concentration would also increase the polyphenols extraction. In order to enhance the recovery of polyphenols extraction, denaturation of enzyme by heating in 90°C for 20 minutes was eliminated. Therefore, after the filtration, soluble enzymatic extracts were directly stored in the freezer of -20 °C in order to stop the reaction. 40 °C has been proven to be the most optimal temperature for the polyphenols compound extraction. An elevated temperature above 40 °C might degrade the compound. All steps of extraction were conducted in the dark in order to prevent the oxidation of the

compound.

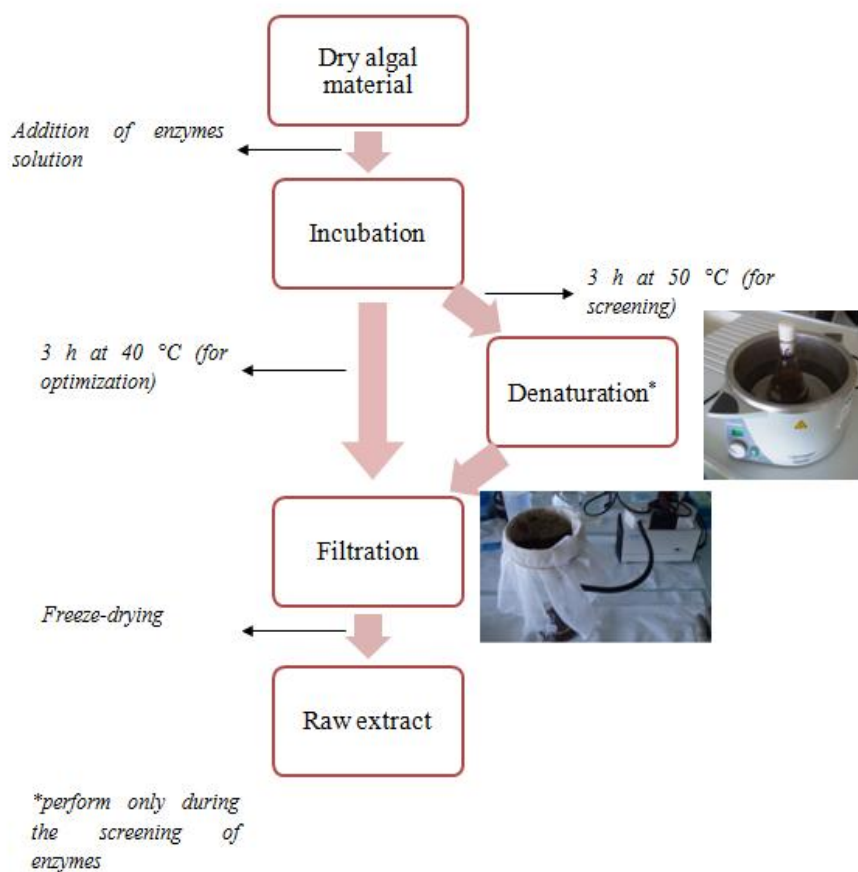


Fig 2-5 Enzyme-assisted extraction of *Sargassum* sp. polyphenols (screening of enzymes).

2.4.2.1 Solid-phase extraction of *Sargassum muticum* enzymatic extract

Solid-phase extraction (SPE) is extraction method using a solid and a liquid phase to isolate and to concentrate a target compound, also called as analyte, from a solution. Main principle of this method is to introduce a sample into compatible sorbent, then to allow it to be absorbed by the sorbent and then to elute it with suitable solvents.

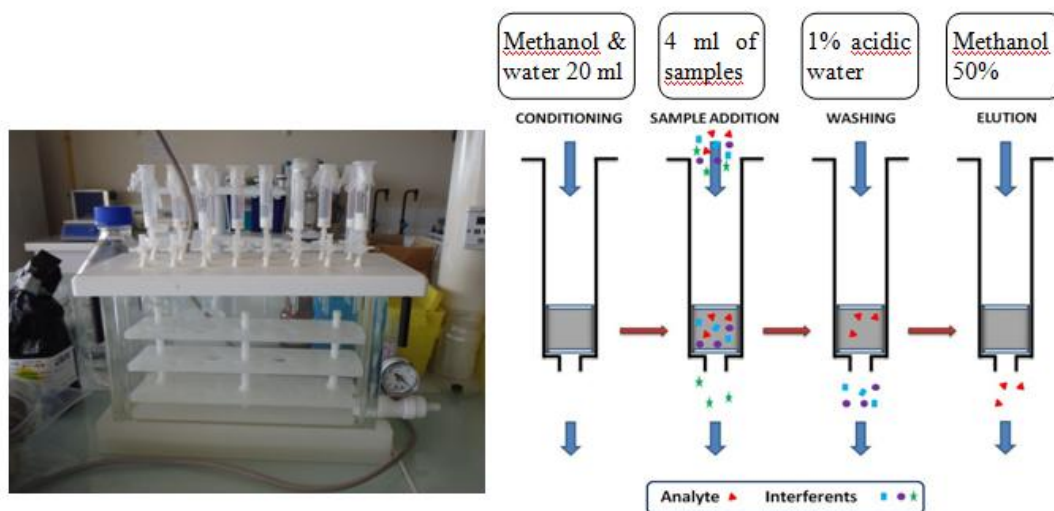


Fig 2-6 Solid-phase extraction of *S. muticum* polyphenols.

SPE was performed by using reversed phase C-18 cartridges based on method performed by Tanniou (2013), with slight modification (Fig 2-6). Prior to the fractionation process, the cartridges were conditioned with the following elution; 20 mL methanol and 20 mL ultrapure H₂O successively. Later, 4 mL of raw extracts (5 mg/mL) was loaded into the cartridge HyperSepTM C18 from Thermo ScientificTM (40-60 μ m, 1 g, 60 Å, 6 mL) and left to absorb the column (time). Prior to elution, the cartridge was washed by 1% acidic water to remove sugars, organic acids, and other water-soluble constituents (Dai and Mumper, 2010). Phlorotannins were then eluted with 40 mL methanol 50% (v/v) (flow rate ~3 mL/min at positive pressure). All processes were repeated four times in order to accumulate sufficient amount of fractions. Same fractions were pooled and evaporated under reduced pressure at 40 °C by a rotary evaporator. Each fraction was re-dissolved using acetonitrile-methanol (2:1; v/v). Polyphenol content of methanol fraction was determined with the Folin-Ciocalteu method and its antioxidant activity was tested via DPPH and FRAP.

2.5 Characterization of enzymatic extracts

2.5.1 Fourier transform infrared spectroscopy (FT-IR)

FT-IR is a vibrational spectroscopic technique and its principle is based on the facts that most molecules absorb light in the infra-red wavelength region of the electromagnetic spectrum. This absorption corresponds specifically to the chemical bonds present in molecules. FT-IR can facilitate to confirm the identity of a pure compound by using the unique collection of absorption bands.

Spectra of *S. muticum* enzymatic extracts was determined using a Nicolet™ iS™ 5 FT-IR spectrometer (Thermo Scientific™) with diamond crystals plates. The spectra were collected with subsequent scanning of samples in triplicate ranging from 4000-400 cm⁻¹. 2 mg of dry enzymatic extracts were introduced onto the crystal plate. The spectra were then acquired as the percentage of absorbance. The detected peaks were then compared with a standard of phloroglucinol.

2.5.2 High performance liquid chromatography (HPLC) analysis

Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Reversed phase HPLC (RP-HPLC) used in this study has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica, which has been surface-modified with RMe₂SiCl, where R is a straight chain alkyl group such as C₁₈H₃₇ or C₈H₁₇. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis).

The analytical chromatographic analysis was performed by UltiMate 3000

Rapid Separation LC (RSLC) System consisted of pump, auto-samplers and diode array detector (DAD-3000RS) with UV-vis detection (Thermo Scientific™). The column was a Nucleodur® RP C₁₈ (250 x 4.6 mm i.d; 5 µm) of Macherey-Nagey. The reversed-phase HPLC analysis was performed based on the modified method of Koivikko et al. (2007) and Sanchez-Camargo et al. (2015). *S. muticum* extracts were eluted by a multi-step gradient of solvents A (1% acid formic in water) and B (acetonitrile). The elution was as following; 0 – 20 min: 5% B, 20 – 35 min: 20 % B, 35 – 45 min: 80 % B, and 45 – 55 min: 0 % B. Polyphenols compounds were detected at 280 nm, while the wavelength was set ranging from 240 – 300 nm. The injection volume was 100µL with 1 mL/min flow-rate at 150 bar. Internal standard range of phloroglucinol was prepared with concentration ranging from 100 – 800 µg/mL.

2.6 Antioxidant activity

2.6.1 DPPH radical scavenging assay

2,2-diphenyl-1-picrylhydrazyl or DPPH is a stable free radical. DPPH assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. DPPH shows a strong absorption maximum at 517 nm.

A series of butylated hydroxyl-anisole (BHA), butylated hydroxyl-toluene (BHT) (respectively 2(3)-*t*-Butyl-4-hydroxyanisole, and 2,6-Di-*tert*-butyl-4-methylphenol, Sigma, Saint Quentin Fallavier, France) solution – serves as positive standard – is prepared in different concentrations (2 – 50 µg/mL). DPPH solution of 0.25 mM is introduced to 100 µL samples solution. Samples solution of *S. muticum* hydrolyzates was made in different concentration by diluting the solution stock in methanol (0 – 10 %). Before reading the optical density at 517 nm, all samples are incubated in 40 °C for 30 minutes. Then, the percentage of inhibition is calculated by using following formula:

$$I (\%) = \left[\frac{(A_{control} - A_{sample})}{A_{control}} \right] \times 100$$

With $A_{control}$ = absorbance of control and A_{sample} = absorbance of samples

IC₅₀ of samples is determined based on the regression obtained from dose response curve. IC is defined at the concentration sufficient to obtain 50 % of a maximum scavenging capacity.

2.6.2 FRAP (ferric reducing antioxidant power)

This method is based on the reduction of a ferroin analog, the complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely blue colored complex $Fe(TPTZ)^{2+}$ by the presence of antioxidants in acidic medium. Then, the results are obtained from the absorbance at 593 nm and expressed as μM Fe^{2+} equivalents (Benzi and Strain, 1996; Benzie and Strain, 1999).

The reagent of TPTZ consisted of 300 mM of acetate buffer with pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) and 20 mM $FeCl_3 \cdot 6H_2O$ solution. 50 μL of *S. muticum* enzymatic extracts was introduced in microplate mixed with 150 μL reagent. After 15 minutes of incubation, the absorbance was read at 593 nm. The results were expressed in μM equivalent to $FeSO_4 \cdot 7H_2O$ calculated from the calibration curve (Shahwar *et al.*, 2012).

2.7 Tyrosinase inhibition activity

Tyrosinase or polyphenol oxidase is an oxidoreductase that participates in the biosynthesis of melanin, a ubiquitous biological pigment found in human's skin, hair, etc. Presence of L-DOPA (L-3,4-Dihydroxyphenylalanine) as substrate results the formation of dopaquinone leading to melanin production. Under

distressed condition, melanine production could cause hypo or hyper pigmentation on human's skin. Potential tyrosinase inhibitors are examined in the presence of L-tyrosine or L-DOPA as enzyme substrate, and tyrosinase activity is assayed in terms of dopachrome formation (Chang, 2012).

Solution of 80 μ L phosphate buffer is introduced to 20 μ L of sample solution added with 20 μ L of DMSO (dimethyl sulfoxide) 50%. Later, 40 μ L of L-DOPA is added to the solution followed with 40 μ L tyrosinase to complete the reaction. Before reading the absorbance at 475 nm, all microplates were incubated at 37 °C for 30 minutes. Kojic acid and quercetin serving as control were prepared at different range of concentration (0.02 – 0.4 %). Percentage of inhibition is calculated by following formula:

$$I (\%)_{at\ x\ min} = \left[\frac{(A_{control} - A_{sample})}{A_{control}} \right] \times 100$$

Where:

$$A_{control} = (DO_{control\ (x\ min)} - DO_{control\ (x\ min)}) - (DO_{control\ (0\ min)} - DO_{control\ (0\ min)})$$

$$A_{sample} = (DO_{sample\ (x\ min)} - DO_{sample\ (x\ min)}) - (DO_{sample\ (0\ min)} - DO_{sample\ (0\ min)})$$

$A_{control}$ = absorbance (optical density/DO) of control

A_{sample} = absorbance (optical density/DO) of samples

2.8 Elastase inhibition activity

As inhibitor, trypsin inhibitor type II-O was used prepared in phosphate buffer in final concentration 1 g/L. All tested samples were dissolved in DMSO 10% with

final concentration same as standard. The activity of porcine pancreatic elastase (Type I; Sigma Chem. Co) was examined using substrate (N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide/ N-Suc-(Ala)₃-nitroanilide), and the release of p-nitroaniline at 405 nm was measured.

The reaction of 20 µL samples was carried out in 80 µL of 100 mM Tris and 500 mM NaCL pH 8, 50 µL of 0.4 U/mL elastase solution, and 50 µL of 0.64 mM substrate. The elastase inhibition was assessed every 10 minutes at 25°C during 40 minutes of reading. Same reactions applied for standard, but the volume of standard introduced in the well ranged between 0 – 100 µL ($c_f = 0 - 0.5$ mg/mL) in order to obtain the calibration curve. All reactions were made in triplicate (Sallenave *et al.*, 1998). The percent inhibition of elastase was calculated as follows:

$$I (\%) = \left[(A - B) / A \right] \times 100$$

Where A = absorbency at 405 nm without the samples/standards, and B = the change in absorbency with samples/standards.

2.9 Antimicrobial activity

2.9.1 Antibacterial activity

Agar diffusion method (Bauer *et al.*, 1996) was chosen as method for evaluating the antibacterial activity of *S. muticum* hydrolyzates. Two gram positive bacteria – *Staphylococcus hominis* and *Bacillus subtilis* – as well as two gram negative bacteria – *Pseudomonas aeruginosa* and *Escherichia coli* – were chosen for this test. These bacteria were prepared in liquid nutrient broth media with bacterial density 1.5×10^8 cfu/mL. Bacterial suspension was poured on to solidified agar media and incubated for 1 h in 37 °C.

Sargassum sp. enzymatic extracts of final concentration 5 mg/mL were prepared in sterile physiological water. 20 µL of tested samples solution – enzymatic extracts solution or positive control (Phosphomycin, Ampicil and Streptomycin) – was introduced to sterile paper disc. All samples were made in triplicate. Later, the impregnated sterile paper discs were placed on the agar media in accordance with samples coding made in advance. The petri dishes were then incubated at 37 °C for 48 h. Zone of inhibition established on the agar media was then measured and expressed in cm. Antibacterial activity was represented by following formula:

Antibacterial activity = Diameter of inhibition zone (cm) – 0.6 cm (diameter of paper disc)

2.9.2 Biofilm Inhibition Activity

This test is based on the Crystal Violet Assay. *E. coli*, *P. aeruginosa* and *B. subtilis* were selected as biofilm forming bacteria. In this assay, the dye that is bounded to adherent cells is resolubilized and measured in optical density (OD). Prior to analysis, bacterial solution of 1.5×10^8 cells in physiologic sterile water 10% was made by incubating the bacteria culture in Lisogeny Broth (LB) sterile medium at 37 °C for 24 h. Later, the bacteria culture was centrifuged for 5 minutes at 5000 rpm and the supernatant was discarded. The remaining pellet was dissolved in physiologic sterile water 10%.

Aliquot of 200 µL bacterial solution was then introduced into a sterile 96-well microplate and left for 24 hours at 37 °C in order to allow the bacteria to form a film layer in the microplate. After 24 hours of incubation, the plate was washed two times with the physiologic sterile water 10%. Then, 200 µL of *S. muticum* enzymatic extracts (1 mg/mL) dissolved in LB were added into the microplate and incubated for 24 h in 37 °C. In the next 24 h, the microplate was rinsed two times with physiologic sterile water 10% and introduced with 200 µL

of 10% crystal violet – prepared in ultrapure water. 15 minutes after the crystal violet was added, subsequently, the microplate was rinsed and the biofilm formed in the microplate was then suspended in 200 μ L of ethanol. The microplate was later read in 600 nm. Negative and positive control consisted of physiologic sterile water 10% and bacteria were applied. Both the control and the samples were made in triplicates.

2.9.3 Antiviral and cytotoxicity activity

Antiviral activity of *S. muticum* enzymatic extracts and their cytotoxicity were analyzed. For cytotoxicity, the kidney cells of African green monkey (Vero cells) were used. Herpes-simplex virus type-1 (HSV-1) model was used to determine the antiviral effect.

Aliquots of 100 μ L cell suspension (3.5×10^5 cells/mL) in Eagle's MEM (minimum essential medium) containing 8 % FCS (fetal calf serum) was incubated with 50 μ L of diluted enzymatic extract (concentration from 10 to 500 μ g/mL) in 96-well plates (72 h, 37 °C, 5 % CO₂). Three replicates were infected using 50 μ L of medium and a virus suspension at a multiplicity of infection (MOI) of 0.001 ID₅₀ cells⁻¹. After incubation, antiviral activity was evaluated by the neutral red dye method. Zovirax, a commercial antiherpetic acyclovir (9-2(2-hydroxyethoxymethyl) guanine), was used as reference inhibitor (positive control).

For antiviral, 50% effective antiviral concentration (EC₅₀) was expressed as the concentration that achieved 50% protection of virus-infected cells from virus-induced destruction. The optical density (OD) was related directly to the percentage of viable cells, which was inversely related to the cytopathic effect (CPE). The linear regression was determined for each assay on the basis of cell controls (0% CPE) and virus controls (100% CPE). Data were expressed as a percentage of protection (%P):

$$\left| \frac{((OD_t)_{virus} - (OD_c)_{virus})}{((OD_c)_{MOCK} - (OD_c)_{virus})} \right| \times 100$$

where $(OD_t)_{virus}$ is the OD of the test sample, $(OD_c)_{virus}$ is the OD of the virus control and $(OD_c)_{MOCK}$ is the OD of the mock-infected control (McLaren *et al.*, 1983; Langlois *et al.*, 1986).

For cytotoxicity, 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced the OD of treated cells to 50% of that of untreated cells. Data were expressed as a percentage of protection (%D):

$$\left| \frac{((OD_c)_C - (OD_c)_{MOCK})}{(OD_c)_C} \right| \times 100$$

where $(OD_c)_C$ and $(OD_c)_{MOCK}$ are the OD values of untreated cells and treated cells, respectively (Langlois *et al.*, 1986).

2.10 Statistical Analysis

Data obtained in this study was based on the average value from triplicate and the data was presented as mean value \pm standard of deviation, with $n = 3$. Analysis of variance was performed to analyze the significant difference caused by the independent variables ($p < 0.05$) by using IBM SPSS Statistics 23. If there was any significant difference observed than Tukey's post-hoc test was used to evaluate which variables showed a significant difference to the other. A significant difference from single independent variable was assigned by different superscript letters. As for analysis with multiple independent variables, the data was presented together with its post-hoc test for better description of which independent variables contributed to the significant difference.

Spectrophotometer data obtained in this study were presented as the mean value with its standard deviation (mean \pm standard deviation), $n = 3$. Boxplot of *S. muticum* biochemical composition from February 2014-February 2016 would be

presented as result. Later, for the seasonal variation data of *S. muticum*, they were statistically analyzed using analysis of variance in order to determine whether there was a significant difference according to the seasons of collection ($p < 0.05$). Tukey's post-hoc test was conducted to evaluate the significant difference. Pearson's correlation was performed to view the relationship between the environmental condition and the biochemical composition of *S. muticum*. Principal Coordinate Analysis was applied to view the tendency of *S. muticum* biochemical composition content in different seasons.

For the spectroscopy data of *S. muticum* from FT-IR analysis, the Principal Component Analysis (PCA) was chosen to group the biochemical composition based on seasons. This grouping was facilitated by selecting certain wavenumbers that corresponded to the analyzed *S. muticum* biochemical composition. Later, for the chromatogram data of *S. muticum* from HPLC analysis, the Principal Component Analysis (PCA) was chosen to group the polyphenols content based on seasons. This grouping was facilitated by selecting certain retention time of *S. muticum* polyphenols that corresponded to phloroglucinols.

Chapter 3 Results and Discussions

3.1 Biochemical composition of *Sargassum*

3.1.1 Indonesian *Sargassum* sp.

Table 3-1 presents the biochemical composition of *S. aquifolium*, *S. ilicifolium*, and *S. polycystum*. *S. aquifolium* and *S. ilicifolium* were species collected on April 2014 at Telukawur, Jepara and *S. polycystum* was collected on October 2013 at Panjang Island, Jepara.

The biochemical compositions of *Sargassum* analyzed in this study were protein, sulfates groups, neutral sugar, uronic acid, nitrogen, polyphenol and ash. Prior to analysis, all dry materials were hydrolyzed using hydrochloric acid (HCl) and ultrapure water (H₂O Milli-Q), except for the nitrogen and ash content that used directly the dry material.

Uronic acid, protein, sugar and polyphenol contents of *S. ilicifolium* were the highest with $7.8 \pm 3.3\%$, $30.5 \pm 1.2\%$, $10.8 \pm 0.2\%$, and $2.1 \pm 0.4\%$ of dry algal material, respectively. In *S. aquifolium*, there were only $4.5 \pm 2.0\%$, $20.9 \pm 1.1\%$, $8.4 \pm 0.1\%$, $10.7 \pm 0.6\%$, and $1.7 \pm 0.3\%$ of uronic acid, protein, sugar, sulfates groups, and polyphenol contents, respectively. *S. polycystum* contained the highest sulfates groups ($13.7 \pm 0.7\%$) However, the nitrogen content of these two species was the same, with 1.6% of dry algal material (Table 3-1).

Ash content of *S. polycystum* ($33.6 \pm 1.9\%$) was higher compared to *S. aquifolium* ($32.5 \pm 0.3\%$) and *S. ilicifolium* ($29.0 \pm 2.6\%$). Different case showed with the protein and nitrogen content of *S. polycystum*, apparently, it had the lowest content with 19.8 ± 0.6 and $1.0 \pm 0.2\%$ of dry algal material, respectively. Nevertheless, *S. polycystum* contained more uronic acid, sugar, and sulfates groups contents than the *S. aquifolium*. In the other hand, they both had 1.7% of polyphenol.

Table 3-1 Biochemical composition of Indonesian *Sargassum* species.

Algae	Content (% dry algal material)								
	Uronic acid	Protein	Sugar	Sulfates groups	Polyphenol	Ash	Nitrogen	Total	Other
<i>S. aquifolium</i>	4.5 ± 2.0	20.9 ± 1.1	8.4 ± 0.1	10.7 ± 0.6	1.7 ± 0.3	32.5 ± 0.3	1.6 ± 0.1	69.2	30.8
<i>S. ilicifolium</i>	7.8 ± 3.3	30.5 ± 1.2	10.8 ± 0.2	11.4 ± 0.5	2.1 ± 0.4	29.0 ± 2.6	1.6 ± 0.1	77.1	22.9
<i>S. polycystum</i>	6.1 ± 2.6	19.6 ± 0.6	10.3 ± 0.1	13.7 ± 0.7	1.7 ± 0.3	33.6 ± 1.9	1.0 ± 0.2	72.0	28.0

Table 3-2 Average of *S. muticum* biochemical composition based on year of collection.

Year	Content (% dry algal material)								
	Uronic Acid	Protein	Sugar	Sulfates groups	Polyphenol	Ash	Nitrogen	Total	Other
2014	2.0 ± 0.4	24.5 ± 6.4	13.5 ± 2.6	12.2 ± 1.7	1.9 ± 0.7	25.1 ± 4.1	2.0 ± 0.7	81.1	18.9
2015	2.6 ± 1.0	27.2 ± 7.1	12.2 ± 4.3	8.2 ± 1.0	3.1 ± 1.1	23.9 ± 2.8	1.9 ± 0.6	79.1	20.9

Conclusion

Indonesian *Sargassum* contained quite high of ash or total mineral. We acknowledge that there was intra-species variation regarding the biochemical composition, unless for the nitrogen content showing almost the same value.

3.1.2 French *S. muticum* – seasonal variation

Seasonal variation of French *Sargassum* was studied, *i.e.* *S. muticum*. In order to acknowledge the seasonal pattern of their biochemical composition, the analysis was conducted for two complete years, *i.e.* from February 2014 to February 2016 (Table 3-2 and 3-3).

In 2014, the highest intensity of sunlight occurred in June with 270.7 hour/month¹ with 46.2 mm/month of precipitation. The water surface temperature recorded in this month was 18 °C and the maximal air temperature (T_{\max}) was 25 °C¹. Meanwhile in 2015, the seawater surface temperature (SST) in June was 16 °C¹, 2° lower than in 2014 with sunlight intensity 20 hours more (290.7 hour/month¹). During this period, the precipitation rate was the lowest with only 4.2 mm/month¹.

In 2014, the average content of *S. muticum* uronic acid, protein, sugar, sulfates groups, ash, and nitrogen contents were 2.0%, 24.5%, 13.5%, 12.2%, 1.9%, 25.0% and 2.0%, respectively. Meanwhile, the average content of *S. muticum* uronic acid, protein, sugar, sulfates groups, ash, and nitrogen in 2015 were 2.6%, 27.2%, 12.2%, 8.2%, 3.1%, 23.9% and 1.9%, respectively. It seems like that there was a decrease in sugar, sulfate groups, ash and nitrogen content of *S. muticum* in 2015. Results of *S. muticum* biochemical composition are presented on Table 3-2.

Moisture and dry material of *S. muticum* showed a seasonal variation. *S. muticum* moisture content ranged from 74.4-84.4% of total weight, while its dry

¹ The meteorological data was based on the real time observation provided the www.meteociel.fr

material was at 12.9-25.6% of total weight. In 2014, the minimum moisture content was from June as the maximum was on September. Then, in 2015, the minimum moisture content of *S. muticum* was obtained on March and the maximum was on September. Appendix 2 shows the figure of *S. muticum* moisture content and dry material from two years of sampling.

Fig 3-1 illustrates the dry material of *S. muticum* in different period of collection. As can be seen from the picture, the dry material from samples collected from February to July tended to increase followed by a decline from August to December in 2014. Unfortunately, this tendency was not followed in the next year of sampling.

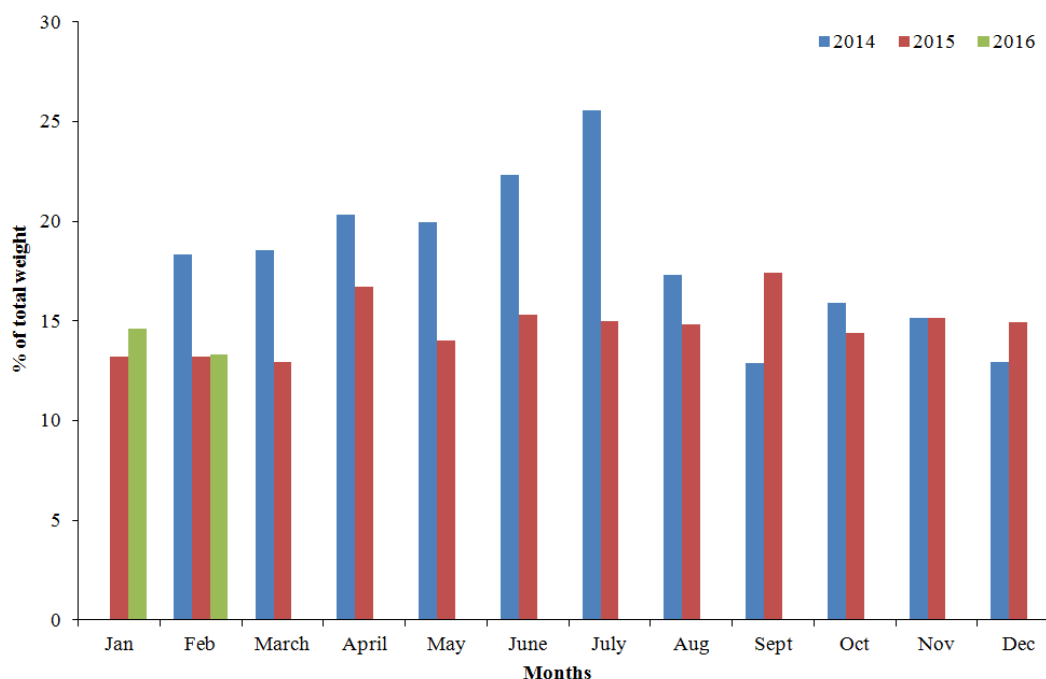


Fig 3-1 Dry material of *S. muticum* in different period of collection (February 2014 – February 2016).

3.1.2.1 Analysis of variance of seasonal effects towards *S. muticum* biochemical composition

Analysis of variance of seasonal effects towards *S. muticum* biochemical composition was analyzed and it revealed that there was a significant difference of biochemical composition caused by season ($p<0.05$). Dry material of *S. muticum*

was significantly influenced by the seasons. As presented in Table 3-3, the significant difference occurred between the spring-summer and the autumn-winter ($p<0.05$).

The protein content of *S. muticum* in summer was significantly lower than spring, autumn and winter ($p<0.05$). In winter and spring, the sugar content was also significantly lower from summer and autumn ($p<0.05$). Polyphenol content of *S. muticum* in autumn appeared to be significantly higher than spring and summer ($p<0.05$). In the contrary with its ash content, *S. muticum* contained significantly higher ash content in autumn and winter than in spring and summer ($p<0.05$). As for its nitrogen content, a significant difference was remarked between summer and spring ($p<0.05$). Apparently, there was no significant seasonal effect towards *S. muticum* uronic acid and sulfates groups content ($p>0.05$). Table 3-4 presents the average value of *S. muticum* biochemical composition in each season of collection from February 2014 – February 2016.

Table 3-3 Seasonal variation of *S. muticum* dry material.

Seasons	Dry material (% of total weight)
Spring 2014-2015	17.1 ± 3.0 ^a
Summer 2014 and 2015	18.4 ± 4.5 ^a
Autumn 2014 and 2015	15.1 ± 1.6 ^b
Winter 2014, 2015, and 2016	14.3 ± 1.9 ^b

Based on the Pearson's correlation, the biochemical composition of *S. muticum* appeared to be related to some of the collected meteorological data. An inversely weak correlation yet significant was shown by the protein content of *S. muticum* towards T_{\max} ($r = -.257$; $p<0.05$) and T_{\min} ($r = -.250$; $p<0.05$). Same case with ash content which had an inverse yet moderate correlation with T_{\max} ($r = -.454$; $p<0.01$), an inversely significant weak correlation with T_{\min} ($r = -.377$; $p<0.01$) and sea surface temperature ($r = -.331$; $p<0.01$). Sunlight gave a significantly inverse but strong correlation with *S. muticum* ash content ($r = -.509$; $p<0.01$). Meanwhile, the correlation of ash content with precipitation was a positive weak correlation ($r = .237$; $p<0.05$).

Regarding the sugar content of *S. muticum*, the effect of SST and precipitation were positively and significantly moderate ($r = ,271$; $p < 0.05$; $r = ,350$; $p < 0.01$). The sugar content also showed a significantly positive yet weak correlation with the T_{\max} ($r = ,267$; $p < 0.05$) and T_{\min} ($r = ,336$; $p < 0.01$). Sunlight had a very weak correlation with sugar content ($r = ,099$; $p > 0.05$ & 0.01). Sulfates groups significantly and moderately correlated to sunlight, T_{\max} and SST with $r = ,378$; $p < 0.01$; $r = ,298$; $p < 0.01$ and $r = ,298$; $p < 0.01$, respectively. In addition, the sulfates groups of *S. muticum* showed an insignificantly very weak correlation with the sunlight ($r = ,099$; $p > 0.05$ & 0.01).

Uronic acid, polyphenol and nitrogen content of *S. muticum*, based on the result obtained in this study, were insignificantly affected with sunlight, T_{\max} , T_{\min} , precipitation and SST ($p > 0.05$ & 0.01). Furthermore, they mostly had a very weak correlation with $r = .00 - .19$. Further detail concerning the Pearson's correlation of *S. muticum* biochemical composition and meteorological data is presented on Appendix 5.

Component loadings and biplot showed the distribution tendency of *S. muticum* biochemical composition related to its seasonal variation as presented on Fig 3-1. Interestingly, the component loading also described the correlation between the biochemical composition and the meteorological as previously described. In the winter, *S. muticum* mostly contained ash and uronic acid in the spring. During winter and spring, *S. muticum* was dominated by the protein content. In the mean time, the polyphenol content was quite abundant in summer and autumn season. As for the sulfates groups, the axe was covered with spring and summer. It indicated that this compound was mostly found during these seasons.

Conclusion

Season, indeed, contributes to the biochemical composition of *S. muticum*, especially in protein, sugar, polyphenols, nitrogen and ash content. Protein and nitrogen content demonstrate same pattern regarding their changing value.

Seasonal effects towards *S. muticum* biochemical composition are usually due to the fluctuating environmental conditions, for example the temperature, salinity, precipitation, and duration of sunlight. Protein and ash content showed an inverse correlation with temperature, however, the ash content of *S. muticum* has a positive correlation with sunlight. Sugar and sulfate groups content in this study had positive correlation with precipitation and sunlight, respectively. In addition, both of these compounds demonstrated a positive correlation with air temperature.

Table 3-4 Seasonal variation of *S. muticum* biochemical composition during February 2014-2016 (content expressed as % of dry algal material). Superscript letters indicate significant difference ($p<0.05$).

Season		Uronic Acid	Protein	Sugar	Sulfates groups	Polyphenol	Ash	Nitrogen	Total	Other
Spring 2014-2015		2.4 ± 1.3^a	27.1 ± 3.4^a	10.9 ± 3.2^a	10.2 ± 2.0^a	2.2 ± 0.4^a	23.5 ± 2.8^a	2.3 ± 0.5^a	78.6	21.4
Summer 2014 and 2015		2.3 ± 0.8^a	21.8 ± 6.4^b	14.8 ± 2.4^b	10.7 ± 3.4^a	2.4 ± 1.0^a	22.4 ± 3.4^a	1.7 ± 0.7^b	76.1	23.9
Autumn 2014 and 2015		2.2 ± 0.4^a	27.2 ± 10.4^a	14.3 ± 3.4^b	9.6 ± 2.4^a	3.2 ± 1.7^b	25.4 ± 3.9^b	2.0 ± 0.6^a	83.9	16.1
Winter 2014, 2015, and 2016		2.2 ± 0.5^a	27.4 ± 2.6^a	11.6 ± 3.7^a	9.3 ± 2.0^a	2.5 ± 0.6^a	25.8 ± 2.3^b	2.0 ± 0.4^a	80.8	19.2

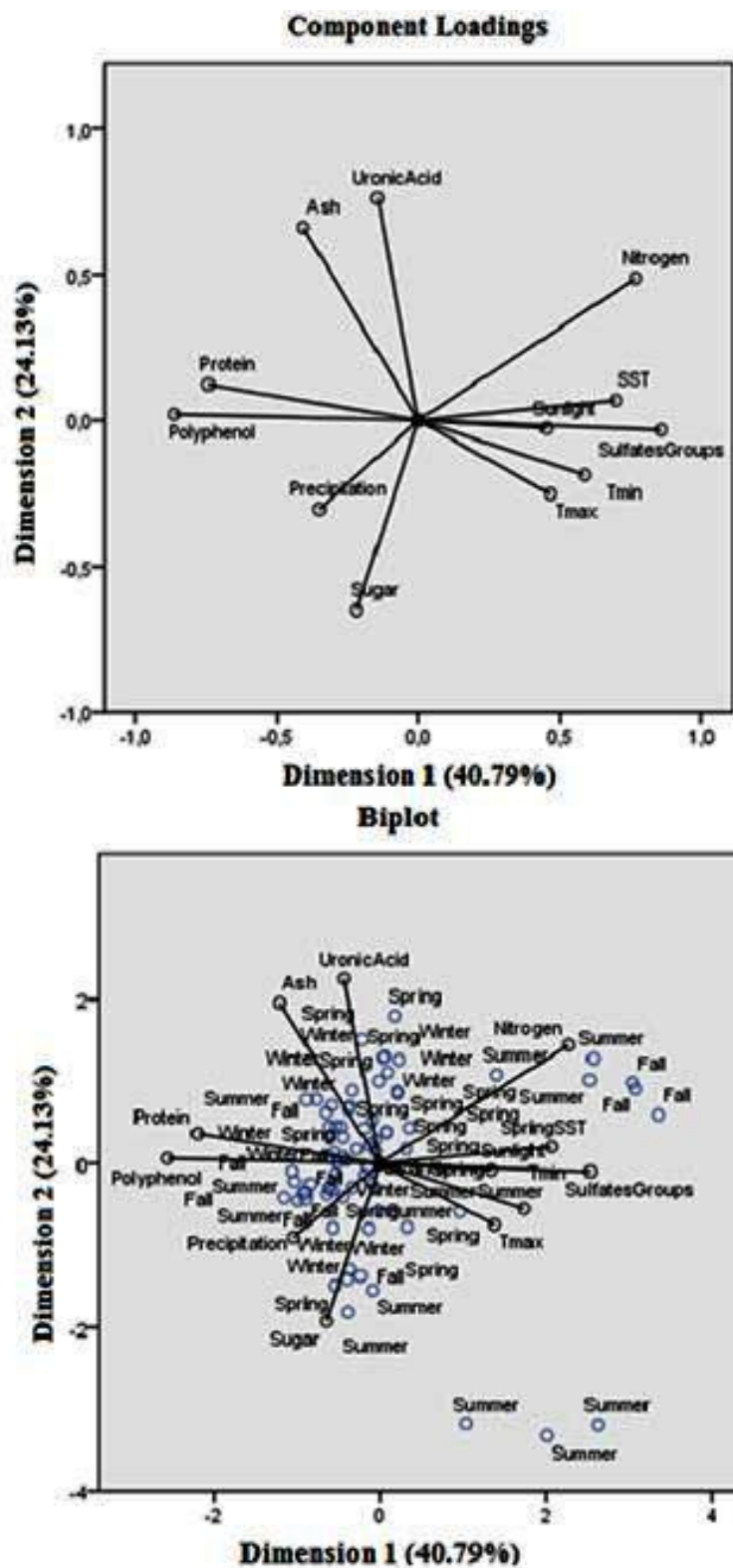


Fig 3-2 Component loadings and biplot of Principal Coordinate Analysis of *S. muticum* biochemical composition

3.2 Extraction of *Sargassum* polyphenol

3.2.1 Seasonal variation of *S. muticum* extracted with different solvents

Extraction yield of *S. muticum* ranged from 8.0% to 24.1% of dry algal material as presented in Table 3-5. Results indicated that there was a seasonal effect towards the extraction yield of *S. muticum* ($p < 0.05$). From four solvents applied for the extraction of *S. muticum*, water resulted higher yield than the other solvents. Aqueous methanol placed in second after water for the extraction yield of *S. muticum*, followed by the aqueous ethanol. Methanol yielded the lowest extraction yield.

Table 3-5 Extraction yield of *S. muticum* extracted with different solvents (expressed in % of dry algal material). Superscript letters indicate significant difference at $p < 0.05$.

Season	Water	Methanol	Aqueous methanol 50%	Aqueous ethanol 75%
Spring	24.0 \pm 5.0 ^a	11.6 \pm 2.6 ^a	14.8 \pm 3.5 ^a	8.7 \pm 3.6 ^a
Summer	21.5 \pm 5.8 ^{ab}	9.8 \pm 2.4 ^a	10.8 \pm 3.9 ^b	12.3 \pm 3.1 ^a
Autumn	17.7 \pm 0.8 ^b	11.3 \pm 1.2 ^a	13.9 \pm 2.8 ^a	14.9 \pm 2.2 ^b
Winter	24.1 \pm 4.5 ^a	10.0 \pm 1.9 ^a	8.6 \pm 2.4 ^b	8.0 \pm 1.4 ^a

3.2.1.1 Polyphenol content of *S. muticum* extracted with different solvents based on season

Seasonal variation of *S. muticum* polyphenol extracted in different solvents, *i.e.* water, methanol, aqueous methanol 50% and aqueous ethanol 75% was evaluated. Fig 3-3, 3-4, 3-5 and 3-6 present the seasonal variation of polyphenol content from water, methanol, aqueous methanol 50% and aqueous ethanol 75% extracts of *S. muticum*. Different letters that is assigned in each bars represented a significant difference between variables ($p < 0.05$).

Results from analysis of variance exhibited that seasonal collection of *S. muticum* gave significant effect to its polyphenol content ($p < 0.05$). The results from Tukey's post-hoc test informed that in water extract of *S. muticum*, its polyphenol showed significant difference in its content between winter-spring and autumn-summer ($p < 0.05$). Then, the content of *S. muticum* polyphenol, extracted

with aqueous methanol 50% and collected in summer, was significantly higher than that of from spring and winter ($p < 0.05$). There was no significant difference in polyphenol content of *S. muticum* from winter and spring-autumn for the same extract ($p > 0.05$). In aqueous ethanol 75% extracts, the significant content of polyphenol was shown by *S. muticum* collected in summer with those from spring and winter ($p < 0.05$). It seems that there was no significant variation of polyphenol content in the methanolic extracts of *S. muticum* ($p > 0.05$).

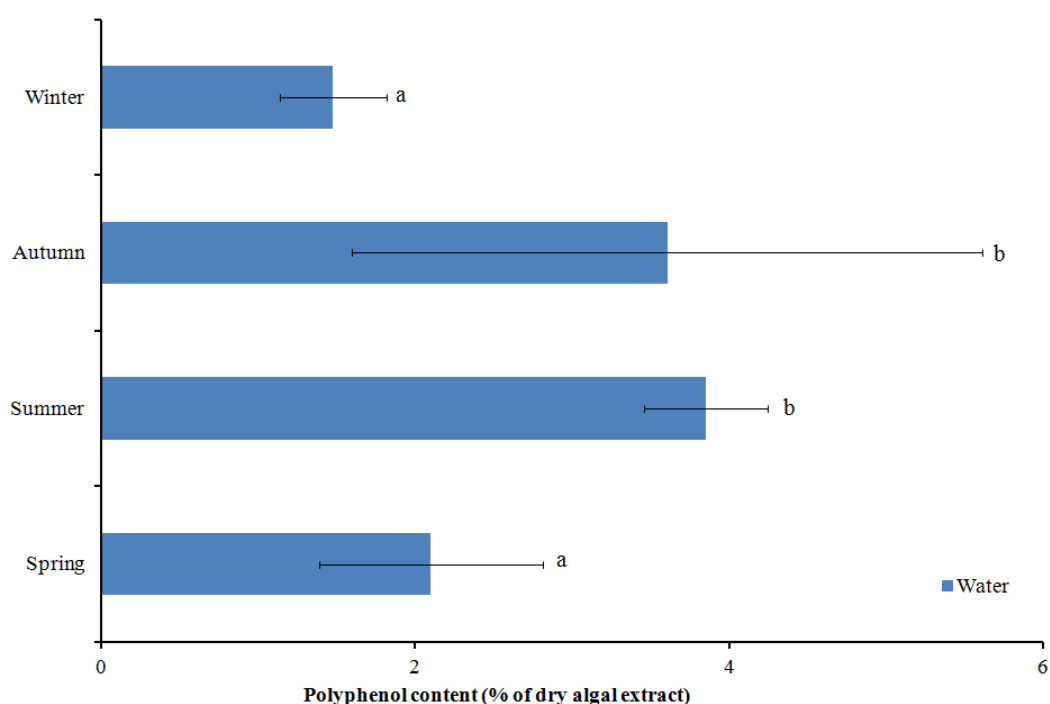


Fig 3-3 Polyphenol content of *S. muticum* water extract.

