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« *Food Technology and Nutrition* »

Par

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« Etude de l'extrusion couplée à l'hydrolyse enzymatique pour l'extraction des composés hydrosolubles de l'algue rouge "Gracilaria gracilis" »

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Résumé de la thèse en français

La population mondiale atteindra 10 milliards de personnes en 2050, ce qui nécessitera d'augmenter de 70 % la production alimentaire (Bleakley and Hayes, 2017; United Nations, 2019). De ce fait, d'autres ressources naturelles ont été proposées afin de répondre à cette croissance rapide. La richesse et les nombreux d'avantages de l'écosystème marin favorisent le développement de divers organismes marins pour la consommation humaine (Pereira, 2018). Actuellement seulement 6.5 % de la consommation mondiale de protéines provient de l'océan avec majoritairement la consommations de poissons et de crustacés alors que les algues et d'autres espèces marines pourraient également constituer une source de protéines intéressantes pour l'alimentation humaine (Mæhre et al., 2016).

Les macroalgues marines sont principalement repérées sur le littoral océanique (Cian et al., 2015). Elles sont tolérantes au sel du fait de leur capacité à ajuster l'osmolarité entre leur cytoplasme et l'eau de mer (De Almeida et al., 2011). Leur métabolisme photosynthétique leur permet de produire des nutriments et de l'oxygène à partir de dioxyde de carbone, de pigments photosynthétiques et d'énergie solaire (De Almeida et al., 2011). Les macroalgues sont des ressources alimentaires incontournables, présentant un faible niveau en calories et riches en vitamines, minéraux, protéines, polysaccharides, stéroïdes et fibres (Circuncisão et al., 2018; De Almeida et al., 2011; O'Sullivan et al., 2010). L'introduction des macroalgues marines dans l'alimentation humaine date, en Chine, de 2700 avant JC (Cian et al., 2015). On constate une forte consommation en Asie tandis que dans les pays occidentaux, la consommation reste limitée en raison de la barrière culturelle et des habitudes alimentaires (Cian et al., 2015; Mæhre et al., 2016). À l'échelle mondiale, la production de macroalgues est en perpétuelle augmentation avec une production de 25 millions de tonnes en 2012 (Mæhre et al., 2016), passant 30.4 millions de tonnes en 2015 (Fao, 2018), pour atteindre 32.4 millions de tonnes en 2018 (Fao, 2020). La plus grande partie de la production mondiale de macroalgues est directement utilisée pour la consommation humaine (Radulovich et al., 2015) et les principaux producteurs sont le Chili, la Chine, la Norvège, l'Indonésie, la République de Corée et les Philippines (Fao, 2018).

Les macroalgues ont été étudiées du fait de leurs teneurs importantes en composés bioactifs. La teneur en protéines de certaines espèces de macroalgues est proche de celle d'autres sources de protéine telles que la viande, les œufs, le soja et le lait (Bleakley and Hayes, 2017). Les macroalgues rouges, l'une des trois classes principales de macroalgues, sont connues pour avoir

une teneur élevée en protéines et en polysaccharides, comparable à celle des ressources terrestres (Gamero-Vega et al., 2020; Rioux et al., 2017). Les hydrocolloïdes dérivés de macroalgues (agar, carraghénane et alginate) sont les molécules les plus exploitées des macroalgues marines pour des applications dans l'industrie alimentaire, pharmaceutique, médicale ainsi que dans le domaine des biotechnologies (Rhein-Knudsen et al., 2015; Xiao et al., 2019). L'extraction traditionnelle du carraghénane est réalisée par le chauffage avec une solution alcaline d'hydroxyde de potassium. Pour l'extraction de l'agar, d'autres procédés comme un traitement alcalin suivi d'une extraction à l'eau chaude sont effectués (Rhein-Knudsen et al., 2015; Rioux and Turgeon, 2015).

Les protéines, quant à elles, sont considérées comme les produits dérivés associés à l'extraction des polysaccharides. La plupart des polysaccharides des macroalgues sont localisés dans la paroi cellulaire (Rioux et al., 2017), qui est connue comme étant la principale barrière à l'extraction des protéines (certaines protéines et polysaccharides se retrouvent piégés dans la matrice extracellulaire) (Mæhre et al., 2016). La dégradation de la paroi cellulaire est donc un moyen de libérer les protéines intracellulaires afin de les utiliser dans des applications diverses (Mæhre et al., 2016).

Enfin, les pigments des macroalgues sont également exploités. La R-phycoérythrine (R-PE), un pigment dominant dans les macroalgues rouges, est une molécule à haute valeur ajoutée (Dumay et al., 2014). Elle peut servir dans l'alimentation et les cosmétiques comme colorant naturel (Francavilla et al., 2013b; Rioux et al., 2017). L'extraction de ce pigment se fait par un simple trempage des macroalgues dans l'eau pendant plusieurs jours, néanmoins cette démarche entraîne une certaine dégradation de la molécule d'intérêt du fait de l'activité des protéases (Dumay et al., 2014).

Ainsi, une méthode d'extraction alternative de ces composés d'intérêt, par rapport aux procédés chimiques et mécaniques, est l'extraction assistée par enzymes (EAE). L'EAE est considérée comme une technique écologique et non toxique, où les solvants organiques et les produits chimiques toxiques sont évités afin de répondre à la réglementation de l'industrie agroalimentaire (Michalak et al., 2017). Cette méthode d'extraction modérée induit une liquéfaction des parois cellulaires, du fait de l'activité enzymatique, provoquant ainsi la libération en plus grande quantité des composés confinés et inaccessibles (Dumay et al., 2014; Sudhakar et al., 2015). Les enzymes ont la capacité de ramollir la paroi cellulaire et de réduire la dégradation des composés cibles (Xiao et al., 2019). Néanmoins, le coût élevé de certaines

enzymes reste le principal facteur limitant leur utilisation (Michalak et al., 2017; Xiao et al., 2019).

Plusieurs études ont montré des résultats prometteurs de l'extraction assistée par enzymes. Une étude menée sur l'extraction du carraghénane hybride kappa/iota de la macroalgue *Mastocarpus stellatus* en utilisant l'enzyme alcalase (protéase commerciale) a mis en évidence des propriétés gélifiantes intéressantes et une amélioration de l'extraction des composés polyphénoliques (Blanco-Pascual et al., 2014). L'extraction du carraghénane iota de la macroalgue *Soliera filiformis* par l'enzyme papaïne a permis l'extraction propre de ce composé, débarrassé des protéines contaminantes extraites lors de l'extraction par la méthode traditionnelle (De Araújo et al., 2012). Le rendement d'extraction du carraghénane le plus élevé a été obtenu avec la macroalgue *Eucheuma cottoni* avec l'utilisation de l'enzyme cellulase novozyme NS50013 par rapport à une extraction traditionnelle par ébullition (Varadarajan et al., 2009). Une combinaison d'enzyme, kappa-carraghénase et d'agarase avec cellulase a montré une amélioration de l'extraction des protéines de la macroalgue *Chondrus crispus* et de *Gacilaria verrucosa*. Avec cette technique d'extraction, les rendements ont été augmentés respectivement de 10 et 3 fois, par rapport aux résultats obtenus en utilisant la cellulase seule (Fleurence et al., 2002, 1995b). Une étude a démontré que les glucosidases commerciales et la protéase peuvent améliorer l'efficacité de l'extraction des matières bioactives de la macroalgue *Chondrus crispus* (Kulshreshtha et al., 2015). La technique EAE varie donc en fonction du type de biomasse algale utilisée et des composés cibles. Les conditions opératoires doivent être adaptées en tenant compte des propriétés des enzymes pour une optimisation de l'extraction.

L'extrusion est un procédé continu qui consiste à combiner plusieurs opérations tel que le mélange, le cisaillement, le chauffage, le refroidissement, la mise en forme, le séchage partiel, la formation ou la transformation d'un matériaux dans un système de vis ou de tunnel (Arora et al., 2020; Makoure et al., 2019a; Vauchel et al., 2008). Les principales opérations impliquées sont des traitements thermomécaniques (Baron et al., 2010). L'extrusion est utilisée depuis le milieu des années 1930 et est devenue l'une des meilleures méthodes de transformation alimentaire permettant d'obtenir différents types de produits tels que les aliments pour le petit-déjeuner, les céréales, les snacks prêts à consommer, etc. (Makoure et al., 2019b; Singh et al., 2018). L'extrusion réactive est un développement de l'extrusion simple à laquelle on ajoute une réaction chimique ou enzymatique. Cette technique consiste à utiliser les extrudeuses comme des réacteurs (bio)chimiques (Choulak, 2004). Ce développement a permis d'améliorer l'extraction simultanée de plusieurs composés d'intérêt. Le premier développement de

l'extrusion réactive date de 1980 avec la modification des polymères synthétiques. Plus tard, elle s'est rapidement répandue et appliquée dans divers domaines tels que la polymérisation, le greffage, la réticulation, etc. (Xie et al., 2006). La texturation par extrusion réactive et/ou enzymatique constitue ainsi une autre voie de valorisation des algues. L'extrusion réactive est une alternative intéressante au procédé de batch car elle permet de travailler en continu (Baron et al., 2010), de traiter des matériaux relativement visqueux (Gatt et al., 2018) et concentrés avec un contact étroit entre les réactifs et les réactants sur des temps relativement courts (inférieur à 5 mn), ce qui permet d'améliorer la purification des composés d'intérêts contenus dans les algues. Le concept d'extrusion enzymatique réactive a été proposé dans le but de généraliser l'utilisation simultanée d'enzyme et de l'extrusion (E. Xu et al., 2020). De plus, la modularité du procédé d'extrusion permet de l'adapter à diverses applications. En effet, le profil des vis peut être adapté en fonction des propriétés de la matière à traiter, telle que la viscosité et la présence de différentes phases (Gatt et al., 2018) et plusieurs zones peuvent être définies afin d'adapter des profils de vis différents le long de la vis (Baron et al., 2010). L'extrusion réactive a été principalement utilisée dans le domaine des polymères (Makoure et al., 2019a) ou de l'industrie agroalimentaire (Wolf, 2010). Plusieurs applications ont été développées avec des matières premières biologiques, principalement pour l'extraction de biomolécules ou pour la valorisation de sous-produits (Baron et al., 2010). L'intérêt d'utiliser l'extrudeuse comme bioréacteur ou réacteur chimique permet d'améliorer la qualité des extraits et de diminuer l'impact environnemental en minimisant l'utilisation de solvants et de réactifs chimiques (Vauchel et al., 2008).

Le genre *Gracilaria* compte plus de 300 espèces dont 160 ont été décrites sur le plan taxonomique. Ce genre est bien connu pour son utilisation industrielle et biotechnologique et présente un intérêt économique du fait de son rendement de production élevé (Francavilla et al., 2013a). La famille *Gracilaria* s'est répandue dans le monde entier et se retrouve aussi bien à des latitudes tropicales qu'à des latitudes tempérées (Iyer et al., 2004; P. Torres et al., 2019). Les *Gracilarias* contiennent une grande quantité de polysaccharides qui peut représenter jusqu'à 62-63 % du poids sec (Rioux et al., 2017). L'utilisation de *Gracilaria* a débuté par la production d'agar dans le but de remplacer le *Gelidium* (Armisen, 1995). Environ 60 % de l'agar est d'origine de la famille *Gracilaria* et les principaux producteurs se trouvent au Chili, en Chine, à Taiwan, et au Vietnam (Skriptsova and Nabivailo, 2009). L'espèce *Gracilaria gracilis* (*G. gracilis*) est une macroalgue rouge présentant également une teneur élevée en polysaccharides (Mensi, 2019; Rioux et al., 2017), en protéines et en R-phycoérythrine (pigment rouge) (Pimentel et al., 2019). Il est à noter que la paroi cellulaire des algues rouges

est importante et contient des polysaccharides (galactant sulfaté environ 70 %) et des protéines (Gajaria et al., 2017 ; Rioux et Turgeon, 2015 ; Rioux et al., 2017 ; Xiao et al., 2019). En France, *Gracilaria gracilis* est autorisée dans l'alimentation humaine (CEVA, 2019). Dans les pays d'Asie du Sud-Est, elle est consommée comme une source principale d'agar-agar qui est utilisé comme agent gélifiant et épaississant dans la préparation d'aliments (Chan and Matanjun, 2017; Souza et al., 2012a). L'agar a de nombreuses applications dans l'industrie agroalimentaire, pharmaceutique, cosmétique, médicale et dans le domaine des biotechnologies. Mais le procédé actuel d'extraction de l'agar consomme beaucoup de solvants. En ce sens l'utilisation de l'EAE et de la technique d'extrusion semble intéressante pour diminuer l'impact environnemental du procédé d'extraction de l'agar et pourraient permettre d'extraire d'autres galactants sulfatés (en plus de l'agar, l'oligoagar peut être extrait, la cellulose, etc). Enfin l'EAE et de la technique d'extrusion pourraient également améliorer les rendements d'extraction des pigments et des protéines des algues rouges.

Ce manuscrit développe les travaux de bio-raffinages de la macroalgue rouge *Gracilaria gracilis* par deux procédés d'extraction différents : l'extraction assistée par enzymes, réalisée en réacteur batch et par extrusion. L'objectif d'extraction d'optimiser l'extraction des produits solubles suivants : la R-phycoérythrine, les protéines et les sucres. Le premier chapitre aborde de manière détaillée les recherches bibliographiques sur l'algue rouge *Gracilaria gracilis* ainsi que sur les méthodes d'extraction et le plan d'expérience mis en œuvre dans cette thèse. Le deuxième chapitre présente en détail le matériel et les méthodes utilisés lors de cette thèse. Le troisième chapitre traite des résultats obtenus lors de l'extraction assistée par des enzymes en réacteur batch et dans le quatrième chapitre, est présenté les résultats obtenus lors de l'extrusion couplée à une hydrolyse enzymatique de *Gracilaria Gracilis*.

En réacteur batch, les biomasses *G.gracilis* lyophilisées (BL) et fraîche/humide (AFM) sont broyées avant l'extraction assistée par enzymes. L'extraction a eu lieu dans un tampon acétate (50 mM) pendant 286 min à l'obscurité. La méthode utilisée s'inspire des travaux de Nguyen (2017). Trois enzymes ont été testées : cellulase, protéase et un cocktail d'enzyme combinant la protéase et la cellulase. Les proportions en enzymes suivantes ont été appliquées : 47 mg/g ms (E1), 70.5 mg/g ms (E1.5) et 94 mg/g ms (E2) (masse d'enzyme par masse d'algue sèche) avec une incubation à 32 °C. Le cocktail d'enzyme, quant à lui, a été testé avec deux concentrations (94 et 47 mg/g ms) et deux pH différents (5.0 et 7.5) pour la solution tampon en fonction des activités catalytiques de chaque enzyme. Après les étapes d'extraction, les

composés hydrosolubles cibles (R-phycoérythrine (R-PE), protéines et sucres) dans le surnagent sont dosés.

L'utilisation des glucosidases et des protéases dans l'extraction assistée par enzyme permet d'améliorer les rendements de libération des protéines, des sucres neutres, des sucres réducteurs et des polyphénols d'autres algues rouges telles que *Solieria chordalis*, *Palmaria palmata* et *Grateloupia turuturu* (Denis et al., 2009a; Hardouin et al., 2014; Kulshreshtha et al., 2015). Il a été montré que l'endo-peptidase donnait le plus grand rendement de protéines (15.20 %) sur *Solieria chordalis* plutôt que l'endo-protéase, la cellulase, la xylanase, la β -glucanase et l'arabanase (Hardouin et al., 2014). L'étude de Kulshreshtha et al., 2015 a montré que la Novozyme-cellulase permettait l'augmentation du rendement des protéines de l'algue rouge *C. crispus* (7.1 %) en comparaison avec les autres enzymes (β -glucanase, ultaflo, et neutrase). Selon l'étude de Nguyen et al. (2017), le rendement des protéines libérées dans des conditions optimales (cellulase 47 mg/g ms, 32 °C et 286 min) a été rapporté à 10.31 % (par rapport au contenu total) sous l'ajout de cellulase avec la biomasse lyophilisée de *G.gracilis*. En comparaison avec Nguyen et al. (2017), nos résultats ont montré que le rendement d'extraction des protéines a augmenté de trois et deux fois respectivement lors de l'utilisation du cocktail d'enzymes (32 %) et de la cellulase (24.18 %) sur la biomasse lyophilisée. Par rapport à la condition de contrôle (en absence d'enzyme), le rendement de libération des protéines en présence de cocktail d'enzymes augmente d'un facteur de 6.7 et d'un facteur 5 en présence de cellulase. Cette augmentation est supérieure à celle obtenue par Fleurence et al. (1995), qui est d'un facteur 3 dans le cas d'un mélange d'enzymes d'agarase et de cellulase sur la biomasse de *Gracilaria gracilis*. Ainsi, l'efficacité de la libération des protéines dépend du type d'enzyme et de l'espèce d'algue.

Les rendements d'extraction des protéines et des sucres (46 % de protéines par hydrolyse par les protéases et 85 % de sucre par hydrolyse par les cellulases) à partir de la biomasse humide sont plus importants en comparaison avec la biomasse sèche. Aussi, les mono-enzymes semblent mieux fonctionner pour l'EAE avec de la biomasse humide, tandis qu'un mélange d'enzymes semble être nécessaire pour traiter de la biomasse lyophilisée. La taille des substrats (surface spécifique) ainsi que la température, le pH, la concentration d'enzyme et le temps d'extraction sont connus comme des facteurs influençant le processus d'extraction (Nadar et al., 2018). La lyophilisation préalable est couramment utilisée pour faciliter le processus d'extraction sur de la biomasse algale (Denis et al., 2009b; Dumay et al., 2013; Munier et al.,

2015; Nguyen, 2017). Tandis que dans notre étude, l'utilisation de la biomasse humide permet également d'améliorer les rendements d'extraction.

Dans cette étude, la récupération des composés solubles augmente de 43 % pour les protéines (lors de l'utilisation de protéase) et de 57 % pour les sucres (lors de l'utilisation de cellulase) avec de la biomasse humide en comparant avec la biomasse lyophilisée (lors de l'utilisation du cocktail enzyme) et un broyage fin ramenant la taille à quelques millimètres. Le broyage de la biomasse humide dans notre étude apparaît plus efficace pour l'extraction que le broyage de la biomasse lyophilisée. Au vu de ces résultats, notre hypothèse pourrait suggérer que la dégradation de la paroi cellulaire de la biomasse humide était supérieure à celle de la biomasse lyophilisée et que le processus de séchage pourrait conduire à des barrières supplémentaires pour la libération.

La cellulase est connue pour catalyser les polysaccharides et donc générer la libération de petits oligosaccharides (Fernandes and Carvalho, 2017; Nadar et al., 2018). La protéase est une enzyme qui cible les liaisons peptidiques dans les protéines. En raison de leur activité catalytique différente, chaque enzyme fonctionne mieux dans des conditions de pH déterminées (5.0 pour la cellulase et 7.5 pour la protéase). Cependant, les conditions opératoires (pH et concentration d'enzyme) du cocktail d'enzymes à une température de fonctionnement fixe (32 °C) dépendent des contenus solubles (soit les protéines, soit les sucres).

Les résultats obtenus pour les deux types de biomasse (lyophilisée et humide) avec l'utilisation du cocktail d'enzyme ont montrés que la libération des protéines était plus importante à pH 7.5, tandis que celle des sucres était plus élevée à pH 5.0. Ce résultat montre que, la libération des protéines peut provenir d'une activité protéique élevée (pH 7.5) alors que la libération des sucres provient d'une activité de cellulase plus important à pH 5.0. Lorsque l'enzyme est utilisée à un pH optimal différent, il est logique que l'activité de l'enzyme soit réduite et moins efficace par rapport à l'activité utilisée dans les conditions de pH optimal. En ce qui concerne la concentration d'enzyme (cocktail d'enzyme), les deux biomasses (lyophilisée et humide) ont montré que sous un pH de 7.5, les protéines libérées pour une concentration d'enzyme de 47 mg/g ms sont du même ordre de grandeur que pour une concentration d'enzymes deux fois supérieure (94 mg/g ms). Par contre le rendement de libération des sucres augmente avec la concentration d'enzymes. Par conséquent, le cocktail d'enzymes 94 mg/g ms contient deux fois plus de cellulase que 47 mg/g ms a permis

d'augmenter le rendement d'extraction des sucres, en accord avec les résultats obtenus par Nguyen (2017).

Les enzymes sont connues pour leur capacité à travailler en synergie. Sur la base du broyat de la biomasse lyophilisée, le mélange de deux enzymes permet une attaque simultanée des polysaccharides (cellulase) et des protéines (protéase) au sein de la paroi cellulaire de l'algue, ce qui entraîne une meilleure rupture de cette barrière cellule-environnement. La cellulase et la protéase sont généralement employées pour solubiliser la paroi cellulaire végétale afin de libérer les biomolécules intracellulaires (Nadar et al., 2018). Fleurence et al., 1995 ont récupéré trois fois plus de protéines de *G. gracilis* en utilisant le mélange enzymatique d'agarase et de cellulase. Il a été démontré que le rendement du R-PE extrait de *Gelidium pusillum* s'améliorait de 26 % avec les consortiums d'enzymes (agarase, cellulase et xylanase) (Mittal and Raghavarao, 2018). Ainsi, la synergie entre les enzymes pourrait induire une forte dégradation des parois cellulaires des algues rouges. Dans cette étude, il est démontré que le cocktail d'enzymes (protéase et cellulase) appliqué dans un tampon acétate (50 mM, pH 5.0) est plus efficace pour l'extraction du contenu soluble de la biomasse lyophilisée qu'on utilisant une enzyme unique. Dans ce cas, l'effet complémentaire des deux enzymes permet une meilleure altération des parois cellulaires de la biomasse lyophilisée.

Sous addition de cellulase (47 mg/g ms à 32 °C), Nguyen (2017) a obtenu la libération de R-PE et de protéines solubles à partir de la biomasse lyophilisée de *G. gracilis* avec un rendement maximal de 15.75 mg/g ms et de 29.30 mg/g ms respectivement. En comparaison avec cette étude, le rendement maximum des R-PE obtenu dans notre étude est plus faible bien qu'un cocktail d'enzymes ait été utilisé. Cependant, les rendements en protéines obtenus dans cette étude sont 2.7 fois plus important en utilisant le cocktail d'enzymes et 2 fois plus importantes en utilisant la cellulase par rapport aux rendements obtenus dans l'étude de Nguyen (2017). Ceci peut s'expliquer par le fait que l'extraction est, probablement, affectée par la composition de la biomasse qui varie en fonction de la saison de récolte, du lieu, de la marée, etc.

Parmi les deux enzymes utilisées dans cette étude, la cellulase (dans un tampon acétate 50 mM, pH 5.0) est théoriquement plus prometteuse puisqu'elle attaque les polysaccharides qui représentent le composant principal des parois cellulaires des algues. La plupart des cellulases attaquent au hasard les sites internes de la région amorphe des chaînes de polysaccharides, ce qui entraîne la génération de petits oligosaccharides et facilite la libération des molécules

piégées (Fernandes and Carvalho, 2017; Nadar et al., 2018). En outre, la cellulase provoquait des microfissures à la surface de la paroi cellulaire, ce qui a conduit à une augmentation des rendements d'extraction des composés solubles (Nguyen, 2017). Des études antérieures d'extraction assistée par enzyme sur *Chondrus crispus* (algue rouge) et *Codium fragile* (algue verte) ont également révélé que l'enzyme carbohydrase commerciale (Novozyme-cellulase, β -glucanase et ultaflo) est plus efficace que la protéase commerciale (neutrane) pour la libération des protéines et des sucres (Kulshreshtha et al., 2015).

Cependant, l'effet de la protéase (dans un tampon acétate 50 mM, pH 7.5) sur la biomasse lyophilisée de *G. gracilis* pour l'extraction des composés solubles est moins efficace pour les protéines et les sucres car leur libération est plus faible que celle obtenue en utilisant la cellulase (dans un tampon d'acétate 50 mM, pH 5.0) et du cocktail d'enzymes (dans un tampon d'acétate 50 mM, pH 5.0). Cette limitation est probablement due à son mécanisme d'action qui diffère de celui de la cellulase (dans un tampon d'acétate 50 mM, pH 5.0).

En utilisant la cellulase (16.50 mg/g ms) à 32 °C avec de la biomasse humide, Nguyen (2017) a obtenu les rendements de R-PE soluble et de protéines à ~ 4.62 mg/g ms et 9 mg/g ms, respectivement. Dans cette étude, avec une concentration d'enzyme 3 fois plus élevée, n'a pas permis d'améliorer significativement le rendement de l'extraction de R-PE. En revanche, la récupération des protéines a augmentée de manière significative de 6 fois en utilisant la cellulase, de 9 fois avec le cocktail d'enzymes et de 12 fois avec la protéase.

Aussi, la libération des R-PE et des protéines à partir de la biomasse humide est plus élevée en présence de la protéase. Le cocktail d'enzymes (dans un tampon acétate 50 mM, pH 5.0), qui contient à la fois une protéase et une cellulase devrait logiquement donner de meilleurs résultats (effet complémentaire entre les enzymes) que la protéase (dans un tampon acétate 50 mM, pH 7.5) seule. Cependant, nous avons observé le contraire dans la présente étude. La combinaison entre les enzymes ne semble pas augmenter la capacité d'extraction des contenus solubles sur la biomasse humide. Dans le dosage des enzymes, il convient de noter que l'état, la pureté et la stabilité du substrat sont reconnus comme des facteurs très importants. Les enzymes, simples ou en mélange, ont une affinité définie avec le substrat en fonction de leur fonction physiologique (Bisswanger, 2014). En plus de la nature de la biomasse, la compétition entre les enzymes pourrait probablement limiter l'efficacité du cocktail d'enzymes pendant le processus d'extraction. L'activité de la cellulase pourrait provoquer une plus grande libération de petits oligosaccharides qui pourraient probablement ralentir l'attaque de la protéase pour la libération des protéines de la biomasse humide. En outre, le cocktail d'enzymes n'a pas stimulé

la libération de sucre plus que la cellulase seule. Cela a permis de confirmer que les consortiums d'enzymes (cocktail) étaient moins efficaces pour la biomasse humide qu'en utilisant une seule enzyme. Par conséquent, l'enzyme appliquée dans l'EAE avec la biomasse humide dépendra des composants cibles, les protéines et les R-PE en utilisant la protéase et les sucres en utilisant la cellulase.

Le quatrième chapitre présente l'extrusion enzymatique appliquée à la biomasse humide (fraîche). La température d'extrusion le long des fourreaux est maintenue constante et fixée à 32 °C et un plan d'expériences, basé sur la méthode des surfaces des réponses, est appliqué en utilisant le plan de Box-Behnken. Trois facteurs ont été étudiés : le débit alimentation en biomasse (X1), la concentration d'enzyme (X2) et la vitesse des vis (X3). Chaque facteur est fixé à trois niveaux (faible, neutre, et élevé), codé par -1, 0 et +1. Les niveaux des 3 facteurs ont été fixés après des essais préalables de la manière suivante : le débit d'alimentation varie entre 1.0 et 5.0 kg/h, la concentration d'enzyme (cellulase) est choisie entre 1 et 4.7 % tandis que la vitesse des vis varie entre 150 et 300 rpm. Le plan d'expérience nécessite alors 15 expériences au total. Les réponses choisies sont : les concentrations en sucre (Y1), en protéine (Y2) et en R-PE (Y3) dans les surnageant récupérés après extrusion.

Les résultats obtenus ont montré que les rendements de libération des R-PE varient de 44 à 61 %/ ms (algues). La libération la plus élevée des R-PE (61.86 %) est observée lors de l'expérience avec le débit de 1 kg/h, l'enzyme 2.85 %, et la vitesse des vis 350 rpm. La libération des protéines, quant à elle, varie de 11 à 15 %/ ms (algues). Le rendement maximal est de 15.14 % avec le débit de 1 kg/h, l'enzyme 2.85 % et la vitesse des vis 350 rpm. En ce qui concerne la libération des sucres (28.53 %), le rendement de libération maximum est obtenu avec le débit 1 kg/h, l'enzyme 4.7 % et la vitesse de la vis 225 rpm.

Sur la base des résultats obtenus, l'analyse ANOVA montre que le modèle d'ajustement des variables de réponses est acceptable avec une dispersion $p < 0.05$ et aucune erreur du modèle n'est significative. En outre, le coefficient de corrélation (R^2) pour chaque réponse est supérieur à 0.8, ce qui correspond à un modèle de prédiction pour les données chimiques (Lundstedt et al., 1998; Sugiono et al., 2019a) relativement cohérent.

Les résultats découlant de l'analyse ANOVA montrent que les deux variables linéaires X1 (débit d'alimentation) et X2 (concentration de l'enzyme) ont un effet significatif sur la libération des R-PE, des protéines et des sucres respectivement. La vitesse des vis (X3) ne semble pas avoir d'effet significatif sur la libération des trois composés. Cependant, cette

variable est en interaction avec la concentration d'enzyme X2 et influe sur la libération des protéines. Le terme quadratique $X1*X1$ a un effet significatif sur la libération des trois composés. Le débit d'alimentation (X1) et la concentration en enzyme (X2) sont plus influents sur les trois réponses ($p < 0.01$) pour la libération des R-PE et des sucres respectivement.

Les rendements de libération les plus élevés pour toutes les réponses sont obtenus avec le débit le plus faible, conséquence de l'augmentation du temps de séjour dans l'extrudeuse (Baron et al., 2010) permettant un contact suffisant entre la biomasse et l'enzyme, en accord avec les résultats obtenus par Vauchel (2007) pour l'extraction des alginates par extrusion à partir de l'algue brune *Laminaria digitata*.