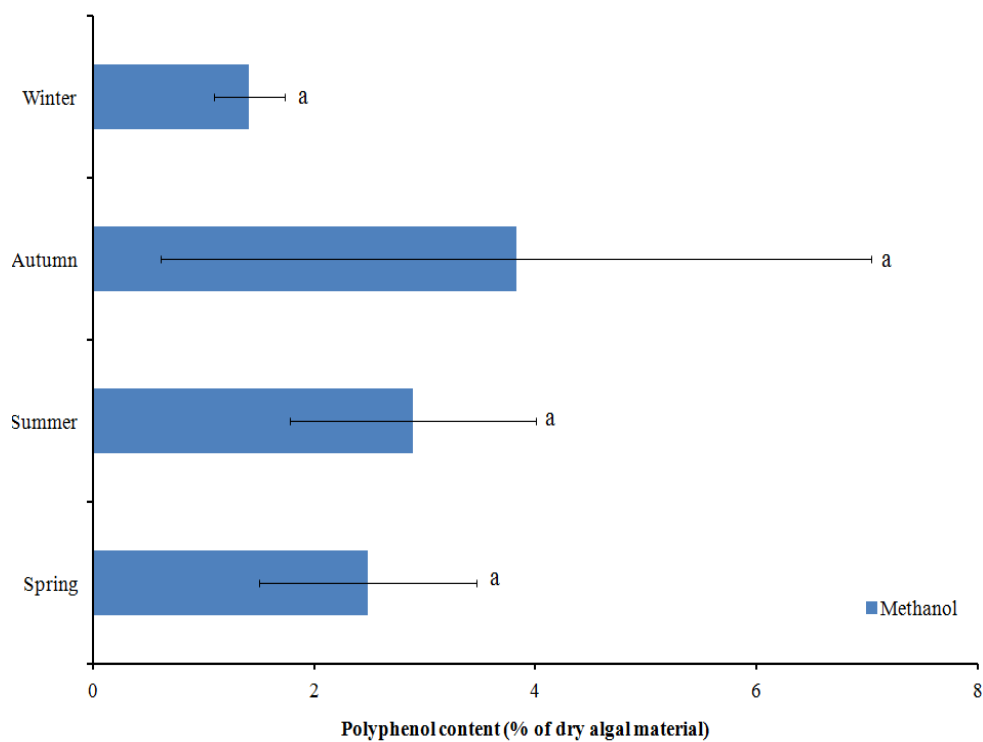


Fig 3-4 Polyphenol content of *S. muticum* methanol extract.

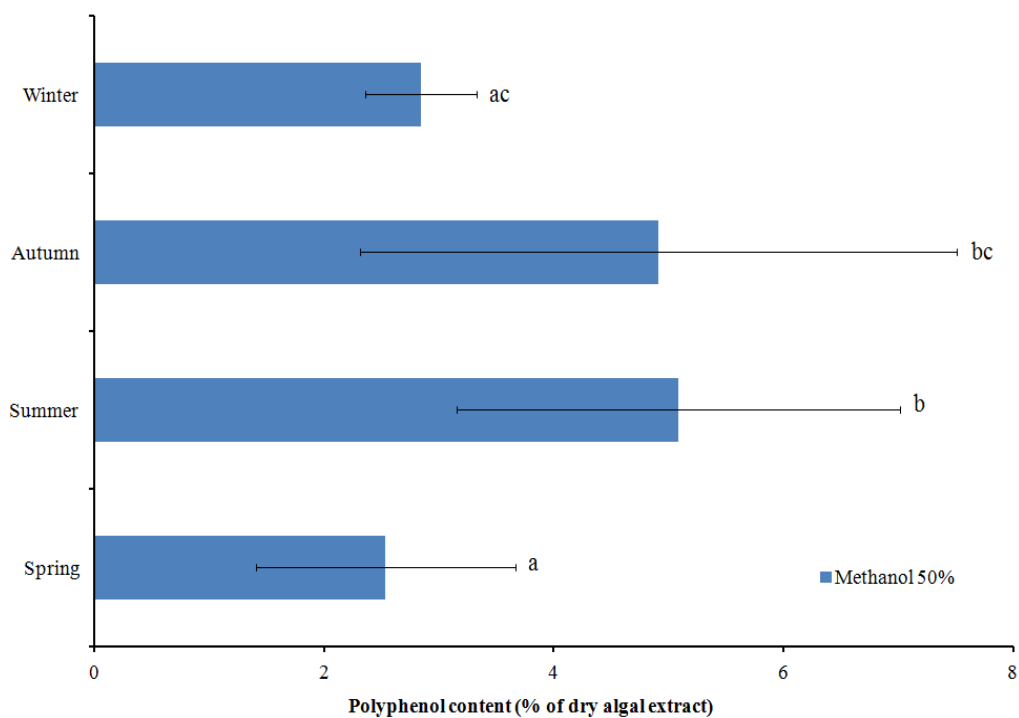


Fig 3-5 Polyphenol content of *S. muticum* aqueous methanol 50% extract.

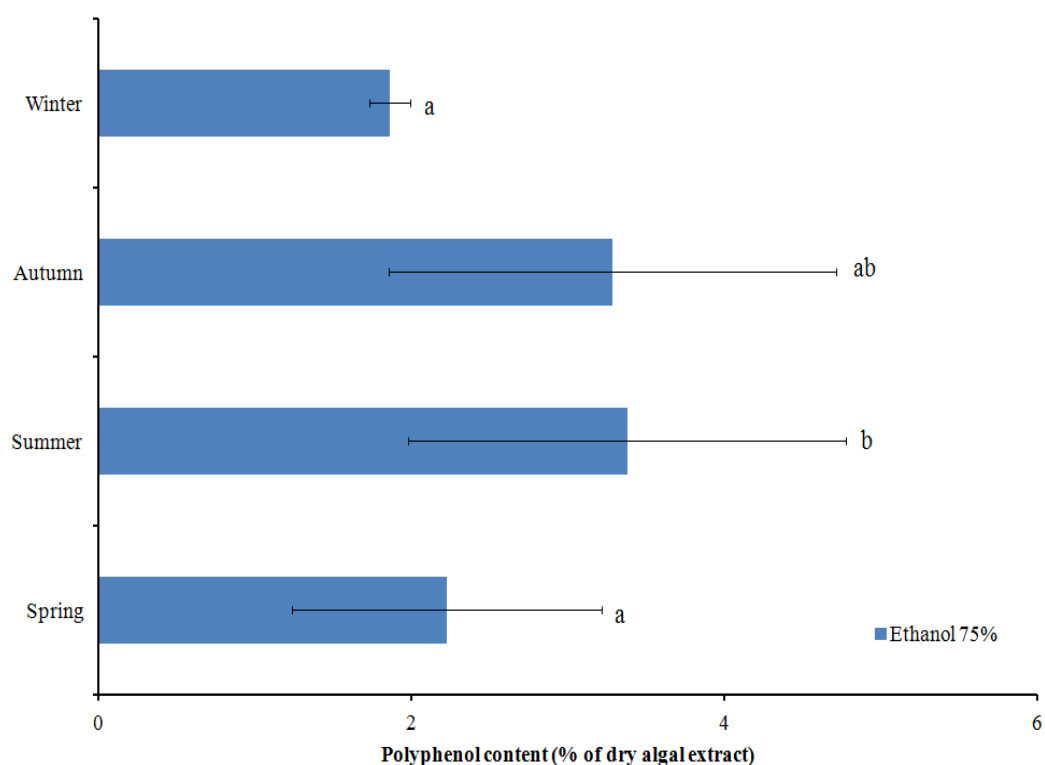


Fig 3-6 Polyphenol content of *S. muticum* aqueous ethanol 75% extract.

Pearson correlation analysis from these data was performed. It helped to inform the correlation between polyphenol content of *S. muticum* extracted with different solvents and the meteorological data. It seems that in all extracts – water, methanol, aqueous methanol 50% and aqueous ethanol 75%, they were inversely correlated with precipitation. However, the correlation was weak since the value of r^2 ranging at -.336 to -.110. Moreover, a significant correlation was only revealed with the water extract at $r = -.336$, $p < 0.01$ while the other had no significant correlation ($p > 0.05$ and 0.01 , 2-tailed) In the contrary, these extracts had significantly and positively weak to strong correlation with the sunlight, the maximum and the minimum air temperature (T_{\max} and T_{\min}) and the sea surface temperature (SST).

Polyphenol content in water extracts showed a significantly strong correlation with sunlight ($r = .700$, $p < 0.01$), T_{\max} ($r = .777$, $p < 0.01$), T_{\min} ($r =$

.757, $p < 0.01$) and SST ($r = .763$, $p < 0.01$). Methanol extracts of *S. muticum* had significantly weak correlation sunlight ($r = .370$, $p < 0.01$), T_{\max} ($r = .324$, $p < 0.05$), T_{\min} ($r = .318$, $p < 0.05$) and SST ($r = .272$, $p < 0.05$). Meanwhile, significantly moderate correlation was exhibited between the aqueous methanol 50% extracts with sunlight ($r = .451$, $p < 0.01$), T_{\max} ($r = .492$, $p < 0.01$), T_{\min} ($r = .516$, $p < 0.01$) and SST ($r = .469$, $p < 0.01$). As for aqueous ethanol 75% extracts, a significantly weak correlation was presented with sunlight ($r = .288$, $p < 0.05$). Further, the correlation of this extract with T_{\max} , T_{\min} and SST was significantly moderate with $r = .432$, $p < 0.01$, $r = .483$, $p < 0.01$ and $r = .409$, $p < 0.01$, respectively. Details to the statistical results for this study are presented on Appendix 7.

3.2.1.2 Polyphenol content of *S. muticum* in different solvents

Aside from the seasonal effect, effect of different solvents in *S. muticum* polyphenol was evaluated as well, as presented in Fig 3-7, 3-8, 3-9, and 3-10. Solvents gave significant effect towards polyphenol content of *S. muticum* collected in summer and winter. In summer, the aqueous methanol 50% extract of *S. muticum* polyphenol was significantly high than water, methanol, and aqueous ethanol 75% ($p < 0.05$). Later, aqueous methanol 50% of *S. muticum* in winter showed a significant difference of polyphenols towards the methanol and water extracts ($p < 0.05$). Aqueous methanol 50% extract of *S. muticum* had the highest polyphenol content in all seasons ranging from $2.5 \pm 1.1\%$ to $4.9 \pm 2.6\%$ of dry algal extract.

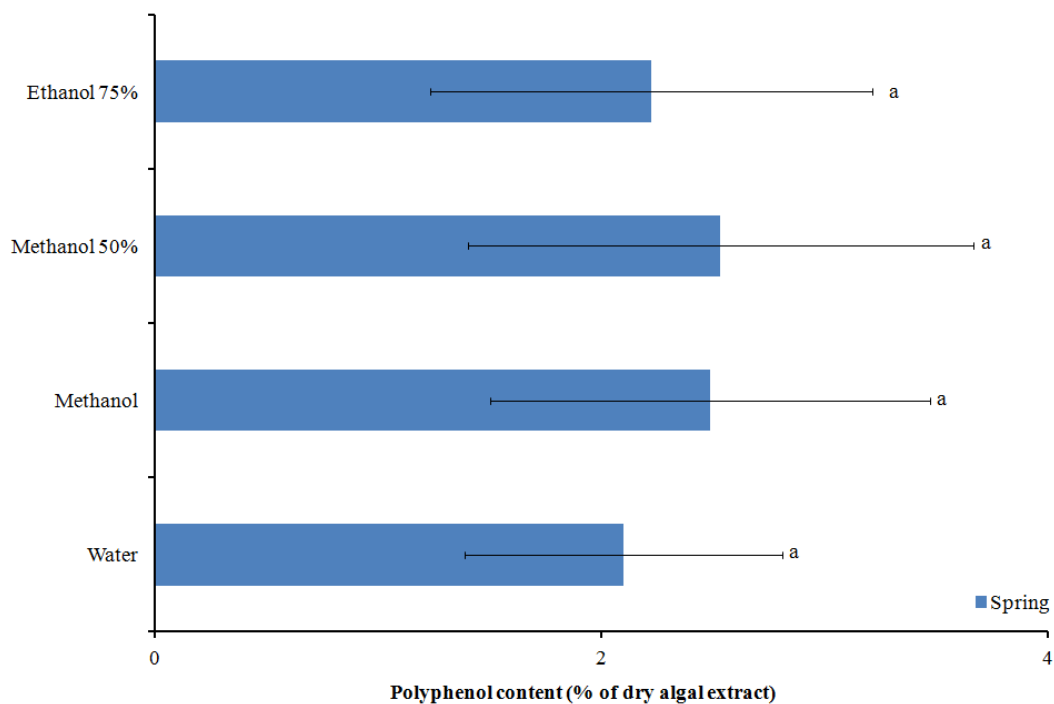


Fig 3-7 Polyphenol content of *S. muticum* collected during spring in different solvents. Alphabets on the bar indicate that there was no significant difference ($p > 0.05$).

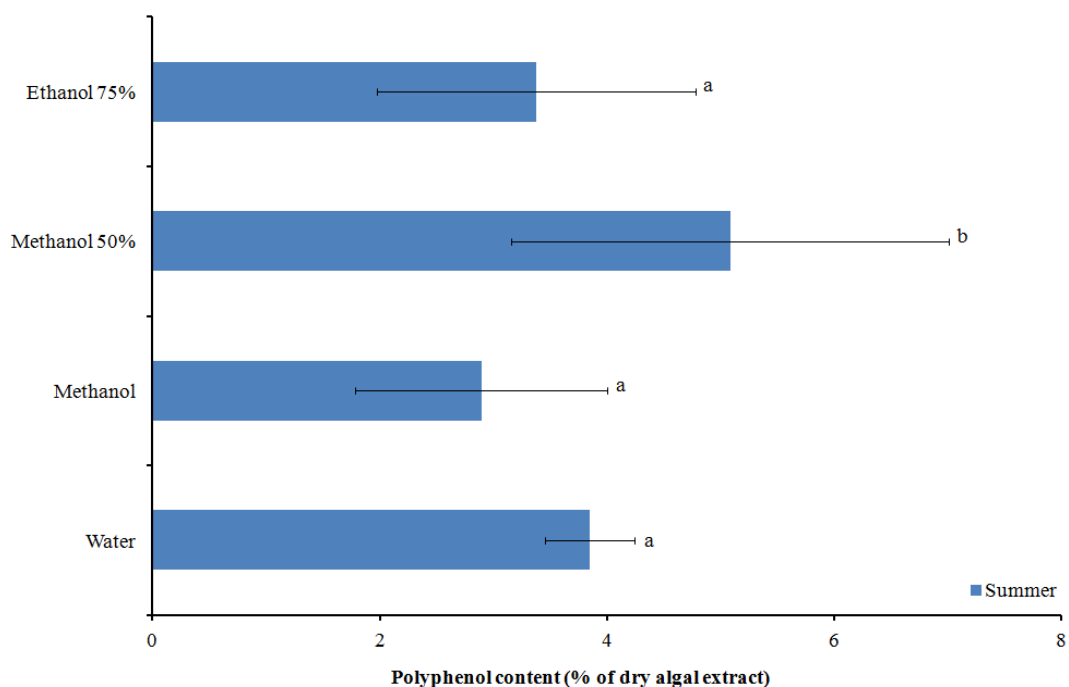


Fig 3-8 Polyphenol content of *S. muticum* collected during summer in different solvents. Different alphabets on the bar indicate that there was significant difference ($p < 0.05$).

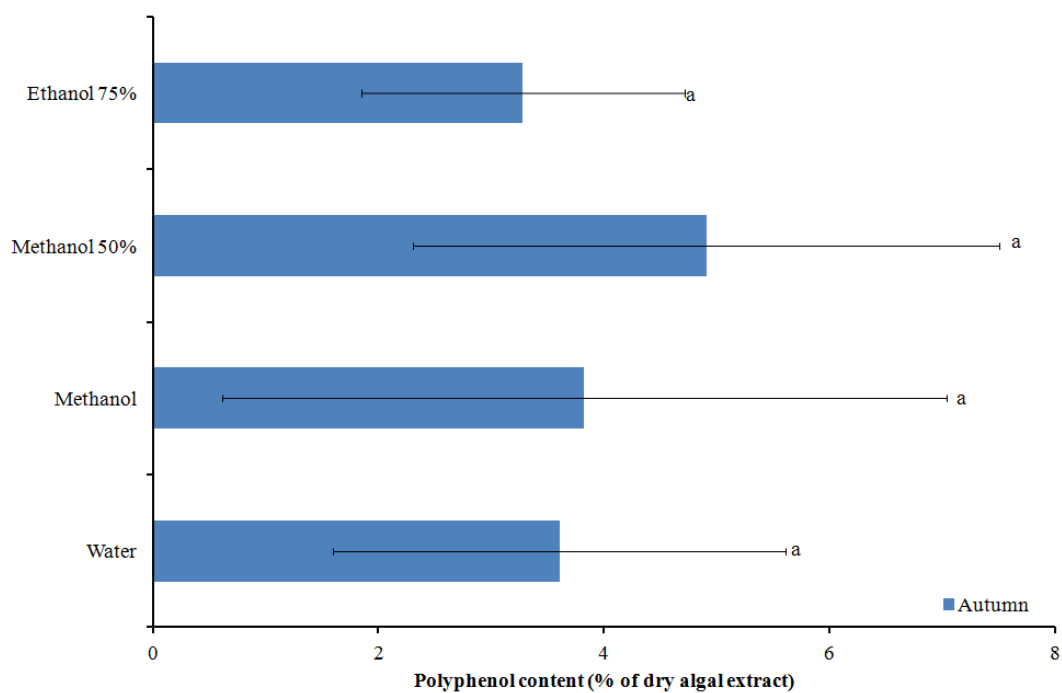


Fig 3-9 Polyphenol content of *S. muticum* collected during autumn in different solvents. Different alphabets on the bar indicate that there was significant difference ($p > 0.05$).

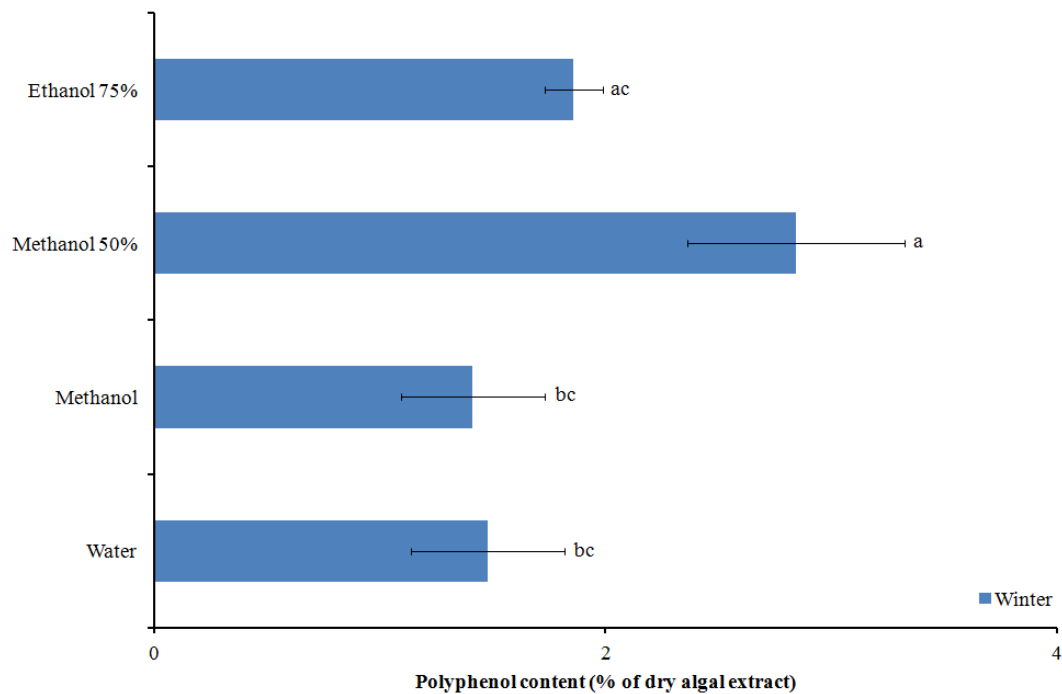


Fig 3-10 Polyphenol content of *S. muticum* collected during winter in different solvents. Different alphabets on the bar indicate that there was significant difference ($p > 0.05$).

Conclusion

From four different solvents used, water had the highest extraction yield with 24% of dry algal material. Seasons and solvents gave significant effects in *S. muticum* polyphenol content. In seasonal variation point of view, *S. muticum* collected in summer contained higher polyphenol when it was extracted with water, aqueous methanol 50% and aqueous ethanol 75%. As for methanol extract, it contained more polyphenol content in autumn sample than the other season. If we look the polyphenol content based on the variation of solvent then the polyphenol from all seasons was more favorable with aqueous methanol 50%.

3.2.2 Solid-liquid extraction of *Sargassum* polyphenol

In solid-liquid extraction of *Sargassum* polyphenol, there were two main treatment of statistical analysis performed. The first analysis concerned about the effect of different solvents in extracting polyphenol in tropical *Sargassum* from Indonesia, i.e. *S. aquifolium*, *S. ilicifolium* and *S. polycystum*. Additionally, effect of different species was also statistically evaluated. Then, the second comparison analysis was performed merely on *S. muticum*. These two different analyses were performed because of the difference in dry material due to different drying treatment. *S. aquifolium*, *S. ilicifolium* and *S. polycystum* were naturally dried, Meanwhile, *S. muticum* was lyophilized. As a result, the characteristics of final products obtained were not the same. The first three species of *Sargassum* were in medium pieces of thalli and *S. muticum* dry form was powder-like material.

Table 3-6 and 3-7 presents the extraction yield and polyphenol content of Indonesian *Sargassum* in different solvents. Water extraction resulted better yielded than the extraction with methanol, aqueous methanol 50% and aqueous ethanol 75% and *S. polycystum* had the highest yield of extracts with 22.8 ± 1.1 % of dry algal material. Methanol extraction showed the lowest yield ranging from 3.1 ± 0.7 to 8.3 ± 3.2 % of dry algal material. Polyphenol of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* was best extracted with methanol (6.4 ± 1.5 % of dry algal material), water (7.0 ± 1.3 % of dry algal material) and aqueous methanol

50% (8.1 ± 2.2 % of dry algal material), respectively. In order to evaluate whether there was a significant effect shown by different solvents or not towards polyphenol content of Indonesian *Sargassum*, statistical analysis of variance was performed to analyze whether there was a significant difference or not.

Table 3-6 Extraction yield of Indonesian *Sargassum* with the resume of significant difference caused by species and type of solvents.

Algae	Solvents	Yield (% of dry algal material)	Sig. ($p < 0.05$) of Species	Sig. ($p < 0.05$) of Solvents
<i>S. aquifolium</i>	Water	17.3 ± 2.2	.005 (df = 2 ; F= 3.399)	.000 (df = 3 ; F= 18.024)
	MeOH	5.7 ± 1.8		
	MeOH 50%	15.8 ± 1.5		
	EtOH 75%	12.3 ± 0.4		
<i>S. ilicifolium</i>	Water	14.7 ± 0.8		
	MeOH	3.1 ± 0.7		
	MeOH 50%	7.3 ± 1.0		
	EtOH 75%	7.5 ± 1.3		
<i>S. polycystum</i>	Water	22.8 ± 1.1		
	MeOH	8.3 ± 3.2		
	MeOH 50%	13.4 ± 1.9		
	EtOH 75%	12.5 ± 0.5		

Statistical analysis showed that there was a significant effect caused by different species and solvents used ($p < 0.05$). This significant effect was related to the extraction yield of Indonesian *Sargassum*. As presented on Appendix 10, extraction yield of *S. aquifolium* and *S. polycystum* was significantly different from *S. ilicifolium* ($p < 0.05$). In addition, water extracts of Indonesian *Sargassum* had a significant difference to methanolic (methanol and aqueous methanol 50%) and ethanolic (aqueous ethanol 75%) extracts ($p < 0.05$). Then, methanol extracts with aqueous methanol 50% and aqueous ethanol 75% extracts showed a significant difference in their extraction yield with methanol ($p < 0.05$).

Table 3-7 Polyphenol content of Indonesian *Sargassum* with the resume of significant difference caused by species and type of solvents.

Algae	Solvents	Polyphenol (% of dry algal extract)	Sig. ($p < 0.05$) of Species	Sig. ($p < 0.05$) of Solvents
<i>S. aquifolium</i>	Water	1.8 ± 0.1	.000 (df = 2 ; F= 50.569)	.000 (df = 3 ; F= 98.976)
	MeOH	6.4 ± 1.5		
	MeOH 50%	3.6 ± 0.2		
	EtOH 75%	6.0 ± 0.4		
<i>S. ilicifolium</i>	Water	5.3 ± 0.3		
	MeOH	6.0 ± 0.4		
	MeOH 50%	1.4 ± 0.0		
	EtOH 75%	4.8 ± 0.3		
<i>S. polycystum</i>	Water	7.0 ± 1.3		
	MeOH	3.9 ± 0.1		
	MeOH 50%	8.1 ± 2.2		
	EtOH 75%	3.5 ± 0.0		

In polyphenol content, there was significant difference caused by different species of *Sargassum* ($p > 0.05$), which was shown by the three species of *Sargassum*. In addition, a significant difference was also observed in different type of solvents that were used to extract polyphenols from Indonesian *Sargassum*. Table 3-7 presents the polyphenol content of Indonesian *Sargassum* using different solvents. As can be seen, type of species and solvents gave significant difference towards polyphenol content. Appendix 8 shows the significant difference in *Sargassum* polyphenol content due to different solvents ($p < 0.05$). Aqueous methanol 50% and aqueous ethanol 75% had a significant difference with water and methanol ($p < 0.05$). It can also be said that in this study water extraction yielded better of dry algal material while aqueous methanol 50% and aqueous ethanol 75% extracted higher content of polyphenol in Indonesian *Sargassum*.

Table 3-8 Extraction yield and polyphenol content of French *S. muticum* June 2014

Solvents	Yield (% of dry algal material)	Polyphenol (% of dry algal extract)
Water	24.8 ± 0.3 ^a	3.8 ± 0.1 ^a
MeOH	13.3 ± 2.9 ^c	2.8 ± 0.4 ^b
MeOH 50%	15.7 ± 1.5 ^b	6.6 ± 0.2 ^a
EtOH 75%	19.0 ± 1.5 ^b	4.0 ± 0.6 ^b

In *S. muticum*, aqueous methanol 50% was more effective in extracting polyphenol containing 6.6 ± 0.2% of dry algal extract as shown in Table 3-8. Nevertheless, water yielded higher dry algal material (24.8 ± 0.3% of dry algal material) than other solvents. Statistical analysis, presented in Appendix 9, revealed that there was a significant difference in extraction yield and polyphenol content of *S. muticum* caused by different solvents ($p < 0.05$). Extraction yield of *S. muticum* water extract was significantly different from methanol, aqueous methanol 50% and aqueous ethanol 75% extracts ($p < 0.05$). A significant difference was also shown by methanol and aqueous ethanol 75% extracts ($p < 0.05$). However, there was no significant difference between aqueous methanol 50% and aqueous ethanol 75% in their extraction yields ($p < 0.05$). Type of solvents also gave a significant effect in *S. muticum* polyphenol ($p < 0.05$). Water and aqueous methanol 50% extracts was significantly different from methanol and aqueous ethanol 75% extracts ($p < 0.05$).

Conclusion

Solid-liquid extraction has been widely used to extract bioactive compound in marine algae, particularly polyphenol. Efficacy of this technique relies in the appropriate selection of extraction conditions, such as type of solvents, temperature, particle sizes of samples, etc. By applying different type of solvents, our study demonstrated that aqueous organic solvents extracted more polyphenol than water only. On the opposite, water is the best media to obtain high extraction yield in comparison to organic solvents since it co-extracted the polysaccharides and salts as well. Regardless, intra-species variation of polyphenol also needs to

be considered. As presented in this study, each *Sargassum* species showed different preference of solvents. *S. aquifolium* and *S. ilicifolium* contained high polyphenol in methanol extract. Meanwhile, 50% aqueous methanol extract of *S. muticum* and *S. polycystum* showed more polyphenol content than the other extracts; water, methanol, 75% aqueous ethanol extracts.

3.2.3 Enzyme-assisted extraction of *Sargassum* polyphenol

3.2.3.1 Screening of enzymes

In enzyme-assisted extraction, the insoluble sludge was separated from the soluble aqueous phase. Only the soluble aqueous phase was studied. Yields and polyphenol content were quantified and compared. Table 3-9 presents the extraction yield and polyphenol content of *S. muticum* enzymatic extracts.

There was no significant difference in the yield of enzyme-assisted extraction. However, compared to the control ($26.5 \pm 4.7\%$ of dry algal material) – an extraction without added enzyme – the enzymatic extracts produced higher yield of dry material; except for Shearzyme extract ($26.2 \pm 1.7\%$ of dry algal material). As a protease enzyme, Neutrase extract yielded $32.6 \pm 4.9\%$ of dry algal material while other protease, Alcalase, only yielded $28.7 \pm 3.5\%$ of dry material. Second highest yield was obtained from Ultraflo extract (carbohydrase), $31.6 \pm 4.5\%$ of dry algal material. The yield of enzyme-assisted extraction from other carbohydrase, such as AMG, Termamyl, Viscozyme and Celluclast ranged between 27.3 ± 0.2 and $30.2 \pm 5.2\%$ of dry algal material.

Insoluble and soluble polyphenol content of *S. muticum* enzymatic extract were determined and results are expressed as % of dry hydrolysate. For the insoluble polyphenol, all *S. muticum* enzymatic extracts showed the same content of polyphenol, *i.e.* 0.9% of dry hydrolysate. As for the control, there was $1.0 \pm 0.0\%$ of dry hydrolysate. Despite the low value, this result indicated that there were still some traces of polyphenol in the residues of *S. muticum* enzymatic extracts.

Neutrase extract had the highest yield of extraction with $32.6 \pm 4.6\%$ of dry hydrolysate; however, the highest polyphenol was shown by Viscozyme extract, $6.4 \pm 0.5\%$ of dry algal material followed by Termamyl $5.0 \pm 0.1\%$ of dry hydrolysate. The protease i.e. Neutrase and Alcalase only extracted polyphenol at $3.8 \pm 0.0\%$ of dry hydrolysate. Nevertheless, in comparison with the control ($2.6 \pm 0.0\%$ of dry hydrolysate); all eight enzymes enhanced the extraction of polyphenol compound in *S. muticum*.

Table 3-9 Yield of extract and polyphenol content of *S. muticum* enzymatic extracts (different letter superscript letters indicates a significant difference ($p < 0.05$)).

Samples	Yield of extracts (% of dry algal material)	Polyphenol (% of dry hydrolysate)
Control	26.5 ± 4.8^a	2.6 ± 0.0^a
Neutrase	32.6 ± 4.9^a	3.8 ± 0.0^{ac}
Ultraflo	31.6 ± 4.5^a	4.2 ± 0.0^{ac}
Alcalase	28.7 ± 3.5^a	3.8 ± 0.0^{ac}
Celluclast	29.9 ± 0.8^a	4.5 ± 0.0^{bc}
AMG	30.2 ± 5.2^a	4.8 ± 0.0^{bc}
Shearzyme	26.2 ± 1.7^a	4.7 ± 0.0^{bc}
Viscozyme	28.1 ± 3.0^a	6.4 ± 0.5^{bc}
Termamyl	27.3 ± 0.3^a	5.0 ± 0.1^{bc}

Beside polyphenol, other biochemical compositions of *S. muticum* enzymatic extracts such as uronic acid, protein, sugar and sulfate groups were also determined. Table 3-10 presents the biochemical composition of *S. muticum* enzymatic extracts. This result informs the presence of polysaccharides in the enzymatic extracts of *S. muticum*.

Table 3-10 Biochemical composition of *S. muticum* enzymatic extracts.

Samples	Content (% of dry hydrolysate)			
	Uronic acid	Protein	Sugar	Sulfates groups
Control	0.5 ± 0.0	33.0 ± 2.3	8.2 ± 2.1	7.0 ± 0.1
Neutrase	0.6 ± 0.1	31.8 ± 0.8	7.7 ± 1.5	8.8 ± 0.0
Ultraflo	0.6 ± 0.0	40.3 ± 0.3	6.8 ± 0.7	6.8 ± 0.1
Alcalase	0.7 ± 0.2	47.4 ± 5.4	12.9 ± 3.6	7.4 ± 0.1
Celluclast	0.6 ± 0.1	52.1 ± 0.6	7.4 ± 0.8	7.6 ± 0.1
AMG	0.5 ± 0.0	53.1 ± 0.0	7.9 ± 0.2	7.5 ± 0.4
Shearzyme	0.6 ± 0.1	54.3 ± 0.7	8.5 ± 0.9	7.5 ± 0.2
Viscozyme	0.5 ± 0.1	27.1 ± 0.7	10.6 ± 1.6	7.4 ± 0.1
Termamyl	0.5 ± 0.1	41.3 ± 0.8	9.9 ± 0.4	7.6 ± 0.2

3.2.3.2 Optimization of *S. muticum* polyphenol enzyme-assisted extraction

According to the screening, Viscozyme and Termamyl extracted more polyphenol than the other enzymes and control – the aqueous phase, as previously presented. Based on the previous result of this study, these two enzymes were further selected to evaluate the efficiency of these enzymes in extracting polyphenol with adjusted conditions. Such conditions referred to the temperature and concentration of enzymes. As mentioned by many previous studies, 40 °C has been the most favorable temperature to extract polyphenol. Additionally, the concentration of enzymes was augmented to 5 and 10% assuming that it might increase the extracted polyphenol content as well. Result is presented on Table 3-10.

This study was meant to acknowledge whether or not by applying same enzyme in samples collected from different month would give same results. As previously described, Viscozyme managed to extract polyphenol until 6% of dry algal material along with Termamyl. Both of these enzymes are carbohydrase. Then, in this part, we decided to attempt an enzymatic extraction using Protamex – a protease.

Based on the observation of soluble enzymatic extracts, there was a distinguish color produced between *S. muticum* from June and November 2014. In screening, we used *S. muticum* from June 2014 while in this optimization; *S.*

muticum from November 2014 was selected. Soluble enzymatic extracts of *S. muticum* from June 2014 had dark brown color. As for that in November, they showed dark green color. As can be seen on Table 3-10, extraction yield of *S. muticum* extracted with 10% of Viscozyme and Termamyl slightly increased from 33 to 35% and from 39 to 42%. However, for *S. muticum* that was extracted with 10% of Protamex did not show any increase in polyphenol content. Viscozyme extracted more polyphenol than Protamex and Termamyl in 5% of concentration. In comparison with 10%, 5% of enzymes had more content of polyphenol.

Table 3-11 Extraction yield and total polyphenol content of *S. muticum* from November 2014 extracted with enzymes in different concentration at 40°C without denaturation.

Enzymes	Concentration (%)	Yield (% of dry algal material)	Polyphenol (% of dry hydrolysate)
Viscozyme	5	33.8 ± 0.6	3.6 ± 1.5
Protamex		42.8 ± 0.2	2.0 ± 0.7
Termamyl		39.8 ± 2.6	1.5 ± 0.0
Viscozyme	10	35.4 ± 1.2	1.8 ± 0.0
Protamex		42.6 ± 0.6	1.7 ± 0.0
Termamyl		42.1 ± 0.4	2.7 ± 0.0
Water		33.4 ± 1.1	3.4 ± 0.0

According to the above result, the next extraction procedure would use Viscozyme and Protamex. As presented in above table, Viscozyme and Protamex extracts of *S. muticum*, using 5% of enzyme, contained higher polyphenol than Termamyl. Table 3-11 presents results of *S. muticum* extraction yield and polyphenol content from June 2014. The extraction was performed at 40 °C for 3 h and without denaturation. The condition of extraction was selected in the assumption that this condition was favorable for polyphenol extraction. Nevertheless, results showed that the selected parameters did not extract more polyphenol in *S. muticum*. Interestingly, the extraction yield of *S. muticum* Viscozyme extract was higher than the yield from the screening. As a reminder, parameters that were applied in polyphenol extraction of *S. muticum* were 50°C, 3

h and with denaturation at 90 °C. By extracting *S. muticum* with Viscozyme and Protamex at 40°C for 3 h and without denaturation, extraction yield attained 45.5 ± 1.4 and $46.4 \pm 0.5\%$ of dry hydrolysate.

Table 3-12 Extraction yield and total polyphenol content of *S. muticum* from June 2014 extracted with 5% enzymes at 40°C without denaturation.

Enzymes	Yield (% of dry algal material)	Polyphenol (% of dry hydrolysate)
Viscozyme	45.5 ± 1.4	2.1 ± 0.2
Protamex	46.4 ± 0.5	3.0 ± 0.0

3.2.3.3 Enzyme-assisted extraction of Indonesian *Sargassum*

Same enzymes and condition of extraction was also applied to Indonesian *Sargassum*, i.e. Viscozyme and Protamex at 40°C. Results are presented in Table 3-13. *S. polycystum* was best extracted with Protamex, $38.2 \pm 6.8\%$ of dry algal material. On the other hand, *S. aquifolium* and *S. ilicifolium* had higher extraction yield with Viscozyme than Protamex. Table 3-14 shows the polyphenol content Indonesian *Sargassum* extracts.

Table 3-13 Extraction yield of Indonesian *Sargassum* extracted with enzymes at 40°C without denaturation.

Algae	Enzymes	Yield (% of dry algal material)	Sig. ($p < 0.05$) of Species	Sig. ($p < 0.05$) of Enzymes
<i>S. aquifolium</i>	Viscozyme	29.5 ± 5.4	.001 (df = 2 ; F = 11.964)	.276 (df 1 ; F = 1.300)
	Protamex	26.2 ± 5.9		
<i>S. ilicifolium</i>	Viscozyme	27.9 ± 1.5		
	Protamex	21.4 ± 2.1		
<i>S. polycystum</i>	Viscozyme	35.8 ± 2.7		
	Protamex	38.2 ± 6.8		

Table 3-14 Polyphenol content of Indonesian *Sargassum* extracted with enzymes at 40°C without denaturation.

Algae	Enzymes	Polyphenol (% of dry hydrolysate)	Sig. ($p < 0.05$) of Species	Sig. ($p < 0.05$) of Enzymes
<i>S. aquifolium</i>	Viscozyme	4.7 ± 0.2	.000 (df = 2 ; F = 18.995)	.000 (df 1 ; F = 77.395)
	Protamex	4.2 ± 0.0		
<i>S. ilicifolium</i>	Viscozyme	3.6 ± 0.1		
	Protamex	5.0 ± 0.3		
<i>S. polycystum</i>	Viscozyme	4.0 ± 0.4		
	Protamex	6.1 ± 0.1		

Analysis of variance exhibited that there was a significant difference in extraction yield and polyphenol content between different species of Indonesian *Sargassum* ($p < 0.05$). Extraction yield and polyphenol content of *S. polycystum* was significantly different from that of *S. aquifolium* and *S. ilicifolium* ($p < 0.05$). In addition, Viscozyme and Protamex gave a significant difference towards polyphenol content in Indonesian *Sargassum* ($p < 0.05$) but not in the extraction yield ($p > 0.05$).

Conclusion

Extraction of polyphenol from *S. muticum* using enzyme-assisted extraction was successfully demonstrated at laboratory-scale. Application of carbohydrase and protease managed to enhance polyphenol content, likewise, extraction yield of *S. muticum* was also improved compared to water extraction. In this study, introduction of Viscozyme increased more polyphenol content yet the extraction yield was improved by Neutrase.

Enzyme-assisted extraction, in certain extent, improves extraction yield and facilitated release of target bioactive compound to the medium. Moreover, this technique is performed under moderate conditions that maintain the bioactivity of target compounds. To do so, it is important to apply the appropriate condition of extraction according to type of enzymes used. Most of the enzymes work optimally at 50 °C, except for Protamex that works optimally at 40 °C.

Interestingly, polyphenol compounds from *S. ilicifolium* and *S. polycystum* extracted with Protamex at 40 °C showed slightly higher polyphenol content than Viscozyme extract. It can be concluded that in optimizing the enzyme-assisted extraction for polyphenol, it is important to consider the optimal condition for each enzymes, inter-species and seasonal variation.

3.3 Characterization of *Sargassum* biochemical composition and extracts

3.3.1 Fourier transform infrared spectroscopy (FT-IR)

In evaluating the seasonal variation of *S. muticum* biochemical composition, dry algal materials were analyzed using the FT-IR spectroscopy. Results from this analysis are presented in sub-chapter 3.3.1.1. Sub-chapter 3.3.1.2 and 3.3.1.3.presents the FT-IR analysis from *S. muticum* and Indonesian *Sargassum* enzymatic extracts, respectively. Presence of polysaccharides (sugars, uronic acid and sulfates groups), proteins, and polyphenols were determined by selecting the corresponding wavenumbers.

3.3.1.1 Biochemical composition of *S. muticum*-seasonal variation

Fig 3-11 presents an example of *S. muticum* biochemical composition spectra from April 2014. The FT-IR spectra managed to detect interesting peaks at 3270-3250, 2940-2925, 2300-1900, 1660-1600, 1500-1200 and 1100-1000 cm^{-1} . Table 3-15 presents details on these peaks. The peaks detection was analyzed based on the single analysis run in each samples.

Certain wavenumbers corresponded to particular biochemical composition could be selected. The interest was to see the seasonal pattern of specific biochemical composition. For that reason, the presence of polysaccharides, protein, uronic acid, sulfates groups and polyphenol were detected by selecting the corresponded wavenumbers. Later, Principal Component Analysis (PCA) was performed to further describe the seasonal pattern in each compound.

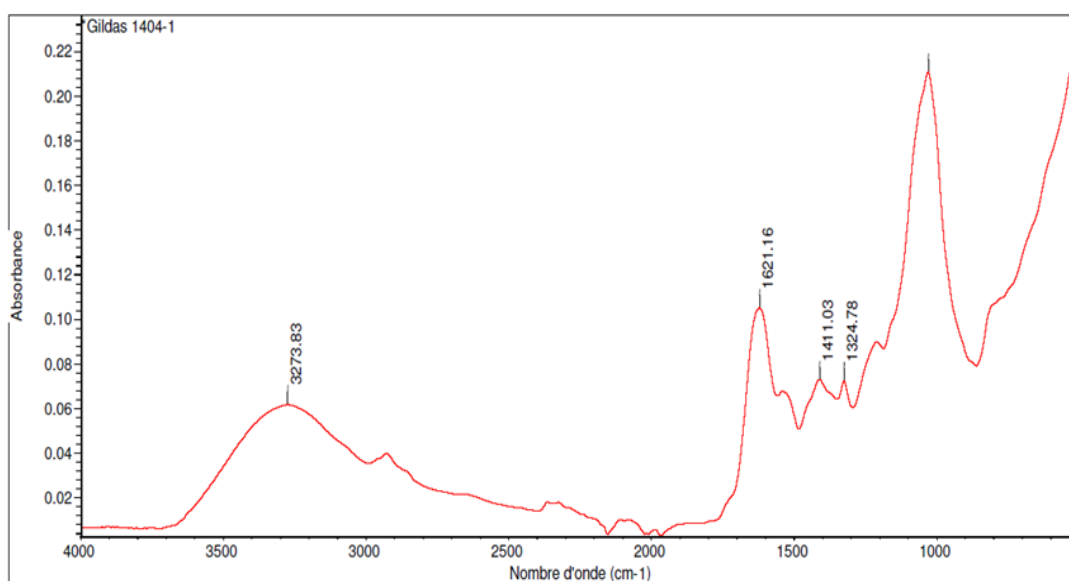


Fig 3-11 FT-IR spectra of *S. muticum* biochemical composition (sample from April 2014).

Wavenumbers chosen for polysaccharides were 1320, 1085-1070, 1030-1025, 895-875, and 825-815 cm^{-1} . As for the protein, 3600-3450 and 1660-1440 cm^{-1} were selected. 1300-1015 cm^{-1} was the range of wavenumbers chosen to detect the presence of sulfates groups. 1705-1595, 1415-1395, 1090-1035, and 940-930 cm^{-1} were wavenumbers for determining the uronic acid compound in *S. muticum*. The detection of polyphenol by FT-IR analysis was indicated by the following wavenumbers 3300-3200, 1620-1605, 1460-1445, 1090-1075, 935-920, 880-865, and 675- 660 cm^{-1} .

Table 3-15 FT-IR characterization of algal biochemical compositions (Bi *et al.*, 2007; Prati *et al.*, 2010; Gomez-Ordenez and Ruperez, 2011; Marimuthu *et al.*, 2012; Giriya *et al.*, 2013; Kannan, 2014; Sugiono *et al.*, 2014; Rodrigues *et al.*, 2015; Kusumaningsih *et al.*, 2016).

Compositions	Wavenumbers (cm ⁻¹)	Liaisons
Polysaccharides	Chapter 4 3423.87-1633	Chapter 5 OH, C=O, and COOH; hydroxyl, carbonyl, carboxyl (alginic acid)
	1730	C=O; carboxylic acid ester
	1675 and 1600	COO ⁻ stretch; carboxylate anion
	1320	C=C stretch
	1200-970	C-C, C-O pyranoid ring and C-O-C stretching of glycosidic
	1139.21-1036.60	Acidic polysaccharides
	1100	Mannuronic units
	1027 (1030-1025)	Guluronic units
	817 (825-815)	Mannuronic units
	1705-1595	C-O stretch
Uronic acid	1609 and 1420	-COO ⁻ asymmetric and symmetric stretch of uronic acid
	1611	Aromatics nucleus
	1415-1395	C-OH stretch
	1090-1035	C-O vibration
	940-930	C-O-C stretch
Sulfates groups	1280-1210	(S=O) Stretch of sulfate groups
	1250-1040	S=O stretching
	1030	S=O stretching
	836.75-836.20	Sulfate groups
	670-600	C-S and C=S stretching
Proteins	3600-3450	N-H stretch
	2347	N-H bond
	1660-1440	C=O, N-H, δ CH ₂ -CH ₃
	939	N-O; nitrogen grouping
Polyphenol	3300-3200	O-H and C-H stretch; phenol hydroxyl carboxylic acid
	2945-2930	C-H stretch
	1620-1605	Aromatic nucleus
	1384	-OH phenolic groups
	1086	C-H stretch; aromatic nucleus

Principal component analysis, also known as PCA, is most commonly used to condense the information contained in a large number of original variables into a smaller set of dimensions. This analysis facilitates the explanation of variance-

covariance structure of a set of variable. This set of variable is converted into principal components. Fig 3-12, 3-13, 3-14, 3-15 and 3-16 show PCA scores plot of *S. muticum* biochemical composition (polysaccharides, protein, uronic acid and sulfates groups) based on FT-IR spectra. Score plot is a projection of data set onto subspace and used for interpreting relations among observations, in this case, a relation between biochemical composition and seasonal variation. In general, the PCA score plot presented on Fig 3-12, 3-13, 3-14, 3-15 and 3-16 distinguished between the spring-summer and autumn-winter samples. In polysaccharides, protein, uronic acid, sulfates groups and polyphenol of *S. muticum*, the summer and winter samples were grouped in the same region in the PCA score plot. However, the pattern of the grouping from these biochemical compositions was different between one to another. Polysaccharides and sulfates groups showed that the autumn-winter samples had grouped in the left region of the score plot towards the PC-2. Meanwhile, protein, uronic acid and polyphenol content of *S. muticum* tended to form a group towards the right region of the plot. It could be suggested that the seasonal variation of polysaccharides and sulfates groups were on the same pattern and the other way around with protein, uronic acid and polyphenol.

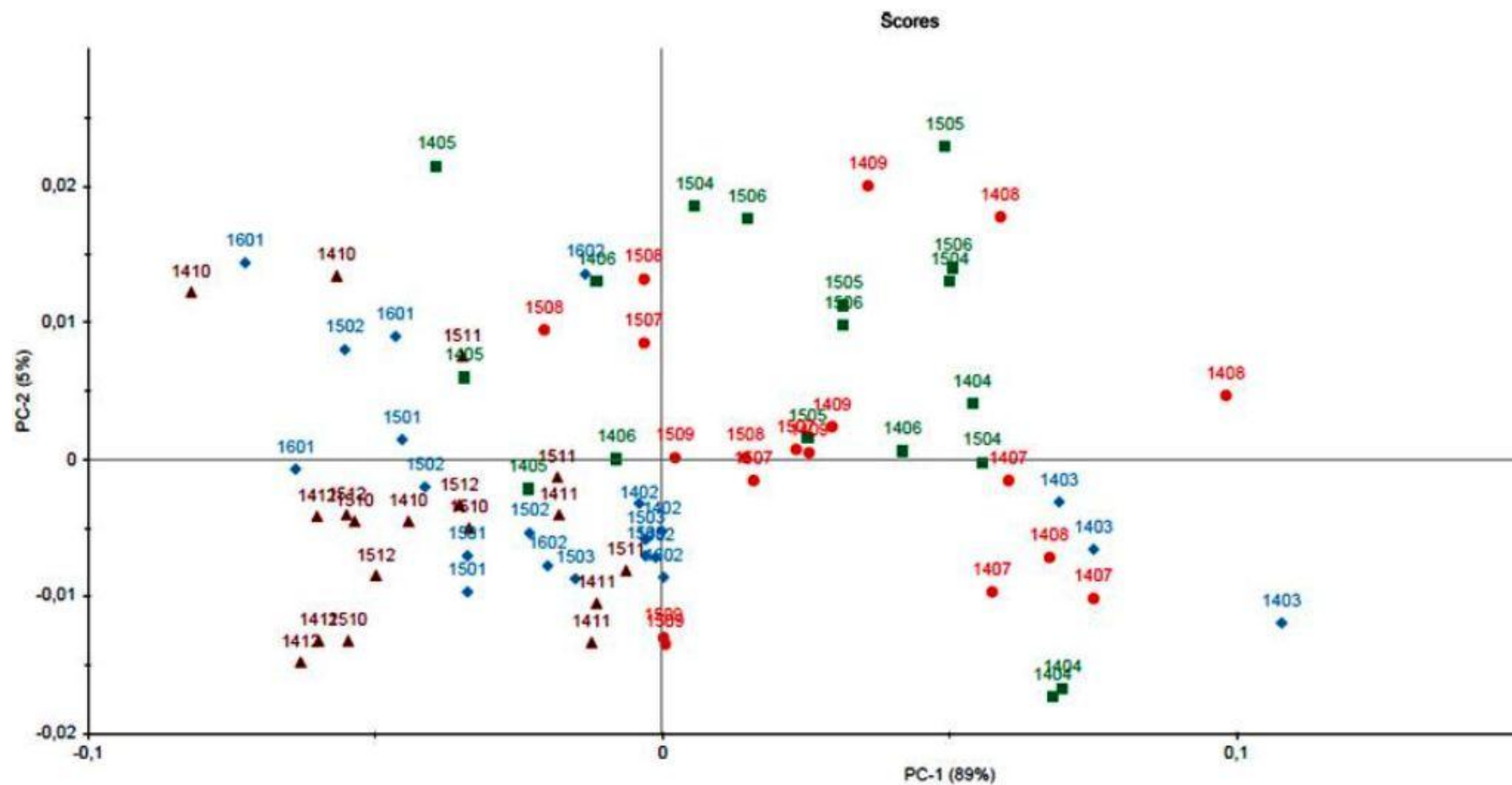


Fig 3-12 Principal component analysis of *S. muticum* polysaccharides from selected wavenumbers of 1320, 1085-1070, 1030-1025, 895-875, and 825-815 cm^{-1} . Green box: Spring, Red circle: Summer, Purple triangle: Autumn, and Blue diamond: Winter.

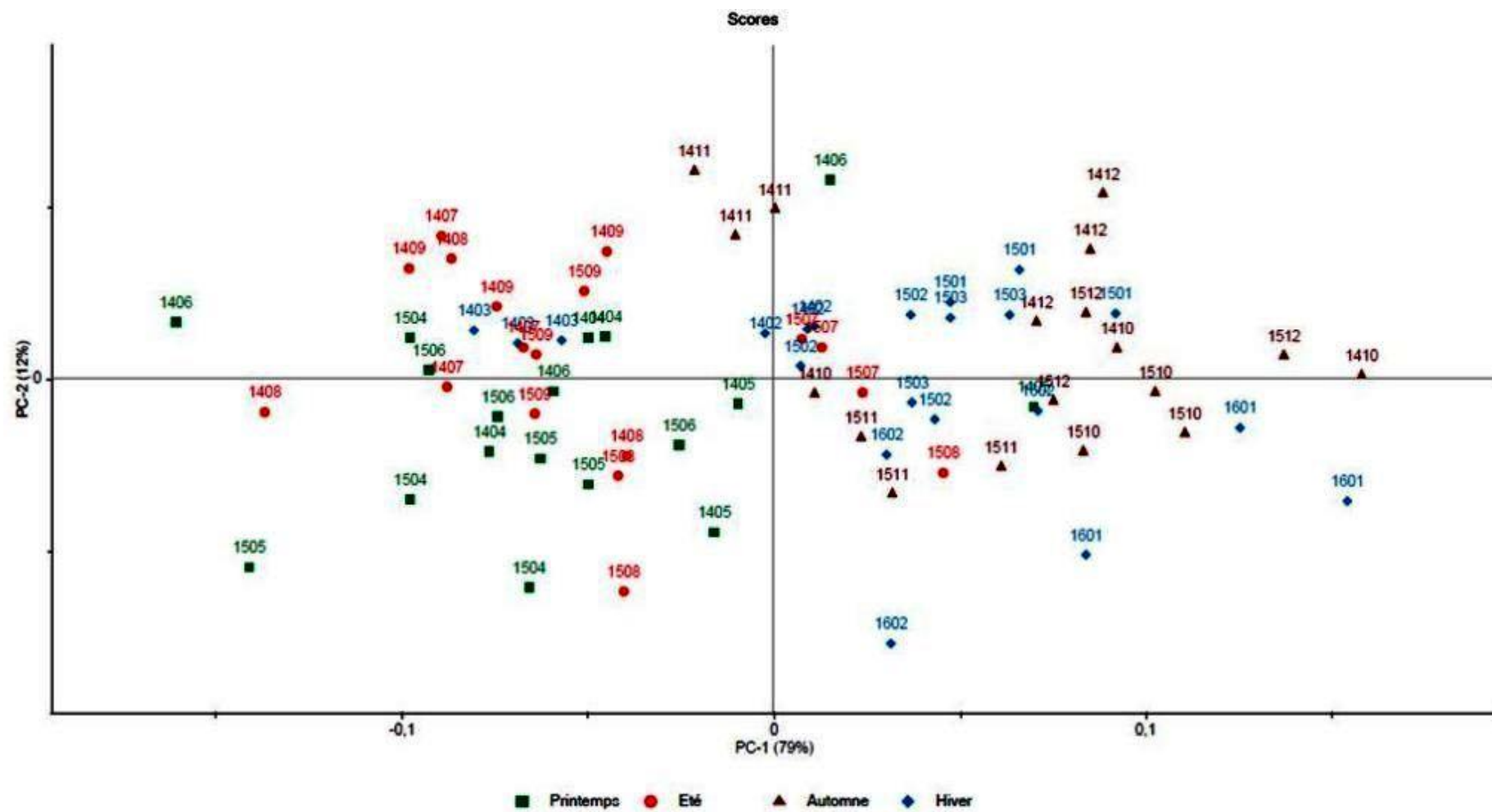


Fig 3-13 Principal component analysis of *S. muticum* proteins from selected wavenumbers of 3600-3450 and 1660-1440 cm⁻¹. Green box: Spring, Red circle: Summer, Purple triangle: Autumn, and Blue diamond: Winter.

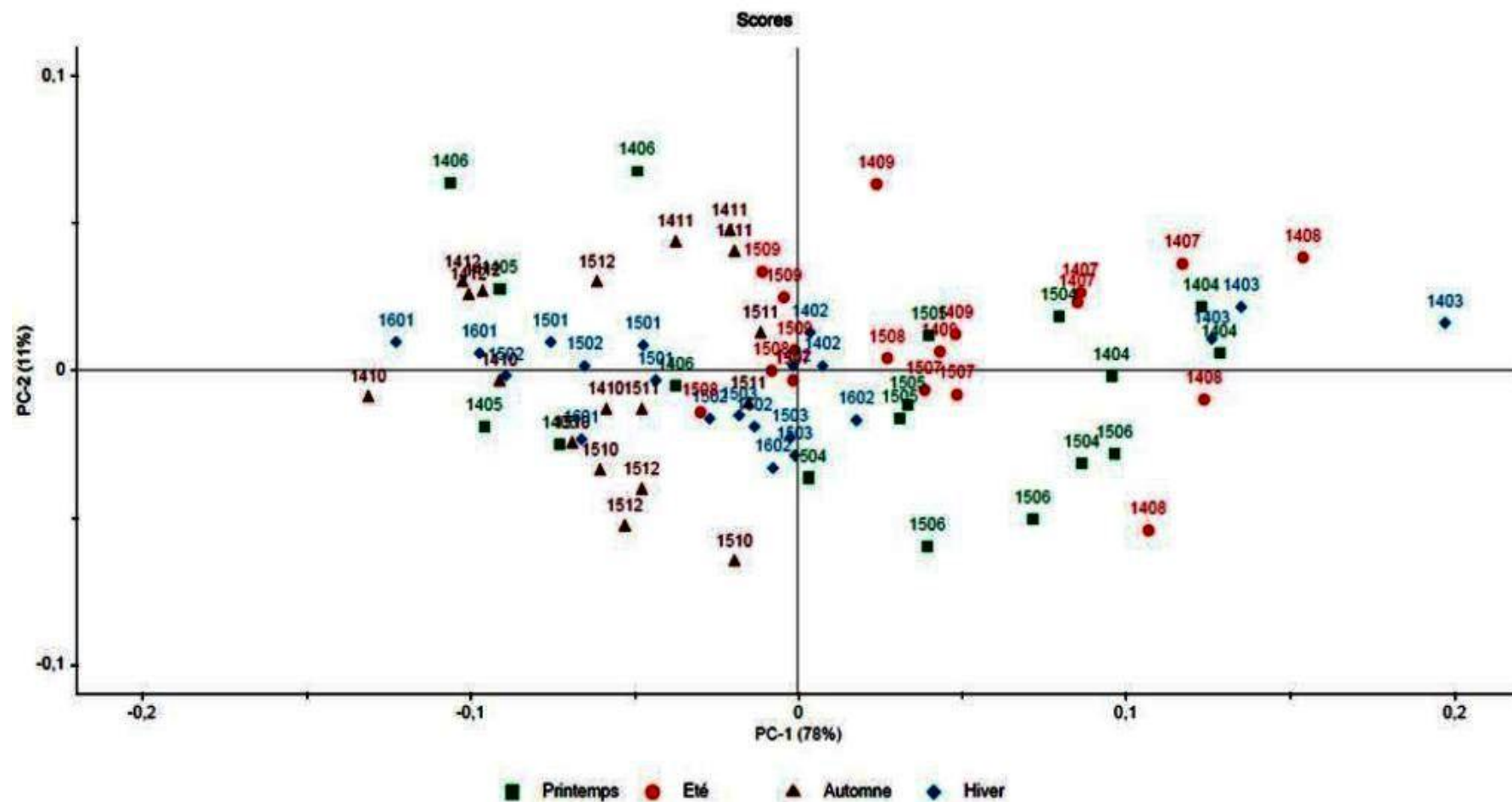


Fig 3-15 Principal component analysis of *S. muticum* sulfates group from selected wavenumbers of 1300-1015 cm⁻¹. Green box: Spring, Red circle: Summer, Purple triangle: Autumn, and Blue diamond: Winter.

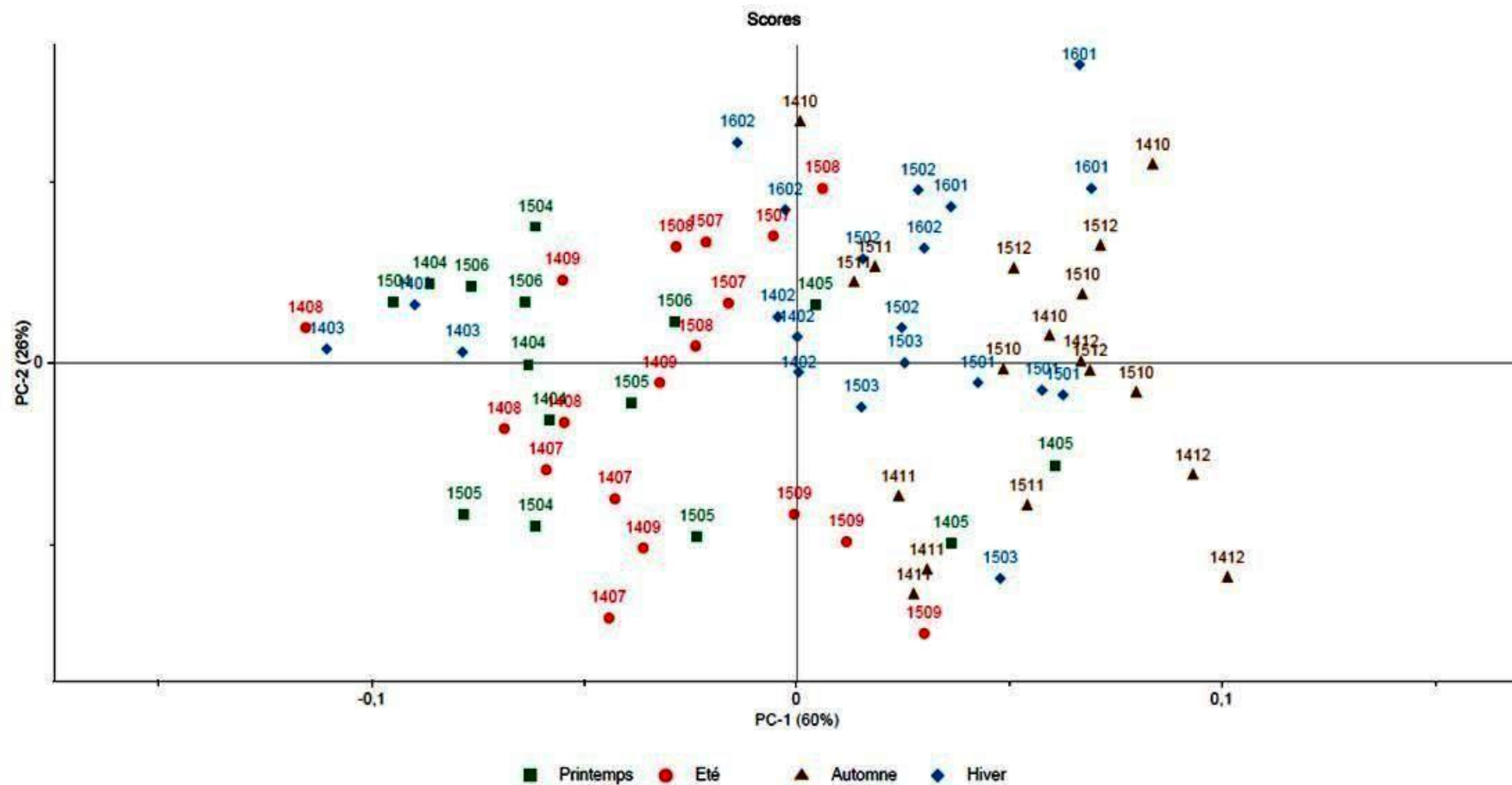


Fig 3-16 Principal component analysis of *S. muticum* polyphenol from selected wavenumbers of 3300-3200, 1620-1605, 1460-1445, 1090-1075, 935-920, 880-865, and 675-660 cm⁻¹. Green box: Spring, Red circle: Summer, Purple triangle: Autumn, and Blue diamond: Winter.

Conclusion

FT-IR analysis is a helpful tool for preliminary identification of certain active compound in algal dry material. This method offers a rapid yet precise analysis. Application of FT-IR in this study is to evaluate the biochemical composition, especially the seasonal variation pattern in dry algal material, and dry enzymatic extract. PCA analysis in the seasonal variation of *S. muticum* biochemical composition reveals that season gives substantial effects towards the contents

Regarding the polyphenol in *Sargassum* enzymatic extracts, FT-IR has detected the presence of several chemical structures resembling those in phloroglucinol (standar).

In this study, the application of FT-IR is used to polyphenol, particularly phlorotannins in *Sargassum*. In addition, this method allows characterizing the seasonal effects towards *S. muticum* polyphenol. Based on the statistical analysis, there are two main group of season influencing the polyphenol production in *S. muticum*, namely the warm/hot season (spring-summer) and cold season (autumn-winter).

3.3.1.2 Enzymatic extract of *S. muticum* polyphenol compounds

Fig 3-17 presents the PCA from selected FT-IR spectra ($1800-600\text{ cm}^{-1}$) of *S. muticum* enzymatic extract. Aqueous and Alcalase extract of *S. muticum* were spotted away from the other samples. Other samples that formed a distinctive group were Neutrase and Ultraflo extracts.

Viscozyme extract was chosen among the other eight as this enzyme had the highest phenolic content based on the Folin-Ciocalteu method. FT-IR managed to detect the presence of phenolic compound based on the identified wavenumbers. The liaison of polyphenol compounds detected from the raw and purified fraction of Viscozyme extract was at 3239.54 , 2934.75 , 1450.96 , 1081.72 , 930.47 , and 873.72 cm^{-1} . These wavenumbers were in accordance to those detected in phloroglucinol (standard) and from previous studies. Additional

bonds which were not present in the purified fraction were detected at 1325 and 899.15 cm^{-1} in raw Viscozyme extract, related to the $\text{C}=\text{CH}_2$ and C-H deformation bonds, respectively. In addition, the PCA analysis of *S. muticum* polyphenol from the FT-IR spectra revealed that there were two groups of variance. The first group consisted of aqueous (control), Alcalase, Neutrase, Ultraflo extracts, and the second group were AMG, Viscozyme, Shearzyme, Termamyl and Celluclast, as shown by Fig 3-18.

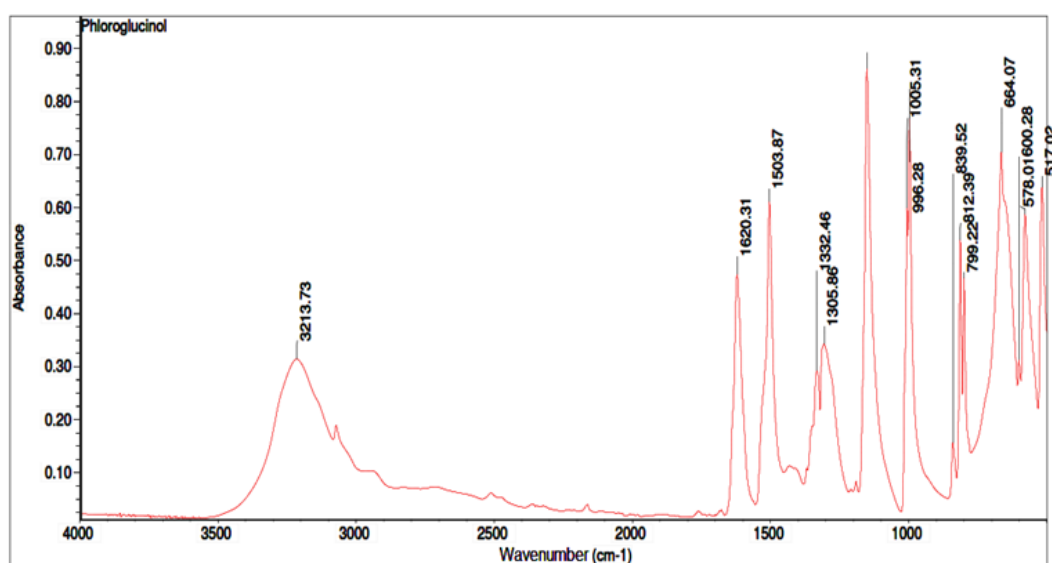


Fig 3-17. FT-IR spectra of phloroglucinol (standard).

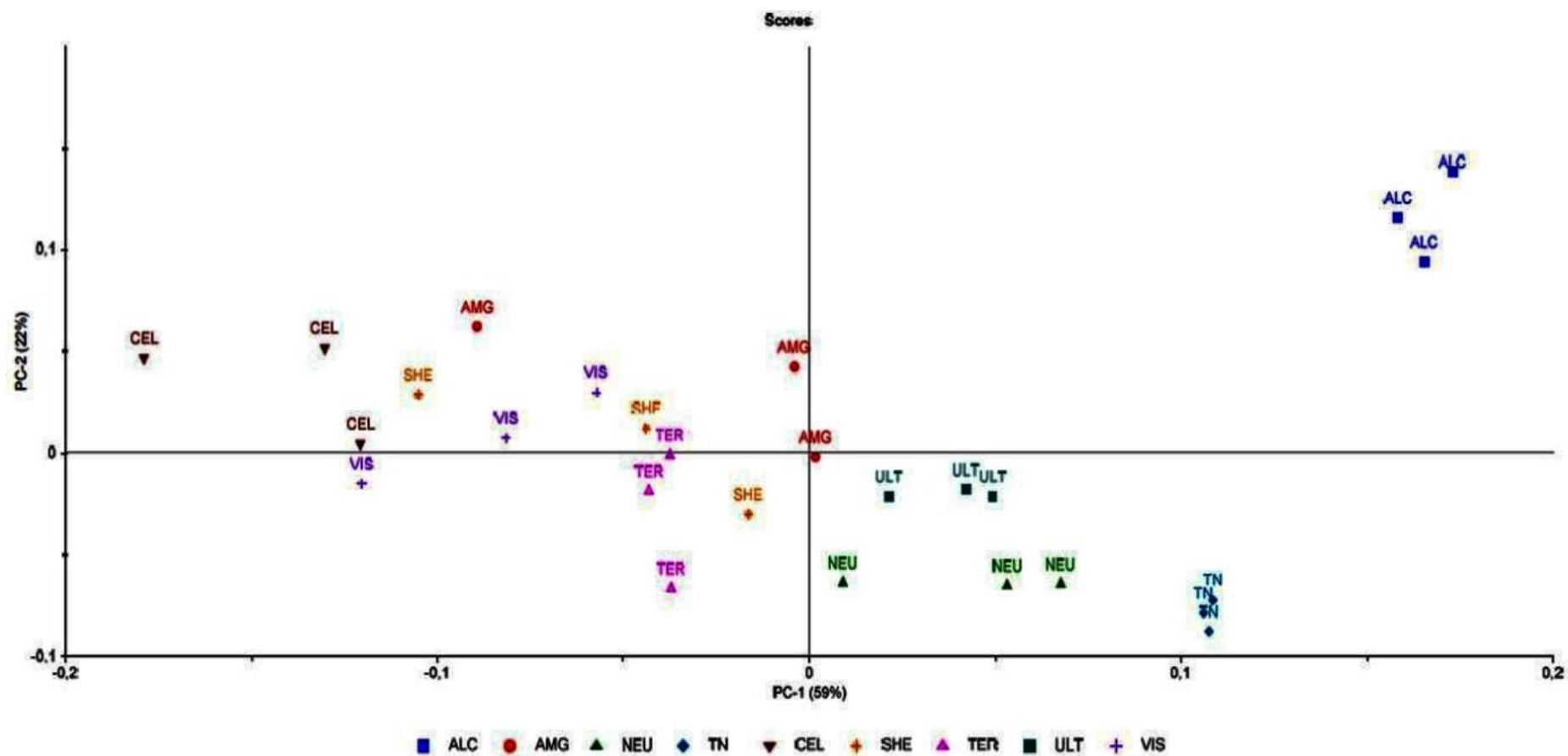


Fig 3-18 Principal component analysis of *S. muticum* enzymatic extract from selected wavenumbers of 1800-600 cm⁻¹. ALC: Alcalase, AMG: Amyloglucosidase, NEU: Neutrase, TN: Aqueous extraction (control), CEL: Celluclast, SHE: Shearzyme, TER: Termamyl, ULT: Ultraflo, VIS: Viscozyme

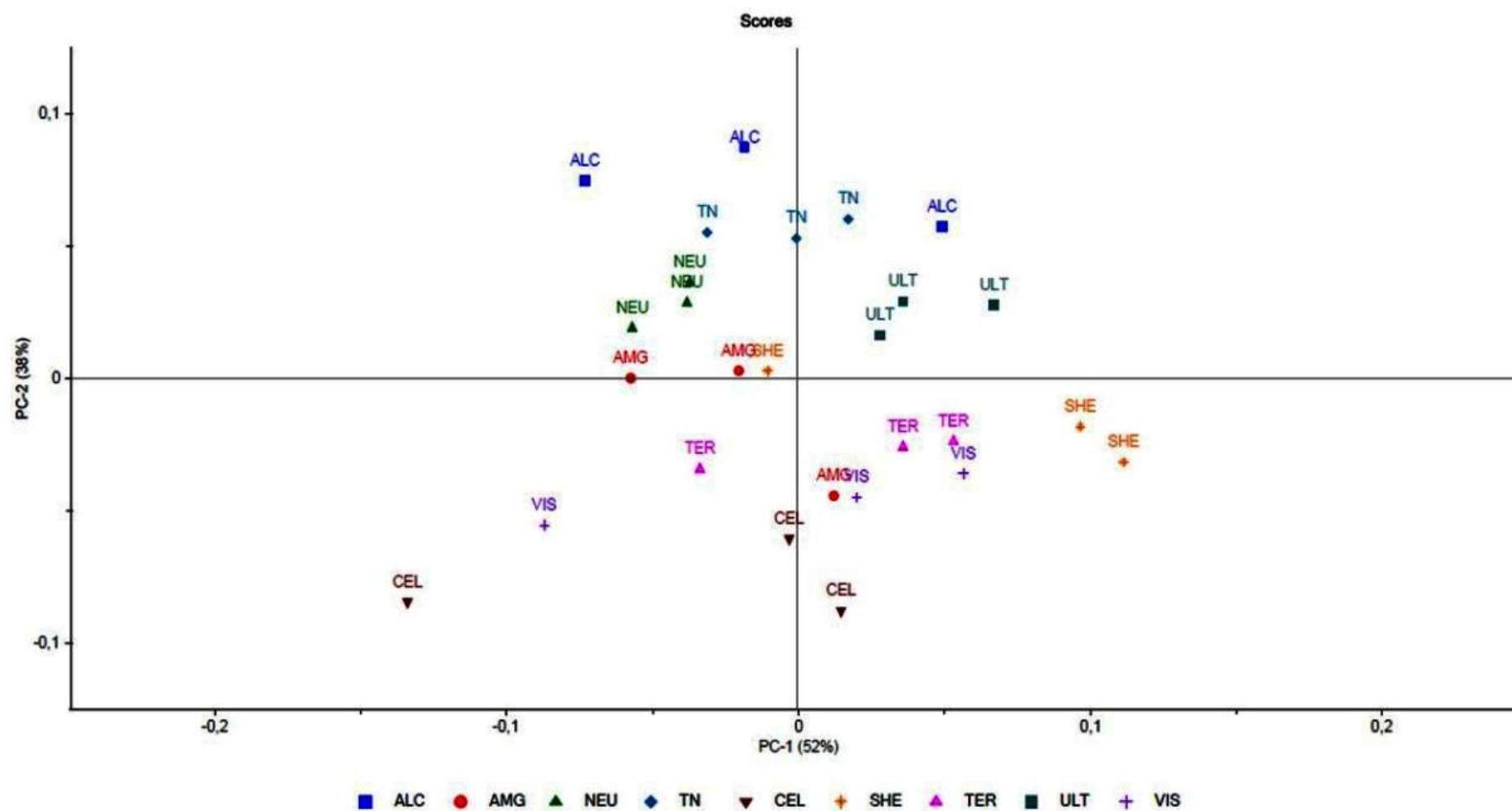


Fig 3-19 Principal component analysis of *S. muticum* enzymatic extract for polyphenol compounds from selected wavenumbers of 3300-3200, 1620-1605, 1460-1445, 1090-1075, 935-920, 880-865, and 675- 660 cm^{-1} . ALC: Alcalase, AMG: Amyloglucosidase, NEU: Neutrase, TN: Aqueous extraction (control), CEL: Celluclast, SHE: Shearzyme, TER: Termamyl, ULT: Ultraflo, VIS: Viscozyme.

3.3.1.3 Enzymatic extract Indonesian *Sargassum* polyphenol compounds

Enzymatic extracts of Indonesian *Sargassum* were characterized using the Fourier-Transform Infrared (FT-IR) spectroscopy as presented on Fig 3-20 and 3-21. In general, the spectra of *S. aquifolium* and *S. ilicifolium* had the same pattern.

The spectra of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* Viscozyme extracts ranged from 3290.89-615.76- cm^{-1} , 3284.48-615.47- cm^{-1} , and 3854.41-1285.15- cm^{-1} , respectively. Then, the detected spectra of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* Protamex extracts ranged from 3304.13-614.73 cm^{-1} , 3277.25-615.51 cm^{-1} , and 3274.49-1259.91. Meanwhile, the phloroglucinols spectrum was detected at 3217.73, 1620.31, 1503.87, 1332.46, 1305.86, 1150.82, 1005.31, 996.28, 839.52, 812.39, 517.02, 799.22, 664.07, 600.28, and 578.01 cm^{-1} .

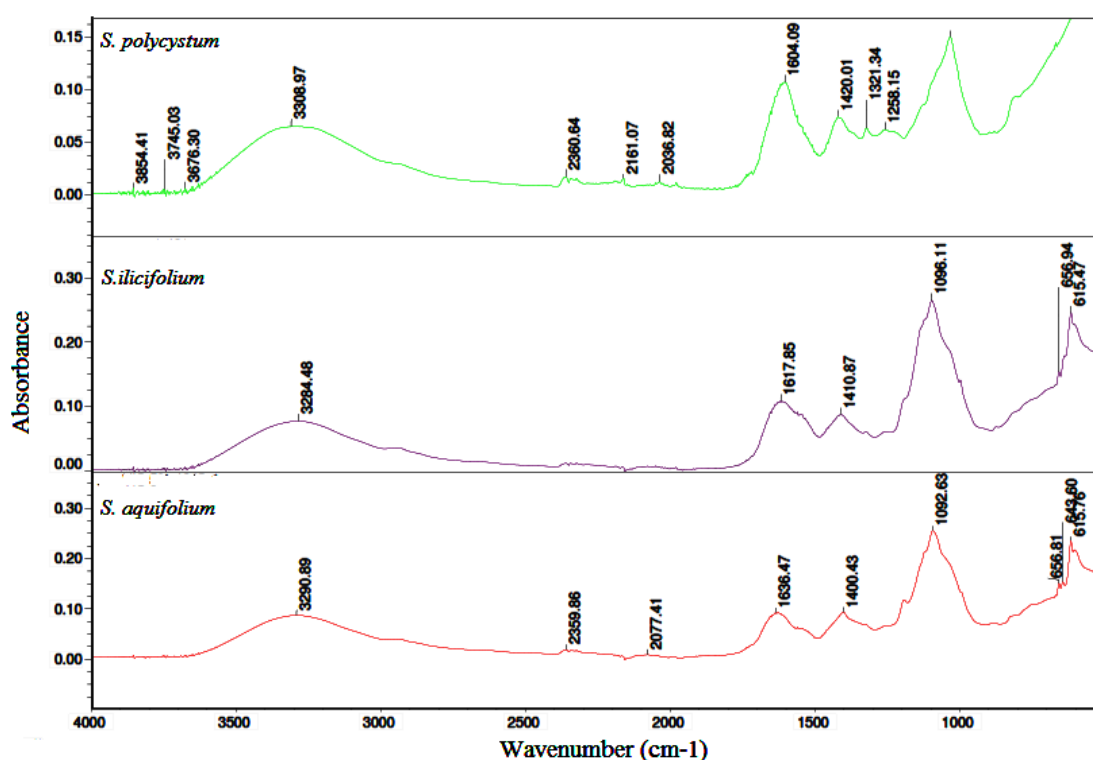


Fig 3-20 FT-IR spectra of *S. polycystum*, *S. ilicifolium*, and *S. aquifolium* extracted with Viscozyme

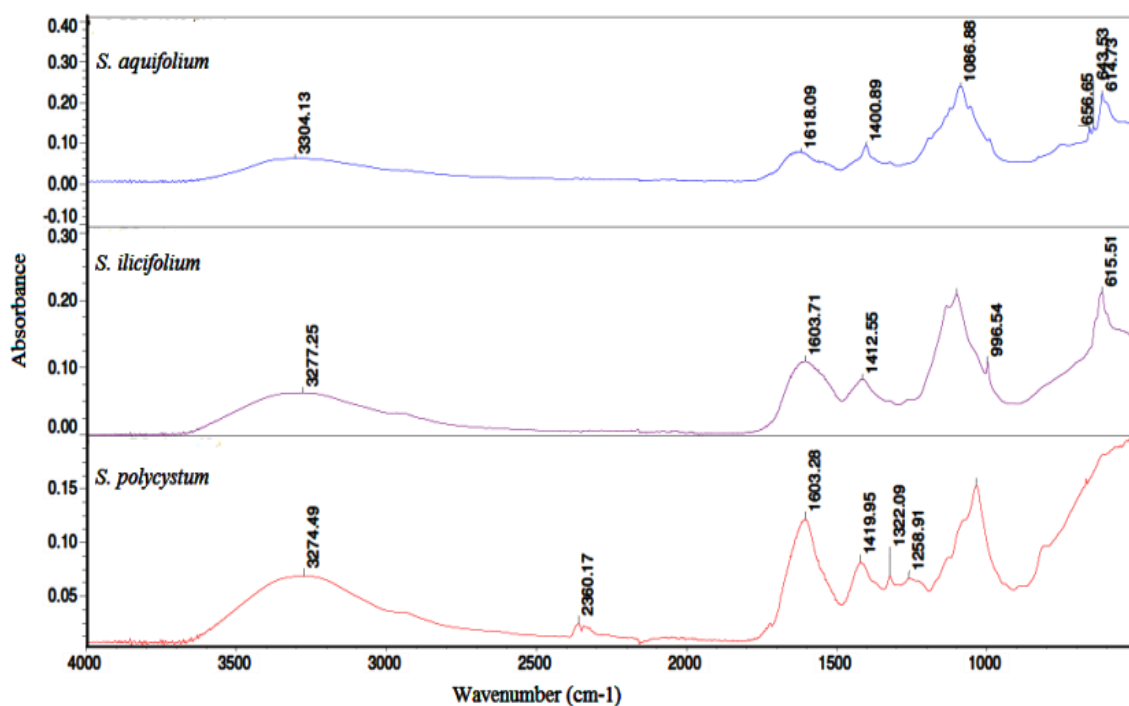


Fig 3-21 FT-IR spectra of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* extracted with Protamex.

The presence of phloroglucinol-like (polyphenol) compounds was detected in *S. aquifolium*, *S. ilicifolium* and *S. polycystum*. The spectra that were assumed to correspond with this compound was 1636.47 and 3290.89 cm^{-1} from *S. aquifolium*, 1617.85 and 3284.48 cm^{-1} from *S. ilicifolium* and 3308.97 cm^{-1} from *S. polycystum*. All of these spectra were from the Viscozyme extracts. As for the Protamex extracts of *S. aquifolium*, *S. ilicifolium* and *S. polycystum*, the spectra that corresponded to the polyphenol were 3304.13 and 1618.09 cm^{-1} ; 3277.25, 1603.71 and 996.54 cm^{-1} ; and 3274.49, 1603.28, and 1322.09 cm^{-1} , respectively.

Conclusion

Preliminary characterization using FT-IR showed the presence of phloroglucinol-like compounds indicating the detection of phlorotannins in enzymatic extracts. Such detection was based on the corresponding peaks obtained from literature and peak of phloroglucinol as standard. However, there was also the presence of other compounds based on their functional structure, such as carbohydrates and proteins, especially in the raw extracts.

3.3.2 High performance liquid chromatography (HPLC)

3.3.2.1 Polyphenol of *S. muticum* – seasonal variation

For further understanding the seasonal variation of polyphenol, the extract of *S. muticum* in different solvents were then analyzed by using HPLC as presented on Appendix 8 Apparently, HPLC chromatograms merely revealed qualitative variation of *S. muticum* polyphenol based on the peaks intensity. The intensity of these peaks varied in function of months and they were detected at 7-8 minutes and 9.5-11 minutes of retention time. Polyphenol compounds referred in this analysis were phlorotannins. In comparison to our samples, the phloroglucinols – commercial standard used to evaluate the presence of phlorotannins – had retention time at 10 minutes. Peaks of *S. muticum* extracts were detected at wavenumbers between 260 and 280 nm; 280 nm was the same wavenumbers in which phloroglucinols peak was detected.

Fig 3-22 and 3-23 are HPLC chromatogram in 3D of *S. muticum* polyphenol extracted in water from the solid-liquid extraction representing autumn, winter, spring and summer in 2014. From these four images, noticeable difference is observed between 6 to 8 min and 10 to 11 min of retention time (sphere-like forms in blue enveloped in red line). 3D image helps to illustrate the detected compound that was elucidated by the HPLC system. *S. muticum* water extracts collected in autumn, winter and spring might contained same character of compound since the HPLC managed to record the detected compound that was elucidated at 6 to 8 min. In summer, the same pattern was not observed (Fig 3-23). In addition, there was a compound detected at 10-11 min; however, it was assumed that the quantity was this compound was fluctuated. In autumn and spring, the compound was clearly distinguished in color yet it was not appeared in *S. muticum* extract collected in summer and winter. We hypothesized that the compound detected in the HPLC system might be a phloroglucinol-like compound, in other words, phlorotannins.