Les rendements de libération des sucres et des R-PE sont également affectés de manière significative par la concentration en enzyme. Le rendement d'extraction augmente lorsque la concentration en enzyme augmente. On peut noter que plus la concentration en enzyme est importante, plus la dégradation des petits polysaccharides de la paroi cellulaire de l'algue est importante, ce qui entraîne une plus grande libération des sucres et d'autres molécules intracellulaires (Fernandes and Carvalho, 2017; Nadar et al., 2018).

La vitesse des vis ne semble pas avoir d'effet significatif sur la libération des contenus solubles. Nos résultats ont montré qu'une libération élevée était obtenue principalement avec une vitesse de vis à haut niveau (350 rpm). L'augmentation de la vitesse de la vis est connue pour provoquer un cisaillement, une augmentation de la contrainte thermique et une pression élevée sur le matériau (Hirth et al., 2014; Sugiono et al., 2019a). L'accessibilité de l'enzyme au substrat devrait logiquement augmenter sous une vitesse de vis élevée, entraînant une plus grande libération. D'un autre côté, plusieurs recherches ont montré qu'une vitesse des vis élevée induit une diminution du temps de séjour (Amalia Kartika et al., 2005; Baron et al., 2010), ce qui diminue les temps de contact entre l'enzyme et le substrat (Bhattacharya et al., 1988).

La condition optimale d'extrusion enzymatique a été obtenue pour un débit d'alimentation de 1 kg/h, une concentration d'enzyme de 4.45 %/ ms (algues), et une vitesse des vis de 350 rpm. La condition optimale a été sélectionnée avec les variables prédites pour atteindre l'efficacité de libération maximale. La désirabilité de cette prédiction était de 0.98 ce qui signifie que celle-ci a un niveau de validité élevé (Sugiono et al., 2019a). Les résultats de la vérification ont montré que les valeurs prédites de concentrations en sucre et en protéines étaient en étroite concordance avec les valeurs observées et ont été jugées suffisamment significatives. Cependant, les rendements observés des R-PE étaient deux fois inférieurs aux

rendements prédis par le modèle. Les R-PE semblent ne pas être libérés totalement et pourraient rester piégés dans le culot pendant le processus de centrifugation.

La condition de contrôle sur la biomasse humide obtenue dans le cas de l'extrusion sans enzyme est également étudiée en vue de rendre compte du rôle de l'enzyme dans la libération des composés d'intérêts. Les résultats montrent que l'ajout d'enzyme améliore significativement les rendements de libération des composés solubles en comparaison avec la condition de contrôle. L'efficacité de libération augmente de 55.71 %, 48.30 % et 40.84 % pour les R-PE, les sucres et les protéines respectivement. Aussi, l'effet thermomécanique généré par l'extrusion associé à l'activité enzymatique permet d'améliorer les rendements de libération.

La macération des produits obtenus à la sortie de l'extrusion, pendant 10 mn et à 4 °C, afin de minimiser l'activité enzymatique, permet d'augmenter d'avantage les rendements de libération des 3 composés. Aussi, les rendements d'extraction des R-PE, des sucres et des protéines ont augmenté de 1.5, 1.3 et 1.3 fois respectivement en comparaison avec l'extrusion enzymatique seule. L'extrusion enzymatique, suivie d'une macération de 10 mn, permet d'augmenter les rendements d'extraction des trois composés d'intérêts.

La comparaison de l'extrusion enzymatique seule et l'hydrolyse enzymatique en réacteur batch, montre que l'extrusion est plus efficace pour la libération des trois composés d'intérêts. Par conséquent, l'extrusion associée à l'hydrolyse enzymatique est une technologie d'extraction prometteuse pour la valorisation et la transformation des algues.

Une macération avec de l'eau pure des produits obtenus après une étape d'extrusion permet d'augmenter les taux d'extraction des différents composés en comparaison avec l'hydrolyse en réacteur batch. Ces taux sont de 56 %/ ms algues pour les R-PE, de 21 %/ ms pour les protéines et de 60 %/ ms pour les sucres. Sur la base de ces résultats, l'extrusion enzymatique suivie d'une étape de macération, améliore d'une manière relativement importante les rendements d'extraction tout en réduisant considérablement les temps de traitements, en comparaison avec l'hydrolyse en réacteur batch, dont les temps peuvent aller au-delà de 5h. Les forts cisaillements générés par les vis d'extrusion, alliés à l'action de l'enzyme, ont permis d'accroître la dégradation des parois cellulaires des algues et d'augmenter les taux de libération (Gatt et al., 2019; Li et al., 2020; Vauchel et al., 2008).

Publications

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Nomenclature

Acronyms and abbreviations

BC	Before Christ
cm	Centimeter
R ²	Correlation coefficient
Da	Dalton
°	Degree
°C	Degree Celsius
dw	Dry weight
DOE	Design of experiments
EAE	Enzyme-assisted extraction
CE	Enzyme cellulase
PE	Enzyme protease
ME	Enzyme cocktail (mixture of cellulase and protease)
BL	Freeze-dried biomass
AFM	Fresh biomass
g	Gravity force /Gram
g/cm ²	Gram per square centimeter
g/cm ³	Gram per cube centimeter
ha	Hectare
h	Hours
>	Higher than
kg	Kilogram
kg/h	Kilogram per hour
kDa	Kilo Dalton
m	Meter
M	Molar
mM	Millimolar
mbar	Millibar

mg/g	Milligram per gram
mg/ml	Milligram per milliliter
min	Minute
mg	Milligram
mm	Millimeter
μm	Micrometer
nm	Nanometer
N	North
%	Percentage
pH	Potential of hydrogen
PBPs	Phycobiliproteins
rpm	Round per minute
R-PE	R-phycoerythrin
B-PE	B-phycoerythrin
b-PE	b-phycoerythrin
C-PE	C-phycoerythrin
RSM	Response Surface Method
m ²	Square meter
sp.	Species
USD	United State dollars
w/w	Weight/weight
W	West

Greek letters

α	Alpha
β	Beta
γ	Gamma
Ι	Iota
Κ	Kappa
λ	Lambda
μ	Mu

v Nu

Chemicals elements and subscripts

Ca	Calcium
Cu	Copper
Fe	Iron
I	Iodine
Mn	Manganese
Mg	Magnesium
K	Potassium
Na	Sodium
Zn	Zinc
OH ⁻	Anion
H ⁺	Proton
Cu ²⁺ & Cu ¹⁺	Copper ions
HCl	Hydrochloride acid
Na ₂ CO ₃	Sodium carbonate
CH ₃ COONa	Sodium acetate
CH ₃ COOH	Glacial acetic acid

Variables

X1	Sample flow rate (kg/h)
X2	Solvent flow rate (kg/h)
X3	Screws speed (rpm)
Y1	Response of soluble sugar concentration
Y2	Response of soluble protein concentration
Y3	Response of soluble R-PE concentration

General introduction

The global population is projected to reach up to 10 billion people by 2050, which requires an increase of 70 % in food production (Bleakley and Hayes, 2017; United Nations, 2019). From this context, other natural resources have been raised in order to respond to this rapid growth. Thanks to the richness and the numerous advantages of the marine ecosystem, various marine organisms have been explored and served as an important source for human consumption (Pereira, 2018). So far, the oceans contributed only 6.5 % of the global food protein consumption which mainly came from fish and shellfish. As one of the marine inhabitants, macroalgae (seaweed) could also consider as a valuable source of protein (Mæhre et al., 2016). This resources locate mainly on the coastline and are salt-tolerant thanks to the adjustment of the osmolality between cytoplasm and seawater (Cian et al., 2015; De Almeida et al., 2011). Their foods and oxygen are produced from carbon dioxide and water using photosynthetic pigments and sunlight (De Almeida et al., 2011).

In addition, macroalgae are reported with low calories content and rich in vitamins, minerals, proteins, polysaccharides, steroids, and dietary fibers which make its a valuable food resource (Circuncisão et al., 2018; De Almeida et al., 2011; O'Sullivan et al., 2010). Macroalgae have been firstly introduced into food since 2700 BC in China and later spread to others Asian countries (Cian et al., 2015). Beside the wisely use in Asia, the consumption of macroalgae in food chain is still limited in western countries due to the cultural and consumption habits (Cian et al., 2015; Maehre et al., 2016). On a global scale, the production of macroalgae was evolved from 25 million tons in 2012 (Mæhre et al., 2016) then 30.4 million tons in 2015 (Fao, 2018), and 32.4 million tons in 2018 (Doumeizel, 2022; Fao, 2020). Most of the world's macroalgae production is applied for food consumption (Radulovich et al., 2015), meanwhile the leading producers are Chile, China, Norway, Indonesia, the Republic of Korea, and the Philippines (Fao, 2018).

Macroalgae/seaweed contain various bioactive compounds. The content of protein in some seaweed species is similar to the other sources such as meat, egg, soybean, and milk (Bleakley and Hayes, 2017). In the meantime, the most exploited compound from seaweed is hydrocolloid which represents a huge market value. Hydrocolloids-derived from seaweed (agar, carrageenan, and alginate) have been used in food, pharmaceutical, medical as well as in biotechnology industry (Rhein-Knudsen et al., 2015). For *Rhodophyta* (one of the three seaweed mains phyla), two main hydrocolloids can be extracted which are known as agar and

carrageenan (Xiao et al., 2019). Following the traditional extraction, carrageenan is usually extracted by heating with an alkaline solution of potassium hydroxide while an alkali treatment followed by hot water extraction are carried out for agar extraction (Rhein-Knudsen et al., 2015; Rioux and Turgeon, 2015). Beside, *Rhodophyta* have been reported with high contents of protein and carbohydrate which are comparable to the terrestrial resources (Gamero-Vega et al., 2020; Rioux et al., 2017). However, protein liberation are obtained as the byproduct associated with polysaccharides extraction since most of seaweed's polysaccharides are located within the algal cell wall (Rioux et al., 2017). This is known as the main hindrance for the release of protein due to the entrapment of certain proteins and extracellular matrix polysaccharides (Mæhre et al., 2016). Base on this phenomenon, the degradation of the cell wall is an optimized way which could enhance the release of intracellular proteins from algal biomass (Mæhre et al., 2016). In addition, R-phycoerythrin (R-PE) also marks as a dominant pigment in *Rhodophyta* family. This hydro-soluble phycobiliprotein has a huge valorization value which can be used widely in research, food, and cosmetics as a natural dye (Dumay et al., 2014; Francavilla et al., 2013b; Rioux et al., 2017). The extraction of this pigment is done simply by soaking seaweed in water for several days. At the same time, this process can induce some degradation due to protease activity (Dumay et al., 2014).

Enzyme-assisted extraction (EAE) approach has become an attractive alternative method compared to chemical and mechanical processes in respect to seaweed biorefinery. EAE is considered as an eco-friendly and nontoxic technique, where organic solvents or other toxic chemicals are avoided to comply with the strict rule for the food industry (Michalak et al., 2017). The disruption of tissue from enzyme activity is known to release more confined and inaccessible compounds, allowing more liberation (yields) compared to the moderate extraction conditions (Dumay et al., 2014; Sudhakar et al., 2015). Enzymes are known with the ability to soften the cell wall and reduce any degradation of the target components (Xiao et al., 2019). Several studies on hydrocolloids extraction have shown promising results when using EAE approach. Kappa/iota-hybrid carrageenan from *Mastocarpus stellatus* has been extracted with alcalase (commercially protease). Results showed that the extracted carrageenan presented good gelling properties and polyphenols extraction yields were also enhanced (Blanco-Pascual et al., 2014). The extraction of iota carrageenan from *Soliera filiformis* with papain was successfully carried out and showed the absence of protein contaminants when extracting with the traditional extraction method (De Araújo et al., 2012). Highest carrageenan yield from *Eucheuma cottoni* was reported with the presence of cellulase novozyme NS50013 compared

to traditional boiling extraction (Varadarajan et al., 2009). Several researches have addressed on using more than one enzyme. A cocktail enzyme (combination of enzyme) of kappa-carrageenase and agarase with cellulase was shown to improve the protein extraction 10 and 3 times respectively, from *Chondrus crispus* and *Gacilaria verrucosa* compared to the results obtained with cellulase alone (Fleurence et al., 2002, 1995b). Commercial carbohydrases and proteases have been reported to enhance the efficiency of extraction of the bioactive materials from *Chondrus crispus* (Kulshreshtha et al., 2015).

EAE technique varies according to the type of biomass and target components. The operating conditions should be adapted and notably take into account the enzyme properties. Furthermore, extrusion technology has been recently introduced into the seaweed bio-refinery. Reactive extrusion either with chemical or with the addition of enzyme represents as another promising method with the possibility to enhance the algal biomass valorization. For certain case, the use of this technique increases the extraction yields (E. Xu et al., 2020) and allows the co-extraction of several interested compounds. Extrusion is known to provoke an intimate contact between substrate and reactants at a very short time, improving the extraction and the purification of the target compounds (Gatt et al., 2018). Using extruder as a bioreactor or chemical reactor, significant gains can be listed not only on product quality but also on environmental impact (Vauchel et al., 2008). Beside the numerous advantages provided in the addition of enzyme, enzyme's cost cannot be neglect and remains the main drawback for this approach (Michalak et al., 2017; Xiao et al., 2019).

The red macroalgae *Gracilaria gracilis* is served as food vegetable for human consumption and this resource is widespread (Fleurence and Guyader, 1995; Iyer et al., 2004; M. D. Torres et al., 2019). It is known as the main source of worldwide agar production with the high production yields (Francavilla et al., 2013a; Rioux et al., 2017). This Rhodophyta also contains high protein content as well as the red pigment R-phycoerythrin (Pimentel et al., 2019). It is worth noting that red algal cell wall is dominant with the presence of polysaccharides (sulfated galactant about 70 %) as well as proteins (Gajaria et al., 2017; Rioux and Turgeon, 2015; Rioux et al., 2017; Xiao et al., 2019). While the extraction of agar is known to consume more solvents, the use of EAE and extrusion technique could be interesting since these techniques consume less solvents and could therefore extract others sulfated galactan (in addition to agar such as oligoagar, cellulose, etc), pigment, and protein from red algae with better extraction yields. In this regard, the works carried out in this manuscript is focusing on two different techniques of enzyme-assisted extraction, aiming to enhance the release of hydrosoluble

compounds known as sugars (mostly neutral sugars), protein, and R-phycoerythrin from red macroalgae *Gracilaria gracilis*. The manuscript is divided into four chapters while the first and the second chapter detailed on literature review, addressing on the description of red macroalgae *Gracilaria gracilis* as well as the extraction methods and the experimental design.

The third chapter illustrated in detail the results of enzyme-assisted extraction conducted in batch mode on fresh and dried *Gracilaria gracilis* biomass. Three enzymes (Cellulase, Protease, and cocktail (combination of Cellulase and Protease)) were tested. The enzyme concentrations and the operating temperatures were also investigated. Optimal conditions including enzyme type and operating parameters were determined based on the extraction yields of hydrosoluble compounds.

In the fourth chapter, enzymatic extrusion was conducted on fresh *Gracilaria gracilis* biomass with the addition of cellulase. In this chapter, experimental design of response surface method was carried out to investigate the effect of three extrusion parameters (biomass flow rate, enzyme concentration, and screw speed). Extraction yields of soluble compounds were used for data interpretation for the determination of optimal condition as well as the relationship between three variables. Optimal extrusion conditions were determined and rapid maceration was investigated on the pellet of enzymatic extrusion. The comparison between enzymatic extrusion and enzymatic hydrolysis (batch without extrusion) were also evaluated.

Chapter I: Literature review

1. Marine macroalgae

1.1. Overview

Oceans cover more than 70 % of the earth and are the habitat to 90 % of the planet's organisms. The ocean provides many unique environment which shelters numerous marine organisms able to produce high potential bioactive compounds for human needs (food, nutraceuticals, pharmaceuticals, and cosmeceuticals) (Pereira, 2018). Macroalgae or seaweed are an extensive group of macroscopic marine organisms (Peng et al., 2015) which play a significant role such as academic, biological, environmental, and economic in coastal ecosystem (Tiwari and Troy, 2015). Currently, around 20,000 species of marine macroalgae are identified meanwhile 221 species among them are marketed (Fao, 2018; Kumar et al., 2015).

Marine macroalgae have been used all over the world for thousands of years for various food and non-food applications (Tiwari and Troy, 2015). They have been used as food for over 2,000 years in China, Korea, and Japan. In Malaysia and Indonesia, they are commonly served fresh as salad (Tiwari and Troy, 2015). In contemporary Japan, 21 species of algae are used in everyday cooking while six of them have been used since the 8th century (Pereira, 2018). On the other hand, macroalgae have been used for non-food application in the western countries. As early as 100 BC, macroalgae were used to feed animals in Greece. Red macroalgae were also utilized for medical purposes in Mediterranean countries (Tiwari & Troy, 2015). Macroalgae have been considered as important traditional remedies since the beginning of 3,000 BC. In Japan and China, brown algae are used in the treatment of hyperthyroidism and other glandular disorders (De Almeida et al., 2011). The use of macroalgae for agricultural application was reported in Ireland and Scotland (Pereira, 2018; Tiwari and Troy, 2015). In Europe, macroalgae as human food have been introduced during the 15th century, particularly by littoral populations which suffer from famine (Fleurence, 2016). Following the difference of their pigmentations, marine macroalgae are classified into three main phyla as below (Peng et al., 2015; Pereira, 2018; Sudhakar et al., 2015):

- Phaeophyta (brown) contain pigments such as fucoxanthin and chlorophyll a and c
- Chlorophyta (green) contain chlorophyll a and b together with various characteristic of xanthophylls
- Rhodophyta (red) contain phycoerythrin and phycocyanine pigments as well as chlorophyll a and carotenoids

1.2. Rhodophyta

The *Rhodophyta* (*red algae*) is a well-characterized and morphologically different lineage of photosynthetic protists. They can be found in unicells, filaments (arranged in rows), large pseudoparenchymatous (up to 3 m), branched or unbranched, terete (cylindrical) to foliose thalli, including crustose and erect forms, and some are calcified (Yoon et al., 2017). Around 7,331 species of *Rhodophyta* are currently reported in www.algaebase.org (M.D. Guiry in Guiry, M.D. & Guiry, 2020). *Rhodophyta* usually compose with some elements as following (Yoon et al., 2017):

- Plastids, water-soluble pigments (allophycocyanin, phycocyanin, and phycoerythrin) which is located on the outer faces of photosynthetic lamellae
- Thylakoids in plastids (as single lamellae)
- Lack of flagellated structures and centrioles
- Floridean starch [α -(1, 4)-linked glucan] in granules which is located outside the plastid and used as food reserve

Two subphyla (*Cyanidiophytina* and *Rhodophytina*) and seven classes were reported under *Rhodophyta* phylum (Figure I-1) whilst *Florideophyceae* is the most dominant class (Yoon et al., 2017). *Rhodophyta* can be found in marine, freshwater, and terrestrial. The dominant classes *Florideophyceae* (largely multicellular) are mostly located in marine habitats (Yoon et al., 2017).

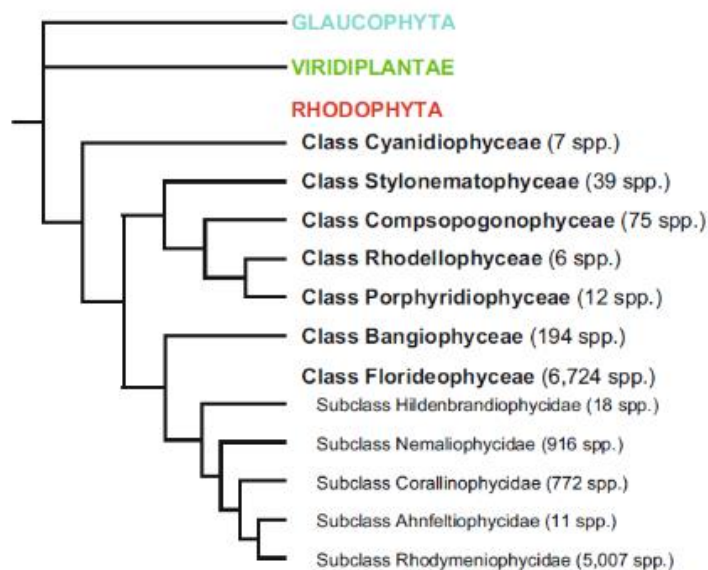


Figure I- 1: Schematic phylogenetic relationships of the red algal classes and subclass (Yoon et al., 2017)

1.2.1. Nutritional compositions

The main constituents of marine macroalgae vary according to species, harvest location and time, wave exposition, water temperature, and also according to the applied methodology for the determination of its constituents (Rioux et al., 2017). High content of water (90 % of fresh weight) was reported in macroalgae compared to the terrestrial plants (Sudhakar et al., 2018).

Among the three main phyla, *Rhodophyta* is known for the high content of polysaccharides and proteins (Fleurence et al., 2012). Agars and carrageenans are the two main cell-wall polysaccharides extracted from red algae (Rioux et al., 2017). They are the most important hydrocolloids extracted from red seaweed together with alginates isolated from brown seaweed (Fleurence, 2016; Roohinejad et al., 2017). Hydrocolloids are generally employed for their physical functions, notably for stabilization of emulsions, viscous behavior, gelation, and control of crystal growth (Roohinejad et al., 2017).

The content of protein in *Rhodophyta* is similar to those observed in meat and high-protein vegetables such as soybean (Gamero-Vega et al., 2020; Rioux et al., 2017). According to Gamero-Vega et al., 's study (2020), 40 % of the studied red seaweed contained more than 20 % dw (dry alage) of protein. An average protein content of 5.2- 40 % dw was reported in the study of Gamero-Vega et al., 2020 and 4-50 % dw was reported by Rioux et al., (2017) respectively. Phycobiliprotein, particularly phycoerythrin (PE), the major light-harvesting pigment was also reported in red algae (Bourgougnon and Stiger-Pouvreau, 2012; Fleurence, 2016). This pigment can be valorized as natural colorant for food, cosmetic, pharmaceutical, and research (Cotas et al., 2020).

Low level of lipids (1-5 % dw) is reported in red algae. Nevertheless, the majority are polyunsaturated fatty acids (PUFAs) notably phospholipids and glycolipids (Gamero-Vega et al., 2020; Rioux et al., 2017; Wells et al., 2017). *Rhodophyta* can contain up to 50 % of eicosapentaenoic acid (EPA, C20:5) (Ladislava, 2012; Rioux et al., 2017) and some can report with more than 30 % of monounsaturated fatty acids (MUFAs) (Gamero-Vega et al., 2020). Most of red algae are claimed to be rich in essential mineral elements for human nutrition such as Na, K, I, Ca, Mg, Fe, Zn, Mn, and Cu (Gamero-Vega et al., 2020; Syad et al., 2013). The amounts of magnesium and manganese in red algae are often higher than those reported in territorial vegetables and cereals. Growing in sea water naturally gives red algae high content of sodium (Gamero-Vega et al., 2020). One of red algae, *Gracilaria Salicornia* contained

2.782 % dw of Ca which is higher than some terrestrial plants such as spinach (0.851 %), broccoli (0.503 %), and cabbage (0.369 %) (Rioux et al., 2017).

Water-soluble vitamins, especially thiamine (B1) and riboflavin (B2), were reported in the majority of red and brown algae (Gamero-Vega et al., 2020; Ladislava, 2012). Several red algae such as *Porphyra umbilicalis* and *Gracilaria changii* contain the amount of vitamin C as high as the most of common vegetables notably tomatoes and lettuces. Red algae, *Gracilaria chilensis* is observed with higher β -carotene content than those reported in carrots (Wells et al., 2017). It is worth noted that phenolic compounds can be found in marine macroalgae. Catechins and flavonoids are the two phenolic compounds which were isolated from brown, green, and red algae. Macroalgae phenolic compounds have strong antioxidant activity and can inhibits α -amylase and/or α -glucosidase, two enzymes which regulate the glycemic index via starch digestion (Rioux et al., 2017).

1.2.2. Consumptions

Red algae represent about 33 % of harvested weight but nearly 50 % of the value of macroalgae aquaculture (about US \$ 6.4 billion in 2012) (Yoon et al., 2017). In 2012, more than 8 million tons of harvested *Eucheuma* sp. and *Kappaphycus* sp. were reported versus 2.7 million tons for *Gracilaria* sp. and *Porphyra* sp., and about 1.8 million tons for *Porphyra* sp. (Fao, 2014a). Red macroalgae are mainly utilized for human food consumption (Fao, 2020, 2014a; Yoon et al., 2017). The global production of red algae of different species was illustrated in Table I-1.

Table I- 1: World aquaculture production of aquatic algae (Fao, 2020)

Rhodophyta species	2000	2005	2010	2015	2016	2017	2018
	(Thousand tons, live weight)						
Eucheuma seaweeds nei* (<i>Eucheuma</i> sp.)	215.3	986.9	3 479.5	10 189.8	9 775.9	9 578.0	9 237.5
Gracilaria seaweeds (<i>Gracilaria</i> sp.)	55.5	933.2	1 657.1	3 767.0	4 248.9	4 174.2	3 454.8
Nori nei (<i>Porphyra</i> sp.)	424.9	703.1	1 040.7	1 109.9	1 312.9	1 733.1	2 017.8
Elkhorn sea moss (<i>Kappaphycus alvarezii</i>)	649.5	1 283.5	1 884.2	1 751.8	1 524.5	1 545.2	1 597.3
Laver (<i>Porphyra tenera</i>)	529.2	584.2	565.2	688.5	713.4	831.2	855.0
Spiny eucheuma (<i>Eucheuma denticulatum</i>)	84.3	171.5	258.7	274.0	214.0	193.8	174.9

nei*: not elsewhere included

According to the updated report of CEVA, (2019), 25 algae (22 macroalgae and 3 microalgae) are authorized in France for food industry. Among the macroalgae, 11 are from red algae including *Palmaria palmata* (Dulse), *Porphyra umbilicalis* (Nori), *Porphyra tenera* (Nori), *Porphyra yezoensis* (Nori), *Porphyra dioica* (Nori), *Porphyra purpura* (Nori), *Porphyra laciniata* (Nori), *Porphyra leucostica* (Nori), *Chondrus crispus* (Pioca, Lichen), *Gracilaria gracilis* (Ogonori), and *Lithothamnium calcareum* (Mäerl).



Gracilaria gracilis
(Ref. 15621)



Lithothamnium calcareum
(Ref. 11268)



Porphyra umbilicalis
(Ref. 33487)



Palmaria palmata
(Ref. 20767)



Chondrus crispus
(Ref. 20781)

Figure I- 2: Red algae authorized in food industry in French
Photos are taken from AlgaeBase (www.algaebase.org) with reference number accordingly

2. *Gracilaria Gracilis*

2.1. General description

The red algae, *Gracilaria gracilis*, belongs to the *Gracilaria* genus and the *Gracilariales* order, one of the three main orders of agarophytes and serves for agar food grade production (Armisen, 1995). *Gracilaria gracilis* (*G. gracilis*) is the current name which is used to replace *Gracilaria verrucosa* (*G. verrucosa*) (Skriptsova and Choi, 2009). The mature *G. gracilis* described by Anna V. Skriptsova & Choi (2009), presented thalli erect, cylindered (up to 25-30 cm tall), and attached to substrate by a discoid holdfast (1-4 mm in diameter), with several thalli arising from a holdfast. The main axes come up with 1.5-2 mm in diameter, usually with 3-4 orders of lateral branches of various sizes at irregular intervals in an alternate to unilateral pattern. The branches are usually slightly constricted at their bases. The cortex has one to three layers of well-pigmented cells. Outer cortical cells are radially elongate, ovoid, and 4.5-7 µm in diameter (Figure I-4).

Table I- 2: Taxonomy classification of *Gracilaria gracilis* (Armisen, 1995)

Phylum	<i>Rhodophyta</i>
Class	<i>Florideophyceae</i>
Order	<i>Gracilariales</i>
Family	<i>Gracilariaceae</i>
Genus	<i>Gracilaria</i>
Species	<i>Gracilaria gracilis</i>

The genus of *Gracilaria* is a part of *Rhodophyta* but it can be found in another colors as black, yellow, and green (Nguyen, 2017). In winter, *G. gracilis* presents marron-red colors while in summer, the color changes to clear red (Perez et al., 1992). *Gracilaria* species can be found all around the world from 60° of Nord latitude to 40° of South latitude, from moderate, cold temperatures to tropical water. The species occupy a place on the foreshore, in sand or muddy basins (Figure I-3) with rocks, and is rarely emerge (no more than 1 h emersion). It can be found at 25 m deep while 98 % of the population are located between 0.5 m to 10 m (optimum 3 m-4 m). Thus, there is no competition with other species except for *Agardhieffa tenera* and *Sargassum muticum* (Perez et al., 1992).

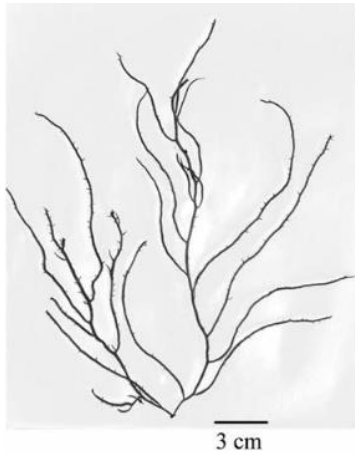


Figure I- 4: Habit of the attach form of *Gracilaria gracilis* (Skriptsova and Choi, 2009)



Figure I- 3: *Gracilaria gracilis* in a sandy basin

2.2. Biochemical Compositions

Gracilaria genus has been known as the source for agar production, hence the content of carbohydrates could reach at 63 % dw (Rioux et al., 2017). Table I-4 summarizes the biochemical components of *G. gracilis* from some previous studies. Dried weight content was reported around 8-15 % of fresh weight (fw) whereas ash content was reported at 34 % dw. Lipids content was observed at a relative low amount, making the seaweed a low energy food (Fook Yee Chye et al., 2017). Fiber content is reported around 25 % dw in *G. gracilis* (CEVA, 2015). Within this content, *G. gracilis* could become a suitable source of dietary fiber (Taboada et al., 2013). This algae *G. gracilis* (ogonori) is one of the authorized red algae for food consumption in French as sea vegetable (Fleurence and Guyader, 1995). The changes of biochemical compositions of the same species of *Gracilaria* depends strongly on environments and seasonal factors (Francavilla et al., 2013a).