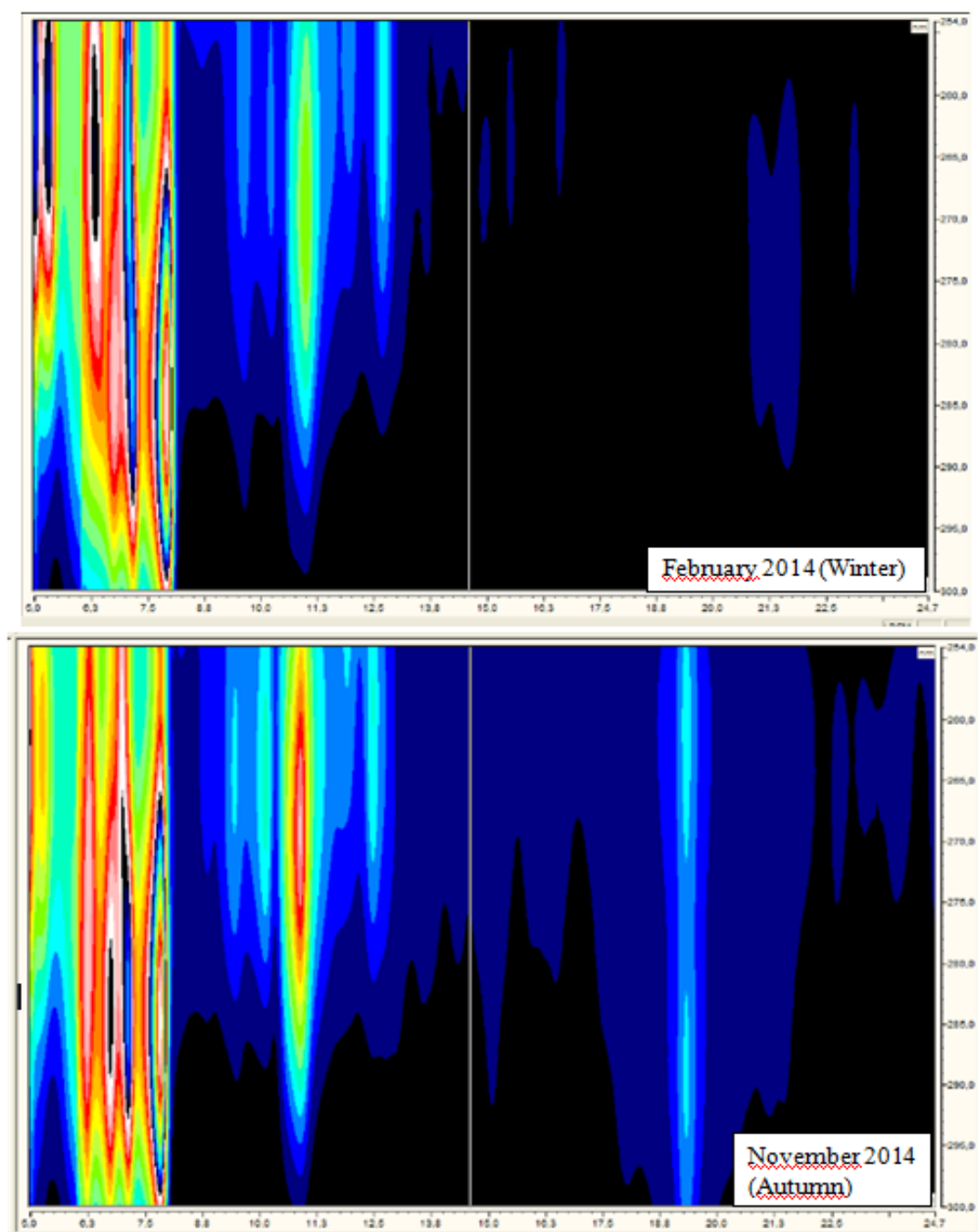


Fig 3-22 HPLC Chromatogram in 3D of *S. muticum* collected in winter and autumn. Axis represents the retention time (vertical) and wavenumbers (horizontal).

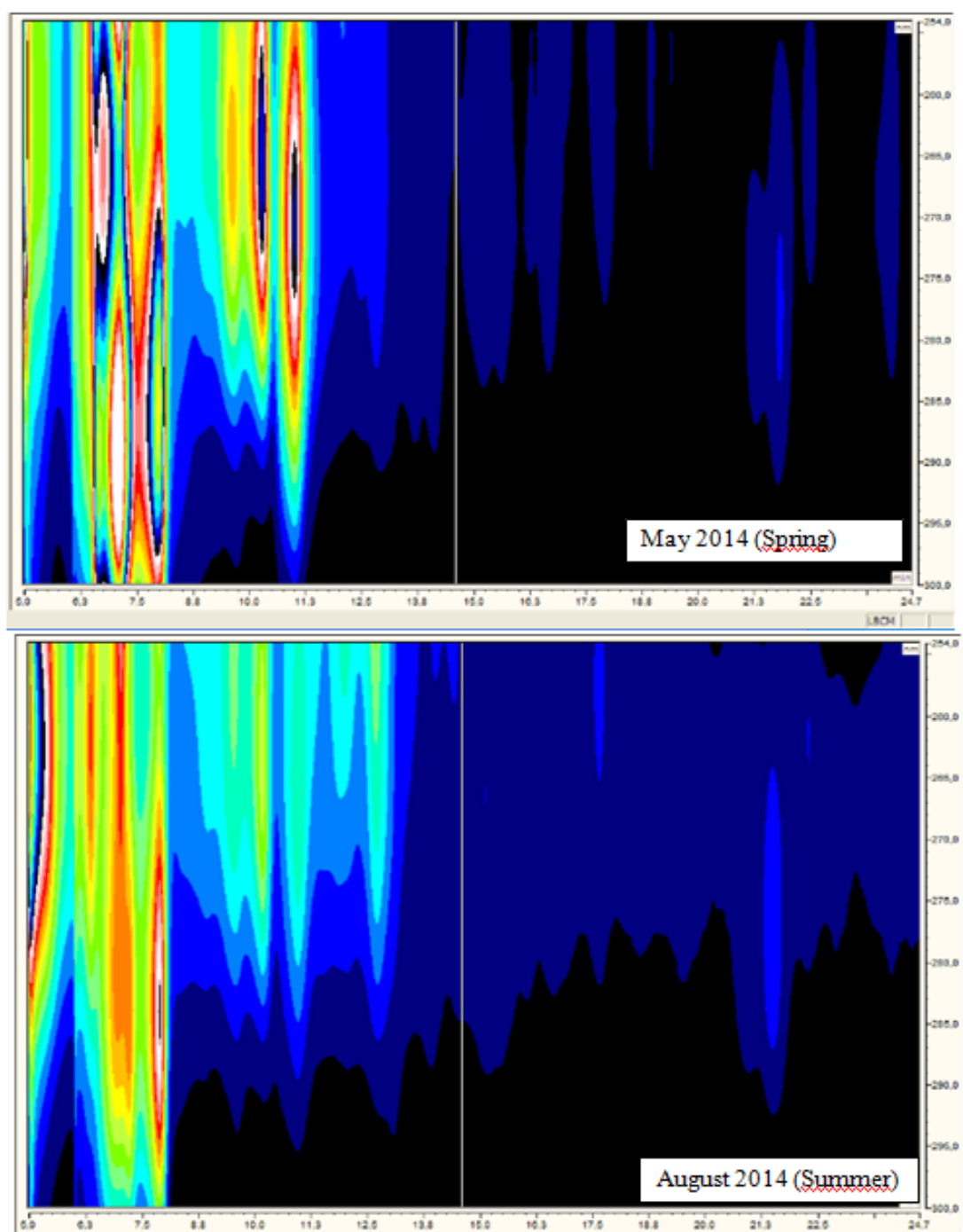


Fig 3-23 HPLC Chromatogram in 3D of *S. muticum* collected in spring and summer. Axis represents the retention time (vertical) and wavenumbers (horizontal).

3.3.2.2 Purified fraction of *S. muticum* Viscozyme extract

Chromatogram of HPLC from the raw extract and the purified fraction of *S. muticum* Viscozyme extract are presented on Fig 3-21. Based on the HPLC analysis, Viscozyme extract of *S. muticum* contained a compound with same characteristic as phloroglucinol (Fig 3-21 (Above)). This was due to the peak that was detected at the same retention time it had as phloroglucinols, *i.e.* at 10.483 min.

Purified fraction of *S. muticum* Viscozyme extract showed a well separated fraction assumed to have similar chemical structure as phloroglucinol (Fig 3-21 (Below)). We attempted to fractionate and collect the fraction from the selected peak. Unfortunately, it was not a successful attempt as the collected fractions was too diluted, thus, the quantity obtained was not sufficient for further analysis. Though, it would be interesting to further evaluate this active fraction.

Conclusion

HPLC analysis performed to the seasonal data of *S. muticum* polyphenol showed that the production of polyphenols was mostly affected by the sunlight, air temperature and sea surface temperature. However, the strength of this correlation varied depending on the solvents used. Water extracts had strong correlation, and aqueous methanol 50% showed a moderate correlation with sunlight, air temperature and sea surface temperature. As for methanol and aqueous ethanol 75% extracts, they demonstrated a weak correlation with those three conditions.

Regarding the pure fraction of *S. muticum* polyphenol extracted with Viscozyme, HPLC managed to separate the peak corresponding to phloroglucinol, even though the intensity was relatively low. It indicated that the eluted compound was not too diluted. As a consequence, this fraction was hardly collected after the fractionation using HPLC.

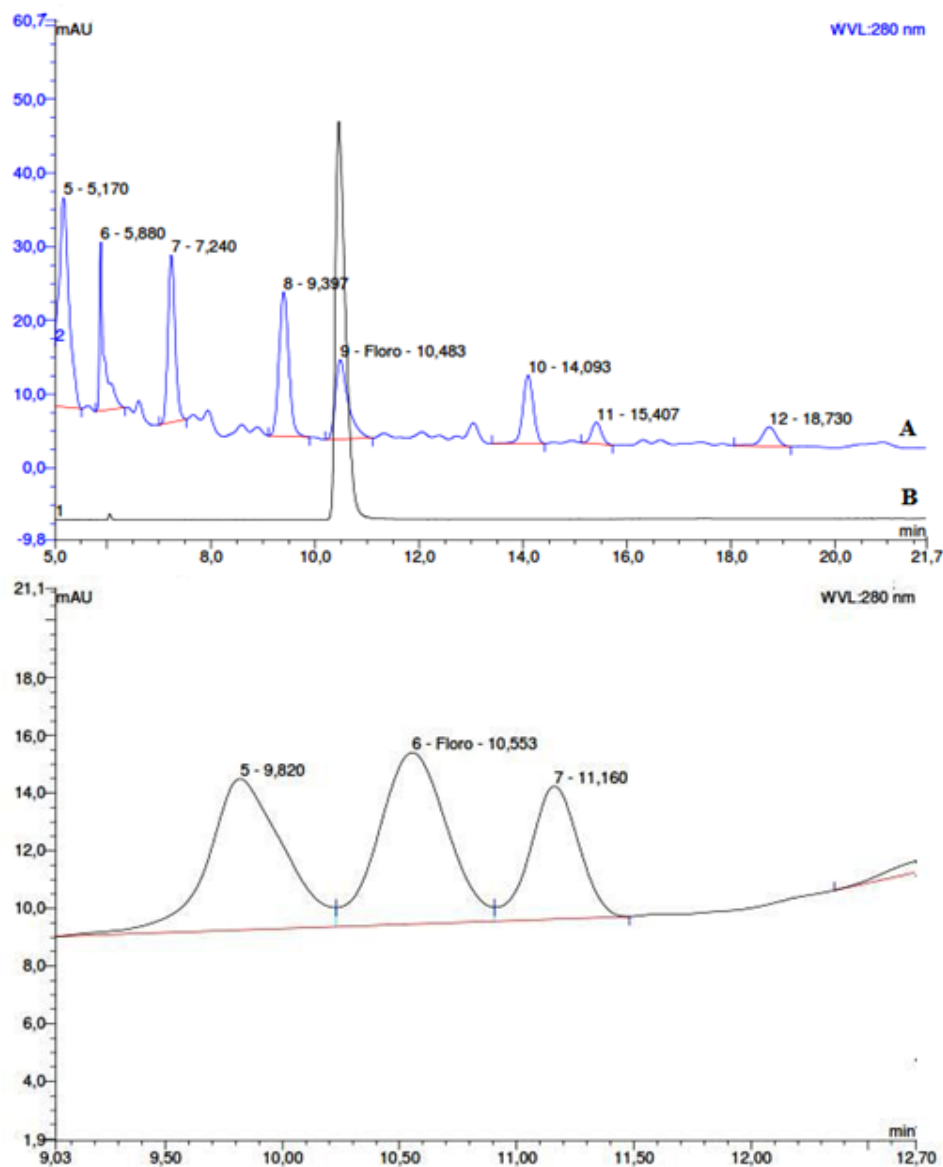


Fig 3-24 Chromatogram of *S. muticum* enzymatic extract (Above) Raw extract (A) with phloroglucinol (B) and (Below) purified fraction.

3.4 Antioxidant activity

3.4.1 DPPH radical scavenging and FRAP

3.4.1.1 Screening of enzymes and optimization

The antioxidant activities of *S. muticum* enzymatic extracts were determined through the free radical scavenging-ability and the reducing power by means of DPPH and

FRAP analysis. Table 3-16 exhibits the antioxidant of *S. muticum* from June 2014 extracted with different enzymes at 50°C for 3 h without denaturation. All samples showed a significant difference ($p<0.05$) of DPPH IC₅₀ compared to control (aqueous extract). Having low value of IC₅₀ indicates that the sample has a strong antiradical activity. In this case, Viscozyme and Celluclast extracts (0.6 ± 0.1 mg/mL) would be mostly effective against the free radical compared to the other. Ultraflo (2.2 ± 0.9 mg/mL) and Alcalase extracts (1.9 ± 0.3 mg/mL) was less effective against the free radical as shown by their high value of DPPH IC₅₀. Nevertheless, the free radical scavenging activity of *S. muticum* enzymatic extracts was inferior to the commercial antioxidant BHA and BHT (IC₅₀: 0.008 ± 0.5 and 0.012 ± 4.8 mg/mL).

All samples had a significantly different reducing capacity from the aqueous extract (65.7 ± 1.0 μ M of Fe²⁺). The most effective reducing capacity was shown by AMG extract in 60.8 ± 1.8 μ M of Fe²⁺. In the contrary, *S. muticum* Viscozyme extract did not show a strong reducing capacity with only 48.7 ± 0.9 μ M of Fe²⁺. The reducing power activity of *S. muticum* enzymatic extracts was still inferior to commercial Vitamin C, 58.1 ± 0.7 μ M of Fe²⁺. Results are presented on Table 3-16. DPPH IC₅₀ of BHA and BHT and reducing power of Vitamin C as standards were $8 \times 10^{-3} \pm 0.5$, $12 \times 10^{-3} \pm 0.5$ mg/ml and 58.1 ± 0.7 μ M of Fe²⁺.

Table 3-16 Antioxidant activity of *S. muticum* enzymatic extracts from June 2014 (different letter superscript letters indicates a significant difference ($p<0.05$)).

Samples	DPPH IC ₅₀ (mg/mL)	FRAP (μ M of Fe ²⁺)
BHA	$8 \times 10^{-3} \pm 0.5$	-
BHT	$12 \times 10^{-3} \pm 0.5$	-
Vitamin C	-	58.1 ± 0.7
Control	0.7 ± 0.2^a	65.7 ± 1.0^a
Neutrase	0.9 ± 0.1^a	58.8 ± 0.6^{bd}
Ultraflo	2.2 ± 0.9^c	58.4 ± 0.8^b
Alcalase	1.9 ± 0.3^{bc}	56.1 ± 3.0^c
Celluclast	0.6 ± 0.1^a	59.3 ± 1.7^{bd}
AMG	0.7 ± 0.3^a	60.8 ± 1.8^{ad}
Shearzyme	1.0 ± 0.3^{ab}	58.4 ± 0.7^{bd}
Viscozyme	0.6 ± 0.1^a	48.7 ± 0.9^c

Termamyl	0.8 ± 0.2^a	57.4 ± 1.0^b
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Antioxidant activity of *S. muticum* from November 2014 that was extracted with Viscozyme, Termamyl and Protamex was evaluated and results are presented on Table 3-17. This time, algal material was extracted at 40°C for 3 h without denaturation and the enzyme concentration was increased to 5%. As for samples that were extracted with 10% enzymes, antioxidant activity was not determined.

Table 3-17 Antioxidant activity of *S. muticum* enzymatic extracts from November 2014 after optimization.

Samples	Concentration (%)	DPPH IC ₅₀ (mg/mL)	FRAP (μM of Fe ²⁺)
Viscozyme	5	7.7 ± 1.7	15.5 ± 0.4
Protamex		3.3 ± 0.8	16.0 ± 0.3
Termamyl		0.6 ± 0.0	17.5 ± 0.5
Viscozyme	10	n.d	n.d
Protamex		n.d	n.d
Termamyl		n.d	n.d
Water		0.7 ± 0.0	31.0 ± 2.1
n.d: not determined			

3.4.1.2 Indonesian *Sargassum* extracted with solid-liquid and enzyme-assisted extraction

A comparison related to the bioactivities between solid-liquid and enzyme-assisted extraction was performed. In order to compare these two methods, 40 °C was chosen as extraction condition. The fact that 40 °C is the most optimal condition for polyphenol extraction in solid-liquid becomes the main fundamental in choosing this condition. Table 3-18 present the results of antioxidant activity from Indonesian *Sargassum* extracted in two different methods.

Based on the result, Protamex extract of *S. aquifolium* had better antiradical activity by its IC₅₀ 1.4 ± 0.7 mg/ml. As for the reducing power, water extract of *S. polycystum* showed promising activity with 46.5 ± 5.3 μM of Fe²⁺. Statistical analysis was performed to know whether there was a significant difference or not in Indonesian *Sargassum* antioxidant activity. Analysis of variance (see Appendix 11),

showed that there was a significant difference ($p>0.05$) in antioxidant activity of Indonesian *Sargassum* due to species and method of extraction. Table 3-18 exhibits the antiradical scavenging activity of Indonesian *Sargassum* represented by IC₅₀.

Table 3-18 Antiradical scavenging activity of Indonesian *Sargassum* in solid-liquid and enzyme-assisted extraction.

Samples	Methods	DPPH IC ₅₀ (mg/mL)	Sig. ($p<0.05$) of Species	Sig. ($p<0.05$) of Solvents
BHA		7.5±0.4		
BHT		11.7±4.5		
<i>S. aquifolium</i>	Water	8.9 ± 2.1		
	MeOH	n.a		
	MeOH 50%	3.2 ± 1.7		
	EtOH 75%	n.a		
	Viscozyme	n.a		
	Protamex	1.4 ± 0.7		
<i>S. ilicifolium</i>	Water	n.a		
	MeOH	3.4 ± 0.8		
	MeOH 50%	9.0 ± 0.7	.014 (df = 2 ; F= 4.802)	.008 (df = 5; F = 3.691)
	EtOH 75%	9.9 ± 1.8		
	Viscozyme	9.9 ± 6.1		
	Protamex	4.4 ± 2.4		
<i>S. polycystum</i>	Water	n.a		
	MeOH	n.a		
	MeOH 50%	20.3 ± 2.5		
	EtOH 75%	5.5 ± 0.3		
	Viscozyme	n.a		
	Protamex	9.5 ± 2.4		

S. aquifolium and *S. ilicifolium* showed a significant difference in DPPH IC₅₀ ($p<0.05$). Later, the DPPH IC₅₀ of methanol extracts was significantly different to the aqueous methanol 50% ($p<0.05$). For the reducing activity, all *Sargassum* species showed significant difference ($p<0.05$). As for the solvents, water, aqueous methanol 50% and Viscozyme extracts had significant difference to each other and with the other methods ($p<0.05$). Water, aqueous methanol 50% and Viscozyme extracts extract were significantly different from aqueous ethanol 75%, methanol and Protamex extracts ($p<0.05$). Such results implied that different methods gave

significant effect towards the reducing activity. Protamex showed no significant difference with methanol and aqueous ethanol 75%.

It might be possible that in certain extracts as in methanol extract of *S. aquifolium* with no antiradical activity, they were not active in small concentrations since the concentration used in this study ranged from 0 to 1 mg/mL.

Table 3-19 shows the reducing antioxidant power (FRAP) activity of Indonesian *Sargassum* in solid-liquid and enzyme-assisted extraction. *S. aquifolium*, *S. ilicifolium* and *S. polycystum* showed a significant difference in FRAP activity ($p < 0.05$). Water extract had a significant difference with the all other extracts ($p < 0.05$). Viscozyme and aqueous methanol extracts showed significant difference with methanol, aqueous ethanol 75%, and Protamex extracts ($p < 0.05$). Interestingly, Protamex extracts did exhibit a significant different with methanol and aqueous ethanol 75% extracts ($p > 0.05$).

Due to different drying treatment between French and Indonesian *Sargassum*, we could not combine the analysis. As a consequence, the statistical analysis was separated. Table 3-20 presents the antioxidant activity of *S. muticum* in different methods of extraction. *S. muticum* extracted in aqueous methanol 50% and Viscozyme showed stronger antiradical activity at 0.5 ± 0.0 mg/ml. Meanwhile, water extract of *S. muticum* had more efficient reducing power with 51.4 ± 0.5 μ M of Fe^{2+} . DPPH IC_{50} of *S. muticum* showed that there was no significant effect generated by different methods of extraction ($p > 0.05$).

A significant difference in consequence of different methods was observed in the reducing activity of *S. muticum*. As shown by Table 3-20, the reducing power activity of water, methanol, aqueous methanol 50% and aqueous ethanol 75% extracts were significantly different from Protamex and Viscozyme extracts. However, there was no significant effect in the reducing power activity of Protamex and Viscozyme extract ($p > 0.05$).

Table 3-20 Reducing antioxidant power (FRAP) activity of Indonesian *Sargassum* in solid-liquid and enzyme-assisted extraction.

Samples	Methods	FRAP (μM of Fe^{2+})	Sig. ($p < 0.05$) of Species	Sig. ($p < 0.05$) of Solvents
Vitamin C		58.1 \pm 0.7		
<i>S. aquifolium</i>	Water	21.8 \pm 1.7	.000 (df = 2 ; F= 46.436)	.000 (df = 5 ; F= 80.014)
	MeOH	12.7 \pm 2.5		
	MeOH 50%	22.4 \pm 4.9		
	EtOH 75%	14.4 \pm 0.3		
	Viscozyme	33.2 \pm 2.5		
	Protamex	16.2 \pm 0.2		
<i>S. ilicifolium</i>	Water	24.3 \pm 1.6	.000 (df = 2 ; F= 46.436)	.000 (df = 5 ; F= 80.014)
	MeOH	11.5 \pm 0.8		
	MeOH 50%	16.0 \pm 0.8		
	EtOH 75%	10.1 \pm 1.2		
	Viscozyme	15.3 \pm 1.0		
	Protamex	8.6 \pm 0.6		
<i>S. polycystum</i>	Water	46.5 \pm 5.3	.000 (df = 2 ; F= 46.436)	.000 (df = 5 ; F= 80.014)
	MeOH	12.7 \pm 3.1		
	MeOH 50%	16.9 \pm 2.6		
	EtOH 75%	13.7 \pm 2.0		
	Viscozyme	27.7 \pm 4.9		
	Protamex	16.3 \pm 0.1		

Table 3-21 Antioxidant activity of French *S. muticum* from June 2014 polyphenol extracted in solid-liquid and enzyme-assisted extraction.

Samples	DPPH IC ₅₀ (mg/mL)	FRAP (μM of Fe^{2+})
BHA	7.5 \pm 0.4	-
BHT	11.7 \pm 4.5	-
Vitamin C	-	58.1 \pm 0.7
Water	1.8 \pm 0.0 ^a	51.4 \pm 0.8 ^a
MeOH	1.9 \pm 0.0 ^a	32.1 \pm 5.0 ^b
MeOH 50%	0.5 \pm 0.0 ^a	44.9 \pm 5.4 ^{ab}
EtOH 75%	0.7 \pm 0.0 ^a	38.9 \pm 6.7 ^{ab}
Viscozyme	0.5 \pm 0.0 ^a	15.0 \pm 1.3 ^c
Protamex	1.6 \pm 1.3 ^a	11.8 \pm 1.6 ^c

Conclusion

Based on the screening, if a sample had strong antiradical activity, it showed an opposite reducing power capacity in which it had an inverse relationship. As an

example, Viscozyme extract of *S. muticum* polyphenol demonstrated a promising antiradical activity but not reducing power. From the eight commercial enzymes used, Celluclast and Viscozyme extracts exhibited similar activity based on their IC₅₀ value. On the contrary, the best reducing power activity was shown by aqueous extracts.

Concerning the antiradical activity of Indonesian *Sargassum* extracted in different methods, in certain case, enzymatic extracts showed stronger activity than the solvent extracts. However, the reducing power activity of enzymatic extracts was less effective than the organic solvents.

3.5 Tyrosinase and elastase inhibition activity

3.5.1 Screening of enzymes and optimization

First result presented in this part is the tyrosinase and elastase inhibition activities of *S. muticum* enzymatically extracted at 50 °C and denaturated at 90 °C with 2.5% of enzymes. Results are presented on Table 3-18. For the tyrosinase inhibition activity of *S. muticum* enzymatic extracts, their activity showed no significant difference ($p>0.05$) and their effects on tyrosinase enzyme were still low compared to kojic acid ($67.6 \pm 6.5\%$ of inhibition) and quercetin ($42.7 \pm 9.4\%$ of inhibition) – serving as positive controls. The inhibition activity of *S. muticum* enzymatic extracts ranged from 6.6 ± 1.5 (Neutrased extract) to $24.5 \pm 2.6\%$ of inhibition (Ultraflo extract).

In elastase inhibition analysis, a significant difference was only showed between the AMG ($21.6 \pm 0.8\%$ of inhibition) and Shearzyme extracts ($32.8 \pm 0.9\%$ of inhibition). It seems that AMG extract has the lowest activity while Shearzyme extract has the highest one. All *S. muticum* enzymatic extracts showed no significant difference with the control ($23.1 \pm 4.8\%$ of inhibition). Furthermore, the elastase inhibition activity of *S. muticum* enzymatic extracts was weaker than that of the trypsin inhibitor ($50.0 \pm 0.0\%$ of inhibition). Results are presented on Table 3-21. Kojic acid and quercetin served as standard and they exhibited 67.6 ± 6.5 and $42.7 \pm 9.4\%$ of tyrosinase inhibition activity.

Table 3-22 Tyrosinase and elastase inhibition activity of *S. muticum* enzymatic extracts, significant difference is assigned with different superscript letter (p<0.05).

Samples	Tyrosinase Inhibition Activity (% of inhibition)	Elastase Inhibition Activity (% of inhibition)
Kojic Acid	67.6±6.5	-
Quecertin	42.7±9.4	-
Trypsin inhibitor	-	50.0±0.0
Control	14.7±3.1 ^a	23.1±4.8 ^{ac}
Neutrase	6.6±1.5 ^b	23.4±3.3 ^{ac}
Ultraflo	24.5±2.6 ^c	24.0±4.2 ^{ac}
Alcalase	10.7±3.5 ^a	30.9±5.9 ^{ac}
Celluclast	19.9±5.1 ^a	29.9±0.5 ^{ac}
AMG	15.4±2.3 ^a	21.6±0.8 ^a
Shearzyme	13.0±0.7 ^a	32.8±0.9 ^{bc}
Viscozyme	23.5±2.6 ^c	29.7±1.9 ^{ac}
Termamyl	14.2±2.5 ^a	28.7±3.8 ^{ac}

Tyrosinase inhibition activity of *S. muticum* (extraction at 40°C with 5% of enzyme) was analyzed and results are presented on Table 3-22. Unfortunately, the activity was less appealing than the previous samples presented on Table 3-21.

Table 3-23 Tyrosinase inhibition activity of *S. muticum* enzymatic extracts.

Samples	Concentration (%)	Tyrosinase inhibition activity (% of inhibition)
Kojic Acid		83.3 ± 5.5
Quecertin		46.7 ± 3.9
Viscozyme		9.4 ± 0.3
Protamex	5	n.a
Termamyl		14.8 ± 0.8
Viscozyme		n.d
Protamex	10	n.d
Termamyl		n.d
Water		n.d

n.d: not determined

3.5.2 Enzymatic extract of Indonesian *Sargassum*

Tyrosinase inhibition activity of *Sargassum* enzymatic extracts was then analyzed and the results are presented on Table 3-23. *S. ilicifolium* extracted with Viscozyme showed no inhibition. Meanwhile, *S. aquifolium* and *S. polycystum* Viscozyme extracts showed an inhibition of 11.5 ± 9.7 and 10.2 ± 4.1 %. For Protamex extracts, the tyrosinase inhibition activity of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* were 9.7 ± 5.0 , 8.2 ± 1.1 and 13.3 ± 6.6 %, respectively. Statistical analysis showed that there was no significant difference ($p > 0.05$).

Table 3-24 Tyrosinase inhibition activity of Indonesian *Sargassum* enzymatic extracts.

Samples	Tyrosinase inhibition activity (% of inhibition)	
Kojic Acid	83.3 ± 5.5	
Quecertin	46.7 ± 3.9	
	Viscozyme	Protamex
<i>S. aquifolium</i>	11.5 ± 9.7	9.7 ± 5.0
<i>S. ilicifolium</i>	n.a.	8.2 ± 1.1
<i>S. polycystum</i>	10.2 ± 4.1	13.3 ± 6.6
n.a.: no activity		

Conclusion

Tyrosinase and elastase inhibition activity of *S. muticum* polyphenol extracted with enzymes might show a weak activity, same case with Indonesian *Sargassum*. However, these results revealed the potential of enzymatic extract that needs further study as whitening and anti-aging agent.

3.6 Antimicrobial activity

3.6.1 Antibacterial activity

Based on the antibacterial activity of *S. muticum* enzymatic extract against four pathogenic bacteria, i.e *S. hominis*, *B. subtilis*, *P. aeruginosa* and *E. coli*, all samples showed no remarkable activity (data was not shown). We assumed that the presence

of polysaccharides in our raw enzymatic extracts provoked the growth of the bacteria instead of suppressing their growth.

Antibacterial activity of *S. muticum* extracts from two methods of extractions was evaluated against *E. coli*, *B. subtilis* and *P. aeruginosa*. Less appealing results were obtained from the enzymatic extracts of *S. muticum*. They did not show any activity against the three bacteria (data was not shown); as a consequence, further analysis was not performed for the other enzymatic extracts.

Table 3-24 presents solely the antibacterial activity of *Sargassum* extracted using different solvents against *E. coli*, *B. subtilis* and *P. aeruginosa*. *S. aquifolium* in aqueous methanol 50% and *S. ilicifolium* in both methanolic extracts showed no activity against *B. subtilis* and *P. aeruginosa*, respectively.

Table 3-25 Antibacterial activity of *S. aquifolium*, *S. ilicifolium*, *S. polycystum* and *S. muticum* against *E. coli*, *B. subtilis* and *P. aeruginosa*.

Samples	Solvents	Antibacterial activity (cm)		
		<i>E. coli</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>
Phosphomycin		2.2 ± 0.1	1.7 ± 0.1	0.9 ± 0.1
Ampicilin		1.7 ± 0.1	1.6 ± 0.1	0.5 ± 0.0
Streptomycin		1.8 ± 0.2	1.1 ± 0.2	2.7 ± 0.1
<i>S. aquifolium</i>	Water	+	+	+
	MeOH	+	+	+
	MeOH 50%	+	n.a.	+
	EtOH 75%	+	+	+
<i>S. ilicifolium</i>	Water	+	+	+
	MeOH	+	+	n.a.
	MeOH 50%	+	+	n.a.
	EtOH 75%	+	+	+
<i>S. polycystum</i>	Water	+	+	+
	MeOH	+	+	+
	MeOH 50%	+	+	+
	EtOH 75%	+	+	+
<i>S. muticum</i>	Water	+	+	+
	MeOH	+	+	+
	MeOH 50%	+	+	+
	EtOH 75%	+	+	+

n.a. = no activity

3.6.2 Biofilm inhibition activity

3.6.2.1 Screening of enzymes and optimization

For the biofilm generated from *P. aeruginosa*, there was no sign of inhibition from the control, Alcalase, Celluclast and AMG extracts. A weak inhibition was presented by Ultraflo and Termamyl extracts with $3.7 \pm 2.0\%$ and $12.4 \pm 3.9\%$, respectively. Highest activity was shown by *S. muticum* enzymatic extracts Viscozyme with $43.7 \pm 4.2\%$ of inhibition. All *S. muticum* enzymatic extracts were less effective in inhibiting the formation of biofilm formed by *P. aeruginosa* compared to *E. coli*, as can be seen in Table 3-22.

Strong biofilm inhibition activity formed by *E. coli* was performed by Shearzyme extract followed by Termamyl extract with $64.3 \pm 10.3\%$ and $49.2 \pm 13.3\%$, respectively. In addition, their activity was significantly better than the control, $14.7 \pm 0.9\%$. Based on the biofilm inhibition activity of *S. muticum* enzymatic extract, the value presented might indicate a promising potential of *S. muticum* enzymatic extracts as an antifouling agent.

Table 3-26 Biofilm inhibition of *S. muticum* enzymatic extract (expressed in % of inhibition, different superscript letters indicates a significant difference ($p < 0.05$)).

Samples	<i>P. aeruginosa</i>	<i>E. coli</i>
Control	n.a	14.7 ± 0.9^a
Neutrase	36.1 ± 7.8^a	29.1 ± 10.0^{ac}
Ultraflo	3.7 ± 2.0^b	32.8 ± 3.7^{ac}
Alcalase	n.a	38.0 ± 7.9^b
Celluclast	n.a	33.8 ± 7.7^a
AMG	n.a	43.2 ± 5.9^{be}
Shearzyme	26.9 ± 2.1^b	64.3 ± 10.3^{de}
Viscozyme	43.7 ± 4.2^a	28.8 ± 9.4^a
Termamyl	12.4 ± 3.9^b	49.2 ± 13.3^{be}

Table 3-23 describes biofilm inhibition of *S. muticum* enzymatic extracts after the optimization. In this work, *B. subtilis* was added as biofilm-forming bacteria that were tested against *S. muticum* enzymatic extracts. Interestingly, 5% of enzymes managed to inhibit the biofilm formation generated by *E. coli* and *B. subtilis* until

40.3 ± 0.3 and 59.0 ± 0.4% of inhibition, respectively. In the contrary with *E. coli* and *B. subtilis*, the enzymatic extracts were less effective against *P. aeruginosa* as they had inhibition activity ranging only from 13.3 ± 0.1 to 26 ± 0.2% of inhibition. Unfortunately, due to time limits, further bioactivities analysis of *S.muticum* extracted with 10% of enzymes could not be conducted.

Table 3-27 Biofilm inhibition activity of *S. muticum* enzymatic extracts (40°C, 3h, without denaturation) (expressed in % of inhibition).

Samples	Concentration (%)	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>B. subtilis</i>
Viscozyme	5	13.3 ± 0.1	40.3 ± 0.3	59.0 ± 0.4
Protamex		26.3 ± 0.2	29.8 ± 0.6	40.0 ± 0.4
Termamyl		16.3 ± 0.2	36.0 ± 0.5	42.3 ± 0.2
Viscozyme	10	n.d	n.d	n.d
Protamex		n.d	n.d	n.d
Termamyl		n.d	n.d	n.d
Water		n.d	n.d	n.d

n.d; not determined

3.6.2.2 Enzymatic extract of Indonesian *Sargassum*

Biofilm inhibition activity of Indonesian *Sargassum* enzymatic extracts formed by *P. aeruginosa*, *E. coli* and *B. subtilis* was evaluated as can be seen on Table 6. *S. aquifolium*, *S. ilicifolium* and *S. polycystum* extracted with Viscozyme and Protamex were more effective against the film formation generated by *B. subtilis* compared to others. *S. ilicifolium* exhibited the highest inhibition activity with 49.0 ± 6.8 %. However, Protamex extract of *S. polycystum* was also showed a promising inhibition against the film formation of *E. coli* with 45.8 ± 3.8 %. The lowest inhibition activity was exhibited by the Viscozyme extract of *S. ilicifolium* against the film formation of *P. aeruginosa* with 9.1 ± 3.0 % of inhibition. Based on the statistical analysis, there was a significant difference between the three species tested and between the types of extractant that effected the biofilm inhibition activity.

Table 3-28. Biofilm inhibition activity of Indonesian *Sargassum* enzymatic extracts (expressed in % of inhibition).

Samples	<i>P. aeruginosa</i>		<i>E. coli</i>		<i>B. subtilis</i>	
	Viscozyme	Protamex	Viscozyme	Protamex	Viscozyme	Protamex
<i>S. aquifolium</i>	11.6 ± 2.3	16.3 ± 4.5	26.3 ± 11.0	9.1 ± 5.3	42.7 ± 9.6	44.5 ± 11.0
<i>S. ilicifolium</i>	9.1 ± 3.0	9.9 ± 4.8	20.0 ± 11.8	18.2 ± 3.1	25.2 ± 14.8	49.0 ± 6.8
<i>S. polycystum</i>	11.5 ± 6.1	12.0 ± 2.7	14.5 ± 4.4	45.8 ± 3.8	36.9 ± 12.9	40.7 ± 13.5
Sig. (<i>p</i> <0.05) of Species		Sig. (<i>p</i> <0.05) of Enzymes		Sig. (<i>p</i> <0.05) of Bacteria		
.207 (df = 2 ; F = 1.647)		.025 (df = 1 ; F = 4.106)		.000 (df = 2 ; F = 51.724)		

3.6.3 Cytotoxicity and Antiviral activity of *S. muticum* enzymatic extract

Cytotoxicity test had revealed that they did not have a toxic effect towards the tested cell, *i.e.* Vero cell kidney of green monkey). As for their potential as an antiviral agent against the HSV-1, only *S. muticum* Neutrase and Alcalase extracts that showed a potential antiviral activity with EC₅₀ at 430.1 ± 16.3 and 225.1 ± 23.3 µg/mL. Zovirax showed no toxicity (CC₅₀>500) and its EC₅₀ was at 0.7 ± 0.3 µg/mL.

Table 3-29 Cytotoxicity and antiviral activity of *S. muticum* enzymatic extracts

Samples	CC ₅₀ (µg/mL)	EC ₅₀ (µg/mL)
Zovirax	>500	0.7 ± 0.3
Control	>500	>500
Neutrase	>500	430.1 ± 16.3
Ultraflo	>500	>500
Alcalase	>500	225.1 ± 23.3
Celluclast	>500	>500
AMG	>500	>500
Shearzyme	>500	>500
Viscozyme	>500	>500
Termamyl	>500	>500

CC₅₀: Cytotoxic Concentration 50% - a concentration of product generating 50% of cell viability compared to control. EC₅₀: Antiviral Effective Concentration – a concentration of product causing 50% of inhibition of HSV-induced destruction towards the cells. n.a = no activity detected.

Conclusion

We acknowledged that the enzymatic extracts of *Sargassum* might have the ability as a probiotic agent instead of antibacterial agent. In comparison with the organic solvent extract, enzymatic extract showed no sign of inhibition. Leading to the assumption that sugar constituents in the enzymatic extracts serve as source of nutrition for the bacteria. However, the enzymatic extracts of *Sargassum* could inhibit the formation of biofilm. Some extracts had promising performance in inhibiting the biofilm with high percentage of inhibition, yet some showed less appealing activity. Considering that the enzymatic extracts of *S. muticum* had no

potential of toxicity towards the Vero cell, it implies the possibilities to develop this method further in *Sargassum*. Additionally, *Sargassum* is marine brown algal with a wide range of pharmacological properties. Present study unveiled the potential of *Sargassum* enzymatic extracts as antifouling and antiviral agents.

3.7 Polyphenol content, and bioactivities of purified fraction

Extraction yield of SPE from the aqueous methanol fraction of Viscozyme extract was $0.5 \pm 0.0\%$ of dry algal material containing $51.5 \pm 0.6 \%$ polyphenol. It showed that there were 10 fold polyphenol content compared to the raw extract ($6.4 \pm 0.5 \%$ dry extract). Antioxidant activity of this fraction was also improved. Value of DPPH IC_{50} was 0.012 ± 0.6 from 0.6 ± 0.1 mg/mL . Reducing power capacity of this fraction improved as well with 62.4 ± 2.0 from 48.7 ± 0.9 μ M of Fe^{2+} .

Table 3-30 Polyphenol content and bioactivities of purified fraction.

<i>S. muticum</i> Viscozyme extract	Polyphenol (% of dry extract)	DPPH (IC_{50} (mg/mL)	FRAP (μ M of Fe^{2+})	Tyrosinase inhibition activity (% of inhibition)	CC ₅₀ (μ g/mL)	EC ₅₀ (μ g/mL)
Purified fraction	51.5 ± 0.6	$12 \times 10^{-3} \pm 0.6$	62.4 ± 2.0	83.2 ± 7.9	>500	259.3 ± 9.6
Raw extract	5.2 ± 0.1	0.6 ± 0.1	48.7 ± 0.9	23.5 ± 2.6	>500	>500

Conclusion

Combination of enzyme-assisted and solid-phase extraction might improve the recovery of polyphenol compounds in brown algae. Working with purified samples has helped to learn about the bioactivities of target compound, in this case, phlorotannins. It was not only enhanced the quantity of polyphenol content but also improved its bioactivities.

3.8 Discussion

3.8.1 Biochemical composition of *Sargassum*

Valorization of marine algae at any significant scale will become a new endeavor and oblige a comprehensive insight as preliminary assessment for a sustain mass production. Although algae have been recognized as a source of variability regarding its chemical profiles, the natural availability or biomass, the impact of natural environmental variation on the presence, form and level of its chemical profile requires attention. Most of algae exhibit great plasticity regarding to biochemical composition in response to a single or combinations of multiple environmental factors (Stengel *et al.*, 2011). This plasticity basically helps them to survive, grow and reproduce in facing numerous important environmental variables like temperature, salinity, desiccation, hydrodynamics and wave exposure, nutrients, carbon dioxide and pH (Harley *et al.*, 2012). It obviously explains the importance of environmental towards the chemical, biological and physiological of algae. Thus, there have been quite studies that concerns about the effect of environmental in the biochemical composition of algae and so did in this study.

By evaluating the biochemical compositions of algae as well as its seasonal variation, it provides several advantages for further application. First, the biochemical compositions of algae represent its nutritional value which is commercially valuable for food industries. Therefore, they have been considered as food supplement for this century as they contain proteins, lipids, polysaccharides, minerals, vitamins and enzymes (Kilinç *et al.*, 2013). Second, due its plasticity towards its surrounding, it was without any doubt the quantity of algae biochemical composition strictly depended on its environmental variables. In some instances, these environmental effects have been previously described to be influential as well to the bioactivity level of algae as reported by many studies (Marechal *et al.*, 2004; Stirk *et al.*, 2007; Kulanthaiyesu and Sivakumar, 2012; Tanniou *et al.*, 2013b). For that reason, it becomes crucial to collect algae at best

period when their chemical constituents are produced in their optimal condition. A seasonal study on algae biochemical composition allows providing the required information particularly for the valorization point of view.

In one of our work, we attempted to evaluate the biochemical composition of tropical brown algae represented by *S. aquifolium*, *S. ilicifolium* and *S. polycystum*. Additionally, seasonal variation of *Sargassum* biochemical composition was also evaluated by using *S. muticum* as a model. Unlike the previous three species, *S. muticum* that was analyzed in this study came from the temperate region.

3.8.1.1 Indonesian *Sargassum*

In general, brown algae contain 5-15 % of proteins, ~ 4% of lipids, ~ 40% of ash, ~ 60% of polysaccharides, and 1-15 % of polyphenols (Park *et al.*, 2000; Burtin, 2003; Lee, 2008; Harnedy and Fitzgerald, 2011; Kim, 2012). Handayani *et al.* (2004) reported that the average of protein content of *S. crassifolium* was 5%. Supirman *et al.* (2012) obtained almost the same value of protein content of *S. filipendula*, i.e. 5%. Kawaroe *et al.* (2013) stated that the protein content of *S. crassifolium* was 8 % of dry weight. *S. ilicifolium* contained 9% of protein (Ganapathi *et al.*, 2013). In this study, the analyzed protein content in all Indonesian *Sargassum*, *S. aquifolium*, *S. ilicifolium* and *S. polycystum*, was higher. However, hot-water extract of *S. hemiphyllum* had 38.5% of proteins (Hwang *et al.*, 2010). It was even higher than our findings. Unfortunately, the authors did not explain further regarding this high protein content. It is probably due to method of extraction. Authors used hot-water extraction at 100 °C for 30 min.

Total sugar detected in *S. aquifolium*, *S. ilicifolium* and *S. polycystum* was lower than that of the previous studies with same species. *S. ilicifolium* contained 38% of carbohydrates (Ganapathi *et al.*, 2013). *S. polycystum* contained 34% of carbohydrates (Ahmad *et al.*, 2012). Same content of carbohydrates was also reported by Matanjun *et al.* (2009) from *S. polycystum* with 34%. *S. polycystum*

had 5% of (Matanjun *et al.*, 2009). Shao *et al.* (2014) studied the chemical composition of different sulfated polysaccharides fractions from *S. horneri* in order to analyze their antioxidant and antitumor activity. Based on the result, the total sugars from three fractions ranged from 19 – 69 % and 11 – 19 % for sulfates. *S. filipendula* crude fucoidan was reported to contain 1.2% of uronic acid, 8.9% of total sugars and 5.3% of sulfates groups (García-Ríos *et al.*, 2012). Uronic acid and sulfates groups content found of Indonesian *Sargassum* in this study were higher.

Ash of *Sargassum* sp. is 34.6 % (Yunizal, 2004). In *S. vulgare*, there are 18.5% of ash content (Marinho-Soriano *et al.*, 2006). In recent study, ash content of *S. aquifolium*, *S. ilicifolium* and *S. polycystum* ranged from 28 to 38 % of dry algal material. Ash content of *S. polycystum* was 42% (Matanjun *et al.*, 2009) and 21% of dry algal material (Ahmad *et al.*, 2012).

Study by Ahmad *et al.* (2012) reported that *S. polycystum* contained 2.3 % of polyphenols. Budhiyanti *et al.* (2012) studied on antioxidant capacity of several *Sargassum* species collected from the coastline of Java. They reported that the total phenolic content of *Sargassum* sp. varied from 0.006-0.65% (in cytoplasmic) and 6.7 – 22% of dried extract (in membrane bound). This previous study showed slightly higher phenolic contents than in this study as it was extracted specifically from the membrane bound. Surprisingly, the polyphenols content found by Ganapathi *et al.* (2013) was similar with recent study, i.e. 2%.

As components of algae experience geographical, inter- and intra-individual variations due to fluctuations in the environmental conditions as well as season, these contents change, either lower or higher. Temperature, salinity, pH, light levels and quality, nutrient levels and composition, contaminants, CO₂ availability and biotic factors due to the distribution of grazers and endo- and epibionts are the fluctuations as mentioned by Stengel *et al.* (2011). These results implied that there were differences in *Sargassum* biochemical composition. Such differences might be due to the abiotic and biotic factors influencing the production of these

constituents (Manivannan *et al.*, 2008). Another hypothesis was that the method used to extract the chemical constituent of *Sargassum* was not the suitable one. Consequently, the obtained results did not represent the biochemical composition of *Sargassum* in general, especially for the polysaccharides. Thus, it would be interesting to determine the polysaccharides of brown algae by using the appropriate method of extraction in the next future.

Conclusion

Biochemical composition of Indonesian *Sargassum* shows inter-species and geographical variation. In addition, seasonal and environmental condition such as temperature, salinity, availability of nutrients and many more, might contribute to the variation of *Sargassum* biochemical composition. Indonesian *Sargassum*, like most *Sargassum* species, is characterized by its high content of ash. Among three species, *S. polycystum* has the highest ash content. Ash content reflects the total mineral of an individual and it is beneficial for the fodder or animal feed industry. Beside ash content, this species is also high in sulfates groups; it indicates the possibility of high fucoidan. Perhaps, this could become preliminary information that this species might contain interesting fucoidan yield that is potential for further application. As for *S. ilicifolium*, it has high uronic content that can be associated with its alginate. Probably, this species has its advantage for hydrocolloid industry.

3.8.1.2 Seasonal variation of *S. muticum* biochemical composition

Dry material of *S. muticum* revealed in this study was up to 25%. In previous study, the dry material of *S. muticum* ranged from 8 to 19% of total weight (Balboa *et al.*, 2016). Dry material of *S. muticum* was affected by season, in which, spring and summer had higher percentage of dry material than autumn and winter. Seasonal pattern of dry material in *Sargassum* did not show same pattern. In certain way, dry material of *S. muticum* collected in February to July 2014 was higher than the same months in 2015.

Biochemical composition of *S. muticum* had been significantly affected by the seasons. Protein, nitrogen and uronic acid content of *S. muticum* increased during winter and spring; while sugar, sulfates groups, and polyphenol increased in summer. Interestingly, the seasonal variation study of *S. muticum* biochemical composition in this study revealed the tendency as expected. Prior to analysis, we assumed that the seasonal variation would give a significant effect to the biochemical composition of *S. muticum*. Besides the seasons, it might be possible that the variation shown by *S. muticum* also followed its bio-physiological functions related to its life cycle. Early fall or autumn is known to be the period when *S. muticum* starts their reproduction followed by the growth of small individuals from October-November (Deysher, 1984; Loraine, 1989). It reaches its optimal growth in the spring then it stops in summer and begins to degrade during this season. This cycle is in accordance as our observation during the collection of *S. muticum*. From September-October, there were no long thalli found along the study site. Most of them were less than 5 cm long – also called as perennial primary shoot; therefore, it was hard to obtain a substantial biomass. Then, in November-February, the primary shoot started to grow and the length of thalli collected ranged from 5-15 cm, approximately. Early spring, which was on March, until May, the biomass of *S. muticum* was quite abundant and fertile as well. During this period of collection, the presence of receptacles on *S. muticum* thalli was observed. The thalli were ramified and their primary lateral reached to 20 cm long bearing numerous small blades and air bladder. Meanwhile, on June to August, the blades and air bladder of *S. muticum* began to fall out leading to a loss of buoyancy (see Appendix 1). A numerous presence of epiphytes such as gastropods and other algae were remarked on the thalli making them hard to wash and clean (see Appendix 1). This observation allows us to establish preliminary correlation between the seasonal variation of *S. muticum* biochemical composition and its life cycle. Unfortunately, the correlation made in this study was merely based on general description since we did not evaluate specifically the effect of *S. muticum* life cycle to the biochemical composition. This kind of correlation needs a comprehensive study involving the measurement of growth rate, morphological

study, biochemical composition analysis and an *in situ* and *ex-situ* time-series environmental variables. In the meantime, it is most likely that the correlation between life cycle and biochemical composition of *S. muticum* is positively related. In theory, sugar content is high during summer and autumn.

Protein synthesis conducted by algae is through the fixation of atmospheric nitrogen and protein. Thus, the protein content in marine algae varies depending on the harvest period. Light, temperature and salinity are environmental factor influencing protein synthesis. Souchet (2004) in Kim (2012) reported the correlation between protein and water nitrogen content as high temperature decrease dissolved nitrogen content in water column. As consequence, the protein content in marine algae is lower during summer as the nitrogen fixation declines in this season. Higher temperature increases consumption energy due to algal respiration. Protein content is relatively lower in summer and higher in winter (Kim, 2012). The protein of *S. muticum* in this study showed the same tendency as well as nitrogen. In addition, the correlation between *S. muticum* protein and sea surface temperature was a significantly inversed yet weak correlation. Balboa *et al.* (2016) reported that the protentin content of *S. muticum* collected in May to June contained 7% of protein and from November to January contained 11% of protein. Additionally, the greater protein content occurred during the decrease of temperature. In other words, there was an inverse correlation between protein content and temperature (Balboa *et al.*, 2016).

In contrary with protein and nitrogen, the content of *S. muticum* polysaccharides was higher in summer than in winter and spring. It suggested that from winter to spring, the low content of polysaccharides was because *S. muticum* utilized them for its growth. In the early summer when it stopped to grow, the polysaccharides served as a reserve. Our result was in accordance with Schiener *et al.* (Schiener *et al.*, 2014) revealing that the carbohydrates in Laminariales were found to accumulate during summer to fall and decrease in winter as they were used as an energy source for new tissue growth. In addition, this accumulation

might also be due to the presence of herbivore or other environmental stress since polysaccharides serve as defense system in marine algae.

Hardouin *et al.* (2013) noted 29.3% of protein content of *S. muticum* was yielded from hydrolysis extracts. Same authors also reported that the compositions of ash, total sugars, uronic acid, sulfates groups and total phenol of *S. muticum* were 27.4%, 9.8%, 8.0%, 5.6% and 2.1%. These results were slightly in accordance with our finding in this study, except for uronic acid and sulfates group. Meanwhile, Jard *et al.* (2013) observed that *S. muticum* had 16.6% of total sugars, 13.6%, 8.4% of protein, 3% of sulfates and 1.9% of phenols.

As for the uronic acid and sulfates groups, these chemical entities highly related to the alginates and fucoidan content in brown algae, respectively. We found that the seasonal variation was not significantly different. Despite of this discouraging result, there was a slight increase of uronic acid and sulfates group in summer. Hypothetically, this tendency was in line with polysaccharides content. Analysis of correlation between the sulfates groups showed that there was a moderate correlation with the sea surface temperature, air temperature and sunshine. Unlike the sulfates, the uronic acid had a weak correlation with these variables.

In *Saccharina japonica*, *S. pallidum*, and *Stephanocystis crassipes*, their fucoidans were mainly influenced by the development of the reproductive structures of the algae itself. It was suggested that the fucoidans did not have a direct relationship with illumination regime, salinity and concentrations of biogenic substances in water (Skriptsova, 2016). Later, they discovered that fucoidan of *S. pallidum* was found in its maximum yield in July (summer) (Skriptsova, 2016). Skriptsova *et al.* (2010) also found that fucoidan yield of *Undaria pinnatifida* increased from April to July. As for the alginate content, represented with its uronic acid, Rodrigues-Montesinos *et al.* (2008) observed an increase of alginate in the cell wall and intercellular matrix of *S. sinicola* in spring as they matured. Then, they were low in alginate during winter since they were

still in juveniles phase. In 2004, study by the same authors reported that the alginate content in whole plant of *U. pinnatifida* increased from March to July followed by a decrease in August. In addition, alginate content of *U. pinnatifida* blades was maximal during spring with 51% and lowest in July with 34%. Increase of alginate content coincides with plant maturation and sporophyte development (Skriptsova *et al.*, 2004). Sugar, and sulfate groups content of *S. muticum* showed quite the same pattern as fucoidan and alginate from other species reported by previous studies. These entities showed an increase in spring-summer then followed by a decrease in fall-winter.

Phenols of *S. muticum* exhibited a significant effect given by the season, *i.e.* between summer-fall-winter and spring with the highest amount of polyphenols was recorded during fall. Looking further to the correlation analysis, we obtained that the polyphenols of *S. muticum* showed a weak correlation with the sea surface temperature, sunshine, air temperature and precipitation. It brought an assumption that polyphenols might have a more complex relationship. Theoretically, polyphenols in brown algae are mostly induced by the presence of predators, the exposure to sunlight, the fluctuation of environmental factors (temperature, lights, nutrient, etc.) and water pollution (Toth and Pavia, 2000; Jormalainen *et al.*, 2003; Koivikko, 2008; Connan and Stengel, 2011). However, it is important to note that they also have primary role as one of the compounds constituting the cell wall of brown algae (Lee, 2008). Thus, they also play a crucial role in algae metabolism and there is a trade-off between polyphenols and annual growth of algae. Accordingly, there are many factors contributing to the production of polyphenols in brown algae. Regardless of these matters, the current study concerned only to the seasonal variation.

Phenols of *S. muticum* from the Callot Island and Point de Diable, coast of Brittany (France) showed an increase occurring between winter and spring in 2003 and 2004. The highest phenols were recorded in spring 2003 and the lowest one was in winter and fall. Meanwhile, at the Point de Diable, the highest phenols were observed in summer 2004 and a decrease to a minimal value was remarked

in winter 2003 (Plouguerné *et al.*, 2006). In this study, the highest phenols were found in fall season. Our assumption suggested that the difference shown in this study was due to different location and perhaps different method of extraction.

The ash content of brown algae is higher compared to most terrestrial vegetables containing from 5 to 10% (Rupérez, 2002). In addition, between the three groups of algae, the ash content of brown algae surpasses the green and red algae. Variations in ash content usually related to the seasonal variations of inorganic compounds and salts in the aquatic environments such as salinity (Kumar *et al.*, 2015). Same authors reported the ash content of *S. wightii* was higher in July and lower in November. In contrary with study conducted by Kumar *et al.* (2015), *S. muticum* ash content was noticed to be high from June till October, while the lowest was from March till May (Balboa *et al.*, 2016). In Japan, *S. horneri* contained 32% of ash in May and 25% in March (Murakami *et al.*, 2011). Present study revealed that the best ash content of *S. muticum* was found during fall and winter then it decreased from spring to summer. Such pattern was quite the same as presented by Balboa *et al.* (Balboa *et al.*, 2016) even though there was a time deviation assuming due to the different locality of *S. muticum*.

Secondary analysis using the Fourier Transform Infrared spectroscopy – abbreviated as FTIR – was conducted as a complementary analysis of colorimetric method. This qualitative analysis helped to describe clearly the seasonal distribution pattern of *S. muticum* biochemical composition and its seasonal grouping as presented on the results. The seasonal grouping managed to distinguish the tendency between the cold (fall and winter) and hot (spring and summer) seasons. However, it is crucial to carefully select the wavelengths corresponding to the target compounds. Even though our samples have been dried with the freeze-drying but they still contain some water.

Water absorbs mostly at $3700\text{--}3100\text{ cm}^{-1}$ based on the table of infrared or to be precise at $3720\text{--}2950\text{ cm}^{-1}$ - absorption of --OH of pure water with strong

intensity. Rodrigues *et al.* (2015) suggested to choose a global analysis based on the spectra at 1800-600 cm^{-1} . This range of spectra is assumed to be the most informative. Moreover, there is also a noise area that related to the crystal of the FTIR at 2200-2000 cm^{-1} . The global analysis mentioned here was related to the PCA analysis. Result revealed that the grouping of PCA using the spectra at 1800-600 cm^{-1} distinguished the season into two groups, *i.e.* cold and hot season (Appendix 6). Such grouping was similar to the grouping of spectra particularly selected for different biochemical composition of *S. muticum*.

Detected peak at 3270-3250, 2940-2925, 2300-1900, 1660-1600, 1500-1200 and 1100-1000 cm^{-1} were wavelengths showing interesting peak based on the global spectra of *S. muticum*. 3270 and 3250 cm^{-1} suggested the presence of two hydrogens bonded ($\text{SO}_2\text{N-H}$) (Gennari *et al.*, 1998). Wavelengths at 3600-3450 cm^{-1} is assigned as the majority of hydroxyl absorption bands in the region intra-molecular hydrogen bonds (Rodriguez, 2010) and the hydroxyl peak usually occurs at 3545 cm^{-1} (Matthews, 2007). Chen *et al.* (2015) adds that absorbance peak at 3600-3450 cm^{-1} ascribed to the release of HCN. Free amine functionalities produce bands either at 3500-3300, 1650-1580, or 1622 cm^{-1} , while N-H bonds absorb at 3350-3070, 1650-1550 or 1594 cm^{-1} (Hamid, 2005; Lawrence-Berkeley, 2009). Strong bands at 1647 and 1540 cm^{-1} are assigned to the amide I and II from protein (Petrone *et al.*, 2011). These bonds show the presence of complexes of amine phenols ligands in our samples. It is not surprising since the polyphenols of brown algae, namely phlorotannins, is known to have strong bond with protein (Stern *et al.*, 1996).

In order to detect the presence of phlorotannins in our samples – type of polyphenols in brown algae – we passed the commercial standard of phloroglucinol as comparison. Peaks at 3213.73, 1620.31, 1503.87, 1332.46, 1305.86, 1150.82, 1005.31, 996.28, 839.52, 812.39, 799.22, 664.07, 600.28, 578.01 and 517.02 cm^{-1} were detected from the standard. There were several peaks detected in our samples corresponding with phloroglucinol, *i.e.* 1619-1617 and 1326-1324. Range of wavelengths at 2940-2925, 1660-1600, 1500-1400,

1100-1000 cm^{-1} observed in our samples also showed to be in accordance with the spectra of gallic acid – commercial standard used to detect the polyphenols – at 1639.70, 1509.58, 140.99, 1153.78, and 1097.08 cm^{-1} by Meenakshi *et al.* (2011). Wavelength at 1620 cm^{-1} indicated that there was aromatic ring as shown in the spectra of our samples (Lim *et al.*, 2002). Rajauria *et al.* (2013) also reported the same spectra at around 1624, 15505 and 1465 cm^{-1} detected in brown algae *Himanthalia elongata* suggesting the presence of aromatic ring. The aromatics groups with C-C stretch absorb at 1500-1400 and 1600-1585 cm^{-1} . Aromatic amines with C-N stretch are functional groups absorbing at 1335-1250 cm^{-1} . Then the wide peak at around 3260 was assumed to correspond with the detection of carboxylic acid with O-H stretch. In general, the maximum transformation of hydroxyl group is at 3300-3500 cm^{-1} region as for the aromatic rings are normally detected between 1400 to 1680 cm^{-1} (Rajauria *et al.*, 2013).

Polysaccharides detection in FTIR are mainly characterized by its C-C and C-O stretching bonds in the pyranose ring as well as to C-O-C stretching of the glycosidics bonds between 1200-970 cm^{-1} (Gomez-Ordenez and Ruperez, 2011). Other author emphasized to the 1800-650 cm^{-1} showing several vibrational modes that are sensitive to the polysaccharides and glycosidic linkages in algae (Shekhar *et al.*, 2012). A strong peak at around 1023 cm^{-1} revealed that our samples might be rich in guluronic acid since the 1025 cm^{-1} is ascribed to the -OH bending of guluronate (Sakugawa *et al.*, 2004). The peak at 1023 and 890 cm^{-1} detected in our samples were the same as those in *Fucus vesiculosus* indicating the presence of alginic acid and fucoidan (Shekhar *et al.*, 2012). Weak peak at 1420 cm^{-1} shown in the spectra of *S. muticum* might be due to the symmetric stretch vibration of C-O-C of uronic acid (Singthong *et al.*, 2004).

Biochemical constituents as a representation of algae's nutritional value have been extensively studies in different time, place and species. Accordingly, many studies have reported the health benefits contained in this marine plant and it seems that algae have been long consumed in the Asia. One of its notoriously unique components is its polysaccharides proven to have a wide array functions

for food, health and beauty. Additionally, other constituents as protein, polyphenols, ash, lipids and pigments are also surprisingly interesting because they are economically potential as well.

This chapter serves as a preliminary report about the biochemical composition of different *Sargassum* species representing the tropical and temperate region. In addition, the seasonal allows us to comprehend the effect of season towards the biochemical composition of brown algae. Then, the application of FTIR analysis as a secondary tool used in this study can facilitate the qualitative analysis by using directly the dry algal material.

Overall, the biochemical compositions of *Sargassum* in this study are in accordance as the previous study. The difference values of biochemical contents have been previously expected as algae show great plasticity to their environmental variables as their way to adapt and survive. FTIR analysis facilitates the preliminary evaluation of algae biochemical composition since this tool is rapid, easy to use and efficient as it does not need substantial amount of dry material. By using this method, the seasonal pattern of a species can be observed and it is also possible to determine their correlation with the environmental parameter.

Conclusion

Seasons play important role in marine algae biochemical composition. Fluctuated environmental conditions enforce algae to adapt their physiological system in order to survive. Certain biochemical entities have their own season by evaluating its seasonal variation will provide information about the most favorable collection time. For example, for hydrocolloid industries that needs high yield of alginate, summer will be the best time to collect algal biomass, in this case *S. muticum*. For fucoidan, it is best to have algal biomass from spring and summer.

This study informs the correlation of sea surface temperature, air temperature, period of sunlight and precipitation to the production of biochemical

composition of *S. muticum*. Some shows linear correlation while other demonstrates inversed correlation. Besides seasons, life cycle of marine algae has been mentioned as contributing factor to the variation of its biochemical composition, particularly to its reproductive cycle. Such tendency can also be seen in this study. Monthly evaluation of *S. muticum* biochemical composition has shown that life cycle contributes to the biochemical profile of this alga. It may also be concluded that in most marine brown algae from the temperate regions, the seasonal variation of their biochemical compositions have the same pattern without considering the other external factor such as grazing, pollution, etc.

3.8.2 Extraction of *Sargassum* polyphenol

3.8.2.1 Seasonal variation of *S. muticum* polyphenol

Polyphenol serves multiple functions for marine brown algae, namely primary and secondary metabolites. As a consequence, the production of polyphenol is determined by an interaction of biotic and abiotic factors (Pavia *et al.*, 1997) and a study related to its variation, in particular the seasonal variation, will give insight into their natural adaptative functions and become basic information for commercial interest. The most favorable collection period of algal material is important to consider. Present study shows that season becomes one of the important factors influencing the production of polyphenols content in *S. muticum*. Results presented in this study reveal that the polyphenols content of *S. muticum* achieved its highest quantity in summer and it decreased during winter. Previous study with *S. muticum* from Point de Diable revealed that its polyphenols content increased between winter and spring 2003, spring and summer 2004 (Plouguerné *et al.*, 2006). Interestingly, same authors also discovered that *S. muticum* collected from Callot Island contained the highest polyphenols during winter and spring in 2003 and 2004. Authors used aqueous methanol extract of *S. muticum*. Polyphenols content of brown algae *Ascophyllum nodosum* also increased during summer, June to September ranging from 0.5 – 1 % of fresh weight (Parys *et al.*, 2009). Mannino *et al.* (2014) reported the same tendency

happened with *Cystoseira amentacea*. Extract of 96% aqueous ethanol of this species contained the highest polyphenols content between spring and summer, with 0.17 ± 0.01 and $0.57 \pm 0.15\%$ of dry weight, respectively. *Cystoseira tamariscifolia* exhibited an exponential increase in its polyphenols from February-June and decreased from June to November (Abdala-Diaz *et al.*, 2006). It can be said that this result is in agreement with several cases from previous studies reporting the variation of polyphenols content in brown algae that occurred in different seasons.

Maximum polyphenols content of *S. muticum* occurring in summer, as presented by this study, has been mentioned to be attributable to the implication of this compounds in the photoprotection of the species (Plouguerné *et al.*, 2006; Le Lann *et al.*, 2012). UV-B radiations provoked the increase of polyphenol content in *A. nodosum* (Pavia *et al.*, 1997), in *Desmarestia* spp. (Fairhead *et al.*, 2006) and in *C. baccata*. In *C. tamariscifolia*, it was demonstrated that the exudation of phlorotannins from its tissue showed a significant correlation with increasing dosage of irradiation (Abdala-Diaz *et al.*, 2006). Light intensity was not measured in this study. Instead, an observation was made for sunlight also known as daylight period in a day, air temperature, sea surface temperature and precipitation. The correlation analysis has described that all the environmental parameters except for precipitation have given significantly positive effect to polyphenols content. As intertidal species, *S. muticum* is often exposed to sunlight radiation during the low tide, particularly in summer where the sun intensity is stronger and the temperature is higher than the other seasons. To prevent themselves from oxidative stress, they produce more polyphenols, *i.e.* phlorotannins. This explains its high content found in this study during summer in all extracts.

In winter, the phlorotannins of *S. muticum* tend to be in its minimum level. It might be explained by their life cycle. The growth period of *S. muticum* occurs between winter and early spring. Thus, it is assumed that there is a trade-off in phlorotannins production of *S. muticum* that is related to its growth. Phlorotannins

of brown algae are produced in the physodes, thus it makes them extractable and reactive. Along their life cycle, these physodes exude phlorotannins to algal cell wall. They are then complexed to alginic acid and deposited at the cell wall. Within this rate, the decrease content of extractable phlorotannins occurs when rates of plant growth, which is cell wall formation, are high. In the opposite, the increase concentration of extractable phlorotannins happens when ever their synthesis exceeds the incorporation into algal cell wall (Arnold and Targett, 2003). As reported in *A. nodosum*, its phlorotannins concentration was high when they experience slow growth rate. Annual shoots of *A. nodosum* having high phlorotannins levels (> 9% of dry weight) grew 25% and 54% less than those with a relatively low phlorotannins level (<6% of dry weight) (Pavia *et al.*, 1999). A negative correlation between growth rate and phlorotannins level was reported in *S. patens* as well. *S. patens* that was cultured at 10°C contained high level of phlorotannins but it had slow growth rate. On the other hand, an individual kept at 30 °C experienced fast growth rate and a decrease in phlorotannins level (Endo *et al.*, 2013). It seems that reproduction cycle of algae contributes indirectly to polyphenol production.

Polyphenol production of marine brown algae shows putative role against the presence of herbivores. This extrinsic factor can significantly induce changes in phlorotannin production of brown algae inhabited nearshore marine communities since it will affect the palatability (Pavia and Brock, 2000). As reviewed by Amsler and Fairhead (2006), snail *Littorina sitkana* avoid the wounded areas of *Fucus gardneri* which showed phlorotannin accumulation, and preferred to feed on undamaged algae with lower phlorotannin contents. Snail *L. obtusata* consumed less *A. nodosum* containing high phlorotannins contents. Turban shell *Turbo cornutus*, is marine herbivorous gastropod that prefers eating dead brown algae than live one. This is due to the inhibitory effect of phlorotannins on glycosidase activity, a digestive enzyme found in viscera of *T. cornutus*. Such activity leads to the feed preference of this species (Shibata *et al.*, 2002).

Conclusion

Inhabiting the temperate region, *S. muticum* has been mentioned to contain polyphenol that is potential for further application. As most species, *S. muticum* contains high polyphenol in summer. Most favorable organic solvent to extract *S. muticum* polyphenol as proven in this study is using 50% aqueous methanol solvent. Therefore, summer will be the best period to collect *S. muticum* for its polyphenol content.

As secondary metabolites in marine brown algae, polyphenol production is highly influenced by the surrounding conditions. One of conditions that have a significant effect towards the production of polyphenol is season as proven in *S. muticum*. This compound is high during spring-summer with maximum content in summer. It suggests the role of this compound as photoprotective agent in *S. muticum* especially when this species is over-exposed to sunlight during the lowest tide. The presence of long interconnected aromatic ring in brown algal polyphenol is the defensive agent mostly responsible generating protection against oxidative stress in brown algae.

Having strong antioxidant activity, polyphenol of brown algae might become an important source of antioxidative agent. As a consequence, polyphenol compounds from marine brown algal have been extensively explored and studied since it is valuable for commercial interest. For commercial purpose, it is crucial to acknowledge the most optimum period of polyphenol production. This study provides the required information. In addition, different type of solvents applied in this study helps to acknowledge best fitted solvent to extract polyphenol.

3.8.2.2 Solid-liquid extraction of *Sargassum* polyphenol

Many studies have attempted to optimize the extraction process by applying different types of solvent, temperature, extraction time, drying process and many more. SLE has become the most used method in extracting polyphenol (ref). This method mostly relies on the polarity of the solvents used due to the chemical nature of target compounds (Hans-Jörg Bart, 2011). In general, the process in SLE

method can be categorized in three parts; (1) changing phase of the solute, in this case the target compounds, as it dissolve in the solvent, (2) its diffusion through the solvent in the pores of the solid to the outside of the particles, and (3) the transfer of the solute from the solution in contact with the particles to the main bulk of the solution (Richardson *et al.*, 2002a). The more dispersed the solute in the solid material, the more difficult the extraction will be and the extraction rate will fall. This is because the solvent will have to penetrate further into the layer of solid material in order to reach the solute (Richardson *et al.*, 2002a). Furthermore, the efficiency of SLE highly depends on the particle size, temperature, and agitation during the process (Richardson *et al.*, 2002b). In the extraction of phenolic compounds, the storage time of dry materials and conditions, as well as the presence of interfering substances are other important factors to consider, influencing the extraction efficiency (Naczki and Shahidi, 2004). In addition, the polarity of target compound becomes the most important factor in choosing the solvent (Azmir *et al.*, 2013). Methanol, ethanol, acetone, ethyl acetate, hexane (Koivikko *et al.*, 2007; Kim *et al.*, 2011; Girija *et al.*, 2013; Sathya *et al.*, 2013; Le Lann *et al.*, 2016) are some examples of organic solvents mostly used for phlorotannin extraction.

Ethanol extract of *S. filipendula*, *S. duplicatum*, *S. crassifolium*, *S. binderi*, and *Padina* sp. showed to contain higher polyphenol with 12.9, 7.9, 6.8, 9.1 and 10.2 mg/g of extracts than ethyl acetate and hexane extracts. Among ethanol and ethyl acetate, hexane extracted less polyphenol in *S. filipendula*, *S. duplicatum*, *S. crassifolium*, *S. binderi*, and *Padina* sp. (Bambang *et al.*, 2013). Methanol extracts of *S. hystrix*, *S. siliquastrum*, and *S. mcclurei* had approximately 20, 17 and 15 g/100 g extract of polyphenol. *S. polyceratium*, *S. angustifolium* and *S. filipendula* extracted with methanol showed less polyphenol content, ranging from 5 to 10 g/100 g of extract, than the three previous species (Budhiyanti *et al.*, 2012). By applying different temperature and extraction time for optimizing the extraction of polyphenols in *S. muticum*, Tanniou (2014) also revealed that 40 °C and 3 hours of extraction resulted better content of polyphenols. Further, polyphenol

extraction of *S. muticum* was more effective with 50% aqueous ethyl acetate than the mixture of 88% hexane- 12% ethanol and 75% of aqueous ethanol as it contained the highest polyphenol content, 18.6% of dry weight. Meanwhile, the lowest polyphenol content in *S. muticum* was obtained from the hexane-ethanol mixture with only 3.7% of dry weight (Tanniou *et al.*, 2013a). In this study, *S. muticum* extracted in methanol 50% had higher polyphenol content than water, methanol and ethanol 75%, same case with *S. polycystum*. On the other hand, *S. aquifolium* and *S. ilicifolium* contained the highest polyphenol content in methanol extract. These findings have proven that type of solvents determine the efficiency of polyphenol extraction. In addition, temperature also play critical role during the extraction of polyphenol. Bambang *et al.* (2013) selected room temperature, meanwhile Budhiyanti *et al.* (2012) and Tanniou *et al.* (2013a) used 40°C as their extraction condition, same with our study.

Other study highlighted in their findings that 40 °C, 180 min, and 40 ml/g of solid-liquid ratio were the most efficient conditions for the extraction of polyphenols in *Sargassum* sp. (Ismail and Abdullah, 2015). Polyphenol compounds are most soluble in less polar solvents than water (Koivikko *et al.*, 2005). Most recommended solvent is a mixture of water with methanol, ethanol or acetone, for example, brown algae *Fucus vesiculosus* have been commonly extracted with aqueous mixtures of ethanol and acetone (Ragan and Glombitza, 1986; Waterman and Mole, 1994; Koivikko *et al.*, 2005). Lopez *et al.* (2011) explained that solvent polarity had no drastic effect to the total amounts of a phenolic groups, but more to the phenolic profile. Regardless of the amount, since this study has proven the contrary, their theory about difference in phenolic profile due to solvent polarity is in agreement with recent result. As can be seen from the chromatogram data (Appendix 8), the spectra of methanol, methanol 50%, water and ethanol 75% extracts are not the same. Water and methanol 50% extracts displayed the most distinguished profile of peaks among the other two solvents.

Most of the temperature suggested for the extraction of terrestrial and marine vegetal polyphenols compound was below 60 °C (Le Lann *et al.*, 2008; Dai and Mumper, 2010; Yoon *et al.*, 2010; Tanniou *et al.*, 2013a; Andrade *et al.*, 2015; Ismail and Abdullah, 2015). Organic solvents such as methanol, acetone, and many others tend to evaporate at high temperatures (approximately > 50°C), thus their efficiency decreases due to the shifting of solvents-to-water ratio leading to the change of sample-to-solvent ratio (Tan *et al.*, 2013). As reported by Dai & Mumper (2010), besides temperature, the sample-to-solvent ratio is another one of the crucial factors that influences the yield of chemical extraction.

Nevertheless, there was several studies that tested the extraction of polyphenols compound using temperature above 60 °C depending in the method and type of solvents applied for the extraction (Siriwardhana *et al.*, 2008; Park *et al.*, 2012; Shitanda *et al.*, 2014). For marine algae, their maceration into a high level of heat might accelerate the extraction process as the heating soften the tissues and facilitate the depolymerisation of polysaccharides (Siriwardhana *et al.*, 2008), phenol-protein, and phenol-polysaccharides linkages (Tan *et al.*, 2013). As proven by Siriwardhana *et al.* (2008), brown algae *Hizikia fusiformis* extracted in water at 100 °C for 60 minutes yielded 4.65 % of polyphenols content while at 25, 50 and 75 °C had only 1.40, 2.02 and 2.74 % of polyphenol, respectively.

Conclusion

Various factors have given significant effect to the extraction of polyphenol in marine brown algae; two of them are type of solvents and temperature. In this study, extraction yield, polyphenol content and chromatographic profiling of *Sargassum* species exhibit variation due to type of solvents and species. Some studies have reported that chemical profile of brown algal polyphenol in one individual is various depend on the polarity of solvents. Interest of this study is to acknowledge what is the most optimum solvent to extract polyphenol in different species of *Sargassum* and. It seems that each species displays its own preference of solvent that might be related to the polarity of polyphenol produced in *Sargassum*.

3.8.2.3 Enzyme-assisted extraction of *S. muticum* polyphenol

In marine algae, the main drawback during the extraction of phenolic compounds occurs due to the presence of complexes polysaccharides as the main component of algal cell wall. Brown algal phlorotannins are known to be incorporated in its cell wall (Schoenwaelder and Clayton, 1999) covalently bonded to the polysaccharides (Koivikko, 2008) and proteins (Stern *et al.*, 1996). Therefore, it requires a strong condition to degrade these bonds and extract the phlorotannins. The SLE method does not meet this requirement since it does not degrade such complex bonds since it works based on the solubility of target compound in to the solvents (Richardson *et al.*, 2002a).

One part of this study was to evaluate the extraction yield and the polyphenol content *S. muticum* digested by various commercial enzymes. Enzyme-assisted extraction has been relatively well-known for the past few years as an alternative method to extract the bioactive compound in marine algae (Heo *et al.*, 2003a, 2003b; Ahn *et al.*, 2004a; Siriwardhana *et al.*, 2004; Park *et al.*, 2005; Athukorala *et al.*, 2006a, 2006b; Je *et al.*, 2009; Charoensiddhi *et al.*, 2014; Sanchez-Camargo *et al.*, 2015). This method has been mostly used to recover bioactive compounds, such as polyphenol that is highly potential as antioxidant (Heo *et al.*, 2003b; Ahn *et al.*, 2004a; Sanchez-Camargo *et al.*, 2015), or in certain case, polysaccharides for its anticoagulant and protein for its antihypertensive effects (Sato *et al.*, 2002).

In order to optimize the extraction yield, optimal conditions of pH and temperature have been chosen for each selected enzymes. Carbohydrase and protease, two main types of enzymes mostly applied in bioactive extraction of marine brown algae, work optimally at pH ranging from 4.5 – 8.0 and temperature at 40-60 °C (Athukorala *et al.*, 2006a; Zou *et al.*, 2008; Je *et al.*, 2009; Charoensiddhi *et al.*, 2014). To achieve optimum degree of hydrolysis, in other word yield of extraction, some studies established a 12 h of extraction (Heo *et al.*, 2003a, 2003b; Athukorala *et al.*, 2006b; Je *et al.*, 2009) and the other study chose

a one-day extraction (24 h) (Charoensiddhi *et al.*, 2014). As an example, AMG (carbohydrase) and Alcalase (protease) extract of *Ecklonia cava* showed higher extraction yield, 41.5 and 42.7%, than the other carbohydrase (Viscozyme, Celluclast, Termamyl and Ultraflo) and protease (Protamex, Kojizyme, Neutrase, Flavourzyme) in 12 h of extraction (Heo *et al.*, 2003a). Still from the same author, AMG was also shown to yield higher degree of hydrolysis than other carbohydrase in *S. fulvellum* (27.9%), *S. coreanum* (33.2%), and *S. thunbergii* (33.9%). Then, Heo *et al.* (2003a) reported that Alcalase yielded high degree of hydrolysis in *Ishige okamurae*, *S. horneri*, *S. coreanum*, *S. thunbergii* and *Scytosiphon momentaria*, with 29.9, 40.0, 40.7, 30.5 and 30.9%, respectively. *E. radiata* extracted with carbohydrase (Ultraflo) and protease (Alcalase and Flavourzyme) for 24 h increased the extraction yield by 5 – 20% compared to aqueous extraction. Almost 70% of algal dry material was achieved when introducing Ultraflo, Alcalase and Flavourzyme to the extraction of *E. radiata* (Charoensiddhi *et al.*, 2014).

Eight commercial enzymes consisted of two proteases (Neutrase and Alcalase) and six carbohydrases (Ultraflo, Celluclast, AMG, Termamyl, Shearzyme and Viscozyme) used in this study were proven to be effective in increasing the yield of extraction in comparison with water extraction, except for Shearzyme. Highest yield was obtained from *S. muticum* digested with Neutrase, one of proteolytic enzymes, though the value was not significantly different from the other enzymes and water extraction. Enzymes from the protease group have been reported from several studies as an effective enzyme in enhancing the extraction yield in algae (Heo *et al.*, 2003a; Wang *et al.*, 2010; Hardouin *et al.*, 2013). Opposite result was obtained from previous study. Extraction of *S. muticum* using Alcalase for 2 and 4 h yielded 13.6 and 17.8% of dry weight. Viscozyme extract of *S. muticum* showed higher extraction yield than Alcalase extract. For 2 h extraction, the extraction yield was 20.6% of dry weight and 4 h extraction resulted 23.5% of dry weight (Sanchez-Camargo *et al.*, 2015). In present study, 3 h extraction of *S. muticum* yielded 28.7%, this value was slightly

higher than the previous study. Such contradictory results might be due to different concentration of enzyme used in the extraction since proportion of substrate to enzyme is one of the most considerable variables (Wijesinghe and Jeon, 2012). Previous study used 0.1 % of enzyme while in present study added 2.5% of enzyme in algal solution. It is assumed that the enzyme concentration is too low, as a consequence, the degradation process is not optimum since the enzyme reaches its limiting concentration but there are still plenty of substrate available to be bound by the enzyme. When the substrate concentration is high, the addition of enzyme can enhance the rate of reaction, till the substrate concentration becomes limiting (Sowbhagya and Chitra, 2010). Suitable amount of enzyme addition is important to consider for achieving the limiting point of substrate for optimum result. In general, our finding regarding the extraction yield of brown algae is in accordance with certain previous studies even though in particular case we had the opposite result as mentioned above.

Beside extraction yield, polyphenol content of *S. muticum* was also enhanced by enzyme-assisted extraction compared to the aqueous extraction. The highest polyphenol content was observed in Viscozyme extract of *S. muticum* containing 6% dry algal material of polyphenol. *S. coreanum* extracted with Protamex contained 9.9 mg/g dry algal material of polyphenols content, but with Viscozyme, its polyphenols content was higher with 11 mg/g dry algal material (Heo *et al.*, 2003a). Study by Sanchez-Camargo also obtained higher polyphenol content in Viscozyme extracts of *S. muticum* from 2 and 4 h of extraction (10.3 and 10 mg/g dry algal material) than the Alcalase extract (7.91 and 6.1 mg/g dry algal material).

The efficiency of Viscozyme in extracting polyphenol content from the brown algae works differently in every species. Siriwardhana *et al.* (2004) studied the effect of different commercial enzymes in antioxidant activity of *H. fusiformis*. Based on their result, Alcalase and Neutrase showed the highest content of polyphenol compound. Alcalase also enhanced the polyphenols content of *S. thunbergii*, *S. horneri*, *S. fullvelum* and *Ishige okamurae* (Heo *et al.*, 2005).

This study reveals that the polyphenols content of *S. muticum* extracted with carbohydrases was higher compared to the proteases. It leads to an assumption that the carbohydrases disrupt algal cell wall and release the metabolites linked to the polysaccharides, in this case the polyphenols compounds. It is also possible that the polyphenols compounds of brown algae are mostly attached to the alginates instead of proteins. As mentioned by previous studies, the polyphenols compounds are not only covalently attached to the proteins but also to the alginates (Bitton *et al.*, 2006; Salgado *et al.*, 2009; Deniaud-Bouet *et al.*, 2014). The enzymatic degradation of alginate fractions always release a significant amount of polyphenols since polyphenols substances form high-molecular weight complexes with alginates due to oxidation with haloperoxidases (Bitton *et al.*, 2006; Salgado *et al.*, 2009). This type of linkage serves as structural component constituting the brown algal cell wall. Polyphenols cross-linking of alginate may be an integral part of initial wall formation as shown in the *Fucus* zygote. The linkages between polyphenols compounds and algal cell wall polysaccharides occurs immediately after the secretion of polyphenols substances into the cell wall, complexing finally with alginic acid (Arnold and Targett, 2003; Koivikko *et al.*, 2005).

From the results of the evaluation of biological activities presented previously, it is apparent that the eight enzymatic extracts of *S. muticum* showed different levels of potential. It indicates that the extracted polyphenols compound might function differently in responding to the test applied. In addition, it is also important to consider the possibility of an intervention from other compound as in this study the enzymatic extracts tested were all crude material.

Conclusion

Based on the results presented in this study, enzyme-assisted extraction enhances the extraction yield and polyphenol content to certain extent compared to the aqueous extracts in *S. muticum*. Some studies applied 12 h of extraction in order to optimize the polyphenol extraction, but here only by applying 3 h of extraction;

we can see an increase of polyphenol content to 2 fold than the aqueous extraction.

3.8.2.4 Optimization of *Sargassum* polyphenol enzyme-assisted extraction

Based on the screening of *S. muticum* polyphenol extraction in different commercial enzymes, Viscozyme and Termamyl extracts came out with their high polyphenol content. As a result, these two enzymes were selected for the next step, which was the optimization part to evaluate the efficiency of these enzymes in extracting polyphenols with adjusted conditions. In addition, Protamex was also used. As mentioned by many previous studies, 40 °C has been the most favourable temperature to extract polyphenols, thus, this temperature was selected.