According to the results of Francavilla et al., 2013 (Table I-3), the protein in *G. gracilis* reached the highest content in January (45 % dw) while the lowest was observed in July (31 % dw). The content of protein in October and April was reported at 41 % dw which is close to the highest content. Carbohydrate amount was reported at 31 % dw and 34 % dw in January and April respectively, while these amounts dropped to 27.5 % dw in July and 24.8 % dw in October. Lipid content was observed in low amount, around 1.12 % -1.98 % dw within this study. Phycobiliprotein which is another interesting compound of red algae would be most abundant in January. R-phycoerythrin (R-PE) reached 7 mg/g dw in January while 3.6 mg/g

dw was reported in October. This study has shown that winter is the best time for harvesting, in particular for the richness of the algae in R-phycoerythrin, arachidonic acid (PUFA ω 6), proteins, and carbohydrates.

Table I- 3: Biological nutritions of *Gracilaria gracilis* from differents seasons (Francavilla et al., 2013a)

	January	April	June	October
Total Protein (% dw)	45	41	31	41
R-Phycoerythrin (mg/g dw)	7	N/A	N/A	3.6
Allophycocyanin (mg/g dw)	3.5	N/A	N/A	1.5
Phycocyanin (mg/g dw)	3	N/A	N/A	0.7
Total Carbohydrate (% dw)	31.1	34.1	27.5	24.8
Total Lipid (% dw)	1.12	1.40	1.98	1.38

dw : Dried weight

G. gracilis sample was taken from Lesina Lagoon, Italy from July and October 2011 to January and April 2012

Table I- 4: Summary of biochemicals compositions of *Gracilaria gracilis* from different studies

	Dried weight (% fw)	Ash (% dw)	Protein (% dw)	Lipid (% dw)	Fiber (% dw)	Carbohydrate (% dw)	References
<i>Gracilaria sp.</i>	15 ¹	8-29 ^{1,2,3}	5-23 ^{1,2}	0.4-2.6 ^{1,2,4,5}	N/A	36 ¹ 62-63 ^{1,2}	1 : (Wen et al., 2006) 2 : (Marinho-Soriano et al., 2006) 3 : (Robledo and Freile Pelegrín, 1997) 4 : (Khotimchenko, 2005) 5 : Khotimchenko and Levchenko (1997) in (Kim, 2012)
<i>Gracilaria dura</i> <i>Gracilaria gracilis</i> <i>Gracilaria longa</i> <i>Gracilaria armata</i> <i>Gracilaria bursa-pastoris</i>	14.2-15.4	27-34	16.7-25.8	0.91-1.28	N/A	35-50.7	(Sfriso et al., 1994)
<i>Gracilaria gracilis</i>	N/A	N/A	21.6-30.2	N/A	N/A	34-35	(Mensi et al., 2009)
<i>Gracilaria gracilis</i>	N/A	N/A	31-45	1.12-1.98	N/A	24.8-34.1	(Francavilla et al., 2013a)
<i>Gracilaria gracilis</i>	7.99 ±0.02	24.8± 0.03	20.2± 0.60	0.60 ±0.01	N/A	46.6	(Rodrigues et al., 2015)
<i>Gracilaria gracilis</i>	8.4	17.8	16.5	3	35	18.8	(CEVA, 2015)

fw: Fresh weight, dw: Dried weight, N/A: Not Available

2.3. World Production

According to the Fao (2018), the global seaweed industry reached more than USD 6 billion per annum) of which 85 % includes food product for human consumption. The global market of macroalgae in 2019 is amounted to 15 billion euros and it is still growing at a high rate. Currently, more than 97 % of the world's production comes from Asia (Doumeizel, 2022). Carrageenan, agar, and alginate extracts from seaweed represent almost 40 % of the world's hydrocolloid market while the rest come from animals, microbes, and land plants. In global scale, seaweed production was reported at 30.4 million tons in 2015 while 29.4 million tons came from aquaculture (Fao, 2018).

The production of *Gracilaria* species have steadily increased in the last decade (Fao, 2014b). In 2011, the production of *Gracilaria sp.* was reported about 2 257 919 tons, in which *Gracilaria gracilis* represented at 1 518 455 tons while 1 513 590 tons was cultured in China and 4 865 tons were cultivated in Taiwan. For the rest of *Gracilaria sp.*, about 94.2 % (697 240 tons) came from cultivation while 42 224 tons are harvested from wild stocks. Chile, Peru, and Argentina are the most productive countries of *Gracilaria* species in America versus Indonesia, Vietnam, the Philippines, and Korea in Asia, and Namiba in Africa (Fao, 2014b).

2.4. Applications

2.4.1. Agar source

The genus of *Gracilaria* is reported with more than 300 species in which 160 have been accepted taxonomically. This genus is well known for industrial and biotechnological use and represents as an important economical resource thanks to its high production yield (Francavilla et al., 2013a). *Gracilaria* genus was firstly introduced for agar production with respect to replace *Gelidium* genus (Armisen, 1995). Various applications of agar have been used in food, pharmaceuticals, cosmetic, medical, and biotechnology industries. Global production of agar has increased from 6,800 tons (US\$ 82.2 million) in 2002 to 9,600 tons (US\$ 173 million) in 2009 (Lee et al., 2017). Market figures of agar has summarized in Table I-5. In 2015, China as the world leader, reported the harvest of 2.7 million tons of farm *Graciliaria* (Fao, 2018). *Gracilaria* represents more than 80 % of the production of agar (Veeragurunathan et al., 2015). About 60 % of all agar was extracted from *Gracilaria* species which mainly came from Chile, China, Taiwan, and Vietnam farms (Skriptsova and Nabivailo, 2009). *Gracilaria gracilis* has demonstrated a broad tolerance to abiotic factors, in particular to light, temperature, salinity, fast growth rate (3-5 % day at 22-27 °C), high production yield for agar (30-35 % dw)

(Skriptsova and Nabivailo, 2009), (25-30 % dw) (Lee et al., 2017), and high gel strength (543.26-630 g/cm²) (Lee et al., 2017).

Table I- 5: Market parameters of agar (Fao, 2014b)

Market parameters for agar	1999	2009
Agar volumes (tons)	7 500	9 600
Total industrial capacity (tons)	9 000	12 500
% utilization	83 %	77 %
Agar from <i>Gracilaria</i>	63 %	80 %
Average agar price (USD/Kg)	17	18
Sale value (millions USD)	128	173

2.4.2. Others uses

Beside the main source for agar extraction, *Gracilaria* genus also contains phycocolloids which is a gelatinous non-toxic colloidal carbohydrate presented in the cell wall and intercellular spaces. These components have been widely used in food industry (ice-creams, jellies, and soups), bacteriological sample, and also in cosmetic (shampoo, soap, hydrating cream, facial mask, lotion, and deodorant) (Francavilla et al., 2013a; P. Torres et al., 2019). The genus of *Gracilaria* has been evaluated for its bioactivities. The bioactivities related to *Gracilaria* sp. summarized from 417 published papers (from 1980 to 2017) was presented in the Figure I-5 below (Torres et al., 2019).

2.4.2.1. Paper making

The solid residues waste produced during agar extraction from *Gracilaria* or *Gracilariopsis* could serve as raw material for pulping and paper making. These have been utilized in China as a fiber source and functional filter (Pei et al., 2013). The higher contents of algal materials in paper resulted in lower permeability and stronger antimicrobial effects. Algal material, when used as a partial substitute for wood pulp, improved paper density, waterproofness, greaseproofness, and antimicrobial effects, indicating its potential use in the food packaging industry (Fao, 2014b).

2.4.2.2. Biofuel

Some species of *Gracilaria* were reported with high carbohydrate content (up to 45 % dw) which could be used for food agar but also as an alternative of ethanol production using fermentation by *saccharomyces cerevisiae* (Amanullah et al., 2013). The study of Kumar et al., (2013) developed an efficient strategy for agar extraction using the resultant pulp for bioethanol production and suggested an integrated bio-refinery process to obtain maximum economic returns from *Gracilaria*. Furthermore, the study noticed that after ethanol production, the leftover residues still contained good amounts of organic matter and useful minerals, and eventually could be used as biofertilizer.

2.4.2.3. Bioremediation capacity

For many decades, seaweed have been recognized as being able to remove inorganic nutrients from the water media. Currently, with the presence of integrated multitrophic aquaculture (ITMA), several species of seaweeds are viewed as renewable biological nutrients scrubbers that take up nutrients (Chopin et al., 2001). Various species of *Gracilaria* have been evaluated for their nutrient removal capacity produced from invertebrate or fish farms (P. Torres et al., 2019). The study of Troell et al. (1997) concluded that a suspended culture of 1 ha (1 kg wet weight per m²) removes 5 % of the dissolved inorganic phosphorus released from a 227 tons mixed salmon farm. Therefore, different species of *Gracilaria* seem to become good candidate for the multitrophic cultivation systems.

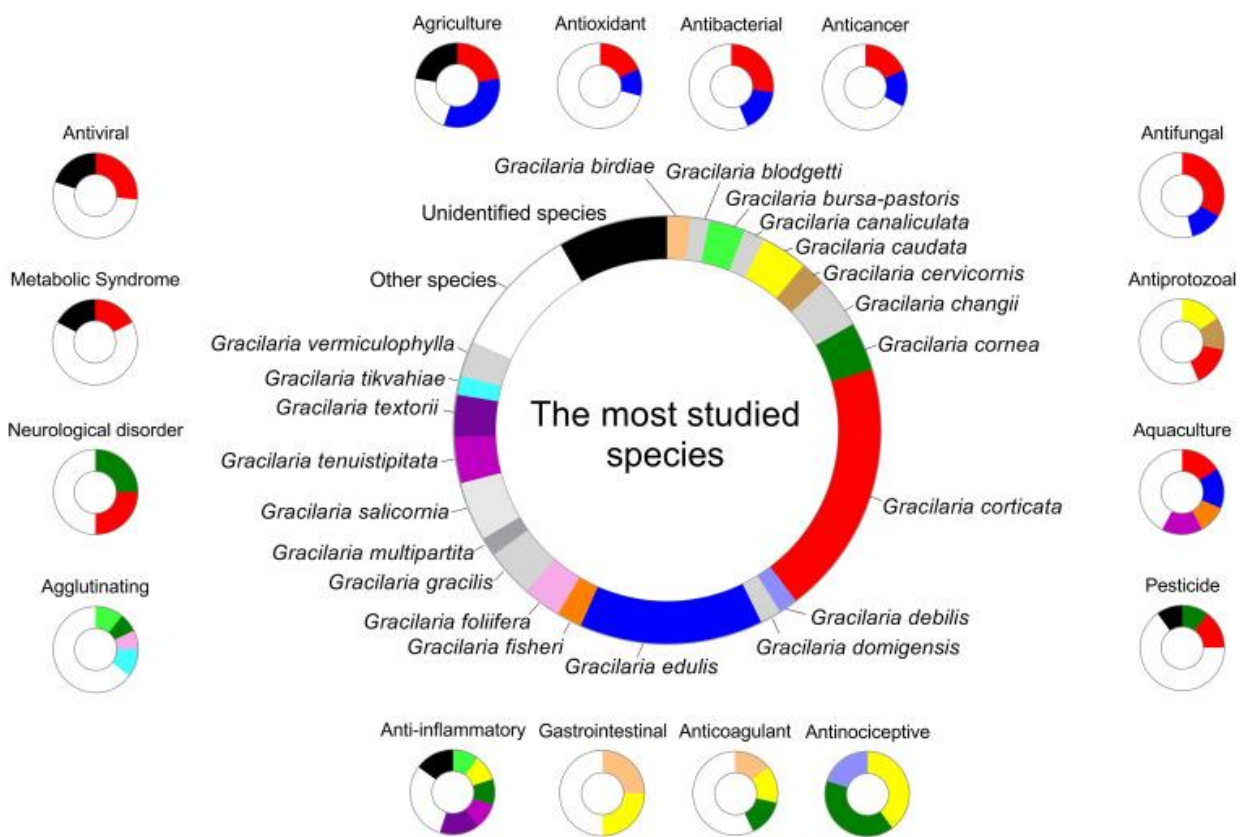


Figure I- 5:Percentage distribution of species of *Gracilaria* analyzed for biological activities (P. Torres et al., 2019)

Central large pie shows the percentage distribution of species of *Gracilaria* within all assays. Small pies show percentage distribution of species of *Gracilaria* within a specific assay. Different color fragments represent distinct species of *Gracilaria* as marked in the central pie. Database obtained for the period of 1980 to 2017.

3. Carbohydrate

3.1. Overview

Carbohydrates are responsible for energy storage and structural elements in marine algae as well as in terrestrial plants. The content of carbohydrate in macroalgae typically ranges around 50-60 % dw. Yields and structures of algae polysaccharides depend on several factors (biological, physical, and environmental) such as harvest period, algal species as well as extraction methods (Rioux and Turgeon, 2015). Meanwhile, the structural characteristics of polysaccharides vary according to the molecular weight, the nature of building units, the content of sulfate groups and their positions, the type of glycosidic bonds, and the geometry of the molecules (Rioux and Turgeon, 2015). The comparison of macroalage carbohydrates contents are somehow complicated due to the different and the challenges in each determination methods (Kumar et al., 2015). Furthermore, some edible marine macroalgae contained higher dietary fiber than those reported in wheat bran. High fiber content was reported in *Codiumreediae* and *Gracilaria* species, presenting 23.5 % dw and 64 % dw respectively (Wells et al., 2017).

3.2. Storage and Cell-wall polysaccharides

Two types of algae polysaccharides have been reported. Structural (cell-wall) polysaccharides are mostly located within algal cell-wall while storage polysaccharides are situated in the plastid (Figure II-6). The algal cell wall has an important structure role. It serves as a physical barrier which acts against wave, ice, and sun dehydration. Meanwhile, it could regulate with solute accumulation, turgor, and cell growth (Rioux et al., 2017). The cell wall also ensures the ionic and gaseous exchanges, and participates into the phenomena of adhesion to the rocky support (Jouanneau, 2010).

In the meantime, storage polysaccharides play a central role in the algae life cycle since they are the main source of energy. They serve as a photosynthetic reserve and some of them as osmoregulators. Their contents vary according to algal species and environmental factors including temperature, water's nutrients, and salinity as well as water movement. For *Rhodophyta*, the main storage carbohydrate is floridean which located in the cytoplasm while sulfated galactan (agar and carrageenan) are recognized as cell-wall polysaccharides. Sulfated galactan represents ~70 % of the cell wall constituents versus ~ 7-24 % of cellulose in *Florideophyceae* class. Their contents varied following the seaweed species (Rioux and Turgeon, 2015; Rioux et al., 2017; Xiao et al., 2019).

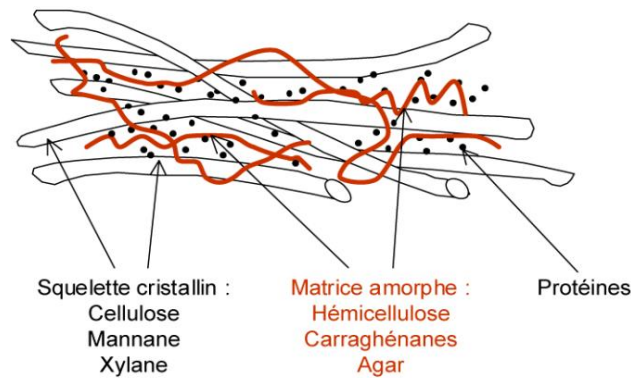


Figure I- 6: Representation schematic of red algae cell wall (Jouanneau, 2010)

3.3. Applications

Carbohydrates have numerous essential roles in living beings. Monosaccharides are the major source of energy for metabolism while polysaccharides are used for energy storage and also for structural components. Furthermore, they have shown with many beneficial health effects, including prebiotic effect and antioxidant or anti-inflammatory activity (Francavilla et al., 2013a). Carbohydrates extracted from *Gracilaria* species have been applied in pharmaceutical and biotechnological industry (Kazir et al., 2019). Agarans and carrageenans are reported with many bioactivities such as: antioxidant, gastroprotective, anti-inflammatory, antinociceptive, anticoagulant, antiviral, anticancer, immunomodulatory, antiproliferative, and antithrombogenic (Barros et al., 2013; Rioux and Turgeon, 2015). The antioxidants activities of sulfated polysaccharides are known as potential agents to reduce cellular damage and to prolong the shelf life of foods (De Castro et al., 2018).

Promising antiviral activity in vitro and low level of cytotoxicity are also reported for sulfated polysaccharides derived from macroalgae (Kwon et al., 2020; Panggabean et al., 2022). So far, sulfated polysaccharides have been presented with antiviral activity to inhibit Herpes Simplex virus (HSV), Human immuno-deficiency virus type-1 (HIV-1), chikungunya virus, and many other enveloped and non-enveloped viruses. Currently, sulfated polysaccharides has shown with the antiviral activity against the on-going pandemic SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) (Hans et al., 2021; Jabeen et al., 2021; Salih et al., 2021). The study of Kwon et al. (2020) has suggested that the treatment of fucoidans (from red algae) nebulized with heparin or possibly TriS-heparin in combination with or without current antiviral therapies, should be assessed first in human primary epithelial cells and then in human patients suffering from COVID-19. In addition, the use of carrageenan nasal spray is also demonstrated to provide efficacy against the patient infected with COVID-19 (Panggabean et al., 2022).

3.4. Food grade carbohydrates in *Rhodophyta*

3.4.1. Agar

3.4.1.1. Description and Structure

Agar was discovered and extracted in Japan by Minoya Tarazaemon since the 17th century (1658) from red algae (Abdul Khalil et al., 2018; Armisen, 1995; Vazhiyil Venugopal, 2011). It is the main cell wall phycocolloids of red algae agarophyte. Agar is a collective term used to describe the mixture of gelling polysaccharides made up of calcium, magnesium, potassium and sodium sulfate esters D- and L-galactose units, linked by alternating α -(1,3)-D-galactose and β -(1,4)-L-galactose units (Lee et al., 2017; Rioux and Turgeon, 2015; Rodríguez et al., 2009).

Agar (Figure I-7) is a complex mixture of different agarans composed of about 70 % of agarose and 30 % of agaropectin (P. Torres et al., 2019). Agarose (high gel strength), agaroids, agarans, and agaropectine (low gel strength) are a part of the red algae galactans which distinguish oneself by different agar components with different conformations and side chain substitution (Lee et al., 2017; Marinho-Soriano and Bourret, 2003). Agarose (molecule of 120 kDa) is the common name of natural polysaccharide composed of repeated D-galactose and 3,6-anhydro-L-galactose units joined by β -1,3- and α -1,4-glycosidic bonds, respectively. This linear structure build up to 70 % of the agar polysaccharides (Lee et al., 2017). On the other hand, agaropectine, a molecule of 12.6 Da, is an acid polymer carrying sulfate, methyl, and methyl pyruvate groups (Rioux and Turgeon, 2015).

Agar is soluble at high temperature (>85 °C) and can form thermoreversible gels. Due to the presence of weak charged groups such as sulfates, the viscosity of agar is lower than that of the carrageenans (Table I-6). Nevertheless, agar seems to be more resistance to hydrolysis under acids conditions (Rioux and Turgeon, 2015). Agar with high molecule weight is desirable to realize stronger gels. Commercial agars comprised between 36 and 144 kDa of the molecular weight whereas agars extracted at laboratory scale have higher masses, presenting at around 340 and 380 kDa (Rioux and Turgeon, 2015).

Table I- 6: Physio-chemical properties of agar and carrageenan (Rhein-Knudsen et al., 2015)

Properties	Agar	Carrageenan
Solubility	Boiling water	Boiling water
Gel Strength (1.5 % at 20 °C)	700-1000 g/cm ³	100-350 g/cm ³
Viscosity (1.5 % at 60 °C)	10-100 centipoise	30-300 centipoise
Melting point	85-95 °C	50-70 °C
Gelling point	32-45 °C	30-50 °C

1 centipoise = N.s.m²

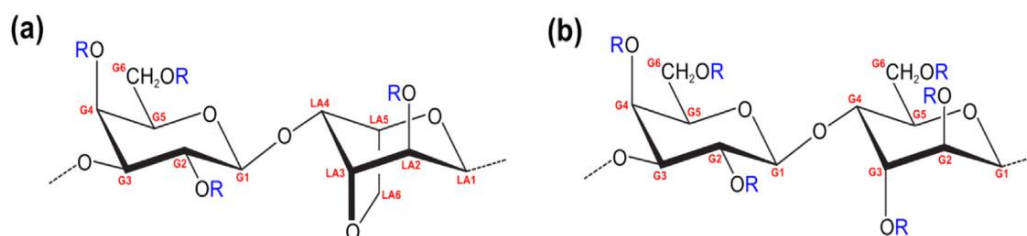


Figure I- 7: Chemical structure of agar repeating unit (a) Agarose structure consisting of alternating β -1,3-linked D-galactose and α -1,4-linked 3,6-anhydro-L-galactose, (b) D disaccharide repeating units of β -1,3-linked D-galactose and α -1,4-linked L-galactose (Lee et al., 2017)

G and *LA* refer to galactose and 3,6-anhydrogalactose units, respectively. *R* can be *H* or side chain substituents such as sulfate ester, methoxyl ether or pyruvic acid

3.4.1.2 Sources

Gelidium and *Gracilaria* genus are the most exploited source for agar extraction. About 20-30 % of agar is observed in cell wall of *Gelidium* versus 15-20 % for *Gracilaria*. The amount of agar in cell wall depends on season, species, and growth conditions (water, temperature, salinity, light conditions, photoperiod, and motion of water) (Lee et al., 2016; Marinho-Soriano et al., 2006; Marinho-Soriano and Bourret, 2003; Rodríguez et al., 2009). The two genus have spread around the globe and can be found in Chile, India, Japan, Madagascar, Mexico, Morocco, Senegal, Spain, Philippines, Portugal, and the south of the United States (Rioux and Turgeon, 2015).

The amount of (3,6)-anhydrogalactoses rings dictates the gelation process, the more there is, the more resistant the gel will be (Rioux and Turgeon, 2015). Low gelling property of agar

is observed by the side chain substituents such as ester, methoxyl group, and pyruvate ketal which can be found in the backbone of agar (e.g. alternating D- and L-galactose). Agar extracted from *Gracilaria* was reported with higher degree of sulfatation than those from *Gelidium* and *Pterocladia*, mainly at the C-6 of the 4-linked-L-galactose which contribute to a poorer gel strength (Lee et al., 2017; Rioux and Turgeon, 2015). Therefore, alkaline treatments have been applied which can convert L-galactose-6-sulfate to 3,6-anhydro-L-galactose, enhancing the gel strength and making *Gracilaria* as an alternative agar resource to *Gelidium* species (Lee et al., 2016; Xiao et al., 2019).

3.4.1.3. Extraction methods

The process of agar extraction is summarized in Figure I-8. For *Gelidium* species, they were pretreated by acid dilution while *Gracilaria* species were pretreated with alkali treatment. This pretreatment step aims to modify the structure of agar by increasing the amount of the (3,6)-anhydrogalactose ring for better gelling properties (Armisen, 1995). After the pretreatment, the extraction is conducted with hot water extraction followed by filtration to remove residual algae fragments. The obtained filtrate is cooled to form a gel then frozen. The ice crystals break the gel during thawing and release water, resulting to a concentrated agar solution. Agar is then dried and milled. Freezing is known as a costly step and can be replaced with syneresis by applying pressure on the gel (Rioux and Turgeon, 2015). This alternative technique is observed with fewer agar impurities than freeze-thaw method (Armisen, 1995).

Many novel and eco-friendly polysaccharides extraction technologies have been highlighted such as pressurized extraction, ultrasound extraction, microwave assisted extraction, and enzyme assisted extraction (Xiao et al., 2019). These methods aim to maximize the yield and maintain highest quality of products. The use of enzyme can soften the cell wall and render target components accessible without any degradation. This method can induce higher extraction yield, lower investment costs and energy, higher reproducibility, low extraction time, and environmental friendly with simple experiment set up (Xiao et al., 2019).

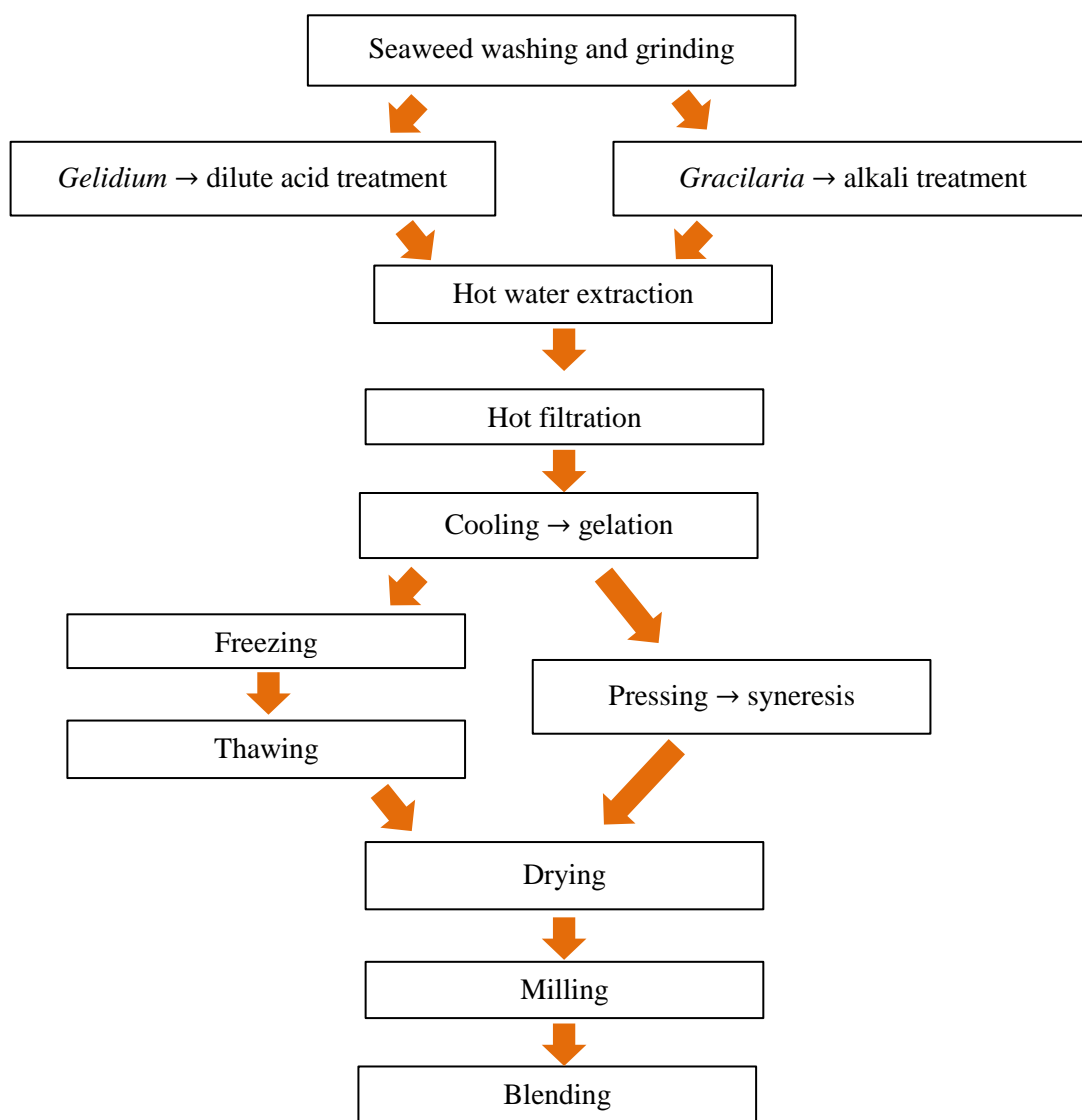


Figure I- 8: Manufacturing steps of Agar from *Gelidium* and *Gracilaria* (Rioux and Turgeon, 2015)

3.4.1.4. Applications

As a hydrophilic gel, agar is the first phycocolloid known as E406 and is approved as GRAS (Generally Recognized as Safe) by the Food Drug Administration (FDA), which can be used as additive in food industry (Abdul Khalil et al., 2018). Agar is employed in food, cosmetic, pharmaceutical, and biotechnological applications due to its gelling, thickening, stabilizing and cryoprotective properties, high biodegradability, and great water holding capacity (Marinho-Soriano and Bourret, 2003; Xiao et al., 2019). The gelling and stabilizing properties of agar allow them to be used in food process with low concentration (0.5-2 %) and

in a wide range of pH. Agar remains stable up to 85 °C and form a gel between 32 °C and 43 °C (Rioux and Turgeon, 2015). The decrease rate of temperature may influence the kinetic of gel formation.

Unlike carrageenan, the gelling mechanism of agar only involves hydrogen bonds whereas for carrageenan, hydrogen bonds and cations are both required (Armisen, 1995). Since agar gel can be induced without addition of potassium or calcium salt, contrary to others polysaccharides, the agar gel is odorless and tasteless, which is interesting for a wide range of applications (Rioux and Turgeon, 2015). The gel syneresis is controlled by agar concentration, holding time, apparent gel strength, rigidity coefficient, pressurization, and total sulfate contents (Rioux and Turgeon, 2015).