It seems that 40°C is not favourable for polyphenol extraction of *S. muticum*, *S. ilicifolium*, and *S. polycystum* using Viscozyme. In the contrary, Protamex extract of these species showed better polyphenol content ranging from 3 to 6% of dry algal material. Such result is not too surprising as 40°C is the most optimum condition for Protamex. Perhaps, in this soft extraction condition, Protamex manages to break protein-phlorotannin linkage of algal cell wall and release more polyphenols. Other possibility is, instead of polyphenol compounds, the protein compound extracted by this proteolytic enzyme is more likely reacted with Folin-Ciocalteu reagent during the determination of polyphenol content. This reagent does not merely measure polyphenol since this it measures the total reducing capacity of a sample. It can also react to any reducing substances such as thiols, nitrogen-containing compound and some inorganic ions (Miyoshi Ikawa *et al.*, 2003; Everette *et al.*, 2010). It might explain partially regarding the high polyphenol content in Protamex extracts of *S. muticum*, *S. ilicifolium* and *S. polycystum*, that there was interference from other compounds, especially when the extracts were not purified. However, it does not exclude the presence of polyphenol, *i.e.* phlorotannins, in those enzymatic extracts.

As previously mention (see 4.2.2), some studies applied high temperature to extract polyphenol in brown algae. An elevated temperature helps the solvents to reach the samples matrices as the viscosity and the surface tension of the solvents are decreased. In addition, it facilitates the solubility of analytes and promotes higher mass transfer rate (Dai and Mumper, 2010). Using high temperature will be mostly compatible with water as solvent. Furthermore, in relation to algae, the introduction of the enzyme followed by heat treatment can further hydrolyze proteins and laminarine (Siriwardhana *et al.*, 2008). As a consequence, this process results in liberating more bioactive compounds to the solvents. However, due to the instability of polyphenol compounds as the elevated temperature stimulate a degradative reaction which is the oxidations of compound resulting in a decrease of polyphenols yields (Akowuah and Zhari, 2010; Dai and Mumper, 2010). As a result, high temperature is not really applicable to polyphenol extraction.

Conclusion

It is unfortunate because polyphenol extraction of *Sargassum* using enzyme-assisted extraction at 40°C does not enhance the polyphenol content compared to the previous screening at 50°C. Even though, the Protamex extract of *Sargassum* indeed shows higher polyphenol content than the Viscozyme extracts. It is not surprising as 40°C is the optimum temperature for Protamex.

3.8.2.5 Purification and characterization of *S. muticum* polyphenol enzymatic extract

FT-IR also detects other peaks that appear to be correlated with polyphenol bonds characterized by its aromatic and hydroxyl groups with the detection of C-H aromatics, C-O-C vibration, C-O stretch, and C=C. However, a protein bond is also exposed through this analysis by the presence of amides bond either in the raw or semi-purified enzymatic extracts. In contrary, the purification method has successfully removed the cellulose and mannuronic acid. Later, the HPLC

analysis has also detected interesting peak that is assumed to be the phloroglucinol-like compound.

Due to the lack of semi-purified extracts, it was impossible to characterize them using mass-spectrometry. This will be performed in future work to help to identify the group of phlorotannins existed in *S. muticum* extracted with enzyme.

3.8.3 Evaluation of biological activities of *Sargassum* polyphenol extracts

3.8.3.1 Antioxidant activities

Polyphenols compounds could be monomeric, oligomeric or polymeric with an aromatic ring having one or more hydroxyl substituents (Balboa *et al.*, 2013). These compounds have been known as a promising and strong antioxidant agent since they have up to eight interconnected rings of hydroxyl groups (Wang *et al.*, 2009). The polyphenols group is known as an excellent hydrogen donor forming hydrogen bonds with the carboxyl group of protein (Ozdal *et al.*, 2013). Later the existence of these hydroxyl groups provides the molecule with the ability of donating a proton to radical leading to its role as a possible chain breaking molecule or antioxidant upon secondary oxidation (Franco *et al.*, 2008).

Therefore, the antioxidant activity of brown algae is strongly related to their polyphenols content. As in this study, *S. muticum* digested with Viscozyme had strongest antiradical activity indicated by the low value of IC₅₀. Reducing the particle size can increase the extraction rate and the yield of polyphenols compounds that can be achieved by grinding and enzyme-assisted extraction (Franco *et al.*, 2008).

S. horneri digested with AMG, Celluclast, Termamyl, Ultraflo and Viscozyme for 12 h of extraction was reported to show DPPH radical scavenging activities at 25 µg/ml (Park *et al.*, 2004, 2005). Heo *et al.* (2005) also studied the antioxidant activity via DPPH assay of *S. horneri* enzymatic extracts. However, their results showed that the Viscozyme, Celluclast, AMG and Ultraflo extracts with final concentration 2 mg/ml did not perform any inhibition. Instead, the

Termamyl extract of *S. horneri* showed 11.2 % of inhibition. This activity was poor compared to the commercial standard BHA and BHT with 87 % and 56 % of inhibition, respectively. Same authors also reported that the antiradical activity of *S. fullvelum*, *S. coreanum*, and *S. thunbergii* extracted with carbohydrase also showed weak activity ranging from 4 – 38% compared to the standards. It might imply that there is an inter-individual and interspecies variation affecting the performance of antioxidant activity.

Antioxidant effects of *S. muticum* enzymatic extracts might also be due to the presence of polysaccharides and protein. Sugar content in *S. muticum* enzymatic extracts in this study ranged from 6 to 10% of dry algal material as shown by the biochemical analysis. Moreover, there was approximately 7% of sulfate groups and 0.6% of uronic acid content in *S. muticum* enzymatic extracts. It is possible that the antioxidative effect of *S. muticum* in this study is provoked mostly by the sulfate groups and alginic acid since it is the sulfated polysaccharides in brown algae known to possess wide range of pharmacological properties. Heterofucans of *S. filipendula* had been reported to show antioxidant activity such as hydroxyl and superoxide radical scavenging, chelating effects on ferrous ions and reducing powers in active concentration at 2 mg/ml (Costa *et al.*, 2011). Lim *et al.* (2014) in their study concerning the antioxidant effects of *S. binderi* also reported that the antiradical effect of this sample was at 2 mg/ml. It indicated that their antioxidant activity was weaker in comparison to the commercial standard, BHA, BHT and ascorbic acid with 6.0×10^{-4} , 9.6×10^{-4} and 3.5×10^{-4} mg/ml. However, it had performed high superoxide (compared to commercial food-grade fucoidan, BHA and BHT) and hydroxycal radical scavenging activities (compared to BHT) (Lim *et al.*, 2014). The ability of *S. graminifolium* polysaccharides to inhibit 50 % of superoxide anions scavenging and antiradical activities was at 1.9 and 0.6 mg/ml, respectively (Zhang *et al.*, 2012).

3.8.3.2 Tyrosinase and elastase inhibition activities

Skin aging causes many changes, including thinning, dryness, laxity, fragility, enlarged pores, skin pigmentation, fine lines and wrinkles (Wang *et al.*, 2015). These alterations in skin structure result from pathomechanism such as enzymatic degradation and disintegration of dermal elastin fibers, which maintain resilience of the skin; the proteolytic breakdown and disorganization of dermal collagen networks which provide the skin with strength and resistance; and the continuous exposure of free radicals against cellular compartments of the skin especially mitochondrial DNA (mtDNA) and excessive synthesis of melanin (Jablonski and Chaplin, 2010; Makrantonaki and Zouboulis, 2010; Dupont *et al.*, 2013).

Tyrosinase is a metallo-enzyme oxidase catalyzing two distinct reactions of melanin synthesis. Melanin helps to protect skin from the damaging ultraviolet radiation of the sun. However, elevated concentrations of melanin in the skin can result in hypigmentation such as freckles and moles (Kang *et al.*, 2012). While tyrosinase is responsible for skin pigmentation, elastase is a proteinase enzyme capable of degrading elastin. Elastin is an extracellular matrix protein providing elasticity to the connective tissues. It forms elastic fiber in skin dermis having an influence on skin elasticity (Kim *et al.*, 2009; Moon *et al.*, 2010). Therefore, inhibition of tyrosinase and elastase activity become a method to protect against skin aging (Kim *et al.*, 2009; Chang, 2012). Many have concerned and interested to the promising potential of marine algal bioactive compound in cosmeceutical industries related to their tyrosinase and elastase inhibition activities. Hexane fraction of *S. polycystum* contained potential skin-whitening agents that could inhibit the melanoma cells in dose-dependent way. The higher concentration of fraction, the stronger activity will be (Chan *et al.*, 2011). Dichloromethane:methanol extract of *Saccorhiza polyschides*, *Cystoseira humilis*, *Bifurcaria bifurcatan* *C. ericoides* and *Laminaria ochroleuca* exhibited an important elastase inhibition activity greater than 70% of inhibition for 100 µg (Oumaskour *et al.*, 2016). In the case of phlorotannins, many studied have reported their potential in inhibiting the tyrosinase activity. Dieckol, isolated

phlorotannins from the brown alga *E. cava* has been examined for its inhibitory effects on mushroom tyrosinase. Dieckol was capable to inhibit the mushroom tyrosinase with IC₅₀ 20 µM and it showed no cytotoxicity (Kang *et al.*, 2012). 7-phloroekol of *E. cava* had been observed to have an inhibitory effect on mushroom tyrosinase at 50 µg/ml. This compound was isolated from the ethanol extract of *E. cava* (Yoon *et al.*, 2009). These examples provide knowledge concerning the tyrosinase inhibition activity from marine brown algal phlorotannins. Some results presented in present study are in accordance with previous studies as previously mentioned. Polyphenol extracts of *S. muticum* extracted in different commercial enzymes showed interesting result regarding the tyrosinase and elastase inhibition activities. Attention was given to the tyrosinase inhibition activity; *S. muticum* Neutrase extract demonstrated the highest activity at 41% compare to other enzymes. Later, Protamex extract of *S. ilicifolium* and *S. polycystum* also showed the potential to inhibit the tyrosinase, slightly better than, the Viscozyme extract. To our best knowledge, there are not any studies yet that evaluate the brown algal polyphenol extracted with commercial enzymes. This finding could serve as basic information regarding the potential of polyphenol enzymatic extract from brown algae in cosmeceutical industries.

3.8.3.3 Antimicrobial activities

As reviewed by Singh (2013), phloroglucinol, eckol, phlorofucofuroeckol (deacetylated 8), dieckol, and 8,8''-bieckol have been tested for antibacterial activity against pathogenic gram positive and negative, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Vibrio parahaemolyticus*, *Propionibacterium acnes*, and many more. All other compounds, besides phloroglucinol, have shown bactericidal activity. Bactericidal effect tends to increase with the degree of polymerization of phloroglucinol. Further, phlorotannins interaction with bacterial proteins is assumed to play a role in the bactericidal action (Nagayama *et al.*, 2002; Shibata *et al.*, 2002). In this study, polyphenol extracts of *Sargassum* extracted using SLE showed positive activity against *E. coli*, *B. subtilis* and *P. aeruginosa*. Unfortunately, the enzymatic

extracts of *Sargassum* polyphenol did not show expected results as they did not demonstrate any antibacterial activity. It is suspected that the presence of sugar could contribute to the growth of bacteria in the media. However, this assumption will need further verification by evaluating the purified enzymatic extract of *Sargassum* polyphenol.

Even though the enzymatic extract of *Sargassum* polyphenol did not show antibacterial activity, biofilm inhibition activity demonstrated the opposite result. It seems that polyphenol of *Sargassum* enzymatic extracts was more active against biofilm formation. This contradictory result is caused by different mechanism of inhibition. In antibacterial activity, the mechanisms of inhibition are most likely thought to rely on inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth, or direct inhibition of oxidative phosphorylation (Scalbert, 1991). Regarding biofilm inhibition activity, two main mechanisms have been proposed, *i.e.* direct inhibition of bacterial settlement or survival of the macrofoulers larvae and regulation of macrofoulers growth, which in turn affects larval settlement of the macrofoulers (Avelin Mary *et al.*, 1993). Some studies have reported the promising potential of brown algal polyphenol compound as antifouling agent. Water extract of *S. vulgare* polyphenol from its pneumatocysts presented 75 to 99% inhibition of mussel *Perna perna* attachment. Polyphenol from leaflets of *S. vulgare* had 67 to 80% inhibition of byssal adhesion (Plouguerné *et al.*, 2012). Phlorotannin extract of *S. tenerrimum* had EC₅₀ of larval settlement inhibition of *Hydroides elegans* at 0.526 ppm. It indicated that this extract was effective in inhibiting the settlement of *H. elegans* larval attachment (Lau and Qian, 1997). Phlorotannins have been reported as rapidly induced compounds toward grazing. One of the studies reporting such result is an induction phlorotannins content in *Styopodium zonale* (Ank *et al.*, 2013). Authors observed that there was an increase of phlorotannins content in *S. zonale* submitted to stimulate herbivory for 2 days compared with uninjured algae. However, after 4 days of exposure to herbivory, drastic decline of phlorotannins content was remarked. A possible explanation made by authors that

phlorotannins induction is likely related to the healing process of the wound, and not an anti-hebivore response.

Antiviral effects of brown algae are usually shown by its polysaccharides as explained by previous studies with active concentration from 0.5 - > 500 µg/ml (Ponce *et al.*, 2003; Lee *et al.*, 2004, 2011; Ahn *et al.*, 2006; Queiroz *et al.*, 2008; Sinha *et al.*, 2010; Elizondo-Gonzalez *et al.*, 2012). The efficiencies of polysaccharides against virus are based on the following mode of actions (1) inhibition of viral reverse transcriptase; (2) the interference with viral adsorption; and (3) enhancement of immune function of host cells (Xianliang *et al.*, 2000; Xin *et al.*, 2000; Zhu *et al.*, 2004; Talarico *et al.*, 2005; Queiroz *et al.*, 2008). However, for the past few years, several studies concerning the antiviral effects of brown algae phlorotannins have appeared. Ahn *et al.* (2004b) reported an inhibitory effect of 8,8'-bieckol and 8-4"-dieckol isolated from *E. cava* on HIV-1 reverse transcriptase and protease *in vitro*. Phloroglucinol, eckol, 7-phloroeckol, phlorofucroeckol and dieckol isolated from *E. cava* were effective against porcine epidemic diarrhea coronavirus at concentration ranging from 10.8 – 14.6 µM (Wang *et al.*, 2008). Ethyl acetate fractions of phlorotannins from brown algae *Eisania bicyclis* have been reported to show antiviral effects against genital human papilloma virus (HPV) at 50 µg/ml (Kim and Kwak, 2015). Dichloromethane :methanol extract of *S. cymosum*, *S. polyceratium*, *S. vulgare*, *Padina gymnospora*, *Lobophora variegata*, *Dictyopteris delicatula*, *Sicetyota menstrualis* and *Stypodium zonale* demonstrated herpes simplex virus-1 (HSV-1) inhibition activity with percentage of inhibition ranging from 20 to 96% at 400 µg/ml. Spectroscopy analysis of these crude extracts detected the presence of polyphenol compounds along with other active compound like halogenated sesquiterpenes obtusol, elatol, meroditerpenoid atomaric acid, triacylglycerols and fatty acids (Soares *et al.*, 2012).

Result in this study regarding to the antiviral effect of *S. muticum* enzymatic extracts seems to be in the contrary to the previous studies. Out of eight, there were only two enzymatic extracts that were quite effective against the

HSV-1; they were *S. muticum* samples digested with Neutrase and Alcalase. However, the antiviral effects of these two enzymatic extracts might not be provoked only by the phlorotannins. It is possible that the presences of other compounds such as protein and polysaccharides that are present in enzymatic extracts are involved in disrupting the viral cells. Based on the colorimetric analysis, the biochemical composition of *S. muticum* enzymatic extracts contained 30 – 55% of protein and 6 – 12% sugar.

Conclusion

Polyphenol extraction of *Sargassum* indeed demonstrates interesting antioxidant activities as reported by many studies. In addition, this study also shows diverse biological activities performed by *Sargassum* polyphenol extracts such as tyrosinase, and biofilm inhibition activities. In the case of *S. muticum*, polyphenol extracts shows quite encouraging result for its elastase inhibition and antiviral activities with no cytotoxicities. However, enzymatic extract of *Sargassum* polyphenol does not show any positive antibacterial activity. Despite of the fact that polyphenol is quite remarkable for its antibacterial activity. This is possible that the sugar content in enzymatic extract of *Sargassum* polyphenol contributes to bacterial growth by providing more nutrients.

Various results obtained from this study imply the vast array of polyphenol potential for commercial interest from *Sargassum* species. Moreover, the bioactivities of polyphenol from this species display between species variation. It is important to consider type of species that has more active polyphenol and method used to extract these compounds. Enzyme-assisted extraction of *Sargassum* polyphenol surely shows remarkable bioactivity in certain extent in comparison to the solid-liquid extraction. One example, reducing power activity of *S. aquifolium* polyphenol in Viscozyme extract is better than the methanol, ethanol or even aqueous extracts. Certain results in this study have proven the efficiency of enzyme-assisted extraction in improving the bioactivities of bioactive compounds, especially polyphenols from marine brown algae.

3.8.3.4 Polyphenol content and bioactivities of purified fraction

Semi-purification fraction of *S. muticum* Viscozyme had yielded an important percentage of polyphenols compounds assuming to be phlorotannins. Antiradical and reducing power capacity of the semi-purified fraction were improved compared to the raw enzymatic extracts. Later, the antiviral activity of polyphenol seems to be enhanced as well with no cytotoxicity remarked. This result suggests that the antioxidant activity showed by *S. muticum* enzymatic extracts is generated by the presence of polyphenols compound. It is supported by FT-IR and HPLC analysis that detects the presence of peaks corresponding to the aromatic rings of phloroglucinol. Chromatography has been used in some previous studies as a procedure to purify phlorotannins in brown algae. Fucophlorethols, fuhhalols from *S. spinuligerum* had been successfully isolated by means of flash chromatography and HPLC followed by ^1H NMR for further identification (Glombitza and Keusgen, 1995; Glombitza *et al.*, 1997). Phlorethol fucodiphlorethol, and Fucophlorethols from *E. cava* was isolated using a flash chromatography (Ham *et al.*, 2007; Li *et al.*, 2011). Column chromatography and RP HPLC were applied to isolate the dieckol and phlorofucofuroeckol-a from *E. cava* (Choi *et al.*, 2014).

Conclusion

This part of study might only succeed to perform semi-purification step, but the results have at least verify the role of polyphenol-rich (phlorotannins) fraction of *S. muticum* as active compound. Bioactivity of this fraction is not limited to antioxidant but also to antiviral activity. It also gives no toxic effect to the tested cell. Preliminary characterization has managed to identify the presence of aromatic groups in purified fraction and the purification procedure has eliminated the presence the polysaccharides.

Working with purified polyphenol fraction of *S. muticum* helps to further comprehend the bioactivity efficiency of this compound. Selection of appropriate method is crucial to obtain sufficient fraction for various assays in order to verify the bioactivity of target compound. Moreover, by purifying the polyphenol of *S.*

muticum will assure that the bioactivity performed indeed come from the polyphenol without any interference from other contaminant compounds such as polysaccharides, proteins, or lipid.

3.8.4 Characterization of Indonesian *Sargassum* species using Fourier-Transform Infrared Spectroscopy

Based on early detection of the spectroscopy analysis, several spectra assumed to correspondent with phloroglucinols were detected. The characteristic of phenolic compounds lies on its aromatic ring (1260, 1620, 2970, and 2850-2970 cm^{-1}) and hydroxyl group (3415 cm^{-1}) (Lim *et al.*, 2002; Ganapathi *et al.*, 2013). Besides phenolic compounds, the detected spectra showed that there was also the presence of other compounds. Broad spectrums between 3500-3000 cm^{-1} usually relate to the presence of polysaccharides and proteins (Gomez-Ordenez and Ruperez, 2011; Kannan, 2014). Spectra ranging from 1200-970 cm^{-1} assumed to be the bonds of C-C and C-O pyranose ring and C-O-C stretching of glycosidic bonds (Gomez-Ordenez and Ruperez, 2011). Medium peak at 1636 cm^{-1} and 1428 cm^{-1} as shown at Figure 1 corresponded to the carbonyl (C=O) and carboxyl (COOH) that might indicate the presence of alginic acid (Ganapathi *et al.*, 2013). Peak signal observed around 648 cm^{-1} might be due to C-Br bond and near 600-670 cm^{-1} due to C-S and C=S stretching vibrations (sulfides) (Girija *et al.*, 2013; Kannan, 2014). As the enzymatic extracts tested in this study were crude extracts, then the presence of other molecules besides phenolic compounds had been expected. Therefore, in polyphenol extract of Indonesian *Sargassum*, there are polysaccharides and polyphenols in the same time.

Conclusion

FT-IR analysis definitely helps in identifying the biochemical entities of algal material through the detection of its functional groups. Enzymatic extract of Indonesian *Sargassum* polyphenol, polysaccharides and proteins are two mostly identified compounds as expected. As enzymes degrade algal cell wall, they release any bioactive compounds that are present in the matrix of algal cell wall.

Without any purification of designated compounds, interferences from other compounds than polyphenol in bioactivity assays must not be excluded.

Chapter 4 Conclusions

Marine brown algal have attracted many attention as they show infinite applications with valuable economic value due to their complexes and distinctive polysaccharides and bioactive compounds. In addition, brown algae have interesting nutritional profiles that are beneficial for food and fertilizer industries. Before going further to commercial exploitation, it is highly suggested to apprehend thoroughly about the variation of algal biochemical compositions and bioactive compositions, in this case, polyphenol. Determination of the best period to collect algal biomass having the highest content of biochemical compositions for certain application is important. Seasonal study of *S. muticum* (collected at Saint Gildas, France) biochemical composition for two years reveals that in certain extent, this marine alga tends to show quite similar pattern. Protein and ash content of *S. muticum* is lower during summer and increases in autumn to spring. On the opposite, sugar content is higher in summer and decreases from autumn to spring. In addition, the reproductive cycle also contributes to the biochemical composition in *S. muticum*. In comparison with other *Sargassum* species, such variation exhibits similar tendency. The seasonal variation study indicates that there is chemodiversity in *Sargassum* species driven by the fluctuated conditions of the surroundings. Present study serves as fundamental information for further possible valorization of algal material in food industries as source of natural nutrition.

Beside the nutritional value of *Sargassum*, this species shows a promising potential for biotechnological application due to the presence of its phlorotannins. This compound is widely known to possess strong antioxidant activity than other polyphenol compounds generated by its long polymers of hydroxyl groups. In addition, this compound also contributes for tyrosinase, elastase and biofilm inhibition, and antiviral activity. It is also proven to be non toxic. Most of the activity showed by *Sargassum* polyphenol originates from the enzymatic extracts. Enzymatic extraction is meant to improve the extraction yield and polyphenol

content in *Sargassum*, and to enhance the bioactivities since it is expected to liberate more polyphenol than the conventional extraction, *i.e.* solid-liquid extraction.

This study has demonstrated that the application of enzymes in extracting the phenolic compound in *Sargassum* is quite effective. In addition, the enzymatic extracts of *Sargassum* demonstrate a vast array of bioactivities. Based on the results presented in this study, different enzymes used give various results concerning the total phenolic content and their bioactivity. Among the eight enzymes, Viscozyme releases higher content of phenolic compounds and it is correlated with its strong antiradical activity. This might be supported by the optimum conditions provided, *i.e.* 50 °C for 3 H, during the extraction process, which is favorable for the Viscozyme, extract activity. Since phenolic compounds are sensitive to heat and easily oxidized by the exposure of light, it is strongly recommended to apply a mild temperature between 40 to 60 °C. Besides the extraction condition, in the case of phenolic compound, it is important to take the period of collection into further consideration. As commonly known, the production of phenolic compound is mostly stimulated by their environmental condition, such as the presence of grazer and direct exposition to sunlight. As a result, the content will vary according to the season.

Another important note to take is that by using enzymes, the active molecules released might not exclusively come from the phenolic groups. It is possible that it also extracts other molecules since the objective of this method is to degrade algae cell walls. Therefore, it will not be surprising that the fucoidan and protein might be responsible for the bioactivity in *Sargassum*. As a consequence, it will be interesting to further work on the purified samples pre-treated by the enzyme-assisted extraction in the future. In addition, the characterization of *Sargassum* phenolic compound might help in understanding more about the mechanisms of their bioactivity.

Exploitation of marine natural bioactive compounds involves the collection of living resources for commercial purposes, sustainable factor must be considered along the process. Some bioactive compounds particularly those induced by the surrounding, in other word, exist as secondary metabolites, often present in low concentrations. In the search of marine bioactive compounds for commercial purposes, a substantial amount of samples is important to consider in providing an adequate amount of target compounds to complete the whole process. In addition, a sustainable resource also becomes important factor in the search of marine natural bioactive compound.

Regarding the exploitation of brown algal phlorotannins derived from *Sargassum*, its massive biomass influx offers certain advantage in terms of valorization. However, considering that the content of phlorotannins is merely up to 20%, it is strongly suggested to determine and evaluate the supply chain management of this species for future exploitation. As an alternative if the natural population could not fulfill the required supply of biomass, cultivation approach might be one of the possible propositions in order to provide algal biomass in substantial amount and sustainably. Furthermore, governmental support as well as reliable and integrated collaboration of inter-institutional research is also required. An understanding and reliable collaboration between government, universities/institutes and stakeholders - private sectors or industries, will bring remarkable output for the interest of marine algal natural compound.

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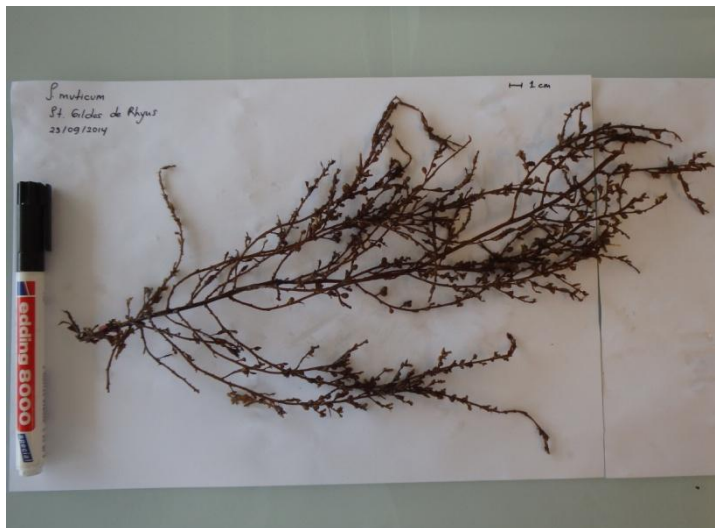
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Appendix 1 Documentations



A. Left: *S. muticum* collected in August 2014 Right: *S. muticum* in August 2014 covered with epiphytes



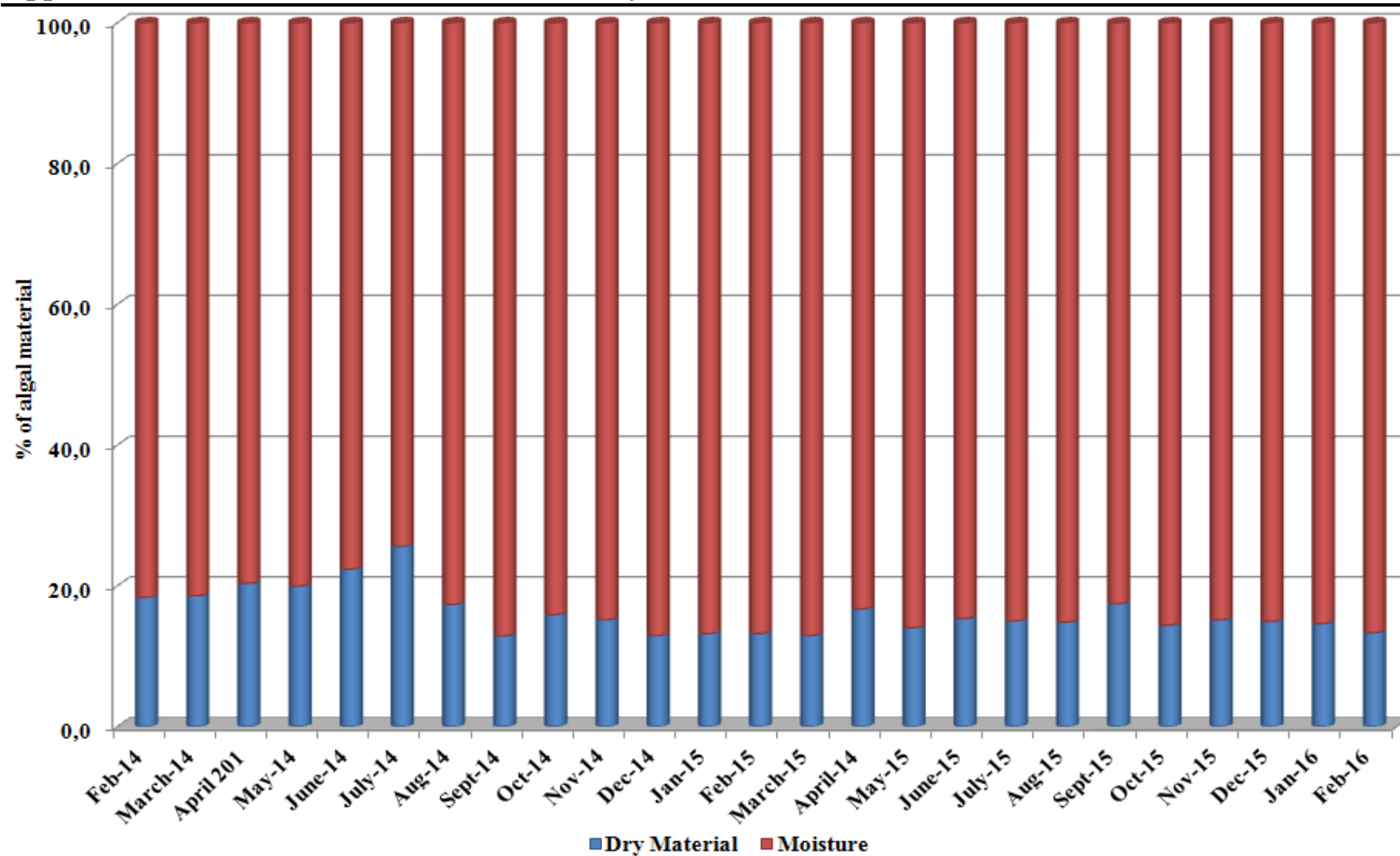
B. Left: *S. muticum* from September 2014 Right: *S. muticum* from January 2015

Extraction assistée par enzyme de phlorotannins provenant d'algues brunes du genre *Sargassum* et les activités biologiques Maria Puspita 2017



C . *S. muticum* collected in January 2015 (winter) (A), *S. muticum* in May 2015 (Spring) (B), *S. muticum* in June 2015 (Summer) (C), and *S. muticum* November 2015 (Autumn) (D).

Appendix 2 *S. muticum* moisture content and dry material



D Histogram of *S. muticum* moisture content and dry material expressed in % of dry material.

Appendix 3 Meteorological data of Vannes, Morbihan

Meteorological data from www.meteociel.fr

Months	Sunlight (h/mois)	Tmax (°C) Vannes	Tmin (°C) Vannes	Precipitations (mm/mois)	Sea surface temperature (°C)
February 2014	75,2	11,8	5,4	202,4	10
March 2014	166,9	14,4	3,7	52,5	11
April 2014	179,6	16,8	7,2	56,7	12
May 2014	213,6	18,1	8,6	52,8	13
June 2014	270,7	23,2	12,4	43,0	17
July 2014	242,1	25,0	14,1	46,2	18
August 2014	198,1	21,7	12,4	119,2	18
September 2014	250,1	24,9	12,5	4,8	19
October 2014	138,1	19,5	10,3	90,0	16
November 2014	61,5	14,8	7,9	173,9	14
December 2014	57,8	10,8	4,7	58,0	12
January 2015	72,7	10,6	3,4	105,1	9
February 2015	127,7	9,8	1,3	42,2	9
March 2015	129,0	12,9	4,8	18,4	9
April 2015	204,2	18,0	7,3	57,4	13
May 2015	171,4	18,0	9,3	64,3	13
June 2015	290,7	22,9	11,4	4,2	16
July 2015	181,8	23,5	14,0	68,7	17
August 2015	179,6	23,0	13,4	101,2	18
September 2015	217,0	20,2	10,0	80,7	17
October 2015	123,8	17,2	8,5	40,0	14
November 2015	66,9	15,8	9,9	68,6	14
December 2015	47,6	14,2	9,6	76,4	12
January 2016	50,2	11,0	4,4	114,5	9
February 2016	84,9	11,1	4,5	95,8	9

Appendix 4 Analysis of variance of *S. muticum* biochemical composition – seasonal variation

Descriptive Statistics

Dependent Variable: DryMat

Seasons	Mean	Std. Deviation	N
Autumn	15,1461	1,61266	18
Spring	17,0818	3,01028	18
Summer	18,3994	4,48324	18
Winter	14,3590	1,93085	21
Total	16,1711	3,30169	75

Levene's Test of Equality of Error Variances^a

Dependent Variable: DryMat

F	df1	df2	Sig.
10,080	3	71	,000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Seasons

Tests of Between-Subjects Effects

Dependent Variable: DryMat

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	192,172 ^a	3	64,057	7,401	,000
Intercept	19708,405	1	19708,405	2277,074	,000
Seasons	192,172	3	64,057	7,401	,000
Error	614,515	71	8,655		
Total	20419,507	75			
Corrected Total	806,688	74			

a. R Squared = ,238 (Adjusted R Squared = ,206)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: DryMat

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
16,247	,340	15,568	16,925

2. Seasons

Dependent Variable: DryMat

Seasons	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Autumn	15,146	,693	13,763	16,529
Spring	17,082	,693	15,699	18,464
Summer	18,399	,693	17,017	19,782
Winter	14,359	,642	13,079	15,639

Post Hoc Tests Seasons

Multiple Comparisons

Dependent Variable: DryMat

Tukey HSD

(I) Seasons	(J) Seasons	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Autumn	Spring	-1,9357	,98065	,208	-4,5157	,6444
	Summer	-3,2533*	,98065	,008	-5,8334	-,6733
	Winter	,7871	,94498	,839	-1,6991	3,2733
Spring	Autumn	1,9357	,98065	,208	-,6444	4,5157
	Summer	-1,3177	,98065	,539	-3,8977	1,2624
	Winter	2,7227*	,94498	,026	,2365	5,2089
Summer	Autumn	3,2533*	,98065	,008	,6733	5,8334
	Spring	1,3177	,98065	,539	-1,2624	3,8977
	Winter	4,0404*	,94498	,000	1,5542	6,5266
Winter	Autumn	-,7871	,94498	,839	-3,2733	1,6991
	Spring	-2,7227*	,94498	,026	-5,2089	-,2365
	Summer	-4,0404*	,94498	,000	-6,5266	-1,5542

Based on observed means.

The error term is Mean Square(Error) = 8,655.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

DryMat

Tukey HSD^{a,b,c}

Seasons	N	Subset		
		1	2	3
Winter	21	14,3590		
Autumn	18	15,1461	15,1461	
Spring	18		17,0818	17,0818
Summer	18			18,3994
Sig.		,846	,194	,523

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 8,655.

a. Uses Harmonic Mean Sample Size = 18,667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = ,05.

Descriptives

Dependent Variable: Biochemical

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Uronic Acid	Spring	18	2,4033	1,27131	,29965	1,7711	3,0355	1,37	6,88
	Summer	18	2,3067	,77705	,18315	1,9203	2,6931	1,36	4,10
	Autumn	18	2,2472	,37386	,08812	2,0613	2,4331	1,57	3,08
	Winter	21	2,1567	,49771	,10861	1,9301	2,3832	1,50	3,05
	Total	75	2,2736	,78580	,09074	2,0928	2,4544	1,36	6,88
Protein	Spring	18	27,0628	3,39211	,79953	25,3759	28,7496	22,35	34,88
	Summer	18	21,8428	6,36032	1,49914	18,6799	25,0057	9,64	30,71
	Autumn	18	27,1700	10,39115	2,44922	22,0026	32,3374	11,69	59,71
	Winter	21	27,4352	2,62323	,57244	26,2412	28,6293	20,70	32,99
	Total	75	25,9400	6,63283	,76589	24,4139	27,4661	9,64	59,71
Sugar	Spring	18	10,8828	3,19887	,75398	9,2920	12,4735	5,57	17,22
	Summer	18	14,7939	2,39312	,56406	13,6038	15,9840	11,69	19,94
	Autumn	18	14,3428	3,38238	,79723	12,6608	16,0248	7,19	19,54
	Winter	21	11,6095	3,67370	,80167	9,9373	13,2818	5,60	17,19
	Total	75	12,8553	3,57511	,41282	12,0328	13,6779	5,57	19,94
Sulfates Groups	Spring	18	10,1683	1,97514	,46554	9,1861	11,1505	7,95	12,43
	Summer	18	10,6561	3,42244	,80668	8,9542	12,3580	7,01	15,26
	Autumn	18	9,5972	2,42427	,57141	8,3917	10,8028	6,90	14,65
	Winter	21	9,2757	2,03859	,44486	8,3478	10,2037	1,84	11,77
	Total	75	9,8984	2,51990	,29097	9,3186	10,4782	1,84	15,26
Polyphenol	Spring	18	2,1550	,34658	,08169	1,9826	2,3274	1,38	2,76
	Summer	18	2,3511	,95636	,22542	1,8755	2,8267	,79	3,87
	Autumn	18	3,1517	1,73673	,40935	2,2880	4,0153	,69	6,43
	Winter	21	2,5462	,64325	,14037	2,2534	2,8390	1,78	3,44
	Total	75	2,5508	1,08540	,12533	2,3011	2,8005	,69	6,43

Ash	Spring	18	23,5256	2,90156	,68390	22,0826	24,9685	17,09	28,04
	Summer	18	22,3694	3,42822	,80804	20,6646	24,0743	16,57	27,69
	Autumn	18	25,4306	3,93399	,92725	23,4742	27,3869	19,00	31,16
	Winter	21	25,8014	2,25975	,49312	24,7728	26,8301	21,14	30,00
	Total	75	24,3425	3,40159	,39278	23,5599	25,1252	16,57	31,16
Nitrogen	Spring	18	2,2906	,49929	,11768	2,0423	2,5388	1,25	2,83
	Summer	18	1,6728	,69131	,16294	1,3290	2,0166	1,10	3,25
	Autumn	18	1,9517	,59993	,14141	1,6533	2,2500	1,37	3,25
	Winter	21	2,0090	,35058	,07650	1,8495	2,1686	1,40	2,59
	Total	75	1,9821	,57429	,06631	1,8500	2,1143	1,10	3,25

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
UronicAcid	3,002	3	71	,036
Protein	5,081	3	71	,003
Sugar	,858	3	71	,467
SulfatesGroups	10,229	3	71	,000
Polyphenol	17,410	3	71	,000
Ash	3,663	3	71	,016
Nitrogen	1,554	3	71	,208

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Uronic Acid	Between Groups	,622	3	,207	,327	,806
	Within Groups	45,071	71	,635		
	Total	45,693	74			
Protein	Between Groups	399,044	3	133,015	3,306	,025
	Within Groups	2856,540	71	40,233		
	Total	3255,585	74			
Sugar	Between Groups	210,099	3	70,033	6,758	,000
	Within Groups	735,726	71	10,362		
	Total	945,825	74			
Sulfates Groups	Between Groups	21,421	3	7,140	1,130	,343
	Within Groups	448,470	71	6,316		
	Total	469,891	74			
Polyphenol	Between Groups	10,037	3	3,346	3,079	,033
	Within Groups	77,142	71	1,087		
	Total	87,179	74			
Ash	Between Groups	148,094	3	49,365	4,949	,004
	Within Groups	708,146	71	9,974		
	Total	856,240	74			
Nitrogen	Between Groups	3,467	3	1,156	3,918	,012
	Within Groups	20,939	71	,295		
	Total	24,406	74			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Uronic Acid	Spring	Summer	,09667	,26558	,983	-,6021	,7954
		Autumn	,15611	,26558	,935	-,5426	,8548
		Winter	,24667	,25592	,770	-,4266	,9200
	Summer	Spring	-,09667	,26558	,983	-,7954	,6021
		Autumn	,05944	,26558	,996	-,6393	,7582
		Winter	,15000	,25592	,936	-,5233	,8233
	Autumn	Spring	-,15611	,26558	,935	-,8548	,5426
		Summer	-,05944	,26558	,996	-,7582	,6393
		Winter	,09056	,25592	,985	-,5828	,7639
	Winter	Spring	-,24667	,25592	,770	-,9200	,4266
		Summer	-,15000	,25592	,936	-,8233	,5233
		Autumn	-,09056	,25592	,985	-,7639	,5828
Protein	Spring	Summer	5,22000	2,11432	,074	-,3426	10,7826
		Autumn	-,10722	2,11432	1,000	-5,6699	5,4554
		Winter	-,37246	2,03741	,998	-5,7327	4,9878
	Summer	Spring	-5,22000	2,11432	,074	-10,7826	,3426
		Autumn	-5,32722	2,11432	,065	-10,8899	,2354
		Winter	-5,59246	2,03741	,038	-10,9527	-,2322
	Autumn	Spring	,10722	2,11432	1,000	-5,4554	5,6699
		Summer	5,32722	2,11432	,065	-,2354	10,8899
		Winter	-,26524	2,03741	,999	-5,6255	5,0950
	Winter	Spring	,37246	2,03741	,998	-4,9878	5,7327
		Summer	5,59246	2,03741	,038	,2322	10,9527
		Autumn	,26524	2,03741	,999	-5,0950	5,6255
Sugar	Spring	Summer	-3,91111	1,07302	,003	-6,7342	-1,0881
		Autumn	-3,46000	1,07302	,010	-6,2830	-,6370
		Winter	-,72675	1,03399	,896	-3,4471	1,9936
	Summer	Spring	3,91111	1,07302	,003	1,0881	6,7342
		Autumn	,45111	1,07302	,975	-2,3719	3,2742
		Winter	3,18437	1,03399	,015	,4640	5,9047
	Autumn	Spring	3,46000	1,07302	,010	,6370	6,2830
		Summer	-,45111	1,07302	,975	-3,2742	2,3719
		Winter	2,73325	1,03399	,048	,0129	5,4536
	Winter	Spring	,72675	1,03399	,896	-1,9936	3,4471
		Summer	-3,18437	1,03399	,015	-5,9047	-,4640
		Autumn	-2,73325	1,03399	,048	-5,4536	-,0129
Sulfates Groups	Spring	Summer	-,48778	,83775	,937	-2,6919	1,7163
		Autumn	,57111	,83775	,904	-1,6330	2,7752
		Winter	,89262	,80728	,687	-1,2313	3,0165
	Summer	Spring	,48778	,83775	,937	-1,7163	2,6919
		Autumn	1,05889	,83775	,589	-1,1452	3,2630
		Winter	1,38040	,80728	,326	-,7435	3,5043
	Autumn	Spring	-,57111	,83775	,904	-2,7752	1,6330
		Summer	-1,05889	,83775	,589	-3,2630	1,1452
		Winter	,32151	,80728	,978	-1,8024	2,4454
	Winter	Spring	-,89262	,80728	,687	-3,0165	1,2313
		Summer	-1,38040	,80728	,326	-3,5043	,7435
		Autumn	-,32151	,80728	,978	-2,4454	1,8024

Polyphenol	Spring	Summer	-,19611	,34745	,942	-1,1102	,7180
		Autumn	-,99667	,34745	,027	-1,9108	-,0825
		Winter	-,39119	,33481	,649	-1,2721	,4897
	Summer	Spring	,19611	,34745	,942	-,7180	1,1102
		Autumn	-,80056	,34745	,107	-1,7147	,1136
		Winter	-,19508	,33481	,937	-1,0760	,6858
	Autumn	Spring	,99667	,34745	,027	,0825	1,9108
		Summer	,80056	,34745	,107	-,1136	1,7147
		Winter	,60548	,33481	,278	-,2754	1,4863
	Winter	Spring	,39119	,33481	,649	-,4897	1,2721
		Summer	,19508	,33481	,937	-,6858	1,0760
		Autumn	-,60548	,33481	,278	-1,4863	,2754
Ash	Spring	Summer	1,15611	1,05272	,692	-1,6135	3,9257
		Autumn	-1,90500	1,05272	,277	-4,6746	,8646
		Winter	-2,27587	1,01442	,122	-4,9448	,3930
	Summer	Spring	-1,15611	1,05272	,692	-3,9257	1,6135
		Autumn	-3,06111	1,05272	,024	-5,8307	-,2915
		Winter	-3,43198	1,01442	,006	-6,1009	-,7631
	Autumn	Spring	1,90500	1,05272	,277	-,8646	4,6746
		Summer	3,06111	1,05272	,024	,2915	5,8307
		Winter	-,37087	1,01442	,983	-3,0398	2,2980
	Winter	Spring	2,27587	1,01442	,122	-,3930	4,9448
		Summer	3,43198	1,01442	,006	,7631	6,1009
		Autumn	,37087	1,01442	,983	-2,2980	3,0398
Nitrogen	Spring	Summer	,61778	,18102	,006	,1415	1,0940
		Autumn	,33889	,18102	,249	-,1374	,8151
		Winter	,28151	,17444	,378	-,1774	,7404
	Summer	Spring	-,61778	,18102	,006	-1,0940	-,1415
		Autumn	-,27889	,18102	,419	-,7551	,1974
		Winter	-,33627	,17444	,226	-,7952	,1227
	Autumn	Spring	-,33889	,18102	,249	-,8151	,1374
		Summer	,27889	,18102	,419	-,1974	,7551
		Winter	-,05738	,17444	,988	-,5163	,4016
	Winter	Spring	-,28151	,17444	,378	-,7404	,1774
		Summer	,33627	,17444	,226	-,1227	,7952
		Autumn	,05738	,17444	,988	-,4016	,5163

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Tukey HSD^{a,b}

Uronic Acid

Season	N	Subset for alpha = 0.05
		1
Winter	21	2,1567
Autumn	18	2,2472
Summer	18	2,3067
Spring	18	2,4033
Sig.		,780

Tukey HSD^{a,b}

Protein

Season	N	Subset for alpha = 0.05	
		1	2
Summer	18	21,8428	
Spring	18	27,0628	27,0628
Autumn	18	27,1700	27,1700
Winter	21		27,4352
Sig.		,059	,998

Sugar

Tukey HSD^{a,b}

Season	N	Subset for alpha = 0.05		
		1	2	3
Spring	18	10,8828		
Winter	21	11,6095	11,6095	
Autumn	18		14,3428	14,3428
Summer	18			14,7939
Sig.		,901	,055	,973

Sulfates Groups

Tukey HSD^{a,b}

Season	N	Subset for alpha = 0.05	
		1	2
Winter	21	9,2757	
Autumn	18	9,5972	
Spring	18	10,1683	
Summer	18	10,6561	
Sig.		,343	

Ash

Tukey HSD^{a,b}

Season	N	Subset for alpha = 0.05	
		1	2
Summer	18	22,3694	
Spring	18	23,5256	23,5256
Autumn	18		25,4306
Winter	21		25,8014
Sig.		,679	,133

Nitrogen

Tukey HSD^{a,b}

Season	N	Subset for alpha = 0.05	
		1	2
Summer	18	1,6728	
Autumn	18	1,9517	1,9517
Winter	21	2,0090	2,0090
Spring	18		2,2906
Sig.		,241	,235

Polyphenol

Tukey HSD^{a,b}

Season	N	Subset for alpha = 0.05	
		1	2
Spring	18	2,1550	
Summer	18	2,3511	2,3511
Winter	21	2,5462	2,5462
Autumn	18		3,1517
Sig.		,662	,097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 18,667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 5 Pearson's Analysis of *S. muticum* biochemical composition – seasonal variation

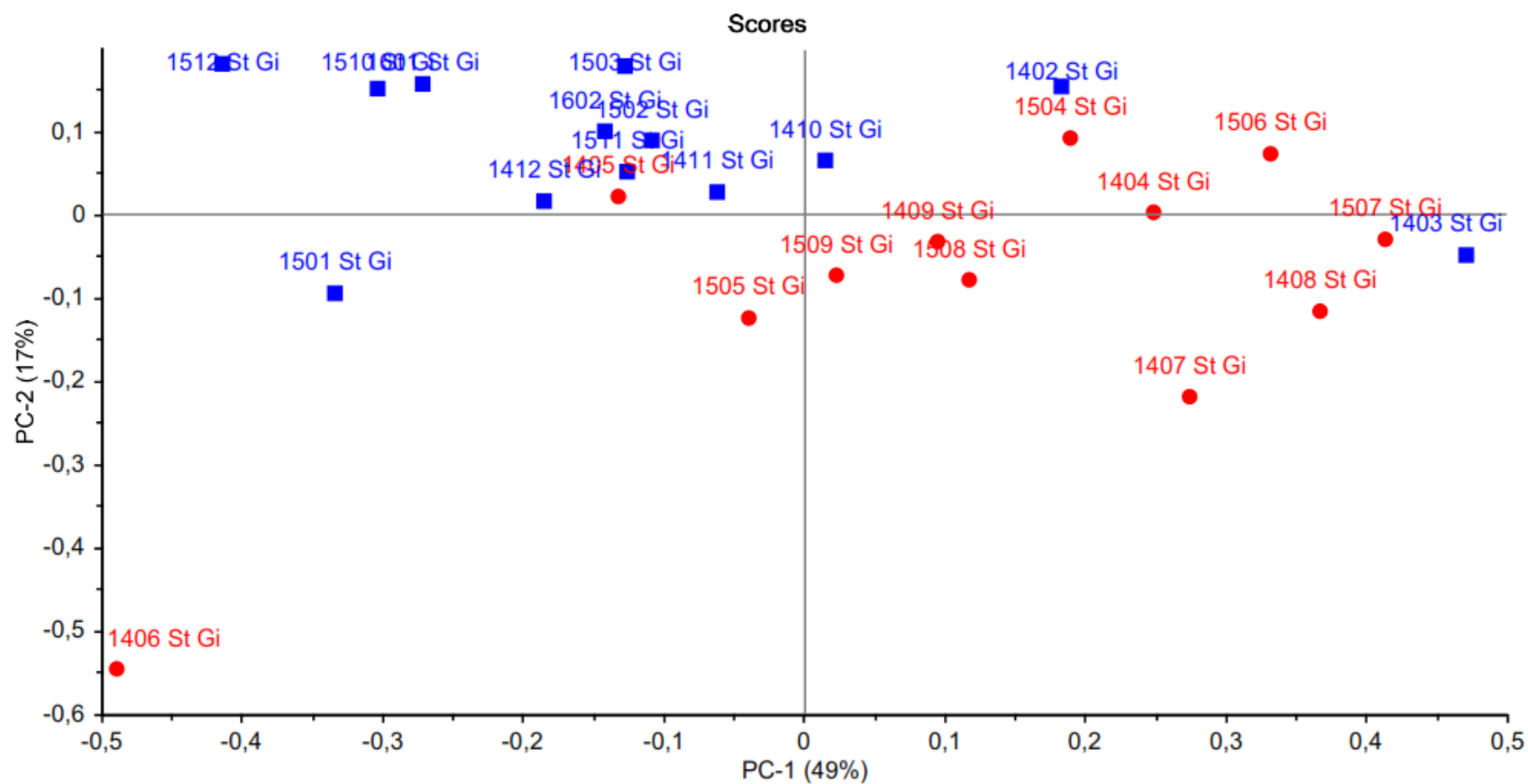
Correlations													
		Uronic Acid	Protein	Sugar	Sulfates Groups	Polyphenol	Ash	Nitrogen	Sunlight	Tmax	Tmin	Precipitation	SST
Uronic Acid	Pearson Correlation	1	,053	-,169	-,317**	,026	,050	,248*	,075	,024	-,016	-,180	-,050
	Sig. (2-tailed)		,654	,147	,006	,825	,668	,032	,521	,838	,892	,123	,668
	Sum of Squares and Cross-products	45,693	20,291	-35,188	-46,482	1,635	9,956	8,293	319,000	6,816	-3,373	-476,006	-9,661
	Covariance	,617	,274	-,476	-,628	,022	,135	,112	4,311	,092	-,046	-6,433	-,131
	N	75	75	75	75	75	75	75	75	75	75	75	75
Protein	Pearson Correlation	,053	1	,177	-,257*	,600**	,181	-,314**	-,162	-,257*	-,250*	,128	-,220
	Sig. (2-tailed)	,654		,129	,026	,000	,121	,006	,165	,026	,030	,272	,057
	Sum of Squares and Cross-products	20,291	3255,585	310,459	-317,395	319,387	301,538	-88,467	-5805,906	-613,701	-446,013	2870,621	-356,740
	Covariance	,274	43,994	4,195	-4,289	4,316	4,075	-1,195	-78,458	-8,293	-6,027	38,792	-4,821
	N	75	75	75	75	75	75	75	75	75	75	75	75
Sugar	Pearson Correlation	-,169	,177	1	-,030	,358**	-,030	-,502**	,099	,267*	,336**	,271*	,350**
	Sig. (2-tailed)	,147	,129		,800	,002	,799	,000	,399	,020	,003	,018	,002
	Sum of Squares and Cross-products	-35,188	310,459	945,825	-19,783	102,673	-26,899	-76,304	1906,394	344,780	322,423	3271,073	304,966
	Covariance	-,476	4,195	12,781	-,267	1,387	-,364	-1,031	25,762	4,659	4,357	44,204	4,121
	N	75	75	75	75	75	75	75	75	75	75	75	75
Sulfates Groups	Pearson Correlation	-,317**	-,257*	-,030	1	-,598**	-,065	,235*	,378**	,298**	,195	-,031	,298**
	Sig. (2-tailed)	,006	,026	,800		,000	,582	,042	,001	,010	,094	,792	,009
	Sum of Squares and Cross-products	-46,482	-317,395	-19,783	469,891	-121,134	-40,925	25,215	5142,651	270,416	131,614	-263,165	183,357
	Covariance	-,628	-4,289	-,267	6,350	-1,637	-,553	,341	69,495	3,654	1,779	-3,556	2,478
	N	75	75	75	75	75	75	75	75	75	75	75	75
Polyphenol	Pearson Correlation	,026	,600**	,358**	-,598**	1	,077	-,452**	-,162	-,046	,067	,054	,000
	Sig. (2-tailed)	,825	,000	,002	,000		,509	,000	,164	,696	,568	,646	,999
	Sum of Squares and Cross-products	1,635	319,387	102,673	-121,134	87,179	21,150	-20,838	-950,945	-17,935	19,526	197,019	,026
	Covariance	,022	4,316	1,387	-1,637	1,178	,286	-,282	-12,851	-,242	,264	2,662	,000
	N	75	75	75	75	75	75	75	75	75	75	75	75

		Uronic Acid	Protein	Sugar	Sulfates Groups	Polyphenol	Ash	Nitrogen	Sunlight	Tmax	Tmin	Precipitation	SST
Ash	Pearson Correlation	,050	,181	-,030	-,065	,077	1	-,089	-,509**	-,454**	-,377**	,237*	-,331**
	Sig. (2-tailed)	,668	,121	,799	,582	,509		,450	,000	,000	,001	,040	,004
	Sum of Squares and Cross-products	9,956	301,538	-26,899	-40,925	21,150	856,240	-12,806	-9340,351	-556,718	-344,421	2720,728	-274,476
	Covariance	,135	4,075	-,364	-,553	,286	11,571	-,173	-126,221	-7,523	-4,654	36,767	-3,709
	N	75	75	75	75	75	75	75	75	75	75	75	75
Nitrogen	Pearson Correlation	,248*	-,314**	-,502**	,235*	-,452**	-,089	1	,113	-,099	-,190	-,172	-,203
	Sig. (2-tailed)	,032	,006	,000	,042	,000	,450		,333	,398	,102	,139	,081
	Sum of Squares and Cross-products	8,293	-88,467	-76,304	25,215	-20,838	-12,806	24,406	351,738	-20,499	-29,366	-333,662	-28,450
	Covariance	,112	-1,195	-1,031	,341	-,282	-,173	,330	4,753	-,277	-,397	-4,509	-,384
	N	75	75	75	75	75	75	75	75	75	75	75	75

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Appendix 6 PCA of *S. muticum* seasonal variation



E FT-IR spectra of *S. muticum* biochemical composition at 1800-600 cm^{-1} ; Red color: spring-summer (hot season), Blue color: autumn-winter (cold season).

Appendix 7 Analysis of variance of *S. muticum* polyphenol content – seasonal variation with solid-liquid extraction

Levene's Test of Equality of Error Variances^a

	F	df1	df2	Sig.
Water	40,221	3	56	,000
MeOH	14,900	3	56	,000
MeOH50	17,337	3	56	,000
EtOH75	7,862	3	56	,000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Season

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Water	50,375 ^a	3	16,792	17,617	,000
	MeOH	18,804 ^b	3	6,268	2,728	,052
	MeOH50	71,240 ^c	3	23,747	8,594	,000
	EtOH75	20,919 ^d	3	6,973	5,701	,002
Intercept	Water	399,614	1	399,614	419,256	,000
	MeOH	337,561	1	337,561	146,937	,000
	MeOH50	777,601	1	777,601	281,402	,000
	EtOH75	385,020	1	385,020	314,783	,000
Season	Water	50,375	3	16,792	17,617	,000
	MeOH	18,804	3	6,268	2,728	,052
	MeOH50	71,240	3	23,747	8,594	,000
Error	EtOH75	20,919	3	6,973	5,701	,002
	Water	53,376	56	,953		
	MeOH	128,650	56	2,297		
	MeOH50	154,745	56	2,763		
Total	EtOH75	68,495	56	1,223		
	Water	541,908	60			
	MeOH	514,943	60			
	MeOH50	1047,977	60			
Corrected Total	EtOH75	503,955	60			
	Water	103,751	59			
	MeOH	147,454	59			
	MeOH50	225,985	59			
	EtOH75	89,414	59			

a. R Squared = ,486 (Adjusted R Squared = ,458)

b. R Squared = ,128 (Adjusted R Squared = ,081)

c. R Squared = ,315 (Adjusted R Squared = ,279)

d. R Squared = ,234 (Adjusted R Squared = ,193)

Estimated Marginal Means

		Season			
Dependent Variable	Season	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Water	Autumn	3,108	,282	2,543	3,672
	Spring	2,099	,230	1,638	2,560
	Summer	3,853	,230	3,392	4,314
	Winter	1,477	,282	,912	2,041
MeOH	Autumn	2,890	,438	2,013	3,767
	Spring	2,487	,357	1,772	3,203
	Summer	2,894	,357	2,179	3,610
	Winter	1,412	,438	,535	2,288
MeOH50	Autumn	4,235	,480	3,274	5,196
	Spring	2,534	,392	1,750	3,319
	Summer	5,085	,392	4,300	5,870
	Winter	2,843	,480	1,881	3,804
EtOH75	Autumn	2,881	,319	2,241	3,520
	Spring	2,224	,261	1,702	2,746
	Summer	3,378	,261	2,856	3,900
	Winter	1,859	,319	1,220	2,499

Post Hoc Tests

Season

Multiple Comparisons

Tukey HSD

			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
Dependent Variable	(J) Season	(I-J) Season				Lower Bound	Upper Bound
Water	Autumn	Spring	1,0086 [*]	,36384	,037	,0452	1,9720
		Summer	-,7453	,36384	,183	-1,7087	,2181
		Winter	1,6308 [*]	,39857	,001	,5755	2,6862
	Spring	Autumn	-1,0086 [*]	,36384	,037	-1,9720	-,0452
		Summer	-1,7539 [*]	,32543	,000	-2,6156	-,8922
		Winter	,6222	,36384	,328	-,3412	1,5856

	Summer	Autumn	,7453	,36384	,183	-,2181	1,7087
		Spring	1,7539 ⁺	,32543	,000	,8922	2,6156
		Winter	2,3761 ⁺	,36384	,000	1,4127	3,3395
	Winter	Autumn	-1,6308 ⁺	,39857	,001	-2,6862	-,5755
		Spring	-,6222	,36384	,328	-1,5856	,3412
		Summer	-2,3761 ⁺	,36384	,000	-3,3395	-1,4127
MeOH	Autumn	Spring	,4028	,56487	,892	-1,0929	1,8985
		Summer	-,0044	,56487	1,000	-1,5001	1,4913
		Winter	1,4783	,61878	,091	-,1601	3,1168
	Spring	Autumn	-,4028	,56487	,892	-1,8985	1,0929
		Summer	-,4072	,50523	,851	-1,7450	,9306
		Winter	1,0756	,56487	,238	-,4201	2,5713
	Summer	Autumn	,0044	,56487	1,000	-1,4913	1,5001
		Spring	,4072	,50523	,851	-,9306	1,7450
		Winter	1,4828	,56487	,053	-,0129	2,9785
	Winter	Autumn	-1,4783	,61878	,091	-3,1168	,1601
		Spring	-1,0756	,56487	,238	-2,5713	,4201
		Summer	-1,4828	,56487	,053	-2,9785	,0129
MeOH50	Autumn	Spring	1,7006 ⁺	,61951	,039	,0602	3,3409
		Summer	-,8500	,61951	,522	-2,4904	,7904
		Winter	1,3925	,67864	,182	-,4045	3,1895
	Spring	Autumn	-1,7006 ⁺	,61951	,039	-3,3409	-,0602
		Summer	-2,5506 ⁺	,55411	,000	-4,0178	-1,0833
		Winter	-,3081	,61951	,959	-1,9484	1,3323
	Summer	Autumn	,8500	,61951	,522	-,7904	2,4904
		Spring	2,5506 ⁺	,55411	,000	1,0833	4,0178
		Winter	2,2425 ⁺	,61951	,003	,6021	3,8829
	Winter	Autumn	-1,3925	,67864	,182	-3,1895	,4045
		Spring	,3081	,61951	,959	-1,3323	1,9484
		Summer	-2,2425 ⁺	,61951	,003	-3,8829	-,6021

EtOH75	Autumn	Spring	,6569	,41216	,390	-,4344	1,7483
		Summer	-,4969	,41216	,626	-1,5883	,5944
		Winter	1,0217	,45150	,119	-,1739	2,2172
	Spring	Autumn	-,6569	,41216	,390	-1,7483	,4344
		Summer	-1,1539*	,36865	,014	-2,1300	-,1777
		Winter	,3647	,41216	,813	-,7266	1,4561
	Summer	Autumn	,4969	,41216	,626	-,5944	1,5883
		Spring	1,1539*	,36865	,014	,1777	2,1300
		Winter	1,5186*	,41216	,003	,4272	2,6100
	Winter	Autumn	-1,0217	,45150	,119	-2,2172	,1739
		Spring	-,3647	,41216	,813	-1,4561	,7266
		Summer	-1,5186*	,41216	,003	-2,6100	-,4272

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

Water

Tukey HSD^{a,b,c}

Season	N	Subset	
		1	2
Winter	12	1,4767	
Spring	18	2,0989	
Autumn	12		3,1075
Summer	18		3,8528
Sig.		,328	,183

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = ,953.

a. Uses Harmonic Mean Sample Size = 14,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used.

Type I error levels are not guaranteed.

c. Alpha = ,05.

MeOH

Tukey HSD^{a,b,c}

Season	N	Subset
		1
Winter	12	1,4117
Spring	18	2,4872
Autumn	12	2,8900
Summer	18	2,8944
Sig.		,053

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2,297.

a. Uses Harmonic Mean Sample Size = 14,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = ,05.

MeOH50

Tukey HSD^{a,b,c}

Season	N	Subset		
		1	2	3
Spring	18	2,5344		
Winter	12	2,8425	2,8425	
Autumn	12		4,2350	4,2350
Summer	18			5,0850
Sig.		,959	,123	,522

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2,763.

a. Uses Harmonic Mean Sample Size = 14,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = ,05.

EtOH75

Tukey HSD^{a,b,c}

Season	N	Subset	
		1	2
Winter	12	1,8592	
Spring	18	2,2239	
Autumn	12	2,8808	2,8808
Summer	18		3,3778
Sig.		,074	,626

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 1,223.

b. The group sizes are unequal. The harmonic mean of the group sizes is used.

Type I error levels are not guaranteed.

c. Alpha = ,05.

Correlations

Descriptive Statistics

	Mean	Std. Deviation	N
Water	2,7023	1,32608	60
MeOH	2,4748	1,58089	60
MeOH50	3,7013	1,95710	60
EtOH75	2,6285	1,23105	60
Sunlight	152,0480	72,96261	75
Tmax	17,1680	4,87348	75
Tmin	8,4400	3,62760	75
Precipitation	73,4800	45,55026	75
SST	13,5600	3,29717	75

Correlations

		Water	MeOH	MeOH50	EtOH75	Sunlight	Tmax	Tmin	Precipitation	SST
Water	Pearson Correlation	1	,672**	,653**	,691**	,700**	,777**	,757**	-,336**	,763**
	Sig. (2-tailed)		,000	,000	,000	,000	,000	,000	,009	,000
	N	60	60	60	60	60	60	60	60	60
MeOH	Pearson Correlation	,672**	1	,692**	,747**	,370**	,324**	,318**	-,202	,272*
	Sig. (2-tailed)	,000		,000	,000	,004	,012	,013	,122	,036
	N	60	60	60	60	60	60	60	60	60
MeOH50	Pearson Correlation	,653**	,692**	1	,698**	,451**	,492**	,516**	-,216	,469**
	Sig. (2-tailed)	,000	,000		,000	,000	,000	,000	,098	,000
	N	60	60	60	60	60	60	60	60	60
EtOH75	Pearson Correlation	,691**	,747**	,698**	1	,288	,432**	,483**	-,110	,409**
	Sig. (2-tailed)	,000	,000	,000		,026	,001	,000	,404	,001
	N	60	60	60	60	60	60	60	60	60
Sunlight	Pearson Correlation	,700**	,370**	,451**	,288	1	,826**	,602**	-,551**	,672**
	Sig. (2-tailed)	,000	,004	,000	,026		,000	,000	,000	,000
	N	60	60	60	60	75	75	75	75	75
Tmax	Pearson Correlation	,777**	,324**	,492**	,432**	,826**	1	,934**	-,341**	,940**
	Sig. (2-tailed)	,000	,012	,000	,001	,000		,000	,003	,000
	N	60	60	60	60	75	75	75	75	75
Tmin	Pearson Correlation	,757**	,318**	,516**	,483**	,602**	,934**	1	-,167	,931**
	Sig. (2-tailed)	,000	,013	,000	,000	,000	,000		,152	,000
	N	60	60	60	60	75	75	75	75	75
Precipitation	Pearson Correlation	-,336**	-,202	-,216	-,110	-,551**	-,341**	-,167	1	-,200
	Sig. (2-tailed)	,009	,122	,098	,404	,000	,003	,152		,086
	N	60	60	60	60	75	75	75	75	75
SST	Pearson Correlation	,763**	,272*	,469**	,409**	,672**	,940**	,931**	-,200	1
	Sig. (2-tailed)	,000	,036	,000	,001	,000	,000	,000	,086	
	N	60	60	60	60	75	75	75	75	75

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Appendix 8 Analysis of variance of Indonesian *Sargassum* – extraction yield and polyphenol content of soli-liquid extraction

Descriptive Statistics

Dependent Variable: Yield

Solvent	Algae	Mean	Std. Deviation	N
EtOH75	<i>S. aquifolium</i>	12,2667	,40415	3
	<i>S. ilicifolium</i>	7,4600	1,25012	3
	<i>S. polycystum</i>	12,5333	,50332	3
	Total	10,7533	2,57082	9
MeOH	<i>S. aquifolium</i>	5,7000	1,75214	3
	<i>S. ilicifolium</i>	3,0667	,70238	3
	<i>S. polycystum</i>	8,2667	3,16439	3
	Total	5,6778	2,90937	9
MeOH50	<i>S. aquifolium</i>	15,7667	1,50444	3
	<i>S. ilicifolium</i>	7,2700	,95692	3
	<i>S. polycystum</i>	13,4333	1,92959	3
	Total	12,1567	4,02227	9
Water	<i>S. aquifolium</i>	17,2667	2,19393	3
	<i>S. ilicifolium</i>	14,6667	,76376	3
	<i>S. polycystum</i>	22,7667	1,12398	3
	Total	18,2333	3,80690	9
Total	<i>S. aquifolium</i>	12,7500	4,85171	12
	<i>S. ilicifolium</i>	8,1158	4,42893	12
	<i>S. polycystum</i>	14,2500	5,77101	12
	Total	11,7053	5,57152	36

Levene's Test of Equality of Error Variances^a

Dependent Variable: Yield

F	df1	df2	Sig.
2,310	11	24	,042

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Solvent + Algae + Solvent *

Algae

Tests of Between-Subjects Effects

Dependent Variable: Yield

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1028,226 ^a	11	93,475	38,522	,000
Intercept	4932,487	1	4932,487	2032,723	,000
Solvent	720,506	3	240,169	98,976	,000
Algae	245,414	2	122,707	50,569	,000
Solvent * Algae	62,307	6	10,384	4,280	,005
Error	58,237	24	2,427		
Total	6018,951	36			
Corrected Total	1086,463	35			

a. R Squared = ,946 (Adjusted R Squared = ,922)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: Yield

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
11,705	,260	11,169	12,241

2. Solvent

Dependent Variable: Yield

Solvent	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
EtOH75	10,753	,519	9,682	11,825
MeOH	5,678	,519	4,606	6,749
MeOH50	12,157	,519	11,085	13,228
Water	18,233	,519	17,162	19,305

3. Algae

Dependent Variable: Yield

Algae	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	12,750	,450	11,822	13,678
<i>S. ilicifolium</i>	8,116	,450	7,188	9,044
<i>S. polycystum</i>	14,250	,450	13,322	15,178

4. Solvent * Algae

Dependent Variable: Yield

Solvent	Algae	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
EtOH75	<i>S. aquifolium</i>	12,267	,899	10,410	14,123
	<i>S. ilicifolium</i>	7,460	,899	5,604	9,316
	<i>S. polycystum</i>	12,533	,899	10,677	14,390
MeOH	<i>S. aquifolium</i>	5,700	,899	3,844	7,556
	<i>S. ilicifolium</i>	3,067	,899	1,210	4,923
	<i>S. polycystum</i>	8,267	,899	6,410	10,123
MeOH50	<i>S. aquifolium</i>	15,767	,899	13,910	17,623
	<i>S. ilicifolium</i>	7,270	,899	5,414	9,126
	<i>S. polycystum</i>	13,433	,899	11,577	15,290
Water	<i>S. aquifolium</i>	17,267	,899	15,410	19,123
	<i>S. ilicifolium</i>	14,667	,899	12,810	16,523
	<i>S. polycystum</i>	22,767	,899	20,910	24,623

Post Hoc Tests Solvent

Multiple Comparisons

Dependent Variable: Yield

Tukey HSD

(I) Solvent	(J) Solvent	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtOH75	MeOH	5,0756*	,73432	,000	3,0498	7,1013
	MeOH50	-1,4033	,73432	,250	-3,4290	,6224
	Water	-7,4800*	,73432	,000	-9,5057	-5,4543
MeOH	EtOH75	-5,0756*	,73432	,000	-7,1013	-3,0498
	MeOH50	-6,4789*	,73432	,000	-8,5046	-4,4532
	Water	-12,5556*	,73432	,000	-14,5813	-10,5298
MeOH50	EtOH75	1,4033	,73432	,250	-,6224	3,4290
	MeOH	6,4789*	,73432	,000	4,4532	8,5046
	Water	-6,0767*	,73432	,000	-8,1024	-4,0510
Water	EtOH75	7,4800*	,73432	,000	5,4543	9,5057
	MeOH	12,5556*	,73432	,000	10,5298	14,5813
	MeOH50	6,0767*	,73432	,000	4,0510	8,1024

Based on observed means.

The error term is Mean Square(Error) = 2,427.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

Yield

Tukey HSD^{a,b}

Solvent	N	Subset		
		1	2	3
MeOH	9	5,6778	10,7533	18,2333
EtOH75	9			
MeOH50	9			
Water	9		12,1567	
Sig.		1,000	,250	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2,427.

a. Uses Harmonic Mean Sample Size = 9,000.

b. Alpha = ,05.

Algae

Multiple Comparisons

Dependent Variable: Yield

Tukey HSD

(I) Algae	(J) Algae	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	4,6342 [*]	,63594	,000	3,0460	6,2223
	<i>S. polycystum</i>	-1,5000	,63594	,067	-3,0881	,0881
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	-4,6342 [*]	,63594	,000	-6,2223	-3,0460
	<i>S. polycystum</i>	-6,1342 [*]	,63594	,000	-7,7223	-4,5460
<i>S. polycystum</i>	<i>S. aquifolium</i>	1,5000	,63594	,067	-,0881	3,0881
	<i>S. ilicifolium</i>	6,1342 [*]	,63594	,000	4,5460	7,7223

Based on observed means.

The error term is Mean Square(Error) = 2,427.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

Yield

Tukey HSD^{a,b}

Algae	N	Subset	
		1	2
<i>S. ilicifolium</i>	12	8,1158	
<i>S. aquifolium</i>	12		12,7500
<i>S. polycystum</i>	12		14,2500
Sig.		1,000	,067

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2,427.

a. Uses Harmonic Mean Sample Size = 12,000.

b. Alpha = ,05.

Descriptive Statistics

Dependent Variable: TPC

Solvent	Algae	Mean	Std. Deviation	N
EtOH75	<i>S. aquifolium</i>	3,4367	,33606	3
	<i>S. ilicifolium</i>	3,5667	,23288	3
	<i>S. polycystum</i>	3,9033	1,42732	3
	Total	3,6356	,77110	9
MeOH	<i>S. aquifolium</i>	3,7933	,90007	3
	<i>S. ilicifolium</i>	4,4800	1,43447	3
	<i>S. polycystum</i>	2,8767	,19399	3
	Total	3,7167	1,10076	9
MeOH50	<i>S. aquifolium</i>	2,1767	,11372	3
	<i>S. ilicifolium</i>	3,1300	,16523	3
	<i>S. polycystum</i>	2,3833	,21127	3
	Total	2,5633	,45809	9
Water	<i>S. aquifolium</i>	1,7696	,05977	3
	<i>S. ilicifolium</i>	2,0888	,10726	3
	<i>S. polycystum</i>	1,4358	,01938	3
	Total	1,7647	,28950	9
Total	<i>S. aquifolium</i>	2,7941	,97262	12
	<i>S. ilicifolium</i>	3,3164	1,09449	12
	<i>S. polycystum</i>	2,6498	1,11772	12
	Total	2,9201	1,07274	36

Levene's Test of Equality of Error Variances^a

Dependent Variable: TPC

F	df1	df2	Sig.
5,845	11	24	,000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Solvent + Algae + Solvent * Algae

Tests of Between-Subjects Effects

Dependent Variable: TPC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29,856 ^a	11	2,714	6,251	,000
Intercept	306,965	1	306,965	706,996	,000
Solvent	23,477	3	7,826	18,024	,000
Algae	2,952	2	1,476	3,399	,050
Solvent * Algae	3,427	6	,571	1,316	,288
Error	10,420	24	,434		
Total	347,242	36			
Corrected Total	40,277	35			

a. R Squared = ,741 (Adjusted R Squared = ,623)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: TPC

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2,920	,110	2,693	3,147

2. Solvent

Dependent Variable: TPC

Solvent	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
EtOH75	3,636	,220	3,182	4,089
MeOH	3,717	,220	3,263	4,170
MeOH50	2,563	,220	2,110	3,017
Water	1,765	,220	1,311	2,218

3. Algae

Dependent Variable: TPC

Algae	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	2,794	,190	2,401	3,187
<i>S. ilicifolium</i>	3,316	,190	2,924	3,709
<i>S. polycystum</i>	2,650	,190	2,257	3,042

4. Solvent * Algae

Dependent Variable: TPC

Solvent	Algae	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
EtOH75	<i>S. aquifolium</i>	3,437	,380	2,651	4,222
	<i>S. ilicifolium</i>	3,567	,380	2,781	4,352
	<i>S. polycystum</i>	3,903	,380	3,118	4,689
MeOH	<i>S. aquifolium</i>	3,793	,380	3,008	4,579
	<i>S. ilicifolium</i>	4,480	,380	3,695	5,265
	<i>S. polycystum</i>	2,877	,380	2,091	3,662
MeOH50	<i>S. aquifolium</i>	2,177	,380	1,391	2,962
	<i>S. ilicifolium</i>	3,130	,380	2,345	3,915
	<i>S. polycystum</i>	2,383	,380	1,598	3,169
Water	<i>S. aquifolium</i>	1,770	,380	,984	2,555
	<i>S. ilicifolium</i>	2,089	,380	1,304	2,874
	<i>S. polycystum</i>	1,436	,380	,651	2,221

Post Hoc Tests

Solvent

Multiple Comparisons

Dependent Variable: TPC

Tukey HSD

(I) Solvent	(J) Solvent	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtOH75	MeOH	-,0811	,31062	,994	-,9380	,7758
	MeOH50	1,0722*	,31062	,010	,2153	1,9291
	Water	1,8708*	,31062	,000	1,0140	2,7277
MeOH	EtOH75	,0811	,31062	,994	-,7758	,9380
	MeOH50	1,1533*	,31062	,006	,2965	2,0102
	Water	1,9519*	,31062	,000	1,0951	2,8088

MeOH50	EtOH75	-1,0722*	,31062	,010	-1,9291	-,2153
	MeOH	-1,1533*	,31062	,006	-2,0102	-,2965
	Water	,7986	,31062	,074	-,0583	1,6555
Water	EtOH75	-1,8708*	,31062	,000	-2,7277	-1,0140
	MeOH	-1,9519*	,31062	,000	-2,8088	-1,0951
	MeOH50	-,7986	,31062	,074	-1,6555	,0583

*. The mean difference is significant at the .05 level.

Homogeneous Subsets

TPC

Tukey HSD^{a,b}

Solvent	N	Subset	
		1	2
Water	9	1,7647	
MeOH50	9	2,5633	
EtOH75	9		3,6356
MeOH	9		3,7167
Sig.		,074	,994

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = ,434.

a. Uses Harmonic Mean Sample Size = 9,000.

b. Alpha = ,05.

Algae

Multiple Comparisons

Dependent Variable: TPC

Tukey HSD

(I) Algae	(J) Algae	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	-,5223	,26901	,149	-1,1941	,1495
	<i>S. polycystum</i>	,1443	,26901	,854	-,5275	,8161
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	,5223	,26901	,149	-,1495	1,1941
	<i>S. polycystum</i>	,6666	,26901	,052	-,0052	1,3383
<i>S. polycystum</i>	<i>S. aquifolium</i>	-,1443	,26901	,854	-,8161	,5275
	<i>S. ilicifolium</i>	-,6666	,26901	,052	-1,3383	,0052

Based on observed means.

The error term is Mean Square(Error) = ,434.

Homogeneous Subsets

TPC

Tukey HSD^{a,b}

Algae	N	Subset
		1
<i>S. polycystum</i>	12	2,6498
<i>S. aquifolium</i>	12	2,7941
<i>S. ilicifolium</i>	12	3,3164
Sig.		,052

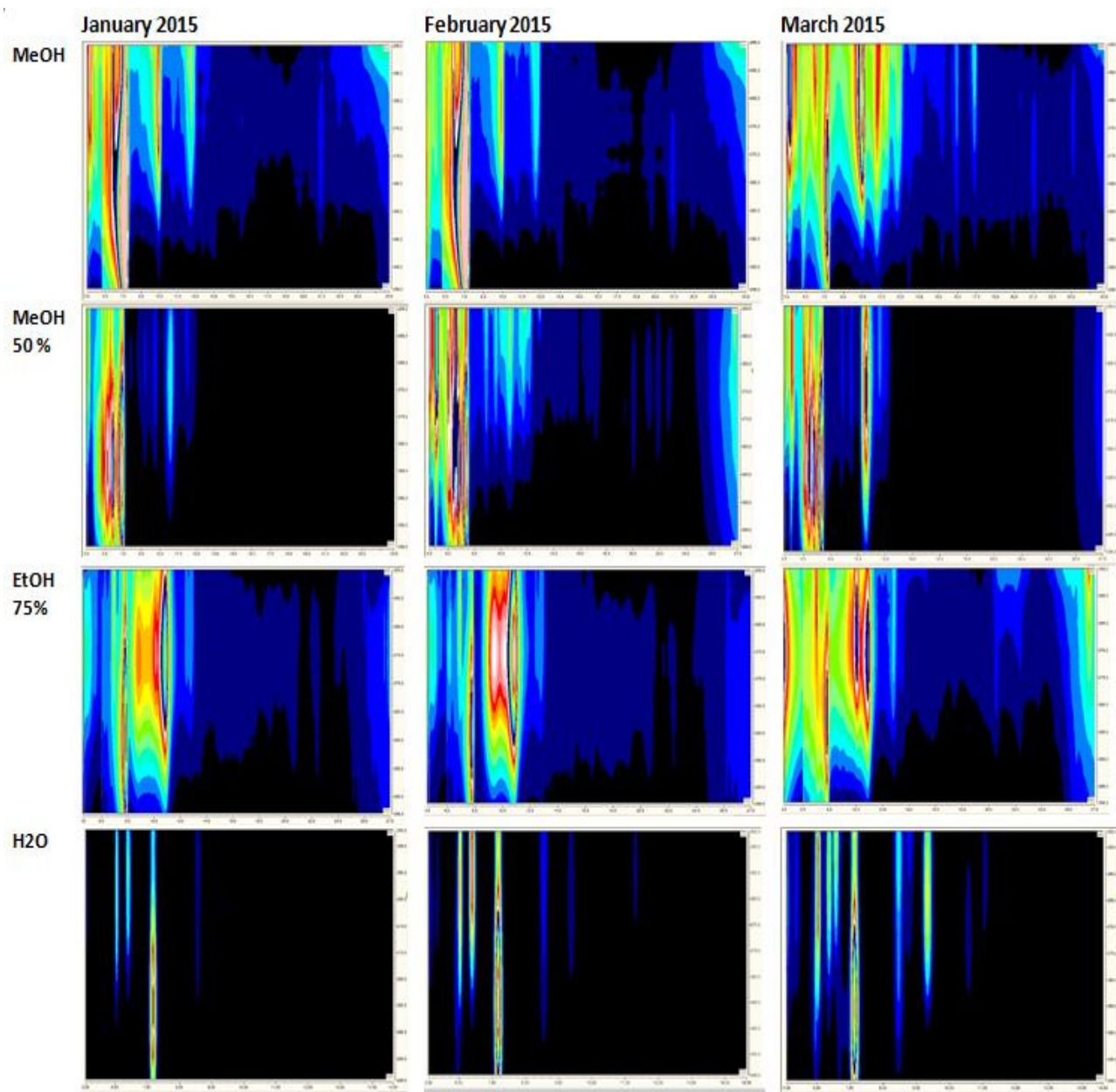
Means for groups in homogeneous subsets are displayed.

Based on observed means. The error term is Mean Square(Error) = ,434.

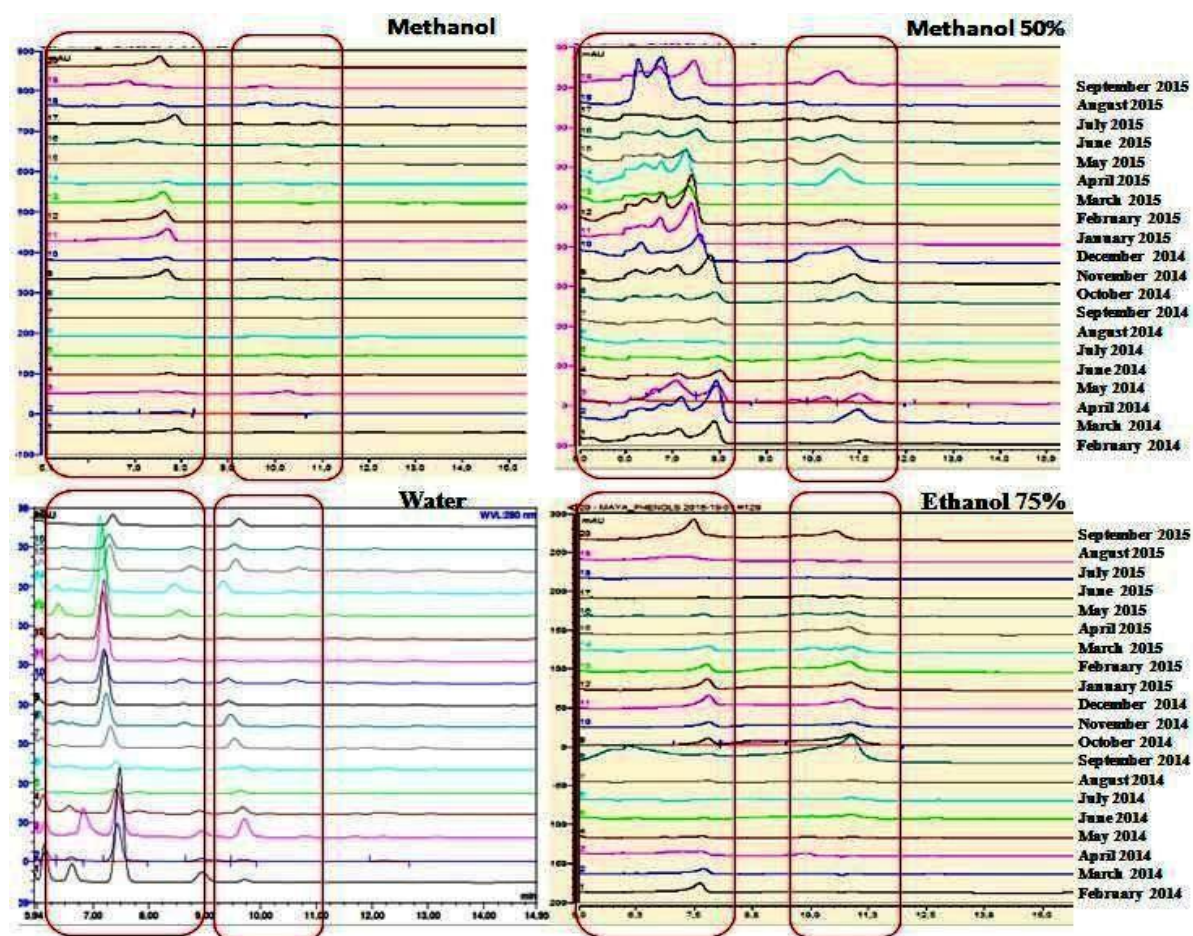
a. Uses Harmonic Mean Sample Size = 12,000.

b. Alpha = ,05.