Agar is used as stabilizing and gelling agent in bakery products, water gels, confectionery, dairy products, canned meat and fish products, soups and sauces, beverages, pie fillings, icings, meringue, ice cream, and sorbet. In candy, agar can be used in combination with large amounts of sugar to increase the gel strength. It can also be used in the broth of canned meat and fish products due to the high melting point and resistance to autoclaving, making it more appropriate than carrageenan for this application. The first gel role is to protect products during shipping and to prevent compounds found in certain fish from attacking the lining of the can, which would blacken the content (Abdul Khalil et al., 2018; Rioux and Turgeon, 2015). The texture of cheese and cream cheese can be improved with the addition of agar. In the beverage industry, it serves as a flocculating and clarifying agent in the preparation of juices and wines. Agar extracted from *Gelidium* species can be used in synergy with locust bean gum to form transparent gels that are firmer and with less syneresis, offering better mouth feel than agar alone. Agar is still used in traditional Japanese food confectionery; “yokan” (agar jelly with red bean paste), “mitsumame” (canned fruit salad with agar jelly), and “tokoroten” (noodle-like agar gel) (Rioux and Turgeon, 2015).

In dentistry, agar is demonstrating as a good biomedical material due to the thermo-reversible properties (melts and liquefies at high temperature and gel once chilling). In addition, agar is used as growth media for laboratory purpose. The most highly purified agar serves for the separation in molecular biology notably for electrophoresis, immune diffusion, and gel chromatography (Abdul Khalil et al., 2018; Rhein-Knudsen et al., 2015).

3.4.2. Carrageenan

3.4.2.1. Description and structure

Carrageenan is another abundant cell-wall polysaccharide of red algae. Their concentrations range between 30-80 % dw of the cell wall constituents of some Rhodophyta such as *Chondrus crispus*, *Kappaphycus alvarezii* (*Eucheuma spinosum*), and *Eucheuma denticulatum* (*Eucheuma cottonii*) as well as the genus of *Gigartina* and *Hypnea* (Rhein-Knudsen et al., 2015). The content of carrageenan varies depending on algae species and season as well as the growth rate condition. Their structures differ following algae species and extraction method (Rhein-Knudsen et al., 2015; Rioux and Turgeon, 2015).

The molecule of carrageenan is made up of ammonium, calcium, magnesium, potassium, and sodium sulfate esters of D-galactose and (3,6)-anhydro-D-galactose units linked by α -(1,3) and β -(1,4) (Jouanneau, 2010; Necas and Bartosikova, 2013; Rioux and Turgeon, 2015). One or two sulfate groups are found on the galactose unit in positions two and/or six. The three main structures of industrial carrageenan are in the form of kappa (κ), iota (ι), and lambda (λ) (Jiao et al., 2011). Mu- (μ) and nu (ν) -carrageenan, are precursors of κ - and ι -carrageenan after alkali modification during the extraction (Rioux and Turgeon, 2015). Carrageenans differ by the number of sulfate groups and (3,6)-anhydro-D-galactose rings, and also its solubility in potassium chloride (Necas and Bartosikova, 2013; Youssouf et al., 2017). These structural variations influence the hydration properties, strength, texture and temperature of gel formation, and gel syneresis (Rioux and Turgeon, 2015).

Table I- 7: Summary of seaweed sources, hydrocolloid carbohydrate products, and applications of seaweed derived hydrocolloids carrageenan (Rhein-Knudsen et al., 2015)

Seaweed source	Type of carrageenan	Applications
<i>Kappaphycus alvarezii</i>	κ -Carrageenan	Gelling agent (stiff and brittle gel)
<i>Eucheuma spinosum</i>	ι -Carrageenan Gelling	Gelling agent (flexible soft gel)
<i>Gigartina spp.</i> <i>Chondrus spp.</i>	λ -Carrageenan	Thickener
<i>Kappaphycus alvarezii</i>	μ -Carrageenan	κ -Carrageenan precursor
<i>Eucheuma spinosum</i>	ν -Carrageenan	ι -Carrageenan precursor

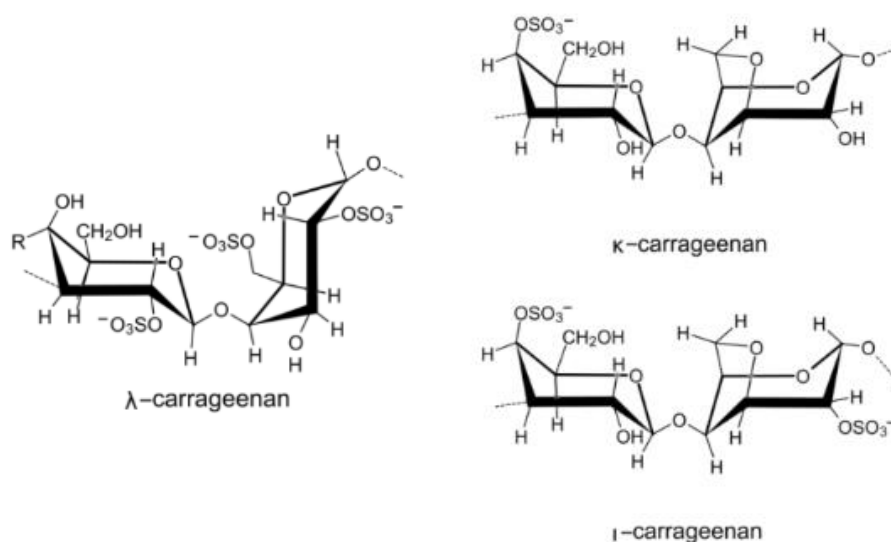


Figure I- 9: Main structures of carrageenan (Necas and Bartosikova, 2013)

3.4.2.2. Applications

Carrageenans have been widely used in food products as gelling, stabilizer, emulsifier, or thickener agents (Rioux and Turgeon, 2015; Youssef et al., 2017). The summary of various application is given in Table I-8 below.

Table I- 8: Principal Food Application of Carrageenan in Dairy Products (Rioux and Turgeon, 2015)

Application	Function	Carrageenan
Flan	Gelling, mouth feel	Kappa, kappa + iota
Cold-prepared custard	Thickening, gelling	Kappa, iota, lambda
Pudding and pie filling	Reduced starch, lower burn-on	Kappa
Ready-to-eat desserts	Syneresis, mouth feel	Iota
Milkshakes	Suspension, mouth feel, stabilize overrun	Lambda
Whipped cream	Stabilize overrun	Lambda
Desserts (mousse)	Stabilize overrun and emulsion	Kappa
Pasteurized chocolate milk	Suspension and mouth feel	Kappa, kappa + lambda
Sterilized chocolate milk	Suspension and mouth feel	Kappa, lambda
Soy beverages	Suspension and mouth feel	Kappa + iota
Evaporated milk	Stabilize emulsion	Kappa
Processed cheese (slice and blocks)	Improve slicing and grating, control melting	Kappa
Cream cheese	Gelling, water binding	Kappa + locust bean gum

3.5. Non-food grade carbohydrates in *Rodhophyta*

3.5.1. Floridean starch

Beside food grade carbohydrates, red algae contain some of non-food grade carbohydrates such as floridean starch, floridoside, isofloridoside, and digeneaside. Floridean starch amylopectin-like glucan is a high molecular weight storage carbohydrate biosynthesized and found in the cytosol of Florideophyceae red algae (Usov, 2011). This carbohydrate has adverse effects on the gel strength of carrageenan and agar, hence it is considered to be an impurity (Rioux and Turgeon, 2015). The Floridean starch is built up of (1, 4)- α -D-glucopyranose residues with a small proportion of (1, 6)-linkage (Figure I-10). It is related to amylopectin and glycogen with intermediate degrees of branching (similar to maize amylopectin) and forms an average linear chain length of 18 glucose units (Rioux and Turgeon, 2015).

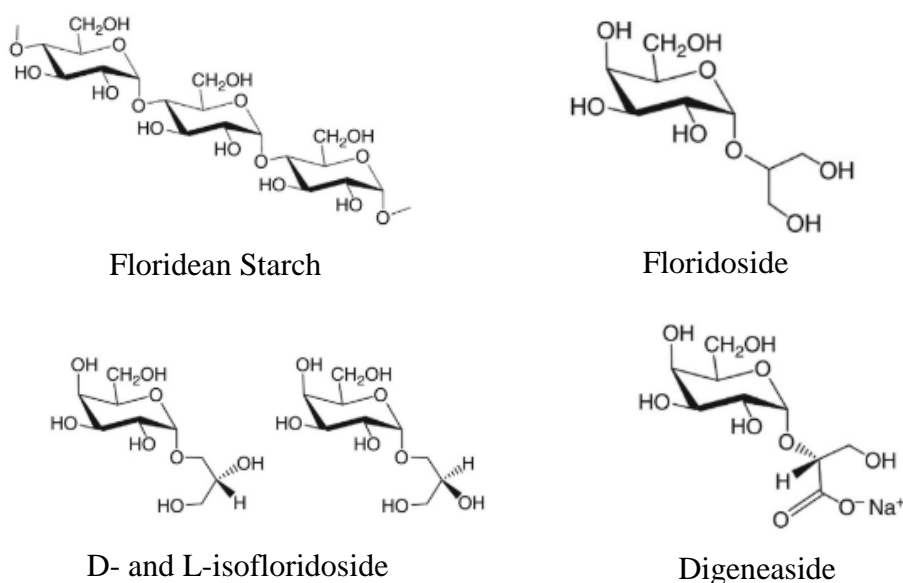


Figure I- 10: Structure of non-food grade carbohydrate from red algae (Rioux and Turgeon, 2015)

3.5.2. Floridoside, isofloridoside and digeneaside

Floridoside, isofloridoside, and digeneaside (Figure I-10) are storage carbohydrates, presenting in low molecular weight. They are served as photosynthetic reserves and osmoregulators. Their synthesis appears to be highly regulated by the nitrogen content of algal tissue (Macler, 1986; Rioux and Turgeon, 2015). Floridoside, 2-o- α -D-galactopyranosylglycerol, can modulate the synthesis of floridean starch and cell wall polysaccharides. The content of floridoside is reported about 1.5 to 8 % dw while the content of digeneaside ranged from 1 to 2.2 % dw (Kirst, 1980). These polysaccharides are reported with several bioactivities such as antioxidant activities, anticomplementary activities (innate immunity), and present the ability to inhibit metalloproteinases (substrate for bifidobacteria fermentation) (Li et al., 2010). Furthermore, they can be used as a prebiotic (Muraoka et al., 2008; Rioux and Turgeon, 2015).

4. Protein

4.1. Overview

Marine macroalgae are reported with many important bioactive compounds including proteins, peptides, peptide derivatives, amino acids, and other amino acid-like active metabolites (Pimentel et al., 2019). Among them, proteins are a fundamental part of macroalgae cell walls. Abundant in algal cell wall, proteins are crucial for growth as well as for regulation of various metabolic processes (Gajaria et al., 2017). They are made up with a long chain of different amino acids which determine the nutritional quality of proteins according to the content, the proportion, and the availability of the amino acids (Francavilla et al., 2013a). Enzymes and bioactive molecules such as pigments and glycoproteins are also classified as proteins (Pimentel et al., 2019).

Protein content defines the whole value of the biomass and its potential as a source of bioactive peptides (Pimentel et al., 2019). Two main factors has reported for protein variation including the chemical composition of the species, and its morphological and structural characteristics (Rosni et al., 2015). The amount of protein in macroalgae varies logically in a wide range according to the species (Kumar et al., 2015), season, geographic distribution, population, cultivation conditions, and nutrients supply during growth phase (Fleurence et al., 2018; Rioux et al., 2017). Furthermore, temperature, light, and water salinity may influence

nutrient supply and nitrogen availability, which turn to influence protein and amino acid content (Pimentel et al., 2019). In winter, macroalgae had low photosynthetic activities, resulting low production of carbohydrate and high protein content (Kadam et al., 2017).

Edible macroalgae with high amount of proteins are a valuable economic source in food industry. Currently, the functional property and the digestibility of macroalgae protein are still limited. Nevertheless, macroalgae proteins are often the by-products of the polysaccharide extraction (Kazir et al., 2019). In addition to polysaccharide extraction, the protein co-extracted protocol can allow the increasing of high added molecules (Ex: Polyphenols) as well as the mitigation of costs and wastes (Kazir et al., 2019).

4.2. Rhodophyta proteins

Some macroalgae may contain up to 50 % dw of protein similarly to the traditional source such as meat, egg, and soybean (Kazir et al., 2019). Brown algae typically have low protein content (1-29 % dw) while the content in green and red algae are higher and comprised between 4 to 50 % dw (Kumar et al., 2015; Rioux et al., 2017; Rosni et al., 2015). Glutamic and aspartic acid represent the highest proportion of amino acids in marine macroalgae (Fleurence et al., 2018; Pimentel et al., 2019). Glutamic acid is the major component of the savory which is known as the fifth basic taste ‘‘umami’’ in Kelp (MacArtain et al., 2007; Wells et al., 2017).

Rodhophyta appears to be an interesting potential source of food proteins (Fleurence, 1999a) and red algae can contain up to 47 % dw of protein (Harrysson et al., 2018). Protein content of different Rhodophyta species are given in Table I-9. In *Gracilaria* species, the protein content is ranged from 5.6 % to 30 % dw (Francavilla et al., 2013a). Red algae proteins are abundant with non-proteinaceous amino acids such as taurine (around 1-1.3 g taurine per 100 grams dw of Nori), a component of bile acids that complex and lower cholesterol in the blood stream (Wells et al., 2017).

4.3. Applications

Proteins play a key role in food processing as well as in the development of food products due to many functional properties which influence consumer acceptance (Suresh Kumar et al., 2014). Phycobiliprotein and lectins are two main known groups of functionally active proteins from algae (Rioux et al., 2017). Protein rich algae can be used for human consumption, food additives, animal feed, fertilizer, and plants growth stimulants as well as bioenergy (Angell et al., 2016; Fleurence et al., 2018). Furthermore, proteins possess many bioactivities such as: antibacterial, antioxidant, immunostimulating, antithrombotic and anti-inflammatory. They can be used for prevention and treatment of hypertension, diabetes, and hepatitis. These health promoting effects make these compounds of great relevance as nutraceuticals (Francavilla et al., 2013a).

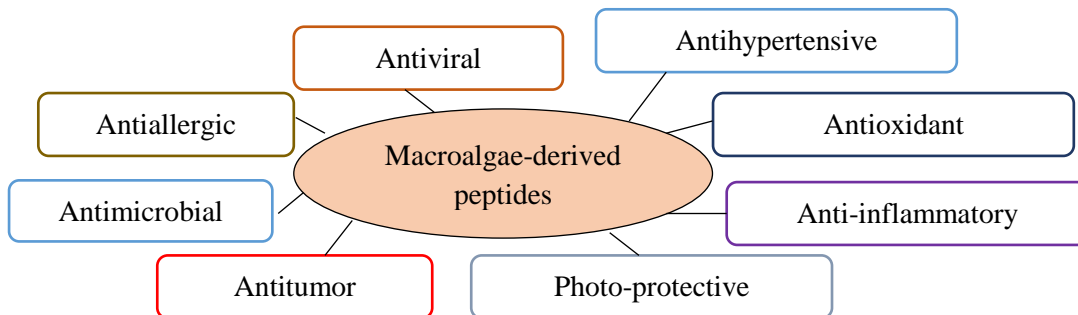


Figure I- 11: Biological activities of macroalgae-derived peptides properties (Pimentel et al., 2019; Pliego-Cortés et al., 2020)

Table I- 9: Protein content, Extraction and quantification of *Rhodophyta* according to the season, source and geographical location (Pliego-Cortés et al., 2020)

Species	Source	Location	Sampling	Protein content % dw	Protein extraction and quantification	References
<i>Rhodymenia pseudopalmata</i>	Wild IMTA	Yucatan, Mexico	July	9.4 ± 0.4 18.7 ± 0.4	Freeze-dried BCA Lowry	(Pliego-Cortés et al., 2019)
<i>Gracilaria conferta</i> <i>Hypnea musciformis</i>	IMTA IMTA Controls	Haifa, Israel Herzliya, Israel	March-August	17.7 ± 1.52 25.32 ± 1.25 ~5 to 10	Dried (60 °C) Kjeldahl N x 6.25	(Ashkenazi et al., 2019)
<i>Chondracanthus chamissoi</i>	Wild	Coquimbo, Chile	January-March	45.2 ± 4.2	Dried (60 °C) EAE, Lowry	(Vásquez et al., 2019)
<i>Chondrus crispus</i>	Wild	North-Central coast, Portugal	April- July October- November	19.5 ± 0.16 19.1 ± 0.33		
<i>Gracilaria sp.</i>	Wild	Portugal	April-July October- November	24.7 ± 0.24 24.4 ± 0.24	Dried (52 °C), Kjeldahl N x 6.25	(Vieira et al., 2018)
<i>Osmundea pinnatifida</i>	Wild	Portugal	April-July October- November	24.3 ± 0.73 22.8 ± 0.33		
<i>Porphyra spp.</i>	Wild	Portugal	April-July October- November	27.4 ± 0.08 28.2 ± 0.16		
<i>R. pseudopalmata</i>	Cultivated	Yucatan, Mexico	30 psu 40 psu	10.5 ± 1.42 4.9 ± 0.43	Freeze-dried BCA Lowry	(Pliego-Cortés et al., 2017)
<i>Palmaria palmata</i>	Wild	Brittany, France	Winter-spring Summer early autumn	21.9 ± 3.5 11.9 ± 2.0	Freeze-dried Kjeldahl N x 6.25	(Galland-Irmouli et al., 1999)

IMTA : Integrated multitrophic aquaculture
N x 6.25 : Nitrogen to Protein conversion factor of 6.25
psu : practical salinity unit

4.4. Proteins Extraction Methods

4.4.1. Principal

The entrapment of certain proteins in the cell wall with extracellular polysaccharides matrix is known as an inevitable obstacle and causes to obtain low protein extraction yield (Kadam et al., 2017; Kazir et al., 2019; Mæhre et al., 2016). Furthermore, protein bioaccessibility and extractability from algae are reported lower than those from animal origin (Mæhre et al., 2016). The extractability of proteins is also affected both by the high viscosity of polysaccharides exert in water solution and by the ionic interactions between the cell wall and proteins. The abundant amount of phenols (mostly in brown seaweed) or trypsin can also decrease the digestibility of proteins by binding the proteins either covalently or by non-covalent forces (Fleurence et al., 2018; Harrysson et al., 2018). In addition, protein digestibility seems to be related to the amount of soluble fiber and therefore prevent bioavailability (MacArtain et al., 2007).

The breakdown of the cell wall for the release of intracellular protein is the optimal way to utilize algal protein (Mæhre et al., 2016). Some protocols such as osmotic shock, mechanical grinding, fermentation breakdown, ultrasonic, and enzymatic degradation of cell wall have been developed for algal protein extraction. The extraction yields vary from one to another (Fleurence et al., 1995b; MacArtain et al., 2007; Mæhre et al., 2016).

4.4.2. Conventional methods

Many algae proteins are extracted by means of aqueous, acid and alkaline extraction, or enzymatic hydrolysis. The recovery of protein is conducted in supernatants part with ultrafiltration, precipitation using ammonium sulfate or chromatographic techniques (Kadam et al., 2017). Alkali soluble protein could represent 7 to 20 % of total algal proteins (Fleurence et al., 1995a).

According to Mæhre *et al.*, (2016), osmotic shock is one of the most common method uses for protein extraction. The cells burst due to the osmotic shock during hypotonic conditions and involve exposing the tissue to water or weak buffer solutions. This method is suitable and efficient for extracting of proteins from animal (Mæhre et al., 2016). On the other hand, protein extraction from plants or algae cell is a complicate process. Mæhre *et al.* (2016) mentioned that the cells hold a defense mechanism against osmotic variations, a mechanism in which intracellular vacuoles containing fluid of high ionic strength are central. When exposed to hypotonic solutions, water or buffer will flow into the vacuole, increasing its size and pushing the other cell organelles towards the cell wall. The intracellular pressure will thus increase, but

the cell wall will prevent the cell from bursting. Mæhre *et al.* (2016) continued that some previous studies have shown that protein extraction protocols based solely on the osmotic shock principle are not very efficient for the extraction of algae proteins. Several extraction protocols aiming the destruction of the cell wall, either by applying mechanical force or by enzymatic treatment, have also been implemented. However, the extraction yields have been limited in most protocols (Mæhre *et al.*, 2016). It is to note that conventional methods are often time consuming, require large amount of solvents, and the efficiency of extraction is limited (Kadam *et al.*, 2017).

4.4.3. Novel methods

New extraction methods such as microwave assisted extraction (MAE), supercritical fluid extraction (SFE), ultrahigh pressure extraction (UPE), pressurized fluid extraction (PFE), pressurized liquid extraction (PLE), and ultrasound assisted extraction (UAE) are being studied and developed in order to improve the protein extraction yields as well as to minimizing the time and resources (Fleurence *et al.*, 2018). Most of the time, the non-conventional techniques improve the mass transfer rate and increase the interactions between solvent and solute, reducing the extraction challenges caused by complex algae matrices (Kadam *et al.*, 2017). Some examples of novel methods are given in Table I-10.

4.4.4. Enzyme approaches

The use of enzyme for cell wall degradation was suggested by Amano and Noda in 1990 in order to facilitate the protein extraction from *Porphyra yezoensis*. Enzymatic mixture including digestive enzymes obtained from the gut of abalone *Haliotis discus* (Disk abalone) and a commercial preparation, Macerozyme R were investigated to improve the protein accessibility (Fleurence, 1999b). This procedure leads to a protoplast production and allows a significantly increase of the protein fraction recovery (+ 5 %) and especially in water soluble proteins (+ 29 %). The protoplast proteins showed an amino acid composition similar to thallus proteins (Fleurence, 1999b).

The degradation of polysaccharides located in cell wall by enzymes is a procedure commonly used for protoplast production and has been used for the isolation of extension, a protein linked to cell wall polysaccharides of higher plants (Fleurence *et al.*, 1995a). Enzymatic approaches are based on the use of cell wall polysaccharide-degrading enzymes to improve extraction yields, modify texture, and facilitate operations (Fleurence *et al.*, 2018). Protease, cellulases, amylases, glucanases, or endoproteases are often used to degrade the algae matrix

and release the proteins. Chemical hydrolysis or subcritical water hydrolysis have been also investigated (Kadam et al., 2017). Different enzymes such as cellulase, xylanase, and β -glucanase were investigated for the release of R-phycoerythrin and protein from red algae *Gracilaria gracilis*. Cellulase was shown to be the most effective enzyme among the three according to the study of Nguyen, 2017.

The enzymatic approaches still need many adaptations according to algae variety (Table I-11). The extraction of proteins under the presence of enzyme is high, meanwhile the digestibility of protein doesn't fully stratify (Fleurence et al., 2002). The use of polysaccharidases as extractive auxiliaries seems to be an alternative method, facilitating access to the algal protein in native or mild conditions. This should be applied in the first treatment of raw material to improve the solubilization of high-value proteins (phycobiliproteins) or to increase the nutritional value of algal proteins by degrading enzymatically the polysaccharides, whose soluble and insoluble fibers have an antinutritional effect (Fleurence, 1999b).

Table I- 10: Novel method for protein extraction from different species

Species	Operating condition	Gain in Protein content	References
Ultrasound Assisted extraction (UAE)			
<i>Ulva sp.</i>	UAE 2h followed by ion exchange purification	70% dw	(Kazir et al., 2019)
<i>Gracilaria sp.</i>		86% dw	
<i>A. nodosum</i>	UAE with amplitude of 68.4 μm	43 % dw (acid treatment) 57 % dw (alkali treatment)	(Pliego-Cortés et al., 2020)
<i>G. pusillum</i>	UAE followed by maceration and buffers	77% dw R-PE 93 % dw R-PC	(Mittal et al., 2017)
<i>Porphyra sp.</i>	UAE with pancreatin hydrolysis	Iodinated proteins raised extraction yields close to 100%	(Romarís-Hortas et al., 2013)
Pulsed Electric Field (PEF)			
<i>U. lactuca</i>	Electric field strength of 7.5 kV/cm with two pulses of 0.05 ms, and a specific energy input of 6.6 kWh/ kg _{prot}	15 % dw	(Postma et al., 2018)
<i>Ulva sp.</i>	50 pulses of 50 kV, applied at a 70.3 mm electrode gap	sevenfold increase in the total protein compared to osmotic shock	(Robin et al., 2018)
Ultrafiltration (UF)			
<i>G. turuturu</i>	An ultrafiltration unit at industrial scale, employing polyethersulfone 25-30 kDa membrane showed	Recovery 100% of R-PE, 32.9% of other proteins and 64.6% of sugars	(Denis et al., 2009b)

Table I- 11: Gains in protein extraction from Enzyme-assisted Extraction of different species

Species	Enzyme used	Protein content and Gains	References
Dry or fresh <i>P.palmata</i>	Pentasonases, Celluclast, Novozym 188, and purified xylanases from <i>Aspergillus niger</i>	Higher liquefaction (>70 %)	(Fleurence et al., 2018)
<i>P.palmata</i>	Xilanase	Increase R-PE extraction from 0.11 to 1.15 g/kg dw (dry ground sample)	(Dumay et al., 2013)
<i>Chondrus crispus</i> , <i>Gracilaria verrucosa</i>	Combinaison agarase/cellulase Carrageenase/cellulase	Increase protein extraction threefold to tenfold	(Fleurence et al., 2018)
<i>Gracilaria verrucosa</i>	Cocktail enzyme (agarase and celluclast)	63 mg/g dw (2 h incubation) 21 mg/g dw (4 h incubation)	(Fleurencel et al., 1995)
<i>Gracilaria gracilis</i>	Cellulase	29.30 mg/g dw (4 h46 min incubation)	(Nguyen, 2017)
<i>Ulva genus</i>	Cellulases, hemicellulases and β -glucanases	Increase the protein solubilisation Recovery yield was close to the extraction in alkali medium	(Fleurence, 1999b)
<i>Chondracanthus chamissoi</i> <i>Macrocystis pyrifera</i>	Cellulose cocktail (Cellic CTec3, Novozymes)	452 mg/g dw of protein 616 mg/g dw of protein Proteolytic activity did not exhibit	(Vásquez et al., 2019)
<i>S. chordalis</i>	Subtilisin protease (Novozymes)	14.7 % of protein extraction	(Burlot et al., 2016)

4.5. Phycoerythrin

4.5.1. Overview

Phycoerythrins (PE) is the most abundant phycobiliprotein (PBPs) in red algae and marine unicellular cyanobacteria (Dumay et al., 2014; Munier et al., 2014). Phycoerythrins from red algae are stable hexameric disk-shaped complexes, presenting 12 nm in diameter and 6 nm high, with the subunit structure $(\alpha\beta)_6\gamma$ (Glazer, 1994). The history of phycoerythrins began from, ‘‘saprocytin’’, a brilliantly blue-colored, red-fluorescent, photo-labile, and water-soluble pigment released by the cyanobacterium *Oscillatoria* species, which described by Nees Esenbeck in the early of 1836. It was renamed in 1843 by Kützing to ‘‘ phykokyan’’ which is more appropriated to the beautiful blue color. In the same year, Kützing also isolated a water-soluble red pigment, the ‘phykoerythrin,’ from a number of red algae (Tandeau De Marsac, 2003). The phycoerythrins were called ‘‘albumineuses substances’’ by Stroke in 1864 (Nguyen, 2017).

Phycoerythrins are divided into four classes which knew as R-phycoerythrin (R-PE), B-phycoerythrin (B-PE), b-phycoerythrin (B-PE), and C-phycoerythrin (C-PE) based on their origin and absorption spectrum (Table I-12). The different types of bilins prothetic groups provoke the different spectral between phycoerythrins (Hemlata et al., 2018; Munier et al., 2014). Phycoerythrins can be easily linked to antibodies and other proteins by conventional protein cross linking techniques, without altering their spectral (Mensi et al., 2014).

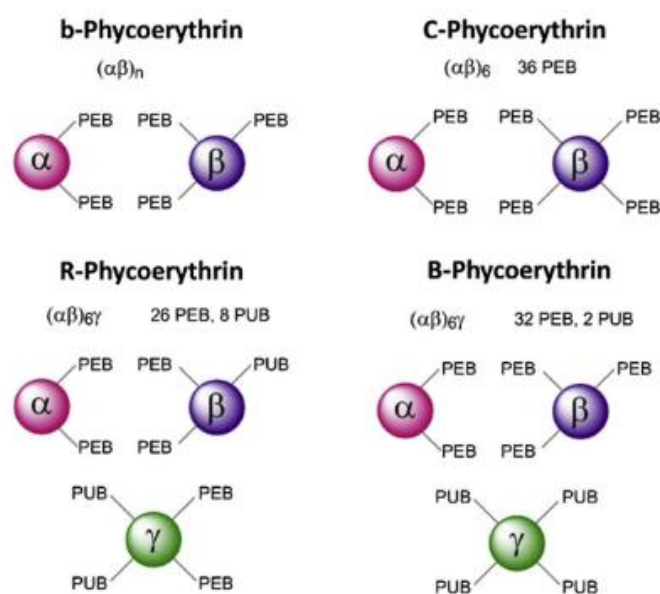


Figure I- 12: 4 classes of phycoerythrins structure (Dumay et al., 2014)

Table I- 12: Spectral Properties of phycobiliproteins (Dumay et al., 2014)

Phycobiliprotein	Color	Prefix	Max. absorption (nm)	Shoulder absorption (nm)	Max. Fluorescence emission (nm)
Phycoerythrocyanin	Orange	–	568/570	595	625
Allophycocyanin	Blue	–	618/671	–	675
Phycocyanin	Blue	C	620	–	640
		R	555/617	–	636
		R	565/540	498	575
Phycoerythrin	Pink	B	565/545	499	576
		b	545	565	570
		C	560	–	577

Prefix: C: Cyanobacteria, R: Rhodophyta, B: Bangiales

4.5.2. Phycoerythrins in *Gracilaria* species

Some species of *Gracilaria* are reported with high phycoerythrins contents. According to values summarized by Nguyen, 2017, the concentration of phycoerythrins in *Gracilaria tenuistipitata*, *Gracilaria lemeneiformis*, *Gracilaria gracilis*, and *Gracilaria folifera* were 22.05 mg/g dw, 8.65 mg/g dw, 1.65-7.5 mg/g dw (Francavilla et al., 2013a) and 3.76 mg/g dw respectively. In *Gracilaria gracilis*, the highest concentration of the three PBPs were reported in winter, the period when light radiation and nutrient concentration are favorable for the biosynthesis of these metabolites (Francavilla et al., 2013a). Phycobliproteins (PBPs) will degrade when nitrogen is deprived and therefore are considered as a source of nitrogen storage (Fernández-Rojas et al., 2014). The molecules of phycoerythrins are unstable to light, low pH, strong ionic strengths, high temperatures, and presence of alcohols. The addition of citric acid or glucose could improve the stability of phycoerythrins and preserve their properties as well as increase the time life (Hsieh-Lo et al., 2019).

Environment factors such as light intensity and quality as well as nutrients can also influence the number and the composition of the phycobilisomes (Barufi et al., 2015; Grossman, 1990). In general, the light intensity is inverse to the total quantity of pigment and chlorophyll a. The phycobilisomes contents vary all over the year and are at maximum in spring

while the minimum is observed from May until end of July. The drop of phycoerythrin content is closely related to the lower biosynthesis due to brightness in summer. In contrast, the content strongly increases when the brightness is low (December, January and February) (Nguyen, 2017).

4.5.3. R-phycoerythrin

R-phycoerythrin (R-PE) is the most abundant PBPs in red algae as well as marine unicellular cyanobacteria (Dumay et al., 2014). It is a 240 kDa oligomeric protein which composed of three polypeptide subunits, forming a complex $[(\alpha\beta)_6\gamma]$: α (18–20 kDa), β (19.5–21 kDa), and γ (30 kDa) (Munier et al., 2015). The γ subunit is located in the center of the molecule and links the $(\alpha\beta)_3$ trimers conferring a high stability pigment (Dumay et al., 2014). This phycoerythrin (Figure I-13) gives a double peak of absorbance at 498 and 565 nm, and a shoulder at 540 nm with a fluorescence emission maximum at 575 nm (Munier et al., 2015). R-PE is a high value compound that costs around 300 euros/mg (Munier et al., 2013a) or 180-250 USD/mg (Fleurence, 2003; Mittal and Raghavarao, 2018) following the purity level.

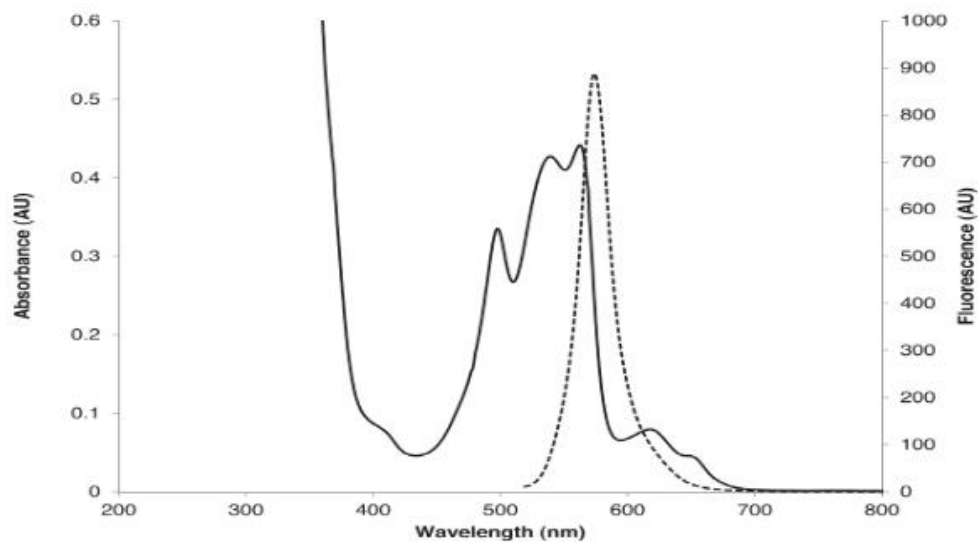


Figure I- 13 : R-PE Absorbance spectrum (solid line) and fluorescence spectrum (dotted line) (Munier et al., 2015)

4.5.4. B-phycoerythrin (B-PE), b-phycoerythrin (b-PE), and C-phycoerythrin (C-PE)

B-PE is observed in red algae of Bangiales order and is commonly discovered in microalgae (Dumay et al., 2014). The B-PE contains the same subunits, forming the same complex $[(\alpha\beta)_6\gamma]$ as R-PE, but presents a different absorption spectrum (Munier et al., 2014). B-PE and R-PE are the most abundant PEs in red algae and their molecular weights are between 240 and 260 kDa, respectively. The content of B-PE can be reported around 2-6 % dw of algae (Dumay et al., 2014).

Beside synthesized by the same organisms as B-PE, b-PE is composed of an $(\alpha\beta)_n$ complex without any γ subunit. The n number can be ranged from 1 to 6. The molecule is smaller than B-PE but presents strong structural similarities (Dumay et al., 2014).

Both C-PE and b-PE are the two phycoerythrins without any γ subunit in $(\alpha\beta)$ complex (Dumay et al., 2014). C-PE is found in cyanobacteria as hexameric or trimeric complexes. In this class of phycoerythrin, the β subunit is linked to four chromophores (all phycoerythrobilin) while in the other PEs, the β subunit carries only three chromophores (Dumay et al., 2014).

4.5.5. Extraction of R-Phycoerythrin (R-PE)

As described in proteins extraction methods, the current protocol focus on how to break the cell wall to allow more protein liberation. The grinding with liquid nitrogen is introduced to facilitate the cell wall destruction. However, this technique doesn't totally guarantee the cell wall destruction and doesn't convince for industry level (Dumay et al., 2014; Le Guillard et al., 2015). Several methods have been developed to obtain PEs from several different algae (Table I-13). PEs extraction can be carried out by soaking algae into water (osmotic shock) for one to several days. This extraction is usually time consuming and PEs can be degraded by protease activity (Dumay et al., 2014). The extraction of phycobiliprotein are also tested with different solvent. Phosphate buffer (5-50 mM, pH 7), distilled water, and seawater are widely used (Pereira et al., 2020; Sudhakar et al., 2015).

Phycoerythrins (PEs) are commonly released in diluted phosphate buffer and then precipitated by salting-out with ammonium sulfate. After desalting by dialysis, PEs are usually purified by various techniques such as expanded bed adsorption chromatography, ion-exchange chromatography, gel filtration, hydroxyapatite chromatography and combination of

two chromatography modes, or preparative electrophoresis. During the extraction and purification procedures, it is uncertain whether the PEs remain in their natural configuration and do not undergo serious structural changes in their conformations and functions (Kannaujiya et al., 2019; Munier et al., 2014). The purification of phycobiliproteins from macroalgae often begins by homogenization in a high speed blender. Cell debris are removed by centrifugation and then ammonium sulfate is added in sufficient amounts to precipitate the phycobiliproteins. One or more ion exchange steps, either on resins or hydroxylapatite, are then used to produce nearly homogenized protein. Later, gel filtration or crystallization is used as a final clean-up step (Kronick, 1986).

So far, enzyme approaches and microwave assisted extraction have introduced into protein extraction methods. The enzyme assisted approaches can be suitable for high added valued compounds, notably R-PE and proteins. Confined and inaccessible compounds can be released due to the enzymatic tissue disruption (Dumay et al., 2014; Sudhakar et al., 2015). Nevertheless, ultrasound assisted extraction, a part of non-conventional method, presents a significant reduction in extraction time and solvent consumption while higher extraction yields can be obtained at lower temperatures which is suitable for thermolabile compounds. Furthermore, high pressure-assisted extraction is highly efficient and short time consuming. This method is easily to operate, highly mechanized, and requires low amount of solvents. It can operate at room temperature which can protect the compound's bioactivity notably heat sensible chromoproteins (Pereira et al., 2020). Therefore, the extraction yield is highly influenced by numerous factors such as the biomass/solvent ratio, the cellular disruption method, the type of solvent used, and the extraction time (Pereira et al., 2020).

4.5.6. Applications of phycobiliproteins

Phycobiliproteins have been recognized as a very useful fluorescent probes due to their excellent spectroscopic properties, stability, high absorption coefficients, and high quantum yields. This protein is highly soluble in water and exhibits a large Stokes shift which is very important for detection. It is reported as a non-harmful substance to human when apply to an external surface or ingested. They have been used as photosensitizers for treatment of tumors and have potential to substitute Photofrin (a kind of light sensitive agent in photodynamic therapy) in common use which is purified from animal blood (Francavilla et al., 2013a).

Other applications of phycobiliproteins can be found in food and cosmetic industry as natural colorants (Francavilla et al., 2013a; MacArtain et al., 2007; Rioux et al., 2017). The food colors market is expected to be worth 3.75 billion USD by 2022, and natural food colors will fill the greatest share of this projection (Aryee et al., 2018). This compound is also used in fluorescent labelling, flow cytometry, fluorescent microscopy, and fluorescent immunohistochemistry (Bleakley and Hayes, 2017; Dumay et al., 2013; Munier et al., 2013b). Moreover, several biological activities have been reported in R-PE including anti-inflammatory, anticancer nature, antitumoral, antioxidant, antidiabetic, immunosuppressive, and antihypertensive (Dumay et al., 2013; Hemlata et al., 2018; Hsieh-Lo et al., 2019).

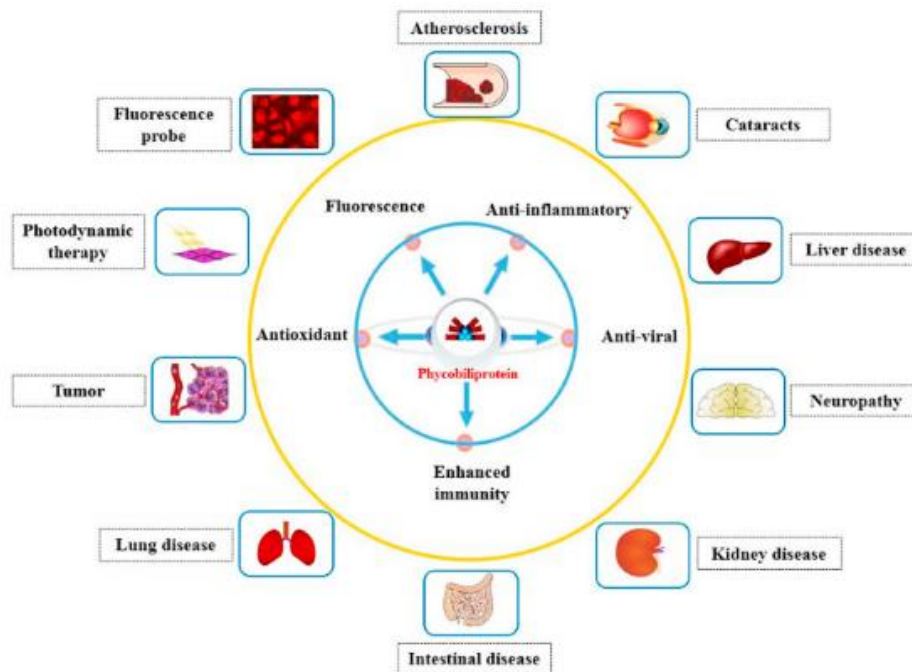


Figure I- 14: Applications of phycobiliproteins in science and health (Li et al., 2019)

Table I- 13: Procedure used for R-phycoerythrin extraction and purification from different *Rodhophyta* (Dumay et al., 2014; Y. Xu et al., 2020)

Protocol	Species	Extraction yield (mg/g dw)	Purity (PI)	References
(NH ₄) ₂ SO ₄ + GF + IE + UF + GF	<i>Corallina officinalis</i>	0.117	4.70	(Hilditch et al., 1991)
Hydroxyapatite + GF	<i>Corallina elongata</i>	0.600	6.67	(Rossano et al., 2003)
(NH ₄) ₂ SO ₄ + IE + UF	<i>Ceramium isogonum</i>	0.038	2.10	(Kaixian et al., 1993)
HIC + IE	<i>Gracilaria verrucosa</i>	0.141	4.40	(Wang, 2002)
(NH ₄) ₂ SO ₄ + GF	<i>Gracilaria longa</i>	0.500	4.50	(D'Agnolo et al., 1994)
(NH ₄) ₂ SO ₄ + IE	<i>Gracilaria corticata</i>	0.780	1.10	(Sudhakar et al., 2014)
(NH ₄) ₂ SO ₄ + IE	<i>Gracilaria crassa</i>	nd	3.79	(Sudhakar et al., 2015)
Aqueous two-phase system (ATPS)	<i>Gracilaria gracilis</i>	nd	3.50	(MENSI et al., 2014)
EI	<i>Grateloupia turuturu</i>	1.410	2.89	(Munier et al., 2015)
ATPS (PEG 1450/K ₃ PO ₄)	<i>Geldium pusillum</i>	0.720	1.10	(Mittal et al., 2019)
2GF + IE	<i>Heterosiphonia japonica</i>	0.940	4.89	(Sun et al., 2009)
Preparative electrophoresis	<i>Palmaria palmata</i>	0.450	3.20	(Galland-Irmouli et al., 2000)
HIC + IE	<i>Porphyridium cruentum</i>	0.825	4.60	(Bermejo et al., 2007, 2003)
(NH ₄) ₂ SO ₄ + IE	<i>Polysiphonia urceolata</i>	0.670	5.60	(Liu et al., 2005)
HIC + hydroxyapatite	<i>Polysiphonia urceolata</i>	0.340	3.90	(Niu et al., 2006)
HIC + IE	<i>Polysiphonia urceolata</i>	0.400	3.26	(Niu et al., 2006)
(NH ₄) ₂ SO ₄ + hydroxyapatite	<i>Porphyra yezoensis</i>	0.940	3.20	(Cai et al., 2012)
(DES-ATPS) (Choline chloride-urea/ K ₂ HPO ₄)	<i>Porphyra yezoensis</i>	0.699	3.82	(Y. Xu et al., 2020)
(NH ₄) ₂ SO ₄ + IE	<i>Portiera hornemannii</i>	0.810	5.21	(Senthilkumar et al., 2013)

HIC : Hydrophobic interaction chromatography, UF : Ultrafiltration, IE : Ion exchange, GF : Gel filtration, ATPS : Aqueous two-phases system
DES-ATPS: Deep eutectic solvent aqueous two-phases system

5. Enzyme-assisted extraction

5.1. Enzymes

Enzymes are the biocatalysts which generate the products from the substrates. They are known as proteins in a single or different chain which contains the active site, an area where the interaction takes place and it contains key amino acids residues for the catalysis reaction (Kumar et al., 2020). The largest group of proteins has been recognized as enzymes. Their role is to accelerate metabolic reactions and are essential in the fight against pathogenic organisms, and in other processes (Martínez Cuesta et al., 2015; Puneekar, 2018; Vandenberghe et al., 2020). Enzymes as other catalysts are not consumed or changed within the reaction and has the possibility to recycle/reuse for many cycles of catalysis reactions (Dumay, 2006).

The activities and functions of enzymes depend on many characteristics, including their sequence, three-dimensional (3D) structure, stability, and interactions with other molecules (Schomburg et al., 2017). The enzymatic reaction occurs under certain energy conditions, corresponding to a short towards high-energy transition state, where the substrate (S) is changed into the product (P). The enzyme catalytic reaction begins with the formation of enzyme-substrate (ES) complex which is dissociated later into a product. The reaction equation is generally described as: $E + S \rightarrow ES \rightarrow E + P$ (Vandenberghe et al., 2020).

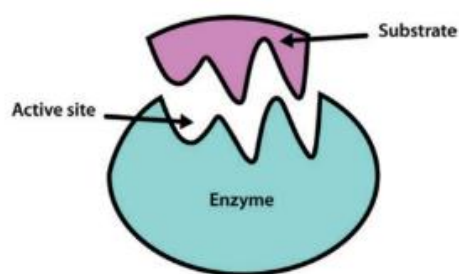


Figure I- 15: Representation of substrate binding to (Lock and key theory) the active site of enzyme (Robinson, 2015)

5.2. Enzymes classification

Enzyme's nomenclature and classification are defined by the standards of Enzyme Commission (EC). Until now, seven classes of enzyme have been recognized including Oxidoreductases (EC 1), Transferases (EC 2), Hydrolases (EC 3), Lyases (EC 4), Isomerases (EC 5), Ligases (EC 6), and Translocases (EC 7) (Figure I-16). Every enzyme is identified with an EC number which is composed of four digits (Dumay, 2006; Vandenberghe et al., 2020). Each number refers to different nomination as below:

- First number represents the class of the enzyme
- Second number represents the subclass
- Third number indicates the sub-subclass
- Fourth number indicates the serial number of the enzyme in its sub-subclass

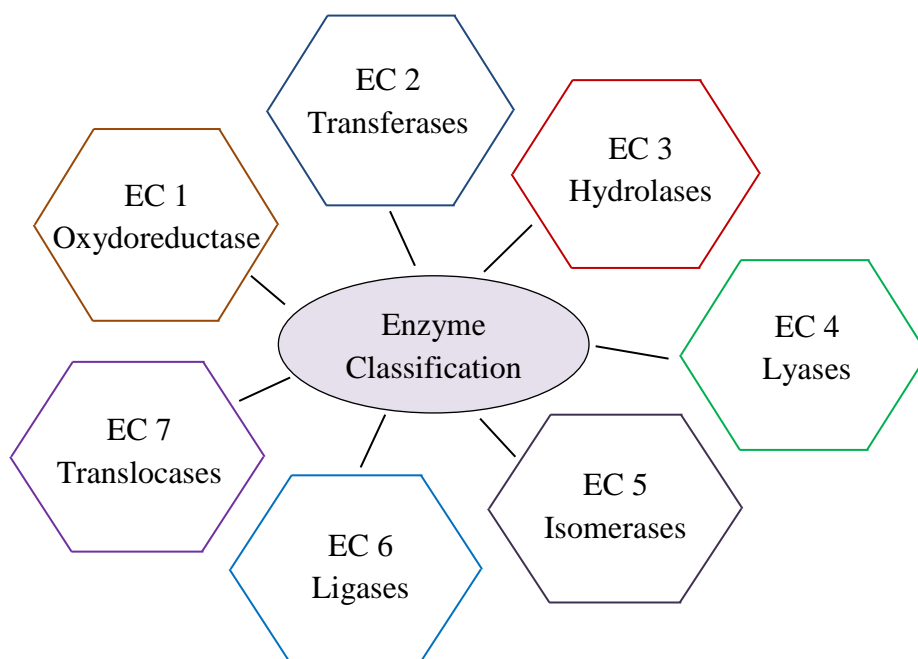


Figure I- 16: Enzymes classification (Vandenberghe et al., 2020)

The catalyzed reactions with each enzyme class have been summarized in the following table.

Table I- 14: Catalysis reaction and examples of each enzyme classification (Vandenberghe et al., 2020)

Enzyme classification	Catalysis reaction	Example
EC 1: Oxydoreductases	Oxydoreaction reaction	Dehydrogenases, Oxydanases, Reductases
EC 2: Transferases	Catalyze group transfer	Transaldoases, Methyltransferases, Transaminases
EC 3: Hydrolases	Catalyze the hydrolytic cleavages of C-O, C-N and C-C	Glycosidases, Peptidases, Nucleases
EC 4: Lyases	Catalyze cleavages of C-C, C-O, C-N and other bonds by elimination	Decarboxylases, Aldonases
EC 5: Isomerases	Catalyze geometric or structural changes in one molecule	Racemases, Epimerases
EC 6: Ligases	Catalyze the junction of two molecules	Synthases, Carboxylases, Ligases
EC 7: Translocases	Catalyze the movement of ions or molecules across membranes or their separation in membranes	ABC-type transporters

The works carried out in this study utilized **Cellulases and Proteases**. Both enzymes are classified under hydrolases class (EC 3) which is able to hydrolyze various bonds. The hydrolases class has devised into different sub-classes as below:

- EC 3.1: Hydrolases acting on ester bonds as well as the thioester hydrolases
- EC 3.2: Glycosylases acting on glycosidic bonds and release at least one osidic compound
- EC 3.3: Hydrolases that act on ether bonds
- EC 3.4: Hydrolases that act on peptide bonds

5.2.1. Cellulase

In this study, the employed cellulase enzymes (EC 3.2.1.4) come from Glycosylases (EC 3.2) subclass and Glucosidases sub-subclass (EC 3.2.1). Cellulase forms a complex of cellulolytic enzymes that act synergistically on the conversion of cellulosic substrates to glucose. They have the ability to hydrolyze the β -1,4-glucosidic linkages of cellulose, producing mainly glucose, cellobiose, and cello-oligosaccharides (Vandenberghe et al., 2020).

This enzyme has been reported as the third largest group of industrial enzymes and their demand has increased since 1995 in several industrial applications such as detergent, textile, animal feed, food, paper, and biofuel industries. Large spectrum of microorganisms such as bacteria, fungi, protozoa, and some animal species (termites and crayfish) are the major sources for cellulase extraction. The production of cellulase is traditionally carried out in batch fermentation processes, mainly using the fungi of the genera *Trichoderma* and *Aspergillus* (Vandenberghe et al., 2020).

5.2.2. Protease

Protease (EC 3.4.21.62) was classified under Hydrolases class that act on peptide bonds subclass (EC 3.4) and Serine endopeptidases sub-subclass (EC 3.4.21). Proteases (peptidases or proteinases) are a group of hydrolytic enzymes that cleave peptide bonds in protein molecules (Vandenberghe et al., 2020). The catalyzed action takes place between two adjacent amino acids in the primary sequence of a protein, thereby generating at least two peptides. This hydrolysis generates the release of H^+ protons, which will induce an acidification of the medium when initial pH value is above 6.5. The reaction is reversed once the pH is lower and therefore OH^- will be dissociated (Dumay, 2006).

According to the EC, the hydrolases subgroup EC 3.4 is classified according to substrate specificity, active site, charge, molecular size, and catalytic mechanisms. The proteases classification is mainly based on the catalyzed reaction and their site of action. Endopeptidases sub-subclass (EC 3.4.21-3.4.25, 3.4.99) are reported to hydrolyze in the middle of the polypeptide chain (Vandenberghe et al., 2020). Furthermore, serine proteases EC 3.4.21 contribute to one-third of all known proteases and are produced from *Bacillus* sp. at industrial production scale.

Table I- 15: EC tree of Cellulases and Proteases employed in the current study

	Cellulases	Proteases
Class	EC 3 : Hydrolases	EC 3 : Hydrolases
Sub-class	EC 3.2: Glycosylases	EC 3.4 Peptidases acting on peptide bonds
Sub-subclass	EC 3.2.1: Glucosidases (hydrolases on both O- and S-glycosidiques)	3.4.21: Serine endopeptidases
EC number	EC 3.2.1.4	EC: 3.4.21.62
Synonym	1,4-(1,3:1,4)- β -D-Glucan 4-glucanohydrolase	Proteinase from <i>Bacillus licheniformis</i> , Subtilisin® A, Subtilisin® Carlsberg
Sources	<i>Aspergillus niger</i>	<i>Bacillus licheniformis</i>
Supplier	Sigma Aldrich	Sigma Aldrich

5.3. Influence factors on enzyme-assisted extraction

5.3.1. Influence of enzyme concentration

For a fix substrate concentration, the variation of the reaction rate does not show the linear relationship with the enzyme concentration. The hyperbolic pattern was reported after a certain enzyme concentration. This phenomenon corresponded to the fact that the entire substrate becomes more complicate at above a certain enzyme concentration. Hence, it is preferable to choose the linear pattern which could limit enzyme concentration and provided maximum efficiency. In the meantime, the costs have been minimized (Figure I-17) (Dumay, 2006).

5.3.2. Influence of substrate concentration

With the fix enzyme concentration, when the substrate concentration is increased, the rate of reaction increases considerably in the beginning of the reaction. However, as the substrate concentration continues to increase, the reaction rate starts to decline, until a stage where increasing the substrate concentration has little further effect on the reaction rate. At this point the enzyme is considered to reach close to the saturation with substrate, which demonstrates its maximal velocity (V_{max}) (Figure I-17) (Robinson, 2015). In addition, higher substrate concentration can influence the enzyme activities in an unspecific manner such as substrate inhibitors (Bisswanger, 2014).

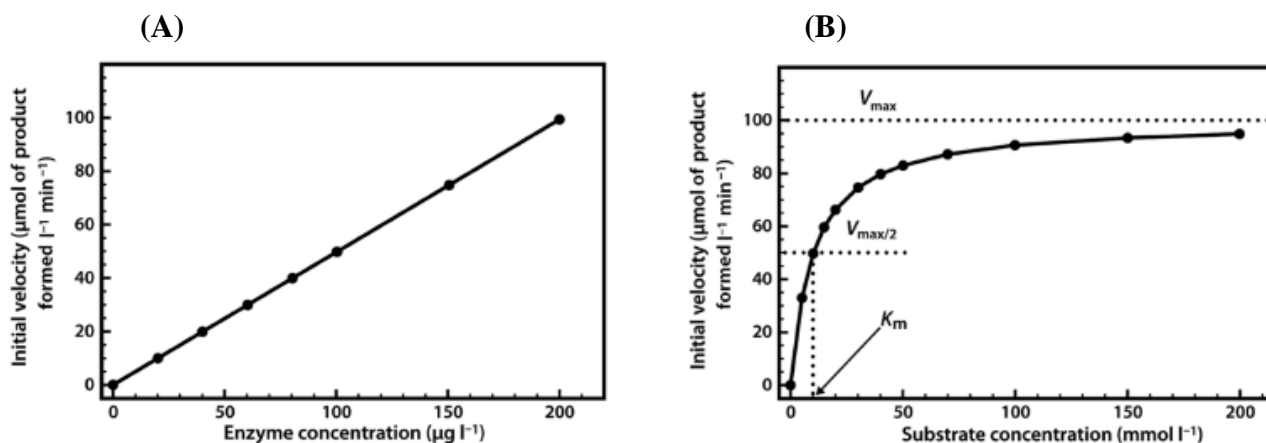


Figure I- 17: Relationship between Enzyme concentration (A) and Substrate concentration (B) with the rate of enzyme catalyzed reaction (Robinson, 2015)

5.3.3. Influence of temperature

The effects of temperature on enzyme activity can be regarded as two forces, acting simultaneously but in counter-acting directions. The rate of molecular movement increases once the temperature surges and therefore the rate of reaction increases. However, as the temperature increased, there is a progressive inactivation caused by denaturation and deactivation of the enzyme protein (Bisswanger, 2014; Dumay, 2006; Robinson, 2015). The result of these two effects represents an asymmetrical curve passing through a maximum value for an optimal temperature (Dumay, 2006). The denaturation by temperature becomes important and varies from one enzyme to another. Generally, it is negligible below $30\text{ }^{\circ}\text{C}$, and starts to become noticeable above $40\text{ }^{\circ}\text{C}$ (Figure I-18). Typically, enzymes derived from microbial sources show much higher thermal stability than those from mammalian sources (Robinson, 2015).

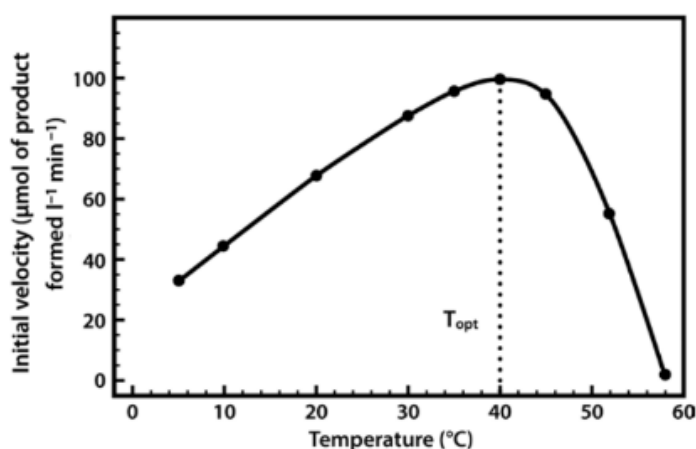


Figure I- 18: The effect of temperature on enzyme activity (Robinson, 2015)

5.3.4. Influence of pH

Enzymes activities depend strictly on the pH (Bisswanger, 2014). As the pH changes, the ionization state of the groups at the enzyme's active site and on the substrate can alter, influencing the binding rate of the substrate to the active site (Dumay, 2006; Robinson, 2015). The optimum pH is defined according to the enzymatic transformation of a substrate in a given composition medium (Dumay, 2006). Most of enzymes activities follow a bell-shaped curve, increasing from zero (strong acid region) up to a maximum value and decreasing to zero to strong alkaline region (Bisswanger, 2014). The velocity of a reaction becomes negligible within ± 2 pH units from pH optimum. Meanwhile, the value of optimum pH varies depending on enzymes (Bisswanger, 2014; Dumay, 2006). The optimum pH of enzyme is recommended not only for catalytic reactions but also for storage since enzyme is fairly stable at its own pH optimum (Bisswanger, 2014).

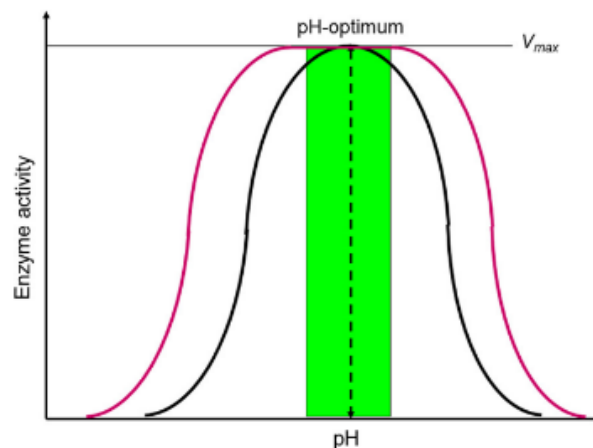


Figure I- 19: pH optimum curve for the activity of an enzyme (black). The green area shows the physiological range. The red line shows the broader pH stability curve of the enzyme (Bisswanger, 2014)

5.3.5. Influence of buffers solutions

Buffers solutions play the role to adjust and stabilize the desired pH during the enzyme assay. Ionic strength and concentration, and the nature of buffer components are two criteria to be taken into consideration of buffer. A more concentrated buffer system has higher capacity to stabilize the pH. Meanwhile, most enzymes accept only moderate ionic strength (commonly between 0.05 and 0.2 M except halophilic and thermophilic enzymes which prefer higher concentrations up to 1 M). Furthermore, low ionic strength can destabilize the protein structure (Bisswanger, 2014).