Appendix 8 3D Image of HPLC chromatogram of *S. muticum* polyphenol compound – seasonal variations in solid-liquid extraction



F. Seasonal variation of *S. muticum* polyphenols in different solvents in 3D image from HPLC analysis. All peaks were detected at wavelength ranging at 260 - 280 nm.



G Chromatogram of *S. muticum* polyphenol compounds in different solvents – seasonal variation profile

Appendix 9 Analysis of variance of *S. muticum* – screening of enzyme assisted extraction for extraction yield, polyphenol content and bioactivities

Multivariate Tests ^a						
Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1,000	4868,315 ^b	8,000	11,000	,000
	Wilks' Lambda	,000	4868,315 ^b	8,000	11,000	,000
	Hotelling's Trace	3540,593	4868,315 ^b	8,000	11,000	,000
	Roy's Largest Root	3540,593	4868,315 ^b	8,000	11,000	,000
Sample	Pillai's Trace	4,260	2,562	64,000	144,000	,000
	Wilks' Lambda	,000	4,766	64,000	69,938	,000
	Hotelling's Trace	41,087	5,938	64,000	74,000	,000
	Roy's Largest Root	20,064	45,145 ^c	8,000	18,000	,000

a. Design: Intercept + Sample

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Levene's Test of Equality of Error Variances ^a				
	F	df1	df2	Sig.
Yield	3,449	8	18	,014
TPC	1,895	8	18	,124
DPPH	6,882	8	18	,000
FRAP	2,967	8	18	,026
Antityrosinase	,706	8	18	,683
Antielastase	3,724	8	18	,010
PAOIBiofilm	5,742	8	18	,001
EcoliBiofilm	2,233	8	18	,075

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Sample

Estimated Marginal Means

1. Grand Mean

Dependent Variable	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Yield	29,011	,701	27,538	30,484
TPC	4,282	,121	4,028	4,536
DPPH	1067,722	68,806	923,166	1212,279
FRAP	58,043	,284	57,447	58,638
Antityrosinase	17,103	1,334	14,300	19,906
Antielastase	27,152	,663	25,758	28,546
PAOIBiofilm	13,181	1,768	9,467	16,895
EcoliBiofilm	28,051	3,953	19,746	36,356

2. Sample

Dependent Variable	Sample	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Yield	ALC	28,700	2,103	24,282	33,118
	AMG	30,200	2,103	25,782	34,618
	CEL	29,867	2,103	25,448	34,285
	CTRL	26,500	2,103	22,082	30,918
	NEU	32,600	2,103	28,182	37,018
	SHE	26,200	2,103	21,782	30,618
	TER	27,333	2,103	22,915	31,752
	ULT	31,567	2,103	27,148	35,985
	VIS	28,133	2,103	23,715	32,552
TPC	ALC	3,797	,362	3,036	4,558
	AMG	4,808	,362	4,047	5,569
	CEL	4,482	,362	3,720	5,243
	CTRL	2,586	,362	1,825	3,347
	NEU	3,819	,362	3,058	4,581
	SHE	4,686	,362	3,925	5,448
	TER	5,045	,362	4,284	5,806
	ULT	4,163	,362	3,402	4,925
	VIS	5,149	,362	4,388	5,911
DPPH	ALC	1980,082	206,419	1546,412	2413,753
	AMG	728,609	206,419	294,938	1162,279

	CEL	602,167	206,419	168,497	1035,838
	CTRL	720,057	206,419	286,387	1153,728
	NEU	900,175	206,419	466,504	1333,846
	SHE	1020,250	206,419	586,579	1453,920
	TER	810,362	206,419	376,691	1244,033
	ULT	2233,168	206,419	1799,498	2666,839
	VIS	614,631	206,419	180,961	1048,302
FRAP	ALC	52,651	,851	50,863	54,438
	AMG	62,405	,851	60,618	64,193
	CEL	59,783	,851	57,996	61,571
	CTRL	65,926	,851	64,139	67,713
	NEU	59,253	,851	57,466	61,041
	SHE	58,830	,851	57,042	60,617
	TER	57,355	,851	55,567	59,142
	ULT	57,530	,851	55,743	59,317
	VIS	48,650	,851	46,863	50,437
Antityrosinase	ALC	14,032	4,002	5,624	22,441
	AMG	18,759	4,002	10,351	27,168
	CEL	17,874	4,002	9,466	26,282
	CTRL	18,102	4,002	9,694	26,510
	NEU	9,974	4,002	1,565	18,382
	SHE	16,353	4,002	7,945	24,762
	TER	17,500	4,002	9,091	25,908
	ULT	21,131	4,002	12,723	29,539
	VIS	20,201	4,002	11,793	28,610
Antielastase	ALC	30,891	1,990	26,709	35,072
	AMG	21,643	1,990	17,461	25,825
	CEL	29,950	1,990	25,768	34,132
	CTRL	23,115	1,990	18,933	27,297
	NEU	23,422	1,990	19,240	27,604
	SHE	32,880	1,990	28,698	37,062
	TER	28,772	1,990	24,590	32,954
	ULT	24,008	1,990	19,826	28,189
	VIS	29,683	1,990	25,502	33,865
<i>P. aeruginosa</i> Biofilm	ALC	3,492	5,304	-7,650	14,635
	AMG	,000	5,304	-11,143	11,143
	CEL	14,271	5,304	3,129	25,414

	CTRL	-3,553E-15	5,304	-11,143	11,143
	NEU	36,090	5,304	24,947	47,232
	SHE	26,996	5,304	15,854	38,139
	TER	12,441	5,304	1,298	23,583
	ULT	3,678	5,304	-7,465	14,821
	VIS	21,661	5,304	10,518	32,803
<i>E.coli</i> Biofilm	ALC	28,333	11,859	3,418	53,248
	AMG	30,856	11,859	5,941	55,771
	CEL	23,807	11,859	-1,107	48,722
	CTRL	14,691	11,859	-10,224	39,606
	NEU	15,697	11,859	-9,218	40,612
	SHE	64,255	11,859	39,340	89,170
	TER	35,814	11,859	10,900	60,729
	ULT	22,885	11,859	-2,030	47,800
	VIS	16,121	11,859	-8,794	41,036

Post Hoc Tests

a. Uses Harmonic Mean Sample Size = 3,000. b. Alpha = ,05.

Yield

Tukey HSD^{a,b}

Sample	N	Subset
		1
SHE	3	26,2000
CTRL	3	26,5000
TER	3	27,3333
VIS	3	28,1333
ALC	3	28,7000
CEL	3	29,8667
AMG	3	30,2000
ULT	3	31,5667
NEU	3	32,6000
Sig.		,472

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 13,269.

TPC

Tukey HSD^{a,b}

Sample	N	Subset	
		1	2
CTRL	3	2,5859	
ALC	3	3,7972	3,7972
NEU	3	3,8193	3,8193
ULT	3	4,1634	4,1634
CEL	3		4,4817
SHE	3		4,6864
AMG	3		4,8080
TER	3		5,0451
VIS	3		5,1493
Sig.		,112	,237

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = ,394.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

DPPH

Tukey HSD^{a,b}

Sample	N	Subset		
		1	2	3
CEL	3	602,1672		
VIS	3	614,6312		
CTRL	3	720,0574		
AMG	3	728,6087		
TER	3	810,3620		
NEU	3	900,1749		
SHE	3	1020,2496	1020,2496	
ALC	3		1980,0824	1980,0824
ULT	3			2233,1682
Sig.		,871	,076	,992

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 127826,654.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

FRAP

Tukey HSD^{a,b}

Sample	N	Subset			
		1	2	3	4
VIS	3	48,6500			
ALC	3	52,6506			
TER	3		57,3548		
ULT	3		57,5300		
SHE	3		58,8297	58,8297	
NEU	3		59,2533	59,2533	
CEL	3		59,7831	59,7831	
AMG	3			62,4054	62,4054
CTRL	3				65,9260
Sig.		,071	,551	,135	,147

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 2,171.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

Antityrosinase

Tukey HSD^{a,b}

Sample	N	Subset
		1
NEU	3	9,9735
ALC	3	14,0325
SHE	3	16,3534
TER	3	17,4996
CEL	3	17,8740
CTRL	3	18,1022
AMG	3	18,7594
VIS	3	20,2015
ULT	3	21,1311
Sig.		,579

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean

Square(Error) = 48,051.

a. Uses Harmonic Mean Sample

Size = 3,000.

b. Alpha = ,05.

Antielastase

Tukey HSD^{a,b}

Sample	N	Subset	
		1	2
AMG	3	21,6433	
CTRL	3	23,1150	23,1150
NEU	3	23,4221	23,4221
ULT	3	24,0075	24,0075
TER	3	28,7722	28,7722
VIS	3	29,6834	29,6834
CEL	3	29,9501	29,9501
ALC	3	30,8906	30,8906
SHE	3		32,8801
Sig.		,076	,054

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 11,886.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

PAOIBiofilm

Tukey HSD^{a,b}

Sample	N	Subset		
		1	2	3
AMG	3	,0000		
CTRL	3	,0000		
ALC	3	3,4924	3,4924	
ULT	3	3,6778	3,6778	
TER	3	12,4405	12,4405	12,4405
CEL	3	14,2713	14,2713	14,2713
VIS	3	21,6607	21,6607	21,6607
SHE	3		26,9964	26,9964
NEU	3			36,0896
Sig.		,157	,101	,098

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 84,389.

a. Uses Harmonic Mean Sample Size = 3,000. b. Alpha = ,05.

Appendix 10 Analysis of variance of Indonesian *Sargassum* – extraction yield of and polyphenol content of enzyme-assisted extraction

Descriptive Statistics

Dependent Variable: Yield

Enzyme	Algae	Mean	Std. Deviation	N
Protamex	<i>S. aquifolium</i>	26,2000	5,85918	3
	<i>S. ilicifolium</i>	21,4333	2,05020	3
	<i>S. polycystum</i>	38,1667	6,78994	3
	Total	28,6000	8,76926	9
Viscozyme	<i>S. aquifolium</i>	29,4667	5,40123	3
	<i>S. ilicifolium</i>	27,8667	1,45717	3
	<i>S. polycystum</i>	35,7667	2,65769	3
	Total	31,0333	4,76156	9
Total	<i>S. aquifolium</i>	27,8333	5,34815	6
	<i>S. ilicifolium</i>	24,6500	3,86614	6
	<i>S. polycystum</i>	36,9667	4,79528	6
	Total	29,8167	6,95881	18

Levene's Test of Equality of Error Variances^a

Dependent Variable: Yield

F	df1	df2	Sig.
1,821	5	12	,183

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Enzyme + Algae + Enzyme *

Algae

Tests of Between-Subjects Effects

Dependent Variable: Yield

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	577,232 ^a	5	115,446	5,632	,007
Intercept	16002,605	1	16002,605	780,636	,000
Enzyme	26,645	1	26,645	1,300	,276
Algae	490,503	2	245,252	11,964	,001
Enzyme * Algae	60,083	2	30,042	1,465	,269
Error	245,993	12	20,499		
Total	16825,830	18			

Corrected Total	823,225	17			
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a. R Squared = ,701 (Adjusted R Squared = ,577)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: Yield

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
29,817	1,067	27,491	32,142

2. Enzyme

Dependent Variable: Yield

Enzyme	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Protamex	28,600	1,509	25,312	31,888
Viscozyme	31,033	1,509	27,745	34,322

3. Algae

Dependent Variable: Yield

Algae	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	27,833	1,848	23,806	31,861
<i>S. ilicifolium</i>	24,650	1,848	20,623	28,677
<i>S. polycystum</i>	36,967	1,848	32,939	40,994

4. Enzyme * Algae

Dependent Variable: Yield

Enzyme	Algae	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Protamex	<i>S. aquifolium</i>	26,200	2,614	20,505	31,895
	<i>S. ilicifolium</i>	21,433	2,614	15,738	27,129
	<i>S. polycystum</i>	38,167	2,614	32,471	43,862
Viscozyme	<i>S. aquifolium</i>	29,467	2,614	23,771	35,162
	<i>S. ilicifolium</i>	27,867	2,614	22,171	33,562
	<i>S. polycystum</i>	35,767	2,614	30,071	41,462

Post Hoc Tests Algae

Multiple Comparisons

Dependent Variable: Yield

Tukey HSD

(I) Algae	(J) Algae	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	3,1833	2,61403	,466	-3,7905	10,1572
	<i>S. polycystum</i>	-9,1333*	2,61403	,011	-16,1072	-2,1595
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	-3,1833	2,61403	,466	-10,1572	3,7905
	<i>S. polycystum</i>	-12,3167*	2,61403	,001	-19,2905	-5,3428
<i>S. polycystum</i>	<i>S. aquifolium</i>	9,1333*	2,61403	,011	2,1595	16,1072
	<i>S. ilicifolium</i>	12,3167*	2,61403	,001	5,3428	19,2905

Based on observed means.

The error term is Mean Square(Error) = 20,499.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

Yield

Tukey HSD^{a,b}

Algae	N	Subset	
		1	2
<i>S. ilicifolium</i>	6	24,6500	36,9667
<i>S. aquifolium</i>	6	27,8333	
<i>S. polycystum</i>	6		
Sig.		,466	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 20,499.

a. Uses Harmonic Mean Sample Size = 6,000.

b. Alpha = ,05.

Analysis of Variance *Sargassum* Indonesia TPC EAE

Descriptive Statistics

Dependent Variable: TPC

Enzyme	Algae	Mean	Std. Deviation	N
Protamex	<i>S. aquifolium</i>	4,1532	,01163	3
	<i>S. ilicifolium</i>	4,9753	,26753	3
	<i>S. polycystum</i>	6,0753	,13383	3
	Total	5,0679	,84847	9
Viscozyme	<i>S. aquifolium</i>	4,7333	,18356	3
	<i>S. ilicifolium</i>	3,5849	,05365	3
	<i>S. polycystum</i>	4,0328	,43502	3
	Total	4,1170	,55472	9
Total	<i>S. aquifolium</i>	4,4433	,33836	6
	<i>S. ilicifolium</i>	4,2801	,78082	6
	<i>S. polycystum</i>	5,0540	1,15515	6
	Total	4,5925	,85026	18

Levene's Test of Equality of Error Variances^a

Dependent Variable: TPC

F	df1	df2	Sig.
6,970	5	12	,003

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Enzyme + Algae + Enzyme *

Algae

Tests of Between-Subjects Effects

Dependent Variable: TPC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11,659 ^a	5	2,332	44,355	,000
Intercept	379,635	1	379,635	7221,242	,000
Enzyme	4,069	1	4,069	77,397	,000
Algae	1,997	2	,999	18,995	,000
Enzyme * Algae	5,593	2	2,796	53,193	,000
Error	,631	12	,053		
Total	391,924	18			
Corrected Total	12,290	17			

a. R Squared = ,949 (Adjusted R Squared = ,927)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: TPC

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
4,592	,054	4,475	4,710

2. Enzyme

Dependent Variable: TPC

Enzyme	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Protamex	5,068	,076	4,901	5,234
Viscozyme	4,117	,076	3,951	4,284

3. Algae

Dependent Variable: TPC

Algae	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	4,443	,094	4,239	4,647
<i>S. ilicifolium</i>	4,280	,094	4,076	4,484
<i>S. polycystum</i>	5,054	,094	4,850	5,258

Post Hoc Tests

Algae

Multiple Comparisons

Dependent Variable: TPC

Tukey HSD

(I) Algae	(J) Algae	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	,1632	,13238	,458	-,1900	,5163
	<i>S. polycystum</i>	-,6108*	,13238	,002	-,9639	-,2576
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	-,1632	,13238	,458	-,5163	,1900
	<i>S. polycystum</i>	-,7739*	,13238	,000	-1,1271	-,4208
<i>S. polycystum</i>	<i>S. aquifolium</i>	,6108*	,13238	,002	,2576	,9639
	<i>S. ilicifolium</i>	,7739*	,13238	,000	,4208	1,1271

Based on observed means.

The error term is Mean Square(Error) = ,053.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

TPC

Tukey HSD^{a,b}

Algae	N	Subset	
		1	2
<i>S. ilicifolium</i>	6	4,2801	5,0540
<i>S. aquifolium</i>	6	4,4433	
<i>S. polycystum</i>	6		
Sig.		,458	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = ,053.

a. Uses Harmonic Mean Sample Size = 6,000.

b. Alpha = ,05.

Appendix 11 Analysis of variance of Indonesian *Sargassum* polyphenol – antioxidant of solid-liquid and enzyme-assisted extraction

Tests of Between-Subjects Effects

Dependent Variable: DPPH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2558046971,177 ^a	17	150473351,246	2,677	,006
Intercept	1967350126,996	1	1967350126,996	34,994	,000
Sample	539919294,348	2	269959647,174	4,802	,014
Solvents	1037452000,309	5	207490400,062	3,691	,008
Sample * Methods	980675676,520	10	98067567,652	1,744	,108
Error	2023883344,430	36	56218981,790		
Total	6549280442,604	54			
Corrected Total	4581930315,608	53			

a. R Squared = ,558 (Adjusted R Squared = ,350)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: DPPH

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
6035,927	1020,339	3966,583	8105,271

2. Sample

Dependent Variable: DPPH

Sample	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	2246,843	1767,279	-1337,366	5831,052
<i>S. ilicifolium</i>	9987,139	1767,279	6402,930	13571,348
<i>S. polycystum</i>	5873,798	1767,279	2289,589	9458,007

3. Methods

Dependent Variable: DPPH

Solvents	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
EtOH 75%	5125,606	2499,311	56,769	10194,443

Methanol	1132,185	2499,311	-3936,651	6201,022
Methanol 50 %	13520,793	2499,311	8451,956	18589,630
Protamex	10196,928	2499,311	5128,091	15265,765
Viscozyme	3285,777	2499,311	-1783,060	8354,614
Water	2954,270	2499,311	-2114,567	8023,107

4. Sample * Methods

Dependent Variable: DPPH

Sample	Solvents	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<i>S. aquifolium</i>	EtOH 75%	,000	4328,933	-8779,483	8779,483
	Methanol	-9,095E-13	4328,933	-8779,483	8779,483
	Methanol 50 %	3202,615	4328,933	-5576,868	11982,098
	Protamex	1415,633	4328,933	-7363,850	10195,116
	Viscozyme	,000	4328,933	-8779,483	8779,483
	Water	8862,810	4328,933	83,327	17642,293
<i>S. ilicifolium</i>	EtOH 75%	9872,120	4328,933	1092,637	18651,603
	Methanol	3396,556	4328,933	-5382,927	12176,039
	Methanol 50 %	17080,624	4328,933	8301,141	25860,107
	Protamex	19716,200	4328,933	10936,717	28495,683
	Viscozyme	9857,332	4328,933	1077,849	18636,815
	Water	5,457E-12	4328,933	-8779,483	8779,483
<i>S. polycystum</i>	EtOH 75%	5504,697	4328,933	-3274,786	14284,180
	Methanol	5,002E-12	4328,933	-8779,483	8779,483
	Methanol 50 %	20279,139	4328,933	11499,656	29058,622
	Protamex	9458,951	4328,933	679,468	18238,434
	Viscozyme	4,547E-12	4328,933	-8779,483	8779,483
	Water	-9,095E-13	4328,933	-8779,483	8779,483

Post Hoc Tests

Sample

Multiple Comparisons

Dependent Variable: DPPH

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	-7740,2956*	2499,31061	,010	-13849,3533	-1631,2379
	<i>S. polycystum</i>	-3626,9548	2499,31061	,326	-9736,0124	2482,1029
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	7740,2956*	2499,31061	,010	1631,2379	13849,3533
	<i>S. polycystum</i>	4113,3408	2499,31061	,240	-1995,7169	10222,3985
<i>S. polycystum</i>	<i>S. aquifolium</i>	3626,9548	2499,31061	,326	-2482,1029	9736,0124
	<i>S. ilicifolium</i>	-4113,3408	2499,31061	,240	-10222,3985	1995,7169

Based on observed means.

The error term is Mean Square(Error) = 56218981,790.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

DPPH

Tukey HSD^{a,b}

Sample	N	Subset	
		1	2
<i>S. aquifolium</i>	18	2246,8431	
<i>S. polycystum</i>	18	5873,7979	5873,7979
<i>S. ilicifolium</i>	18		9987,1387
Sig.		,326	,240

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 56218981,790.

a. Uses Harmonic Mean Sample Size = 18,000.

b. Alpha = ,05.

Methods

Multiple Comparisons

Dependent Variable: DPPH

Tukey HSD

(I) Solvents	(J) Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtOH 75%	Methanol	3993,4204	3534,55896	,866	-6640,5643	14627,4051
	Methanol 50 %	-8395,1870	3534,55896	,192	-19029,1717	2238,7977
	Protamex	-5071,3223	3534,55896	,706	-15705,3070	5562,6624
	Viscozyme	1839,8285	3534,55896	,995	-8794,1562	12473,8132
	Water	2171,3357	3534,55896	,989	-8462,6490	12805,3204
Methanol	EtOH 75%	-3993,4204	3534,55896	,866	-14627,4051	6640,5643
	Methanol 50 %	-12388,6073*	3534,55896	,015	-23022,5920	-1754,6226
	Protamex	-9064,7427	3534,55896	,133	-19698,7274	1569,2420
	Viscozyme	-2153,5918	3534,55896	,990	-12787,5765	8480,3929
	Water	-1822,0847	3534,55896	,995	-12456,0694	8811,9000
Methanol 50 %	EtOH 75%	8395,1870	3534,55896	,192	-2238,7977	19029,1717
	Methanol	12388,6073*	3534,55896	,015	1754,6226	23022,5920
	Protamex	3323,8647	3534,55896	,933	-7310,1200	13957,8494
	Viscozyme	10235,0155	3534,55896	,065	-398,9692	20869,0002
	Water	10566,5227	3534,55896	,052	-67,4620	21200,5074
Protamex	EtOH 75%	5071,3223	3534,55896	,706	-5562,6624	15705,3070
	Methanol	9064,7427	3534,55896	,133	-1569,2420	19698,7274
	Methanol 50 %	-3323,8647	3534,55896	,933	-13957,8494	7310,1200
	Viscozyme	6911,1509	3534,55896	,387	-3722,8338	17545,1356
	Water	7242,6580	3534,55896	,336	-3391,3267	17876,6427
Viscozyme	EtOH 75%	-1839,8285	3534,55896	,995	-12473,8132	8794,1562
	Methanol	2153,5918	3534,55896	,990	-8480,3929	12787,5765
	Methanol 50 %	-10235,0155	3534,55896	,065	-20869,0002	398,9692
	Protamex	-6911,1509	3534,55896	,387	-17545,1356	3722,8338
	Water	331,5071	3534,55896	1,000	-10302,4776	10965,4918
Water	EtOH 75%	-2171,3357	3534,55896	,989	-12805,3204	8462,6490
	Methanol	1822,0847	3534,55896	,995	-8811,9000	12456,0694
	Methanol 50 %	-10566,5227	3534,55896	,052	-21200,5074	67,4620
	Protamex	-7242,6580	3534,55896	,336	-17876,6427	3391,3267
	Viscozyme	-331,5071	3534,55896	1,000	-10965,4918	10302,4776

Based on observed means.

The error term is Mean Square(Error) = 56218981,790.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

DPPH

Tukey HSD^{a,b}

Solvents	N	Subset	
		1	2
Methanol	9	1132,1855	
Water	9	2954,2701	2954,2701
Viscozyme	9	3285,7773	3285,7773
EtOH 75%	9	5125,6058	5125,6058
Protamex	9	10196,9281	10196,9281
Methanol 50 %	9		13520,7928
Sig.		,133	,052

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 56218981,790.

a. Uses Harmonic Mean Sample Size = 9,000.

b. Alpha = ,05.

FRAP

Descriptive Statistics

Dependent Variable: FRAP

Sample	Solvents	Mean	Std. Deviation	N
<i>S. aquifolium</i>	EtOH 75%	14,4353	,29421	3
	Methanol	12,6588	2,54162	3
	Methanol 50 %	22,4471	4,92682	3
	Protamex	16,1725	,24924	3
	Viscozyme	33,1961	2,46124	3
	Water	21,8089	1,72733	3
	Total	20,1198	7,38959	18
<i>S. ilicifolium</i>	EtOH 75%	10,1375	1,24743	3
	Methanol	11,4638	,79286	3
	Methanol 50 %	16,0303	,75736	3
	Protamex	8,6480	,56784	3
	Viscozyme	15,2844	,98845	3
	Water	24,3450	1,58457	3
	Total	14,3182	5,42148	18
<i>S. polycystum</i>	EtOH 75%	13,7451	1,97886	3
	Methanol	12,7451	3,07423	3
	Methanol 50 %	16,9137	2,60771	3
	Protamex	16,3030	,11792	3
	Viscozyme	27,6863	4,90511	3
	Water	46,4706	5,31729	3
	Total	22,3106	12,53955	18
Total	EtOH 75%	12,7726	2,32053	9
	Methanol	12,2893	2,12591	9
	Methanol 50 %	18,4637	4,12110	9
	Protamex	13,7078	3,80838	9
	Viscozyme	25,3889	8,42009	9
	Water	30,8748	12,10221	9
	Total	18,9162	9,43207	54

Tests of Between-Subjects Effects

Dependent Variable: FRAP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Extraction assistée par enzyme de phlorotannins provenant d'algues brunes du genre <i>Sargassum</i> et les activités biologiques Maria Puspa 2017					

Levene's Test of Equality of Error Variances^a

Dependent Variable: FRAP

F	df1	df2	Sig.
3,348	17	36	,001

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Sample + Solvents + Sample

* Solvents

Tests of Between-Subjects Effects

Dependent Variable: FRAP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4477,074 ^a	17	263,357	39,833	,000
Intercept	19322,399	1	19322,399	2922,554	,000
Sample	614,026	2	307,013	46,436	,000
Solvents	2645,063	5	529,013	80,014	,000
Sample * Solvents	1217,985	10	121,799	18,422	,000
Error	238,013	36	6,611		
Total	24037,486	54			
Corrected Total	4715,088	53			

a. R Squared = ,950 (Adjusted R Squared = ,926)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: FRAP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
18,916	,350	18,207	19,626

2. Sample

Dependent Variable: FRAP

Sample	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>S. aquifolium</i>	20,120	,606	18,891	21,349
<i>S. ilicifolium</i>	14,318	,606	13,089	15,547
<i>S. polycystum</i>	22,311	,606	21,081	23,540

3. Methods

Dependent Variable: FRAP

Solvents	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
EtOH 75%	12,773	,857	11,034	14,511
Methanol	12,289	,857	10,551	14,028
Methanol 50 %	18,464	,857	16,725	20,202
Protamex	13,708	,857	11,970	15,446
Viscozyme	25,389	,857	23,651	27,127
Water	30,875	,857	29,137	32,613

4. Sample * Methods

Dependent Variable: FRAP

Sample	Methods	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<i>S. aquifolium</i>	EtOH 75%	14,435	1,485	11,425	17,446
	Methanol	12,659	1,485	9,648	15,670
	Methanol 50 %	22,447	1,485	19,436	25,458
	Protamex	16,173	1,485	13,162	19,183
	Viscozyme	33,196	1,485	30,185	36,207
	Water	21,809	1,485	18,798	24,820
<i>S. ilicifolium</i>	EtOH 75%	10,138	1,485	7,127	13,148
	Methanol	11,464	1,485	8,453	14,475
	Methanol 50 %	16,030	1,485	13,020	19,041
	Protamex	8,648	1,485	5,637	11,659
	Viscozyme	15,284	1,485	12,274	18,295
	Water	24,345	1,485	21,334	27,356
<i>S. polycystum</i>	EtOH 75%	13,745	1,485	10,734	16,756
	Methanol	12,745	1,485	9,734	15,756
	Methanol 50 %	16,914	1,485	13,903	19,924
	Protamex	16,303	1,485	13,292	19,314
	Viscozyme	27,686	1,485	24,676	30,697
	Water	46,471	1,485	43,460	49,481

Post Hoc Tests Sample

Multiple Comparisons

Dependent Variable: FRAP

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	5,8016*	,85709	,000	3,7066	7,8966
	<i>S. polycystum</i>	-2,1909*	,85709	,039	-4,2859	-,0959
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	-5,8016*	,85709	,000	-7,8966	-3,7066
	<i>S. polycystum</i>	-7,9925*	,85709	,000	-10,0874	-5,8975
<i>S. polycystum</i>	<i>S. aquifolium</i>	2,1909*	,85709	,039	,0959	4,2859
	<i>S. ilicifolium</i>	7,9925*	,85709	,000	5,8975	10,0874

Based on observed means.

The error term is Mean Square(Error) = 6,611.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

FRAP

Tukey HSD^{a,b}

Sample	N	Subset		
		1	2	3
<i>S. ilicifolium</i>	18	14,3182		
<i>S. aquifolium</i>	18		20,1198	
<i>S. polycystum</i>	18			22,3106
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 6,611.

a. Uses Harmonic Mean Sample Size = 18,000.

b. Alpha = ,05.

Methods

Multiple Comparisons

Dependent Variable: FRAP

Tukey HSD

(I) Solvents	(J) Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EtOH 75%	Methanol	,4834	1,21211	,999	-3,1634	4,1301
	Methanol 50 %	-5,6911 [*]	1,21211	,001	-9,3378	-2,0443
	Protamex	-,9352	1,21211	,971	-4,5820	2,7115
	Viscozyme	-12,6163 [*]	1,21211	,000	-16,2630	-8,9695
	Water	-18,1022 [*]	1,21211	,000	-21,7489	-14,4555
Methanol	EtOH 75%	-,4834	1,21211	,999	-4,1301	3,1634
	Methanol 50 %	-6,1744 [*]	1,21211	,000	-9,8212	-2,5277
	Protamex	-1,4186	1,21211	,848	-5,0653	2,2281
	Viscozyme	-13,0996 [*]	1,21211	,000	-16,7464	-9,4529
	Water	-18,5856 [*]	1,21211	,000	-22,2323	-14,9388
Methanol 50 %	EtOH 75%	5,6911 [*]	1,21211	,001	2,0443	9,3378
	Methanol	6,1744 [*]	1,21211	,000	2,5277	9,8212
	Protamex	4,7559 [*]	1,21211	,005	1,1091	8,4026
	Viscozyme	-6,9252 [*]	1,21211	,000	-10,5719	-3,2785
	Water	-12,4111 [*]	1,21211	,000	-16,0578	-8,7644
Protamex	EtOH 75%	,9352	1,21211	,971	-2,7115	4,5820
	Methanol	1,4186	1,21211	,848	-2,2281	5,0653
	Methanol 50 %	-4,7559 [*]	1,21211	,005	-8,4026	-1,1091
	Viscozyme	-11,6811 [*]	1,21211	,000	-15,3278	-8,0343
	Water	-17,1670 [*]	1,21211	,000	-20,8137	-13,5202
Viscozyme	EtOH 75%	12,6163 [*]	1,21211	,000	8,9695	16,2630
	Methanol	13,0996 [*]	1,21211	,000	9,4529	16,7464
	Methanol 50 %	6,9252 [*]	1,21211	,000	3,2785	10,5719
	Protamex	11,6811 [*]	1,21211	,000	8,0343	15,3278
	Water	-5,4859 [*]	1,21211	,001	-9,1326	-1,8392
Water	EtOH 75%	18,1022 [*]	1,21211	,000	14,4555	21,7489
	Methanol	18,5856 [*]	1,21211	,000	14,9388	22,2323
	Methanol 50 %	12,4111 [*]	1,21211	,000	8,7644	16,0578
	Protamex	17,1670 [*]	1,21211	,000	13,5202	20,8137
	Viscozyme	5,4859 [*]	1,21211	,001	1,8392	9,1326

Based on observed means.

The error term is Mean Square(Error) = 6,611.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

FRAP

Tukey HSD^{a,b}

Methods	N	Subset			
		1	2	3	4
Methanol	9	12,2893	18,4637	25,3889	30,8748
EtOH 75%	9	12,7726			
Protamex	9	13,7078			
Methanol 50 %	9				
Viscozyme	9				
Water	9				
Sig.		,848	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 6,611.

a. Uses Harmonic Mean Sample Size = 9,000.

b. Alpha = ,05.

Appendix 12 Analysis of variance of Indonesia *Sargassum* polyphenol – biofilm inhibition activity of enzyme-assisted extraction

Descriptive Statistics

Dependent Variable: Antibiofilm

Bacteria	Species	EAE	Mean	Std. Deviation	N
<i>Bacillus subtilis</i>	<i>S. aquifolium</i>	Protamex	44,4620	11,00078	3
		Viscozym	42,6692	9,59290	3
		Total	43,5656	9,28335	6
	<i>S. ilicifolium</i>	Protamex	48,9907	6,81602	3
		Viscozym	25,2466	14,84923	3
		Total	37,1187	16,61076	6
	<i>S. polycystum</i>	Protamex	40,7081	13,48363	3
		Viscozym	36,8840	12,85987	3
		Total	38,7960	11,96917	6
	Total	Protamex	44,7203	10,01106	9
		Viscozym	34,9333	13,36151	9
		Total	39,8268	12,51127	18
<i>Escherichia coli</i>	<i>S. aquifolium</i>	Protamex	9,1169	5,30387	3
		Viscozym	26,3226	10,98586	3
		Total	17,7198	12,17942	6
	<i>S. ilicifolium</i>	Protamex	18,1880	3,14442	3
		Viscozym	19,9577	11,81075	3
		Total	19,0729	7,79051	6
	<i>S. polycystum</i>	Protamex	45,8097	3,80399	3
		Viscozym	14,4961	4,36150	3
		Total	30,1529	17,53736	6
	Total	Protamex	24,3716	16,94320	9
		Viscozym	20,2588	9,80190	9
		Total	22,3152	13,59349	18

<i>Pseudomonas aeruginosa</i>	<i>S. aquifolium</i>	Protamex	16,3049	4,47868	3
		Viscozym	11,6177	2,30003	3
		Total	13,9613	4,09029	6
	<i>S. ilicifolium</i>	Protamex	9,9637	4,75706	3
		Viscozym	9,0844	2,98026	3
		Total	9,5240	3,58282	6
	<i>S. polycystum</i>	Protamex	12,0483	2,65659	3
		Viscozym	11,4821	6,07117	3
		Total	11,7652	4,20272	6
	Total	Protamex	12,7723	4,50231	9
		Viscozym	10,7280	3,77902	9
		Total	11,7502	4,16723	18
Total	<i>S. aquifolium</i>	Protamex	23,2946	17,43621	9
		Viscozym	26,8698	15,34457	9
		Total	25,0822	16,03918	18
	<i>S. ilicifolium</i>	Protamex	25,7141	18,36269	9
		Viscozym	18,0962	11,96439	9
		Total	21,9052	15,53710	18
	<i>S. polycystum</i>	Protamex	32,8554	17,29853	9
		Viscozym	20,9541	14,13361	9
		Total	26,9047	16,50199	18
	Total	Protamex	27,2880	17,50645	27
		Viscozym	21,9734	13,84989	27
		Total	24,6307	15,86321	54

Levene's Test of Equality of Error Variances^a

Dependent Variable: Antibiofilm

F	df1	df2	Sig.
2,118	17	36	,029

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Bacteria + Species + EAE + Bacteria * Species + Bacteria * EAE + Species * EAE + Bacteria * Species * EAE

Tests of Between-Subjects Effects

Dependent Variable: Antibiofilm

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10817,682 ^a	17	636,334	9,093	,000
Intercept	32760,262	1	32760,262	468,132	,000
Bacteria	7239,420	2	3619,710	51,724	,000
Species	230,461	2	115,231	1,647	,207
EAE	381,315	1	381,315	5,449	,025
Bacteria * Species	521,218	4	130,305	1,862	,138
Bacteria * EAE	144,639	2	72,320	1,033	,366
Species * EAE	574,733	2	287,367	4,106	,025
Bacteria * Species * EAE	1725,894	4	431,474	6,166	,001
Error	2519,309	36	69,981		
Total	46097,253	54			
Corrected Total	13336,991	53			

a. R Squared = ,811 (Adjusted R Squared = ,722)

Estimated Marginal Means

1. Grand Mean

Dependent Variable: Antibiofilm

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
24,631	1,138	22,322	26,939

2. Bacteria

Dependent Variable: Antibiofilm

Bacteria	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
<i>Bacillus subtilis</i>	39,827	1,972	35,828	43,826
<i>Escherichia coli</i>	22,315	1,972	18,316	26,314
<i>Pseudomonas aeruginosa</i>	11,750	1,972	7,751	15,749

3. Species

Dependent Variable: Antibiofilm

Species	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound

<i>S. aquifolium</i>	25,082	1,972	21,083	29,081
<i>S. ilicifolium</i>	21,905	1,972	17,906	25,904
<i>S. polycystum</i>	26,905	1,972	22,906	30,904

4. EAE

Dependent Variable: Antibiofilm

EAE	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Protamex	27,288	1,610	24,023	30,553
Viscozym	21,973	1,610	18,708	25,238

5. Bacteria * Species

Dependent Variable: Antibiofilm

Bacteria	Species	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<i>Bacillus subtilis</i>	<i>S. aquifolium</i>	43,566	3,415	36,639	50,492
	<i>S. ilicifolium</i>	37,119	3,415	30,192	44,045
	<i>S. polycystum</i>	38,796	3,415	31,870	45,722
<i>Escherichia coli</i>	<i>S. aquifolium</i>	17,720	3,415	10,793	24,646
	<i>S. ilicifolium</i>	19,073	3,415	12,147	25,999
	<i>S. polycystum</i>	30,153	3,415	23,227	37,079
<i>Pseudomonas aeruginosa</i>	<i>S. aquifolium</i>	13,961	3,415	7,035	20,888
	<i>S. ilicifolium</i>	9,524	3,415	2,598	16,450
	<i>S. polycystum</i>	11,765	3,415	4,839	18,692

6. Bacteria * EAE

Dependent Variable: Antibiofilm

Bacteria	EAE	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<i>Bacillus subtilis</i>	Protamex	44,720	2,788	39,065	50,376
	Viscozym	34,933	2,788	29,278	40,589
<i>Escherichia coli</i>	Protamex	24,372	2,788	18,716	30,027
	Viscozym	20,259	2,788	14,604	25,914
<i>Pseudomonas aeruginosa</i>	Protamex	12,772	2,788	7,117	18,428
	Viscozym	10,728	2,788	5,073	16,383

7. Species * EAE

Dependent Variable: Antibiofilm

Species	EAE	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<i>S. aquifolium</i>	Protamex	23,295	2,788	17,639	28,950
	Viscozym	26,870	2,788	21,215	32,525
<i>S. ilicifolium</i>	Protamex	25,714	2,788	20,059	31,369
	Viscozym	18,096	2,788	12,441	23,752
<i>S. polycystum</i>	Protamex	32,855	2,788	27,200	38,511
	Viscozym	20,954	2,788	15,299	26,609

8. Bacteria * Species * EAE

Dependent Variable: Antibiofilm

Bacteria	Species	EAE	Mean	Std. Error	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>Bacillus subtilis</i>	<i>S. aquifolium</i>	Protamex	44,462	4,830	34,667	54,257
		Viscozym	42,669	4,830	32,874	52,464
	<i>S. ilicifolium</i>	Protamex	48,991	4,830	39,195	58,786
		Viscozym	25,247	4,830	15,451	35,042
	<i>S. polycystum</i>	Protamex	40,708	4,830	30,913	50,503
		Viscozym	36,884	4,830	27,089	46,679
<i>Escherichia coli</i>	<i>S. aquifolium</i>	Protamex	9,117	4,830	-,678	18,912
		Viscozym	26,323	4,830	16,527	36,118
	<i>S. ilicifolium</i>	Protamex	18,188	4,830	8,393	27,983
		Viscozym	19,958	4,830	10,162	29,753
	<i>S. polycystum</i>	Protamex	45,810	4,830	36,014	55,605
		Viscozym	14,496	4,830	4,701	24,291
<i>Pseudomonas aerugino</i>	<i>S. aquifolium</i>	Protamex	16,305	4,830	6,510	26,100
		Viscozym	11,618	4,830	1,822	21,413
	<i>S. ilicifolium</i>	Protamex	9,964	4,830	,168	19,759
		Viscozym	9,084	4,830	-,711	18,880
	<i>S. polycystum</i>	Protamex	12,048	4,830	2,253	21,844
		Viscozym	11,482	4,830	1,687	21,277

Post Hoc Tests Bacteria

Multiple Comparisons

Dependent Variable: Antibiofilm

Tukey HSD

(I) Bacteria	(J) Bacteria	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	17,5116 [*]	2,78848	,000	10,6957	24,3275
	<i>Pseudomonas aeruginosa</i>	28,0766 [*]	2,78848	,000	21,2607	34,8925
<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	-17,5116 [*]	2,78848	,000	-24,3275	-10,6957
	<i>Pseudomonas aeruginosa</i>	10,5650 [*]	2,78848	,002	3,7491	17,3809
<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	-28,0766 [*]	2,78848	,000	-34,8925	-21,2607
	<i>Escherichia coli</i>	-10,5650 [*]	2,78848	,002	-17,3809	-3,7491

Based on observed means.

The error term is Mean Square(Error) = 69,981.

*. The mean difference is significant at the ,05 level.

Homogeneous Subsets

Antibiofilm

Tukey HSD^{a,b}

Bacteria	N	Subset		
		1	2	3
<i>Pseudomonas aeruginosa</i>	18	11,7502		
<i>Escherichia coli</i>	18		22,3152	
<i>Bacillus subtilis</i>	18			39,8268
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 69,981.

a. Uses Harmonic Mean Sample Size = 18,000.

b. Alpha = ,05.

Species

Multiple Comparisons

Dependent Variable: Antibiofilm

Tukey HSD

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>S. aquifolium</i>	<i>S. ilicifolium</i>	3,1770	2,78848	,497	-3,6389	9,9929
	<i>S. polycystum</i>	-1,8225	2,78848	,792	-8,6384	4,9934
<i>S. ilicifolium</i>	<i>S. aquifolium</i>	-3,1770	2,78848	,497	-9,9929	3,6389
	<i>S. polycystum</i>	-4,9995	2,78848	,186	-11,8154	1,8164
<i>S. polycystum</i>	<i>S. aquifolium</i>	1,8225	2,78848	,792	-4,9934	8,6384
	<i>S. ilicifolium</i>	4,9995	2,78848	,186	-1,8164	11,8154

Based on observed means.

The error term is Mean Square(Error) = 69,981.

Homogeneous Subsets

Antibiofilm

Tukey HSD^{a,b}

Species	N	Subset
		1
<i>S. ilicifolium</i>	18	21,9052
<i>S. aquifolium</i>	18	25,0822
<i>S. polycystum</i>	18	26,9047
Sig.		,186

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 69,981.

a. Uses Harmonic Mean Sample Size = 18,000.

b. Alpha = ,05.