5.3.6. Influence of inhibitors

5.3.6.1. Reversible inhibition

- Reversible inhibitors/ competitive inhibitors

Reversible inhibitors are recognized when they bind reversibly to an enzyme. Competitive inhibitor is usually a molecule that is structurally similar to the substrate which may be able to bind reversibly to the enzyme's active site (Robinson, 2015). As an example, malonate is a competitive inhibitor of the enzyme succinate dehydrogenase due to the similar structure with succinate. This type of inhibitors can be evacuated from the active site with the presence of high concentration of substrate, thereby restoring enzyme activity (Robinson, 2015). However, for some enzymes, high concentration of either substrate or product can induce an inhibition. The presence of high concentration of sucrose, substrate of Invertase, considerably reduces the Invertase activity, whereas the β -galactosidase of *Aspergillus niger* is strongly inhibited by its own product (the galactose) (Robinson, 2015).

- Non-competitive inhibitors

Non-competitive inhibitors on the other hand react at a distinct site from the active site. The binding of this type of inhibitor does not physically block the substrate and active site binding, but it does prevent subsequent reaction. Most of non-competitive inhibitors are chemically unrelated to the substrate, and their inhibition cannot be overcome by increasing the substrate concentration. These inhibitors reduce the concentration of the active enzyme in solution. Uncompetitive inhibition is rather rare, occurring when the inhibitor is only able to bind to the enzyme once a substrate molecule is itself bound (Robinson, 2015).

5.3.6.2. Irreversible inhibitors and poison

Irreversible inhibitors have been known as an inhibitor that binds permanently to an enzyme. Many irreversible inhibitors are recognized therefore as potent toxins (Robinson, 2015). For example, diisopropyl fluorophosphate (DFP) inhibits acetylcholinesterase activity by reacting covalently with an important serine residue found within the active site of the enzyme, resulting in the constant propagation of nerve impulses and can lead to death (Robinson, 2015).

5.3.7. Influence of substrates and cofactors

Substrates, co-substrates, and cofactors as well as enzyme itself are the most important components of the enzyme assay. It is very important to look at their state (intactness or degradation), their purity, and their stability. Naturally, enzymes have a defined substrate

according to its physiological function. However, many enzymes present broad specificity, accepting also the substances which structurally related to the physiological substrate. As an example, alcohol dehydrogenase could react with various alcohols. This is also the same observation for cofactors (Bisswanger, 2014).

Cofactors are non-protein components and can be categorized into prosthetic groups and coenzymes, depending on their type of association with the enzymes. Prosthetic groups gather small molecules, which remain bounded to the enzyme (Kumar et al., 2020) and are indispensable for catalytic reaction (Bisswanger, 2014). Cofactors are commonly metal ions or some organic molecules or vitamins (Vandenberghé et al., 2020). Meanwhile, coenzymes are dissociable non-protein components and usually carry chemical group (Kumar et al., 2020).

5.3.8. Influence of solvent

Water is the standard solvent for enzyme assays following the cellular milieu. However for some special cases, organic solvents cannot be completely inevitable (apolar organic solvent are needed when applying lipases enzymes) (Bisswanger, 2014). Solvent play an important role on protein dynamics. Protein motion is considered to be Brownian; therefore, the diffusive motion depends upon the friction resulting from the surrounding solvent environment. Ideally, a mobile solvent should support faster motion and flexibility. In contrast, a cage of more static/viscous solvent (glycerol, ethanol, and ethylene glycol) should promote stability of protein by restricting its motion (Kumar et al., 2020).

6. Reactive extrusion

6.1. Principal

Extrusion technology is a combination of thermal and mechanical treatments where the material could be subjected to high temperature and shear for a short time (Aktas-Akyildiz et al., 2020). This technology involves numerous independent and dependent variables that provides thermal and mechanical energy to the extruding materials, inducing chemical and physical changes, at both macroscopic, and microscopic scale. In the meantime, independent variables are temperature, moisture content, blend composition, screw speed, die size and configuration, and screw configuration while dependent variables are related with specific mechanical energy, residence time, torque, pressure, etc. (Singh et al., 2019). The extrusion generates many different roles. This process consists of conveying and manufacturing product with specific cross-sectional profiles, imparting of temperature and/or stress to induce morphological changes. It can use for induction and/or acceleration of chemical reactions such

as reactive extrusion. Further roles are also reported such as mixing, separation, heating or cooling, co-extrusion, reduction in moisture and volatile levels, flavor generation, encapsulation, and sterilization (Wolf, 2010).

Reactive extrusion induces chemical or biological reactions together with the thermo-mechanical treatment inside the extruder (Berzin & Hu, 2004). The concept of enzymatic reactive extrusion is recently proposed in order to generalize the simultaneous enzyme and extrusion behaviors in the wider fields (E. Xu et al., 2020). As a continuous process, reactive extrusion becomes an interesting alternative toward batch process (Baron et al., 2010) and is shown to handle better the very viscous material (Gatt et al., 2018). Reactive extrusion has especially been studied in continuous monomers polymerization in bulk reactions, avoiding the use of solvents while enhancing process productivity and flexibility (Gatt et al., 2018).

6.2. Extruder

Extruder generally consists of single-screw or twin-screw (either co-rotating or counter-rotating) located inside a stationary cylindrical barrel. At the end-plate, die is connected to the end of the barrel which determines the shape of extruded sample (Maniruzzaman et al., 2012). The ratio between length and diameter (L/D) of the screws is one of the important characteristic for extruder. The typical ratio (L/D) ranges from 15-50:1 (mm) (Berzin & Hu, 2004) or 20-40:1 (mm) (Maniruzzaman et al., 2012) can be observed either in a single-screw or twin-screw extruder.

6.2.1. Single-screw extruder

Classically, single-screw extruder consists of one rotating screw inside a stationary barrel (Maniruzzaman et al., 2012). Extrusion in single-screw extruder depends on friction force which ensures the transport of the material (Guy, 2001; Makoure, 2019). The efficiency depends on material adhesion to the barrel. If the material sticks to the screw, the material and the screw will turn together in the barrel, and the material will not reach the die (Vauchel et al., 2012). The more material sticks to the screw, the more difficult progress in the screws barrel will occur. In contrast, the material easily progress to the outlet when the high coefficient of friction is reported (Makoure, 2019). Single-screw extruder is reported with relatively poor mixing ability; hence, it is usually supplied with preconditioned premixed material with added steam and water. Having only one shaft, single-screw extruder will not self-clean as completely at the end of the operation (Guy, 2001).

6.2.2. Twin-screw extruder

A more advanced extruder, twin-screw extruder consists of two rotating system positioned in the stationary barrel where extrusion can be performed by either co-rotating or counter-rotating from the screw configuration (Makoure, 2019; Maniruzzaman et al., 2012).

Twin-screw extruder is naturally more complicated than single-screw extruder, but at the same time provides much more flexibility and better control (Vauchel et al., 2012). It can handle very viscous, oily, sticky, and wet materials. Twin-screw extruder provides greater ability and flexibility for controlling both product and process parameters. Containing two shafts, the inside of the barrel is swept to clean and the two screws swipe each other. At the end of the operation, some steam and water usually can clean the extruder barrel from the inside (Guy, 2001). Feed rates and screw speed are independent and can be individually chosen to yield desired shear levels, energy inputs, and product characteristics (Altomore and Ghossi, 1986). Twin-screw extruder can regulate the operating temperature via external heat transfer and it has the ability to regulate the throughput (Altomore and Ghossi, 1986).

6.2.2.1. Principal components of twin-screw extruder

Typical twin-screw extruder consists of following assembles as shown in Figure I-20 (Vauchel, 2007; Vergnes and Chapet, 2001).

- A shearlock (barrel) assembly with its heating and cooling accessories
- Screws (single or twin)
- Main motor with variable speed and a gearbox that drives the screws in rotation
- Feeding device with variable flow rate (Hopper)
- Die (a device for cutting or shaping the extruded material), if necessary
- Control cabinet

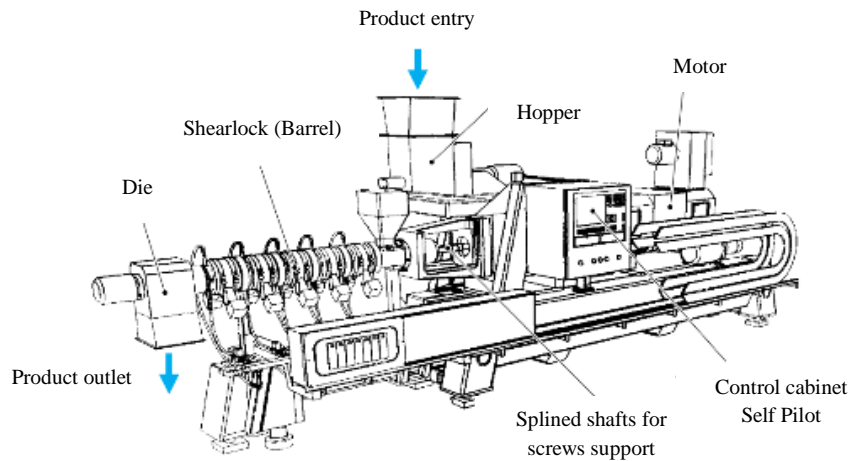


Figure I- 20: Diagram of twin-screw extruder (Vergnes and Chapet, 2001)

6.2.2.2. Screws profiles

The screws of an extruder are characterized by a highly versatile screw configuration which allows various constraint profiles. It can handle different substrates with different viscosities, rheological behaviors and phases, making easy adapted conditions (Gatt et al., 2018). In twin-screw extruder, screws configuration can be generated as co-rotating or counter-rotating with intermeshing or non-intermeshing (Figure I-21). The kneading bloc and the screw section are presented in Figure I-22 below.

For non-intermeshing twin-screw extruder, the counter-rotating type is more effective at pumping material than the co-rotating type that is often operated to push a low viscosity non-cooked feed material (Guy, 2001; Shuyang, 2018). Extruders with intermeshing usually have a very short barrel due to the intermeshing design generates more wear and tear on the screws and barrels. However, this type of screw design are known for better at pumping and mixing (Guy, 2001; Shuyang, 2018). Co-rotating and intermeshing extruders have the most advance control panel as they are designed to protect the extruder and could operate from dangerous conditions, resulting this type of extruders are the most used extruder-type. This type of extruder can operate with the most variables of food material (from high to low of fat, sugar, starch, and protein as well as from high to low of viscous materials) (Shuyang, 2018).

Twin-screw extruders are usually composed with different screws such as forward pitch screw, monolobe paddle, bilobe paddle, and reversed pitch screw. Forward pitch screws are responsible for conveying action, while monolobe paddle screw exert a radial compression and shearing action. Bilobe paddle screws boost significant mixing and shearing as well as conveying, and axial compressing in combination with the forward pitch screws. Reverse pitch

screws are used for an intensive shearing and moderate mixing but also exert strong axial compression in combination with forward pitch screws (Amalia Kartika et al., 2005).

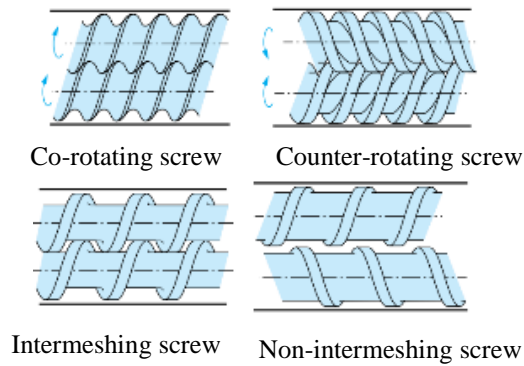


Figure I- 21: Principal configurations of twin-screw extruder (Vergnes and Chapet, 2001)

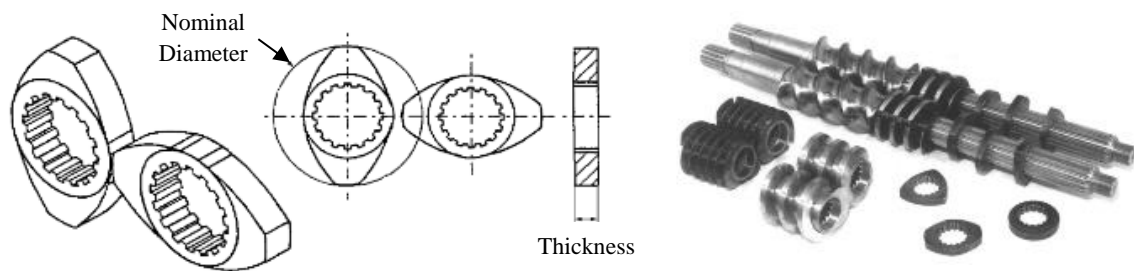


Figure I- 22: Kneading bloc (left), Screw sections and splined shafts (right) (Vergnes and Chapet, 2001)

6.3. Influence of various variables

The performance of extrusion varies from one to another according to biomass properties and process variables such as extruder type, feed moisture, screw speed, feed rate, and others ingredients. These variables will affect the physical and textural properties of an extruded product for the acceptance of final products (Singh et al., 2018). Several examples of extrusion process conditions are given in Table I-16.

Table I- 16: Summaries of extrusion process conditions conducted on different materials

	Extruder type	Extrusion conditions	Gains	References
Alginate extraction from <i>Laminaria digitata</i>	Clextral BC 45 twin screw extruder	Biomass flow rate 1 kg/h, Solvent flow rate 1 kg/h Screw speed 400 rpm	Alginate yields 40.2 %	(Vauchel, 2007)
Alginate extraction from <i>Sargassum cristaefolium</i>	Berto Industry BEX-DS-2256 Twin-screw	Algae/solution ratio (1:5) Feed rate 27.5 rpm pH 12 Temperature 40-80 °C	Alginate yields 36-45.54%	(Sugiono et al., 2019a) (Sugiono et al., 2019b)
Oil extraction from sunflower seed	Consecutive intermeshing and non-intermeshing zone, co-rotating (Clextral)	Feed rate 70 kg/h High pressing temperature (120 °C)	Oil recovery 85-90 % (dw)	(Uitterhaegen and Evon, 2017)
	Intermeshing, co-rotating (Clextral) BC45	Feed rate 22 kg/h Feed rate 18 kg/h Spacing of reverse screw elements. Two filtration sections.	Oil recovery 79% (dw) Seeds with reduced moisture content Oil recovery 85.3% (dw)	
Oil extraction from coriander seed	Intermeshing, co-rotating (Clextral) BC21	Feed rate 2.3 kg/h	Oil recovery 45 % (dw) High foot contents (at least 48 %)	
		Feed rate 3.9 kg/h High pressing temperature (120 °C)	Oil recovery 46.9 % (dw)	
Biodiesels production from fish by-products	Clextral BC 45 twin screw extruder	Biomass low rate of 4 kg/h Solvent flow rate 1 L/h Screws speed 400 rpm	Oil recovery Salmon 6.5 % (dw) Trout 70 % (dw) Sardine 6.4 % (dw)	(Makoure et al., 2019a)
Protein extraction from alfalfa (<i>Medicago sativa</i>)	Clextral BC 45 twin screw extruder	Alfalfa flow rate 14.2 kg/h Liquid/solid ratio 10.4	Protein yields 50.8 % (dw)	(Colas et al., 2013)

Table I- 16: Summaries of extrusion process conditions conducted on different materials (Cont')

Extruder type	Extrusion conditions	Gains	References
Corn stover	Screw speed: 80 rpm, Moisture content: 27.5%, Enzyme dosage: 0.028 g (enzyme/g dry corn stover)	Glucose: 48.79%, Xylose: 24.98%, Combined sugar: 40.07%	(Zheng and Rehmann, 2014)
	Extrusion physical pretreatment		
	Screw speed: 80 rpm, Temperature: 140 °C, NaOH loading ratio: 0.04 g/g biomass	Glucose: 86.8%, Xylose: 50.5%	
	Extrusion alkali pretreatment		
	Die temperature: 55–90 °C, Ammonia loading: 0–2.0 g/g biomass	Digestibility: increased up to 32%, Lignin reduction: 12.5%	
	Extrusion alkali pretreatment		
	Screw speed: 325 rpm, Temperature: 99 °C NaOH loading: 0.06 g/g biomass	Glucose: 83%, Xylan: 89%, Lignin removal: 71%	
	Extrusion alkali pretreatment		

6.3.1. Screw configuration

The arrangement of screw configuration (different pitches, lengths, stagger angles, positions, and spacing) is known as a main factor which influent the extent of mixing, product transportation, extruded properties, mechanical energy input, and residence time distribution during extrusion processing (Zheng and Rehmann, 2014). Many studies have shown that the screw configuration is a key importance for the performance of extrusion process, its energy consumption, residence time distribution, and the obtained product quality (Colas et al., 2013; Evon et al., 2013; Uitterhaegen and Evon, 2017). Some example of the optimal screw configuration conducted with twin screw extruder on different material were presented in Table I-17 below:

Table I- 17: Different screw profile investigated on different material of twin-screw extruder

Material	Screw profile	Reference
Alginate extraction from <i>Laminaria digitata</i>	Clextral BC 45 twin screw extruder T2F 50, C2F 50, C2F 33.33, C2F 25, MAL2 -25.7°, C2FC 16.6, C2F 16.6, MAL2 -25.7°, C2FC 16.6, C2F 16.6, C2FC 16.6, C2F 16.6	(Vauchel, 2007)
Alginate extraction from <i>Sargassum cristaefolium</i>	Berto Industry BEX-DS-2256 Twin-screw extruder TC1 300, TC2 220, TC3 140, TC4 120, TM 80	(Sugiono et al., 2019a)
Protein extraction from alfalfa (<i>Medicago sativa</i>)	Clextral BC 45 twin screw extruder T2F 66, C2F 50, C2F 33, MPS 45°, C2F 25, BB 90°, C1F 33, C1F 25, C1F 25, CF1C -25, C1F 33, C1F 33, C1F 33, CF1T 15, C1FTC -15, C1F 33, C1F 25	(Colas et al., 2013)

T2F: trapezoidal double-thread screw, C2F: conveying double-thread screw, C1F: conveying simple screw
C2FC and CF1C, CF1Ct: reverse screw, MAL2 and BB: Bilobe paddle-screw, TC = groove transfer direct pitch element, TM: groove mixing pitch element
Screw type and screw pitch (°) or length (mm) are represented

According to Colas et al. (2013), the best screw profile (Table I-17) was the most favorable for the highest protein release (more than 50 % of global protein) from alfalfa biomass. However, the energy consumption with this screw profile was the highest among the others screw profile. The study of Vauchel, 2007 on the release of alginate from brown algae *Laminaria digitata* showed that the best screw profile (Table I-17) allowed the release of alginate at 40.2 % under the biomass flow rate of 1 kg/h, solvent flow rate 1 kg/h with the screw rotation speed at 400 rpm. With a fix screw profile, Sugiono et al., 2019 obtained the

best alginate recovery 45.54% at algae to solution ratio (1:5), feed rate (27.5 rpm), and pH 12 from *Sargassum cristaefolium* (Sugiono et al., 2019a). It is worth noticed that the screw configuration varied according to different feed material. Operating with the best screw profile will lead to obtain the optimal yields. Furthermore, under the same screw profile, other parameters such as flow rate or solid/liquid ratio could logically affect the obtained results (Colas et al., 2013).

6.3.2. Feed rate

Feed rate has emphasized as important during extrusion process. As shown in Table I-16, the feed rate varies according to the nature of the material. For oil extraction from sunflower seed, the feed rate varied from 18 to 70 kg/h while small feed rate (2.3 -3.9 kg/h) was conducted when extracting with coriander seed (Uitterhaegen and Evon, 2017). The best alginate extraction from macroalgae *Laminaria digitate* was operated at sample flow rate 1 kg/h and solvent flow rate 1 kg/h (Vauchel, 2007). High dilution of biomass/solvent will generate low shear stress, resulting biomass cannot be fully destructed. On the contrary, when the biomass/solvent was not diluted enough, a plug due to biomass accumulation was generated and could block the operation (Vauchel, 2007).

In oil extraction, when an insufficient feed flow rate occurred, the extrusion is unable to form an effective oil expression through pressure build up. This is due to the extruder, in particular the space around the reverse screws does not fill up with material and the dynamic plug. On the other hand, with an excessive inlet flow rate, the extruder becomes too filled, leading to a blocking of the filtration element by solid particles and further to the backflow of material to the feeder and consequently the clogging of the extruder. This effect could be overcome with the increase of the screw rotation speed, as it reduces the degree of fill inside the extruder. Therefore, it is worth noticed that the performance of the extrusion-pressing process strongly depends on the degree of filling of the extruder, rather than a sole parameter such as the feed rate or the screw speed (Uitterhaegen and Evon, 2017).

6.3.3. Temperature

The barrel temperature of an extruder is often easy to control. The heat can be added to the extrusion via steam injection with a full control. This action will reduce the requirement of a large drive motors. The temperature either too hot or too cool of the head at the final section of the extruder could provoke material to stick to the inner barrel and interfere the viscous flow, resulting the product with a torn burnt appearance (Shuyang, 2018). Temperature applied in

extrusion process could have an effect to the heating rate of the material and their melt stage (Wang et al., 2016).

Temperature also shows several impacts on the performance of extrusion pressing for oil extraction. Increasing temperature will enhance the oil expression capacity and facilitate its release through the fibrous matrix. This effect is due to the decrease of oil viscosity and the coagulation of the protein fraction of the seed. However, high processing temperatures may provoke undesirable changes in the oil such as the destruction of thermosensitive compounds and oil discoloration. Furthermore, a strong reduction of the oil viscosity with high pressing temperatures may exert a negative impact on the pressing capacity of the extruder (Uitterhaegen and Evon, 2017). On brown rice pasta, the study of Wang et al., (2016) showed that increasing the temperature is shown to increase the die pressure and motor torque in the extrusion. The best quality was obtained at a high extrusion temperature of 120 °C and screw speed of 120 rpm. Thus, the authors concluded that the extrusion temperature and screw speed had significantly effect on the cooking quality and textural properties of brown rice pasta (Wang et al., 2016).

Beside, increasing temperature was reported to improve alginate extraction yield from brown algae *Sargassum cristaefolium* (Sugiono et al., 2019b). The optimal temperature for the best release of alginate (34.01 %) was reported at 58.18 °C. Increasing temperature allowed to form more porous structure on the algal cell wall and degraded the alginate chain, therefore resulting more release of alginate (Fertah et al., 2017; Sugiono et al., 2019b). It is noticed that barrel temperature and moisture content are reported as the most important parameters to influence the expansion and related characteristics of extruded product. Generally, the expansion ratio increases alongside with the increase in temperature (Shuyang, 2018). When involving with enzyme, operating extrusion temperature will take into consideration with enzyme requirement as describe in above section.

6.3.4. Moisture content

The moisture content of biomass is a key parameter for the efficiency of the extrusion process. In oil extraction, moisture acts as lubricant and shows a large impact on the rheological properties of the cellular material inside the extruder. The optimal moisture content is largely depend on the raw material as well as the operating conditions (degree of filling, temperature, screw profile, feed rate, and screw speed) (Uitterhaegen and Evon, 2017). Food with low moisture content is more viscous and therefore positively favors expansion during extrusion

process. It will experience greater pressure differential rather than those with high moisture content (Singh et al., 2007). Low moisture content will restrict the melt flow inside the extruder barrel, resulting the increase in shear and residence time. Therefore, it favors the expansion due to greater starch gelatinization and/or melting. In starch gelatinization, the effect of moisture content could be somehow conflicting since water is required for starch gelatinization but too much water can also act as a lubricant to reduce shear, pressure, and the temperature in the barrel of the extruder (Shuyang, 2018).

Rodríguez-Miranda et al. (2014) found that moisture content at the moment of feeding affected significantly on the expansion index on the extrudates for balanced feed (with high fishmeal contents as well as bean flour and soy protein). The authors concluded that high expansion index was obtained in a range of 186- 279 g/kg bean flour (diet with a partial fish flour substitution) and 93-279 g/kg soy protein under the temperature 120 °C with a screw speed 80 rpm. This operating condition favors the saving for production costs. The authors continued that a high amount of moisture is required in order to obtain a low sinking velocity when extrusion was conducted with a high starch and low fishmeal contents (Rodríguez-Miranda et al., 2014).

6.3.5. Screw speed

Screw speed has a great impact on specific mechanical energy, residence time for the feed, and the capacity of the extruder. When screw speed is increasing, more friction will occur inside the extruder, resulting the increase of specific mechanical energy. Under a fix feed rate, increasing screw speed will decrease the filling in the barrel. This phenomenon will reduce the mechanical energy and thus lower the barrel and product temperature (Shuyang, 2018). The study of extrusion on brown rice pasta showed that screw speed has negative relationship with die pressure and motor torque, observing at low temperature. Since fast screw speed will generate a shorter heating time and high shearing force, resulting low viscosity as well as low die pressure (Wang et al., 2016). Furthermore, extrusion treatment at high screw speed can alter water-binding capacity (WBC) and water solubility (WS) of wheat bran (Aktas-Akyildiz et al., 2020).

Single screw extrusion of corn-gluten showed that increasing screw speed positively increased the protein digestibility (Bhattacharya and Hanna, 1985). As high screw speed favored the shear force in the extruder and therefore denatured the protein more easily and

facilitated the enzymatic hydrolysis (Singh et al., 2007). According to Bhattacharya et al., 1988, the screw speed presented two opposing effects occur simultaneously which made it difficult to predict its effect on nutritional status of food. High screw speed will reduce the residence time and therefore the food constitution due to heat injury is less. However, the shear damage is improved when the increase in the rotational speed of the screw (Bhattacharya et al., 1988). In snack food, increase in screw speed reduced the quantity of total antioxidant activity in particular at low feed rate (Ozer et al., 2006). The authors concluded that the effect of shearing was dominant than the short residence time when high screw speed was operated, resulting in more damage on the total antioxidant activity of the product.

6.3.6. Residence time distribution

Residence time distribution is a dependent variable and is generally one of the main parameters which influence the extrusion performances (Altomare and Ghossi, 1986). It strongly depends on process parameters including screw speed, feed rate, viscosity, screw configuration, and die design (Vauchel, 2007; Vauchel et al., 2012; Wolf, 2010). Residence time also takes into consideration of moisture content, die diameter, and temperature since they are subjected to have the effect on process parameters (Wolf, 2010).

Residence time is obtained by injecting an instantaneous pulse (tracer) into the system and determining the output concentration of tracer and the time it takes to exit from the extruder (Altomare and Ghossi, 1986). The tracer must have the same hydrodynamic properties as the fluid in the purpose not to disturb the flow. So far, dyes are reported as the most used tracers (Vauchel 2007).

It was shown that the mean residence time was shortened when screw speed was increased. In addition, low barrel temperature generated also the shorter residence time (Altomare and Ghossi, 1986). The authors mentioned that moisture contents (10-28 %) of rice flour had a very little effect on residence time when using screw with conveying element in a twin-screw extruder (Altomare and Ghossi, 1986). The authors concluded that screw profile and flow rate were reported as the most effect parameters on residence time distribution of rice flour in twin-screw co-rotating extruder. Chuang and Yeh (2004) found that the mean residence time was much longer when conducting single-screw extrusion on rice flour at 60–80 °C and moisture content 35 %. The authors stated that using mixing disc and pin-mixing element generate longer residence time with high extrudate temperature rather than forward screw element. Additionally, the variation of die diameter did not affect significantly the mean residence time

in case using forward screw element. However, it was found that increasing die diameter of mixing disc (from 20-40 mm) reduced significantly the mean residence time (Chuang and Yeh, 2004). A study on starch extrusion showed that the mean residence time was increased when moisture was increased (from 16 to 28 %) and screw speed was decreased (from 160 to 80 rpm) (Kumar et al., 2008).

6.3.7. Specific mechanical energy (SME)

Specific mechanical energy (SME) represent the amount of energy transmitted to the extruded material per unit of mass (Vauchel, 2007). Similar to residence time distribution, SME depends on various variables and the change of SME could generate the change in texture of extruded product (Shuyang, 2018). SME concept has been widely applied in extrusion cooking as it is correlated to the rheological state and the level of transformation of the products (N'Diaye et al., 1996).

SME negatively correlated with the bulk density in the extrusion of corn starch-soy protein concentrate extrudates (De Mesa et al., 2009). Various studies involving corn meal, corn starch, rice starch, rice flour, and protein-starch mixtures are well-documented with the fact that increasing SME along the increasing of screw speed will generate higher shear rates (De Mesa et al., 2009). High SME generally generates greater driving force for the expansion of product. It is worth noticed that increasing SME favors positively the Maillard reaction, resulting to a darker color of the final product (Fang et al., 2014; Rausch, 2009). As SME increases, the friction and temperature inside the extruder are logically increased, therefore this phenomenon links and favors the Maillard reaction (Van Lengerich, 1990). The study of Fang et al. (2014) on soy protein showed that increasing SME would positively correlate to tensile strength, hardness, and proportion of smaller fractions, however high SME would have a negative effect on melt viscosity at extruder die (Fang et al., 2014). Extrusion on animal feed (mainly soybean and wheat meal) showed that SME had a positive relationship with temperature, meanwhile it had a negative correlation with screw speed and moisture content (Wang and Copeland, 2013).

6.4. Advantages and limitations

6.4.1. Advantages

The extrusion technology offers various advantages such as improvement the efficiency of mixing of concentrated solutions (gelatinized starches), throughput and conversion efficiency, heat transfer characteristics, and regulated introduction of modifiers, plasticizers or reactants in a continuous process (Arora et al., 2020). The good mixing capacity is able to remove

softened parts while exposing the interior surface to thermal and chemical action, thus improving the material deconstruction (Gatt et al., 2018).

Table I- 18: Advantages and Limitations of Reactive and Extractive Extrusion Process (Vauchel et al., 2012)

Advantages	Limitations
<ul style="list-style-type: none"> • High mixing capacity, even with highly viscous materials, enabling reduction of reaction or extraction time • Efficient heat transfer between barrel and material and in material bulk owing to mass transfer between screws • Limitation of solvent consumption • Possibilities to inject or to degas products all along the screws • High tolerance to temperature and pressure (up to 400–500 °C and 500 bars) • Continuous process • Solvents are confined (process and environment safety) 	<ul style="list-style-type: none"> • Corrosive products are banned • Highly exothermic phenomena are banned • Diffusion is less efficient in a viscous material (extrusion) than in a diluted material (batch) • Residence time being reduced, extraction and reaction kinetics have to be fast

Physical and nutritional characteristics are related to the quality of food products. Strong interrelationship has been reported between the effect of extrusion processing on physical and nutritional properties of the feed/food due to the complexity of the process (Singh et al., 2019). Beside, extrusion cooking can minimize heat degradation of the food nutrients and enhance the digestibility of the major ingredients, mainly protein and starch (Singh et al., 2019). Extrusion cooking can boost the digestibility of protein, reduce gossypol and inactivate proteinase inhibitors through protein denaturation (Singh et al., 2019). Oil-soluble vitamins such as vitamins D and K are quite stable during extrusion process with a small loss observed at 15-20 % according to the applied conditions. On the other hand, chemical change was reported on vitamins A and E as well as their derivatives (carotenoids and tocopherols) when the oxygen and heat are presented (Shuyang, 2018).

The extrusion process is used to modify or texturize food materials. Studies have shown that extrusion cooking might have positive effects on total and soluble dietary fiber (Aktas-

Akyildiz et al., 2020). Furthermore, reactive extrusion induces total free or small amount of solvents, making more benefits for environmental protection. In addition, process safety and security are ensured. Flexible and multi-purpose are also reported in reactive extrusion where extruder serves as an excellent multi-function reactor (Berzin & Hu, 2004).

6.4.2. Limitations

Regarding many advantages provided by extrusion technology, some limitations are still inevitable. The difficulty to regulate the temperature (limit of cooling capacities) and the limited residence time (1-5 min) which only allows fast reaction to occur are two main drawbacks highlighted in extrusion process technology (Gatt et al., 2018). In single screw extruder, the transport and mixing capacity are limited, notably with low viscous reagents or products (Berzin & Hu, 2004).

The extrusion cooking effect on nutrient retention in the food product is still a major concern as protein denaturation, starch gelatinization, lipid oxidation, and Maillard reaction can occur which induce the change in the nutritional qualities of the extruded products (Singh et al., 2019). The Maillard reaction is accelerated when low feed moisture and high barrel temperature was applied. The reaction was due to the produce of dextrin and free sugar from the high shear inside the barrel and others substance are presented to favor the reaction (Shuyang, 2018). Overheating provokes degradation and reduces the bioavailability of the protein and essential amino acids like lysine, and loss of heat-labile vitamins (Singh et al., 2019). Water-soluble vitamin such as vitamins B and C are known for their less stable properties with heating. High loss of vitamin C has been reported as 90 %, and therefore, it is usually to apply it on the final product after the extrusion (Shuyang, 2018). The retentions of vitamins decrease when increasing the temperature, screw speed, and specific mechanical energy while decreasing moisture, throughput/feed rate, and die diameter (Shuyang, 2018; Singh et al., 2007). In addition, the complex interactions between operating variables, formulation and process variables highlighted the unstable and the level of reproducible for operating food extrusion, particularly during transients where the product deviates from specifications (Farid et al., 2007).

6.5. Applications

6.5.1. Food industry

The use of extrusion in food industry dated since mid-1930s with the production of pasta and cereal (Gatt et al., 2018). Extrusion-cooking technology has been widely used in food sector for the manufacturing of expanded, unexpanded, and texturized food products (Arhaliass et al., 2009; Arora et al., 2020) as can be seen in Figure I-23. Many ready-to-eat products (breakfast cereals and snack foods) are manufactured from extrusion process (Farid et al., 2007). Starch is the most widely extruded food. The most common is to break down the starch granule to render it digestible and to produce a shaped product (Wolf, 2010). In the animal feed industry and veterinary science, extrusion is commonly applied as a means of producing pelletized feeds, implants, or injection molding (Maniruzzaman et al., 2012).

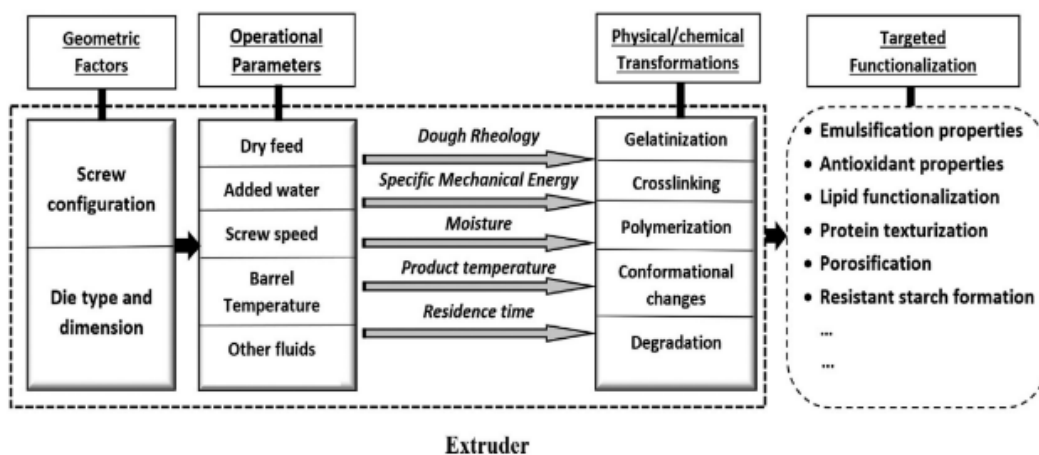


Figure I- 23 : Strategies for food ingredient functionalization by extrusion wherein extruder can be configured to function as a reactive, supercritical, or enzymatic process- based system (Arora et al., 2020)

In the meantime, reactive extrusion is a popular technique for chemical modification of starches. Extruders also served as chemical reactors for non-thermoplastic polysaccharides such as in the acid hydrolysis of cellulose to glucose, partial hydrolysis of cellulose to microcrystalline cellulose using mineral acids and hydrogen peroxide, and alkaline extraction of alginates from seaweeds (Bhandari et al., 2012; Vauchel et al., 2008). The surface esterification of wood particles with maleate polyethylene and maleate polypropylene was also investigated via reactive extrusion (Bhandari et al., 2012). Several applications of reactive extrusion were summarized in Table I-19.

Table I- 19: Examples of Reactive Extrusion Application and Associated Benefits and Limits (Vauchel et al., 2012)

Reaction types	Applications	Benefits and Limits
Depolymerization	Oligomer production from starch, guar, cellulose	Continuous process
Sugar hydrolysis	Levulinic acid production	Reaction time reduced, satisfying yield; corrosion problems
	Flavor precursors (Millard reaction)	Reaction time reduced by eightfold without loss in yield
Specific grafting	Continuous halogenization	Substrate degradation and secondary reactions reduced
	Modified starch transglycosylation	Reactant consumption considerably reduced, high yields, solvent free
Acetylation	Starch acetate production	Gelatinization yield enhanced and secondary reactions reduced
Oxidation	Oxidized starch production	Hydrosoluble compound production, rapid and continuous process
Saponification	Industrial soap production	Continuous process, better work conditions, reaction control enhanced
Cross-linking	Phosphorylated starch production	Phosphorylation yield enhanced, reaction time reduced, no drying
Polymerization	Gluco-oligosaccharides and polydextrose production from glucose	Selective control of polymerization, rapid and continuous reaction
	Oligocondensation of phenolic resins	Continuous process, better work conditions, reaction control enhanced
	Production of high polymers from ϵ -caprolactone	High molecular weight (“on demand”)
	Copolymerization of starch with cationic methacrylate, acrylamide, and acrylonitrile	Reaction time reduced, polymerization yield enhanced, sustainable process
Acid-base reaction	Casein to caseinate conversion	Energy consumption reduced (easier drying, more concentrated solution), investment costs reduced
Chemical treatment and whitening	Natural fibers treatment (cotton, flax, hemp, etc.)	Energy, reactants, and solvent consumption reduced; high yields; no wetting agent use

6.5.2. Pharmaceuticals

Extrusion is also applied in pharmaceutical industry for changing product microstructure, product chemistry, or the macroscopic shape of products (Wolf, 2010). Hot melt extrusion (HME) has been widely applied in the pharmaceutical industry and academia due to several advantages over traditional processing methods such as roll spinning and grinding (Maniruzzaman et al., 2012). The main application of HME is to disperse active pharmaceutical ingredients (APIs) in a matrix at the molecular level, thus forming solid solutions. Others applications have been reported with HME such as enhancing the dissolution rate and bioavailability of poorly soluble drugs by forming a solid dispersion or solid solution, controlling or modifying the release of the drug, taste masking of bitter APIs, and formulation of various thin film (Maniruzzaman et al., 2012).

6.5.3. Others applications

Extrusion is also widely developed in the oilseed-processing industry regarding the fractionation capacities of the machine (Gatt et al., 2018). It is also the most common and economic technology used in the aqua-feed industry (Singh et al., 2019). Extrusion processing is also often applied to encapsulate flavors, nutrients, and drugs (Wolf, 2010). In plastics processing, the polymers (granules) are transformed in a molten and homogenous phase of extrusion and then shaped through the die. Furthermore, reactive extrusion is able to elaborate new products and to carry out difficult operations to be made by conventional reactors such as mass polymerization, polycondensation, chemical and rheological modification of polymers, compatibility of polymer mixtures, elaboration of composites, and recycling of polymers (Berzin and Hu, 2004; Makoure, 2019; Vauchel, 2007).

In chemistry, extrusion has been used to overcome the problems encountered with conventional methods such as low yields, incomplete reactions, long reaction times, working in a dilute medium (leads to the discharge of effluents), product's quality control, safety, and the size of batch installations (Vauchel, 2007). Various applications have also been investigated with reactive extrusion technologies (synthesis of copolymerization, polymerization, and encapsulation) (Makoure, 2019).

7. Response surface methodology

The response surface (RSM) methodology is a group of empirical techniques which describe and develop the adequate relationship between several independent experimental variables with one or more responses (Khuri and Mukhopadhyay, 2010; Yetilmezsoy et al., 2009). This technique was developed by Box and Wilson in 1951 and has been widely used for designing the experiments as well as optimizing the responses (Raissi and Farsani, 2009; Raymond et al., 2016; Witek-Krowiak et al., 2014). RSM technique is based on the fit of mathematical models (linear, square polynomial function, and others) to the experimental results and verifies the model by statistical techniques (Witek-Krowiak et al., 2014).

According to Witek-Krowiak et al. (2014), the optimization via RSM approach has been devised into different stages as below:

- Selection of independent variables and possible responses
- Selection of experimental design strategy
- Execution of experiments and obtaining results
- Fitting the model equation to experimental data
- Obtaining response graphs and verification of the model
- Determination of optimal conditions

RSM commonly involves two important models (first and second order). In the first step, a suitable approximation is used to define the true relationship between the responses and the set of variables. When the interpretation of the preliminary model (first-order) is still insufficient, the model is upgraded with the addition of high-order term for a better interpretation (Yetilmezsoy et al., 2009). The relationship of response “Y” and independent variables “ x_i ” of response surface can be expressed as:

$$Y = f(x_1, x_2, \dots, x_k) + \varepsilon$$

For better approximation, the second-order equation has been wisely applied, with the integration of linear effect, interaction effect, and quadratic effect of studied variables. The second-order model is a very flexible, easy to estimate the parameters, and is considerable practical experience in solving real response surface problems (Raymond et al., 2016).

RSM is typically explained by the quadratic equation below:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} x_i x_j + \varepsilon$$

Where Y is the response, k is the number of the independent variables, β_0 is intercept coefficient, x_1, x_2, \dots, x_k are the coded independent variables, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and ε is the random error between predicted and measured values.

Multiple linear regression analysis was used to evaluate the mathematical models of each response. The significant terms in the model were determined by analysis of variance (ANOVA). A good model will present high value of R^2 and less errors (Raissi and Farsani, 2009). The response can be graphically represented, either as three-dimensional or as contour plot. This representation allows the observation of the curvature of the response surface and is the fastest way for modelling if the optimal response is located within the experimental boundaries (Witek-Krowiak et al., 2014).

7.1. Design of experiment

Design of experiment (DOE) is being known as a powerful methodology which helps to avoid experimental biases and reduce the required number of experiments (Mandenius and Brundin, 2008). The basic idea is to diversify all significant parameters simultaneously over a set of designed experiments and then to combine the results through a mathematical model. The model attempts to describe a relationship between one or more independent variables and a response variable (Mandenius and Brundin, 2008). The model can be used for optimization, prediction or interpretation. Therefore, process performance can be improved by taking into account the most significant factors. These will help to reduce operation costs as well as experimental time (Witek-Krowiak et al., 2014).

In this study, the experimental design used Box-Behnken design for response surface analysis.

7.2. Box-Behnken Design

The design was created in 1960 by Box and Behnken (Box and Behnken, 1960; Witek-Krowiak et al., 2014). It is a 3 levels incomplete factorial design (rotatable or nearly rotatable) as an alternative to the labor extensive of full factorial design (Raymond et al., 2016). This design was created to minimize the number of experiments, particularly in quadratic model fitting. The employed model is second-order polynomial which ensures linear, quadratic, and interaction effects accuracy (Witek-Krowiak et al., 2014).

Box-Behnken design doesn't contain experimental points, where all variables have extreme values. This aspect might be beneficial since some undesired phenomena might occur in

extreme conditions (Ferreira et al., 2007). This design reports to be more efficient than full factorial design and slightly more labor efficient than central composite design. Two restrictions have been known, the number of experimental factor has to be equal or higher than three, and the model should only use to fit second order polynomial equation (Witek-Krowiak et al., 2014).

For industrial research, the use of Box-Behnken design is preferable since it is consider as an economical design and requires only three levels of each factors, coding as -1, 0, and +1 (Khuri and Mukhopadhyay, 2010; Yetilmezsoy et al., 2009).

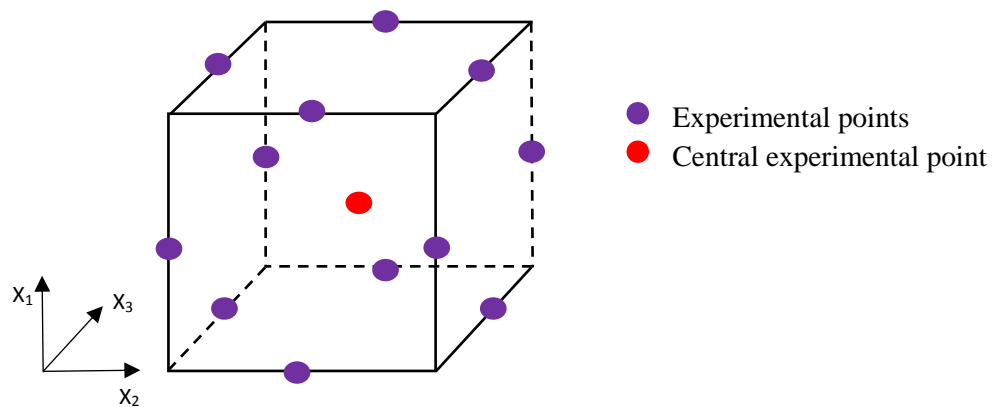


Figure I- 24 : Box-Behnken design of three variables

Chapter II: Materials and Methods

1. Sample collection

The biomass of *Gracilaria gracilis* (*G. gracilis*) was collected from Atlantic coast in French at Piriac-sur-Mer (47° 22' 38.0'' N, 2° 33' 20.2'' W) in mid-April 2019 and Pornichet (47° 15' 06'' N, 2° 20' 03'' W) in November 2021. These two locations are the habitat of *Gracilaria gracilis* which is accessible and allow to harvest sufficient biomass for the extraction procedure.

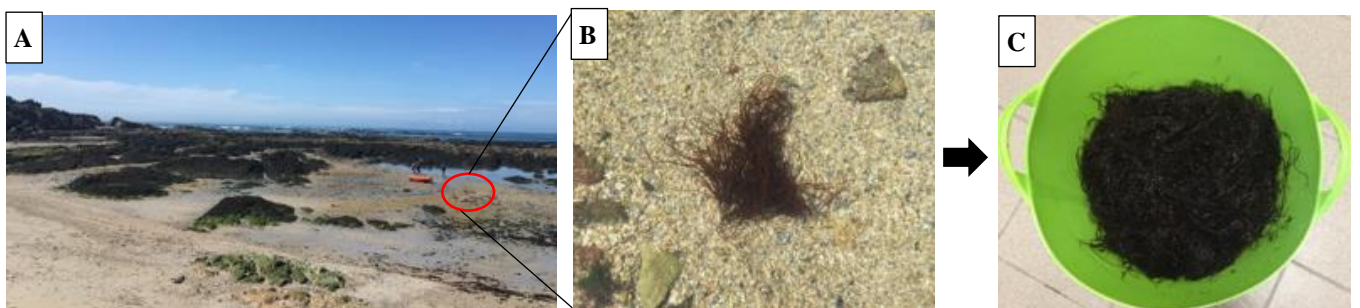


Figure II- 1: Biomass of *G.gracilis* at costal area (A and B), and after cleaning (C)

2. Sample preparation

2.1. Cleaning

The harvested biomass needs to be properly clean prior further analysis. In order to deeply remove sand, epiphytes, and foreign matter, biomass was rinsed with tap water two times and finalize with distilled water for one more time. Salad spinner was used to remove the surface water before storage. The cleaned sample (biomass) were immediately stored at -20 °C prior to further analysis.

2.2. Freeze-drying

The cleaned biomass was undergone in freeze-drying process which was conducted with the freeze-drier (CHRIST alpha 1-2 LD plus, Germany) with two desiccation steps (10 hours at -30 °C and 0.37 mbar for first desiccation and -52°C and 0.03 mbar within 50 hours for the second desiccation).

2.3. Grinding

Freeze-Dried sample (BL)

G. gracilis biomass was ground before extraction process. The grinding helps to enlarge the contact surface between sample and enzyme in the solvent in order to increase the liberation of targeted compounds. Freeze-dried biomass (BL) was ground with liquid nitrogen using mortar and pestle according to the method realized in the study of Nguyen (2017).

Fresh sample (AFM)

The fresh biomass (AFM) was ground two times in order to obtain the homogenous biomass. Firstly, biomass was ground with a kitchen mixer (Moulinex FP542110) that helps cut down the long filamentous into a small size. After this grinding, biomass was later ground with a mixer (Retsch GM 200) at 7000 rpm for 1 minute in order to archive small and homogeneous sample (3-4 mm). On the other hand, the grinding of fresh biomass for extrusion extraction was conducted with a grinder Fabio Leonardi TC12 (Italy) which comply with the huge quantity of biomass for extrusion extraction. The process of sample preparation was summarized in Figure II-2 below.

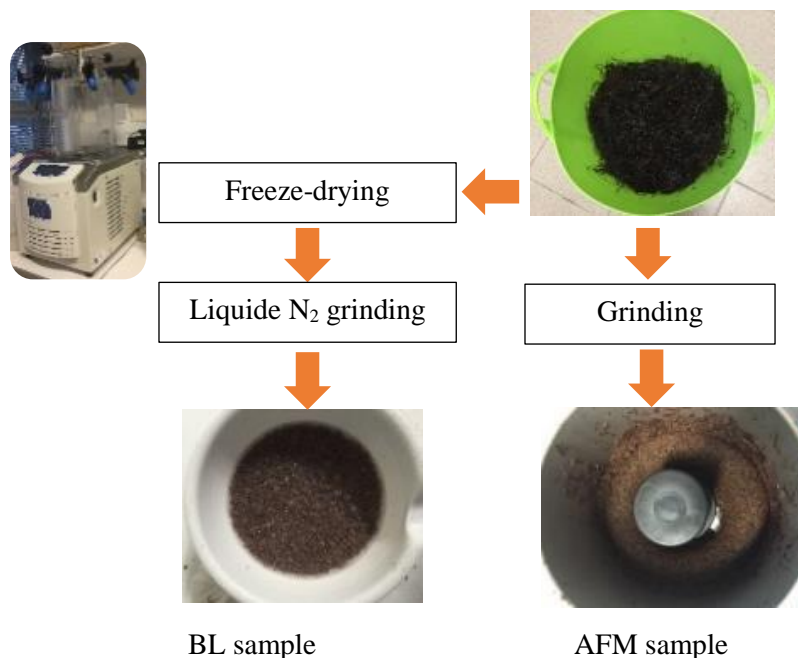


Figure II- 2: Sample preparation process

3. Chemicals

All chemicals are for analytical grade. Concentrated hydrochloric acid (HCl 37 %) came from VWR, sodium carbonate powder (Na₂CO₃, 99.8 %) came from ACROS Organics. Sodium acetate (CH₃COONa) and glacial acetic acid (CH₃COOH) from Sigma Aldrich were used to prepare the acetate buffer for enzyme. Enzyme cellulase (ref. 22178 from *Aspergillus Niger*) and protease (ref. P5459-5G from *Bacillus Licheniformis*) were purchased from Sigma Aldrich.

4. Enzyme-assisted extraction

4.1. Enzymatic hydrolysis (Batch)

4.1.1. Acetate buffer preparation

The solution of acetate buffer 50 mM was prepared from sodium acetate (CH₃COONa) and glacial acetic acid (CH₃COOH). The pH either 5.0 or 7.5 was strictly adjusted with the addition of NaOH solution in order to provide the optimal working medium for enzymes. For ensuring the quality of acetate buffer, the shelf life must not exceed 1 month.

4.1.2. Enzyme preparation

The preparation of cellulase, protease, and enzyme cocktail were conducted on the same day of experiments to avoid the changes related to storage condition. The concentration was respected with the values presented in the operating conditions.

4.1.3. Experimental design (Operating conditions)

Freeze-dried biomass

Nguyen (2017) worked on the optimization of enzymatic hydrolysis (Batch) on dried *G. gracilis* by using three different enzymes: cellulase, xylanase, and β-glucanase. The optimal condition for a high release of R-phycoerythrin and protein was obtained with biomass/solvent ratio 30 g dw (algae)/l and cellulase concentration 47 mg/ dw (algae) with a constant temperature 32 °C for 5 h and acetate buffer 50 mM with pH 5.0.

Following the study of Nguyen (2017), the current study adapted this optimal condition by testing two more enzymes: protease and enzyme cocktail (mixture of cellulase and protease). Protease activity works best with higher pH (7.5). In this regard, acetate buffer 50 mM with pH 7.5 was used when conducting extraction under protease addition. The concentration of protease (47 mg/g dw algae) was adapted the same as cellulase in this study. In addition, two operating temperature 37 and 32 °C were investigated when applying protease on freeze-dried biomass. The catalysis activity of protease is known to work better at 37 °C according to the

supplier instruction. In this regard, this study investigated two temperatures 37 and 32 °C for a better understanding the effect of temperature on studied algal.

The investigation on enzyme cocktail (the mixture of two enzymes) was operated with pH 5.0 and 7.5, respectively with respect to the pH required by each enzyme. Moreover, two concentrations (47 and 94 mg/g dw algae) were tested for better understanding the effect of enzyme concentration. Meanwhile, the operating temperature was fixed at 32 °C in the current study. The experimental design of all operating conditions was summarized in Table II-1 below.

Fresh biomass

The same enzymatic hydrolysis set up as well as enzyme preparation of freeze-dried biomass were applied on fresh biomass with some minor modifications. The operating temperature was tested at 32 °C for all enzymes. Enzyme cocktail (combination of cellulase and protease) was investigated with different buffer pH (5.0 and 7.5) and two concentrations (47 and 94 mg/g dw). The operating conditions applied on fresh biomass were summarized in the Table II-2.

Table II- 1: Experimental design of enzymatic hydrolysis of freeze-dried (BL) biomass

Biomass	Enzyme	Enzyme concentration mg/g dw algae	pH Buffer 50mM	Temperature °C	
Freeze-dried	Cellulase	47	5.0	32	
	Protease	47	7.5	32	
				37	
	Cocktail	94	47	5.0	32
				7.5	32
				5.0	32
				7.5	32

Table II- 2: Experimental design of enzymatic hydrolysis of fresh (AFM) biomass

Biomass	Enzyme	Enzyme concentration mg/g dw algae	pH Buffer 50mM	Temperature °C	
Fresh	Cellulase	47	5.0	32	
	Protease	47	7.5	32	
	Cocktail		94	5.0	32
				7.5	32
				5.0	32
				7.5	32

4.1.4. Extraction procedure

Enzymatic hydrolysis (Batch) was conducted in a batch mode following a method optimized in the study of Nguyen (2017) (Figure II-3). The mixture of seaweed biomass, enzyme, and buffer solution was agitated under a controlled temperature in the dark for 286 min. The supernatant was collected after the separation process at 4 °C, 25 000 g, and 20 min (Sorvall Lynx 6000 centrifuge, USA). The ratio of algae and solvent (acetate buffer) was set at 3 g per 100 ml (adapted from Nguyen, 2017). A control sample (without enzyme) was always carried out in each condition simultaneously. Triplicate experiments were conducted to assess the reproducibility. The procedures to recover the soluble compounds were summarized in Figure II-4.

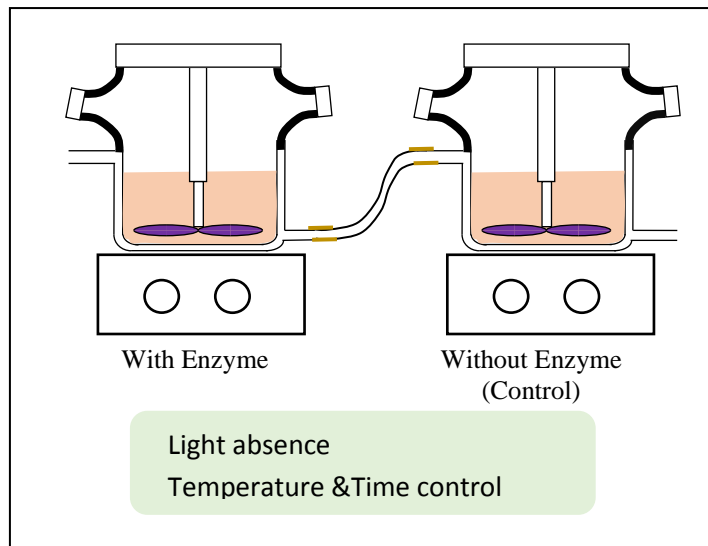


Figure II- 3: Representation of enzymatic hydrolysis extraction

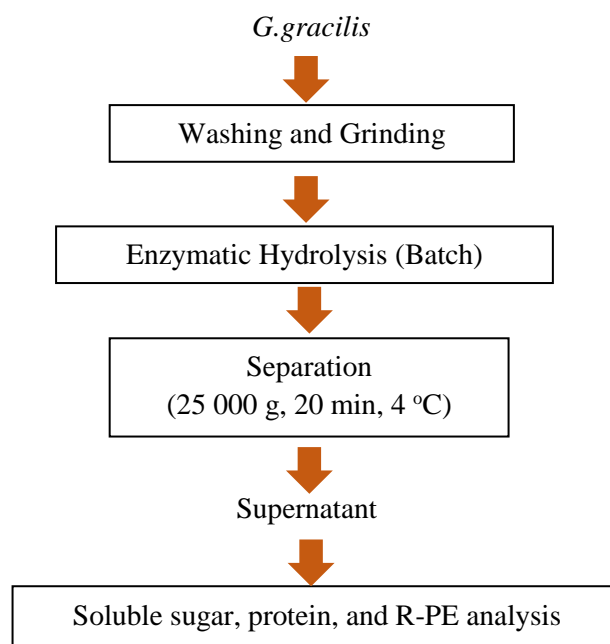


Figure II- 4 : Procedure to recover soluble contents from *Gracilaria gracilis*

4.2. Enzymatic extrusion

4.2.1. Twin screw extruder

The extrusion process was conducted in a co-rotative intermeshing twin-screw extruder (BC21 type Cleextral, Firminy, France). This extruder could generate biomass flow rate from 1 to 20 kg/h according to the nature of raw material. The maximal screw speed can be up to 672 rpm. The twin-screw extruder consists of different screw segment elements placed at different zones. The principal screws of the extruder consist of trapezoidal double-thread, conveying double thread, reverse screw, and bilobe paddle screw. The screw diameter and its length to diameter (L/D) were 25 mm and 36:1 (mm) respectively. The same screw configuration was validated from Vauchel's thesis (2007) and was adapted in the current study. This configuration presented three bilobe paddle screws and one reverse screw as presented in Table II-3 and Figure II-5.

Table II- 3: Screws profile configuration

Segment	Screw profile	Length (mm)	Zone
1	T2F 50/50	150	Conveying
2	C2F 50/50	150	
3	C2F 25/50	150	
4	MAL2 45°	25	Mixing with kneading bloc
5	C2F 25/50	15	Conveying
6	MAL2 45°	20	Mixing with kneading bloc
7	C2F 33.3/50	50	Conveying
8	C2F 25/50	100	
9	C2F 16.6/50	50	
10	C2F 33.3/50	50	
11	MAL2 45°	15	Mixing with kneading bloc
12	C2F 25/50	50	Conveying
13	C2FC 12.5/50	25	
14	C2F 25/50	50	

T2F: trapezoidal double-thread screw
 C2F: conveying double-thread screw
 C2FC: reverse screw
 MAL2: Bilobe paddle-screw

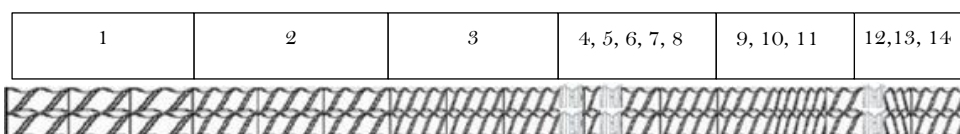


Figure II- 5: Screw profile configuration in a twin-screw extruder

4.2.2. RSM experimental design

Choice of variables

Three influents variables were selected to investigate in the current study, knowing as sample (biomass) flow rate (X1), enzyme (cellulase) concentration (X2), and screw speed (X3). In this study, the investigation of enzymatic extrusion on fresh *G.gracilis* began with enzyme cellulase as it was shown with promising extraction yields from batch condition. The studied variables were well-known to affect the performance of extrusion and the release efficiency. Other variable such as screw profile or biomass/solvent ratio were adapted from the previous study of Vauchel, 2007. Using response surface method (RSM) with Box-Behnken design on three variables, a total 15 experiments were investigated.

Level of variables

All variables were divided into three levels (low, basal, and high), coded as -1, 0, and +1 (Table II-4). This study focused more with low flow rates and medium screw speed. Moreover, the level of each variable was selected base on previous experimental tests and was inspired from literature review. In this study, the biomass flow rate was ranged from 1 to 5 kg/h (Sugiono et al., 2019) while the screw speed ranged from 100 to 350 rpm. Meanwhile, enzyme concentration was ranged form 1 to 4.7 %/ dw (algae) where 4.7 %/ dw (algae) was the optimal enzyme concentration apply in hydrolysis enzymatic (alone) in Batch mode 5 h (Nguyen, 2017). A minimum of enzyme dose was investigated due to enzyme high price.

Table II- 4: Variables for Box-Behnken design

Variables	Units	Level		
		-1	0	+1
Sample (biomass) flow rate (X1)	kg/h	1.0	3.0	5.0
Enzyme concentration (X2)	%	1.0	2.85	4.7
Screw speed (X3)	rpm	100	225	350

kg/h: kilogram per hour
rpm: round per minute

Box-Behnken design

The Box-Behnken design of three variables had a total of 15 runs in a single block, randomly ordered, and 3 replications at the central points of each variable. The mathematical relationship between the three independent variables can be fitted as a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^3 \beta_{ii} x_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} x_i x_j$$

Where Y is the predicted responses (soluble R-PE, protein, and sugar), β_0 is the intercept coefficient (model constant), β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient of variables i and j , x_i and x_j are independent variables.

Moode software 12.1 was used to analyze the experimental data for regression analysis and plot the response surface. Analysis of variance (ANOVA) was conducted to investigate the statistical parameters. Correlation coefficient (R^2) was determined for all polynomial models. The p value were calculated to confirm the significance of the studied variables (Mensi, 2019; Sugiono et al., 2019a; Wang et al., 2020).

4.2.3. Calibration of sample and solvent flow rate

The calibration of sample and solvent flow rate before the conduct of extrusion extraction were compulsory and were carried out with grinded fresh biomass and pure water (for solvent). The calibration equations were determined. The index of extruder hopper and external pump was determined from the calibration equation as given in Figure II-6 below. Table II-5 summarized the applied index which were used during the extrusion process in this study.

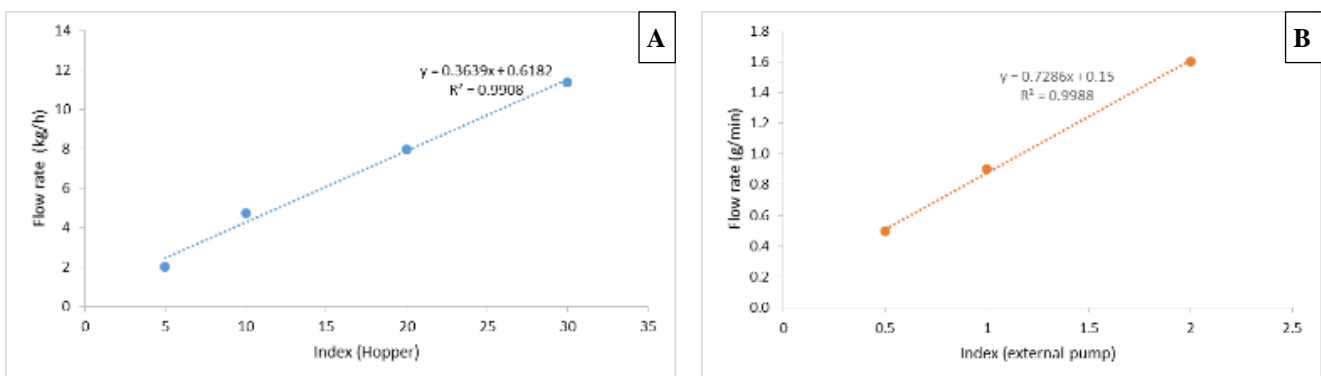


Figure II- 6: Calibration curve for (A) Sample flow rate and (B) Solvent flow rate

Table II- 5: Applied index during extrusion extraction

Sample flow rate (kg/h)	Index (Hopper)
1	1
3	7
5	12
Solvent flow rate (g/min)	Index (External pump)
0.5	0.514
1.5	1.242
2.5	1.971

4.2.4. Extraction procedure

Enzymatic extrusion

The extrusion was carried out at enzyme optimum temperature of 32 °C. The solvent solution consisted of enzymes and acetate buffer (50 mM, pH 5). Thus, in order to test the different enzyme concentration, different enzyme/buffer ratio were prepared at each experiment depending on solvent and biomass flow rates.

Pre-grinded *G. gracilis* biomass was introduced in the hopper of the extruder with the sample (biomass) flow rate (X1) ranged from 1 to 5 kg/h. The enzyme concentration (X2) varied from 1 to 4.7 %/ dw algae and was supplied into the extruder by an external pump (Ismatec RS232, Germany). The solvent flow rate was adjusted to maintain a constant pH and enzyme concentration within the high and low levels according to the experimental design. Screw speed was ranged from 100 to 350 rpm (Figure II-7).

The extruded sample was collected after 10 min (in order to ensure the stability state inside extruder) of each and every 15 conditions. After sampling, extruded sample was put immediately at 4 °C (to minimize the enzyme activity) and later was centrifuged at 11 000 rpm, 4 °C, and 15 min (Eppendorf centrifuge 5804R, France). The supernatant was collected for soluble R-Phycoerythrin, protein, and sugar analysis. Each chemical analysis was carried in triplicate.

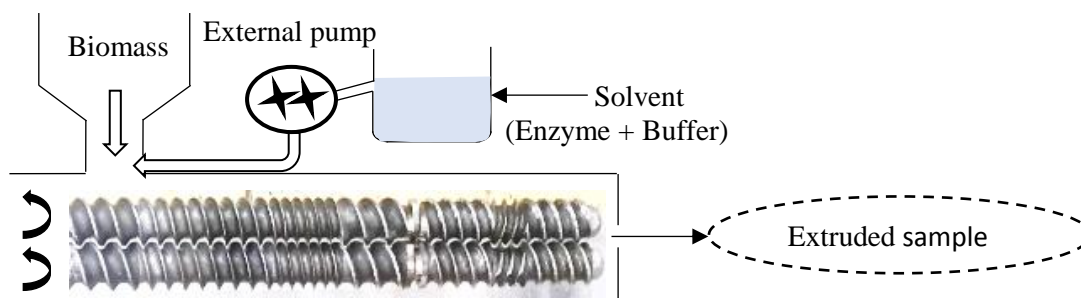


Figure II- 7: Representation of Enzymatic Extrusion

Enzymatic extrusion with maceration

For one experiment (the optimal condition of enzymatic extrusion), a quick maceration of the pellet after centrifugation was performed. The maceration was carried out with a pellet/pure water ratio equal to 30 g dw (algae)/l (to replicate the enzymatic hydrolysis in batch condition according to Nguyen(2017)) at 4°C (to minimize the reactivation of enzyme activity), in dark during 10 min with low agitation (minimizing the effect of agitation). This rapid action aimed to recover the rest of soluble compounds which were trapped during the first separation process. The procedure of enzymatic extrusion was summarized in Figure II-8 below.

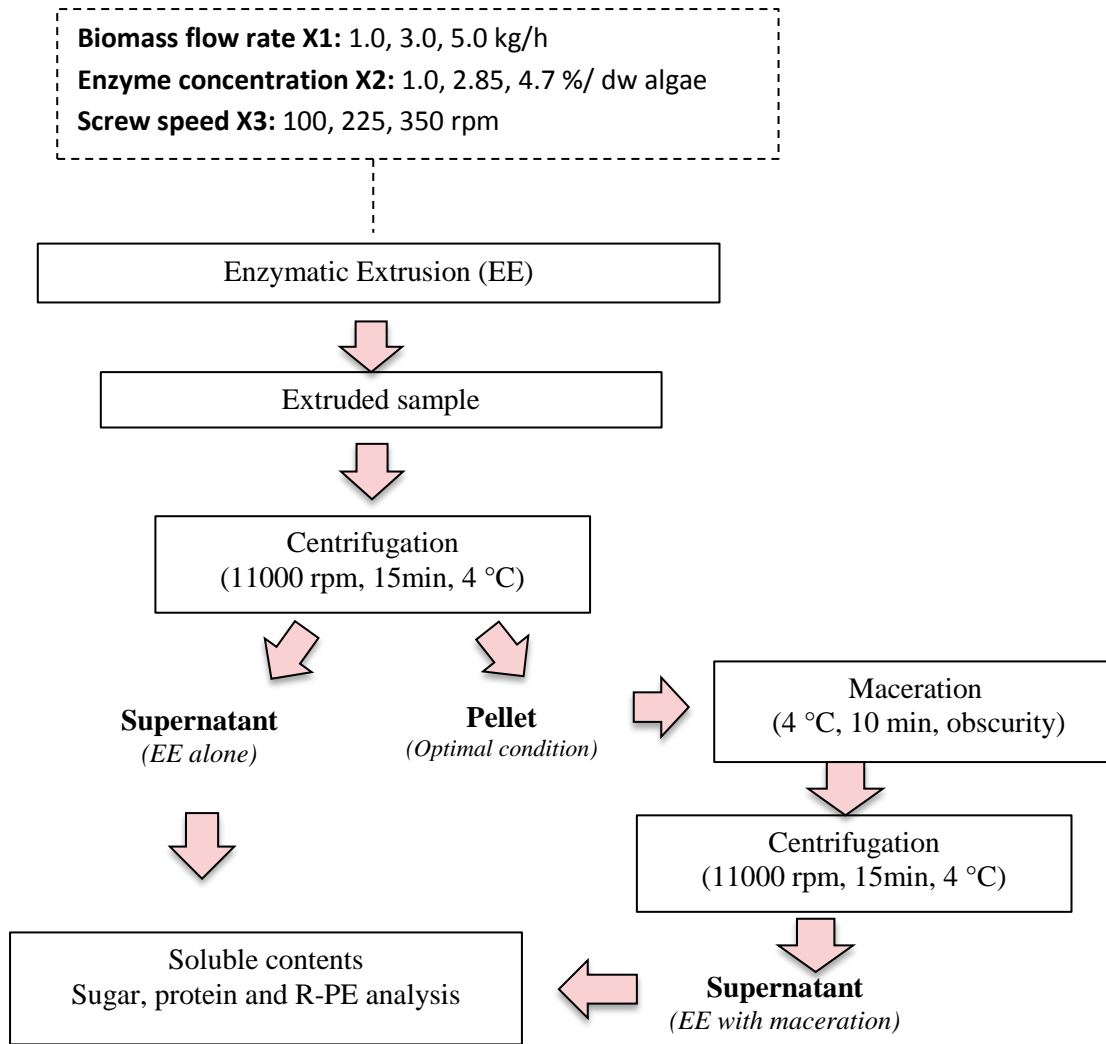


Figure II- 8: Recovery process of soluble contents by enzymatic extrusion

5. Analytical methods

5.1. Biochemical contents in raw material

Dry weight and ash content

Fresh biomass was used for the determination of dry weight by heating at 105 °C during 24 h in oven. The ash content was determined after heating at 550 °C for 24 h in furnace. The analyses were conducted in triplicate and results were express as percentage.

Carbohydrate

Extraction of total carbohydrate was performed according to (Francavilla et al., 2013a) with some modifications. Briefly, 100 mg of dried sample was mix with 5 ml of HCl (2.5 M) and the extraction took place at 90 °C within 5 h. After extraction, the mixture was neutralized with Na₂CO₃ powder before being centrifuged at 10,000 rpm during 15 min at room temperature (25 °C). The supernatant was collected and the total carbohydrate was determined by the phenol-sulfuric acid method (Dubois). All extractions were conducted in triplicate and the results were expressed in percentage per algal dry weight.

Protein

The total protein was determined by multiplying the total nitrogen (from Kjeldahl method) with conversion factor of 6.25. Results were expressed in percentage per algal dry weight.

5.2. Analyze of soluble contents

R-Phycoerythrin

The concentration of the red pigment R-phycoerythrin (R-PE) was determined by spectral analysis adapted from Beer and Eshel (Beer S. and Eshel A., 1985). Briefly, the supernatant was injected into quartz curve for the measurement of absorbance at 455 (A₄₅₅), 565 (A₅₆₅) and 592 (A₅₉₂) nanometers. The R-PE concentration was calculated from the following equation:

$$[\text{R-PE}] \text{ mg/ml} = [(A_{565} - A_{592}) - (A_{455} - A_{592}) * 0.20 * 0.12] \quad (\text{Equation II-1})$$

The final R-PE concentration results were expressed as milligrams of R-PE per gram of algal dry weight (mg/g dw).

Soluble protein

The concentrations of soluble proteins were analyzed by the BCA (Bichinchoninic acid) method which combines the well-known reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of

the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. Reactant Pierce BCA Protein Assay Kit came from Pierce Thermo (Ref product 23225). The calibration curve (Figure II-9) was obtained from the seven diluted concentrations of common protein BSA (bovine serum albumin): 1.5, 1.0, 0.8, 0.5, 0.25, 0.125, and 0.063 mg/ml. To start, 200 μl of working reagents were added with 25 μl of prepared sample and incubated at ambient temperature for 30 minutes in absence of light. The absorbance was measured at a wavelength of 570 nm. The concentration was calculated by the equation obtained from calibration curve and was expressed in milligrams per milliliter (mg/ml) then expressed in milligram per gram of dry weight sample (mg/g dw). The protein concentration of enzyme was subtracted in order to count only the released protein from biomass.

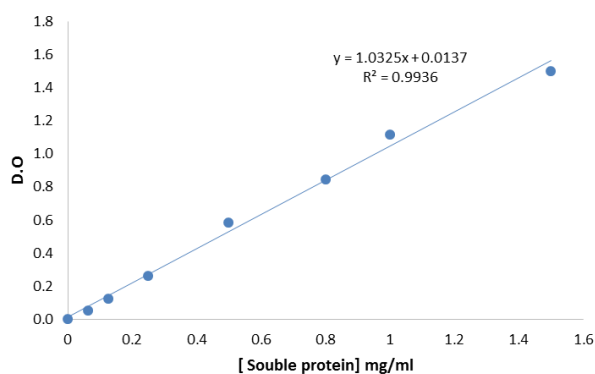


Figure II- 9: Calibration curve of soluble protein concentration

Soluble sugar

Soluble sugar was determined by phenolsulphuric acid (the Dubois method) (Dubois et al., 1956). Simple sugar, oligosaccharides including methyl ethers with free or potentially free reducing groups, give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The obtained monosaccharides are dehydrated and rearranged to form furfural (from pentose) or hydroxymethyl furfural (from hexose). These compounds lead to a characteristic yellow coloration by condensation with phenol. The reaction is sensitive and the color is stable.

The calibration curve (Figure II-10) was prepared at different concentrations of glucose (100, 75, 50, 25 and 10 mg/l). Briefly, 500 μl of prepared sample were introduced into a flask, then 500 μl of phenol solution at 5 % were added. Then, 2.5 ml of concentrated sulfuric acid (95 %-98 %) were added. The reaction lasted 10 minutes followed by vortex (Heidolph Reax 2000) for 10 seconds and 15 minutes of incubation at ambient temperature. Next, the flask was placed in water bath at 35 °C for 30 minutes. The reading wavelength was set at 490 nm. The

results were obtained from the equation provided by the calibration curve and were expressed in milligrams of glucose equivalent per liter of prepared sample (mg/l). By taking into account the dry weight, the concentration was then expressed in mg/g dw of dry weight sample.

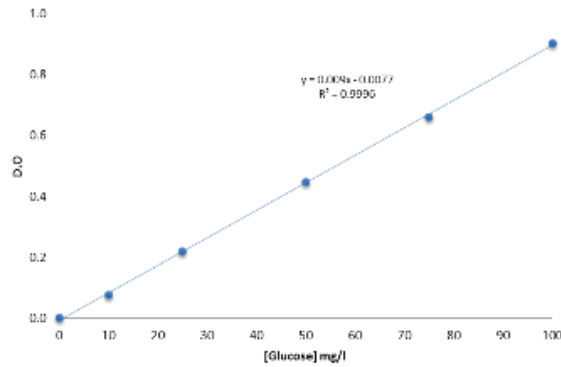


Figure II- 10: Calibration curve of soluble sugar concentration

Yields

The yields of soluble sugar, protein, and R-PE released in liquid part after extraction process were defined as the amount (mass) of the extracted soluble contents divided per their initials contents in dry biomass. The extraction yields were expressed in percentage.

Productivity

The productivity of soluble sugar, protein, and R-PE released by different extraction technique was determined by the following equations:

Enzymatic hydrolysis:

$$\text{Productivity (kg/l/h)} = \frac{\text{Released soluble concentration (mg/l)}}{\text{operating time (h)}}$$

Extrusion:

$$\text{Productivity (kg/l/h)} = \frac{\text{Released soluble concentration (mg/g dw algae)} * \text{Flow rate(kg/h)}}{\text{volume of extruder (m}^3\text{)}}$$

Statistical analysis

Results were accessed using one-way ANOVA test using Minitap 19 software. A significant difference was considered when the p-value was smaller than 0.05 (p< 0.05).

Chapter III:

Enhanced liberation of soluble sugar, protein, and R-phycoerythrin under enzyme-assisted extraction on dried and fresh *Gracilaria gracilis* biomass

Summary

This chapter investigated the enzyme-assisted extraction (EAE) on freeze-dried and fresh macroalgae *Gracilaria gracilis*, for the release of hydrosoluble components (R-Phycoerythrin, proteins, and sugars). Three enzymes: cellulase, protease, and enzyme cocktail (mixture of cellulase and protease) were applied in the study. Results showed that freeze-dried biomass released the highest target components in the presence of enzyme cocktail while fresh biomass could work better with a single enzyme, either protease for the release of R-PE and protein, or cellulase for the release of sugar. Results also revealed that the extraction of protein and sugar was improved by 43 % and 57 % from fresh biomass compared to dried biomass. The difference of biomass status was shown to affect the required enzyme and the release efficiency of target components during the extraction process. Employing an enzyme cocktail on freeze-dried biomass boosted the extraction yield which was probably from the complementary effect between enzymes. On the other hand, single enzyme handled better on fresh biomass, resulting from the already hydrated cell-wall degradation as well as the smaller biomass size (after grinding) if compared to freeze-dried biomass. Based on these results, working with fresh biomass could provide economic benefits (enzyme limitation, drying stage elimination) for further implementation of the bio-refinery process. Furthermore, biomass treatment (fresh or freeze-dried) as well as target components determined enzyme type and the efficiency of enzyme-assisted extraction.

1. Introduction and objective

Rhodophyta, one of the three main phyla of marine macroalgae, is reported to contain various biologically active compounds i.e. polysaccharides, sterols, phycobiliproteins, and vitamins (Bedoux et al., 2014; Francavilla et al., 2013a). Polysaccharides are often the main extracted product derived from macroalgae and have become a high-value raw material of industrial interest (Buschmann et al., 2017; Kazir et al., 2019; Khan et al., 2019; Wu et al., 2014). The *Gracilaria* genus is recognized as one of the main resources for macroalgae polysaccharides and is reported with 62-63 % of polysaccharides per dry weight (Rioux et al., 2017). The genus is widespread and can be found in tropical to temperate latitudes (Iyer et al., 2004; P. Torres et al., 2019). Polysaccharides of red algae gather agar, carrageenan, xylans, water-soluble sulfated galactans, and porphyrins (mucopolysaccharides) (Charoensiddhi et al., 2017) as well as cellulose, xylanase, or mannan fibrils (Mittal and Raghavarao, 2018). These polysaccharides serve as food emulsifiers, gelling agents and can be used as media for bacterial growth identification, or used as therapeutic agents (Cicinskas et al., 2019). In South-East Asian countries, agar-agar (extracted from *Gracilaria*) is used in food preparation as a gelling and thickening agent (Chan and Matanjun, 2017; Souza et al., 2012b).

Besides the high content of polysaccharides, the *Rhodophyceae* family is reported with phycoerythrin, especially R-phycoerythrin (R-PE) which can be found up to 50 % w/w of total protein (Pimentel et al., 2019). This R-phycoerythrin is classified as phycobiliproteins and is of great interest in the food industry (natural colorant), research sector (fluorescent dye, immunology, cell biology, and flow cytometry), and cosmetics (Bedoux et al., 2014; Dumay et al., 2014, 2013; Mensi, 2019; Munier et al., 2014). The purified powder of R-PE has a high market value (between USD 180 and 250 per milligram), which varies according to the purity level (Mittal and Raghavarao, 2018; Nguyen, 2017; Wang et al., 2020).

Several extraction methods such as the conventional one (solid-liquid extraction using phosphate buffer), hydrothermal, acid, base, organic solvent, as well as the physical method (freeze-thaw cycles, freeze grinding, ultrasound, and liquid nitrogen grinding), have been used to extract bioactive compounds from macroalgae (Sudhakar et al., 2015; Wang et al., 2020). However, these techniques are mostly time-consuming (several days), costly, and unsuitable for industrial setup (Le Guillard et al., 2015; Wang et al., 2020). In addition, protein extraction from macroalgae is challenging due to the complexity of the cell wall structure with strong cohesion and mechanical properties with the complex ionic and hydrogen-bonding interactions (Deniaud et al., 2003; Wang et al., 2020). Moreover, various polysaccharides in algal cell walls

serve as a barrier that prevents biomolecules release as well as act as anti-nutritional factors to limit the digestibility of protein fractions (Hardouin et al., 2016; Wang et al., 2020). Many studies have confirmed the complexity of protein extraction due to the cell membrane and the entrapment with polysaccharides (Kazir et al., 2019). It is highlighted that unwanted protein-polysaccharide interactions can limit the efficiency of water-soluble protein in red algae. As a result, the protein extraction in red algae *Gracilaria gracilis* can be improved by degrading agar in the algal cell walls (Fleurence et al., 1995b; Wang et al., 2020).

In this regard, the use of enzymes would allow the penetration of extraction solvents into the cell to increase the release of bioactive compounds (Mittal and Raghavarao, 2018) and act as a surfactant (Vandanjon et al., 2016). Furthermore, enzyme-assisted extraction could prevent any degradation of the targeted compounds (Hardouin et al., 2016; Mæhre et al., 2016). This type of extraction takes place in mild conditions and provides strong substrate specificity, high yield, and less by-products (Zhang et al., 2019). Given the dominance of polysaccharides present in red algal cell walls, polysaccharidase enzymes are mostly investigated. Some enzymes/enzyme consortia have been tested. Cellulase was demonstrated to deliver the best results for R-PE and protein extraction from dried *Gracilaria gracilis* compared with xylanase and β -glucanase (Nguyen, 2017). The enzymatic pre-treatment followed by alkaline extraction allowed high protein extraction yield from *Palmaria Palmata* (Mæhre et al., 2016). The enzyme consortia of agarose, cellulase, and xylanase also improved the extraction yield of R-PE by 26 % from *Gelidium pusillum* (Mittal and Raghavarao, 2018). Protease addition has shown the ability to enhance protein extraction in *S. chordalis* (Burlot et al., 2016) as well as to improve the release of bioactive components from *Chondrus crispus* (Kulshreshtha et al., 2015). In this regard, it would be worth to apply protease on *Gracilaria gracilis* for better comprehension and utilization.

To our knowledge, only a few studies have worked with fresh biomass with respect to dried biomass. In addition, the combination of enzymes is still in the investigation phase. This work aims to investigate the effect of enzyme types (cellulase, protease, and combination of both – cocktail) and enzyme concentrations of enzyme-assisted extraction on fresh and dried *Gracilaria gracilis* seaweed biomass. Here, the extraction yields of aqueous soluble compounds (R-PE, protein, and sugar) were determined.

2. Biochemical contents of raw materials

The biochemical compositions of *G. gracilis* were summarized in Table III-1. According to the literature, *G. gracilis* contains around 16-31 % dw (dry matter) of protein while the carbohydrate varies from 18-34 % dw (CEVA, 2015; Francavilla et al., 2013a; Mensi, 2019; Rodrigues et al., 2015; Sfriso et al., 1994). Some studies have shown that the content of polysaccharides of this algal genus could reach up to 62-63 % dw (Rioux et al., 2017). In this study, the amount of carbohydrate and protein were reported at 43.68 % and 24.81 % respectively which were in accordance to the quantities observed in this seaweed species. Ash content was reported at 2.85 % which was lower compared to the value reported in some previous studies 17-24 % (CEVA, 2015; Rodrigues et al., 2015). Therefore, the amount of minerals content is not much appreciable in our biomass. The variation of these contents is strongly related with the preparation methods and the environmental factors which vary according to season, geographical location, water quality, climate, tide movement, etc.

Table III- 1: Biochemical content of *G. gracilis* biomass

Compositions	Content (%)
Dry weight *	13.41 ± 0.20
Water *	86.59 ± 0.20
Ash **	2.85 ± 0.03
Carbohydrate **	43.68 ± 0.20
Protein **	24.81 ± 0.50

*: Contents are expressed per fresh weight

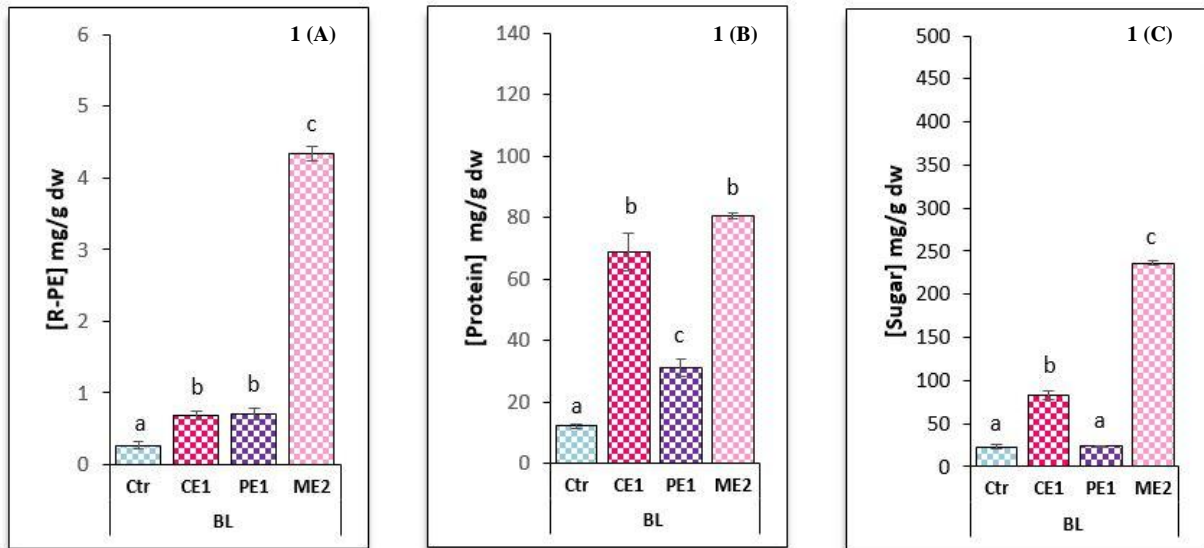
**: Contents are expressed per dry weight

3. Results of enzyme-assisted extraction on freeze-dried biomass

3.1. R-PE release efficiency

The objective of the study was to determine the best enzyme, single or cocktail for freeze-dried biomass that would allow the best recovery of soluble components including R-Phycocerythrin (R-PE), sugars, and proteins. The results of R-PE release from enzyme-assisted extraction (EAE) using cellulase (CE), protease (PE), and enzyme cocktail (ME) at operating temperature 32 °C were presented in Figure III-1A. The addition of either protease or cellulase resulted in a significant increase in R-PE extraction compared to the control experiment without enzyme ($p < 0.05$). The use of a single enzyme released a similar amount (~0.70 mg/g) dw of R-PE against the recovery of 0.27 mg/g dw from the control condition. Increasing two times

the enzyme /substrate ratio (mass basis) alongside the use of an enzyme mixture released almost three times higher R-PE as obtained with enzyme cocktail (4.34 mg/ g dw). Based on this phenomenon, synergistic relationships between enzymes seemed to affect R-PE liberation from dried biomass.



Ctrl: control condition (without enzyme addition)
 CE1: Cellulase at 47 mg/g dw algae (Acetate buffer 50 mM, pH 5)
 PE1: Protease at 47 mg/g dw algae (Acetate buffer 50 mM, pH 7.5)
 ME2: Enzyme cocktail (cellulase and protease) at 94 mg/g dw (Acetate buffer 50 mM, pH 5)
 Results were obtained at operating temperature 32 °C

Figure III- 1: Soluble contents released from freeze-dried biomass (A) R-PE, (B) proteins, and (C) sugars

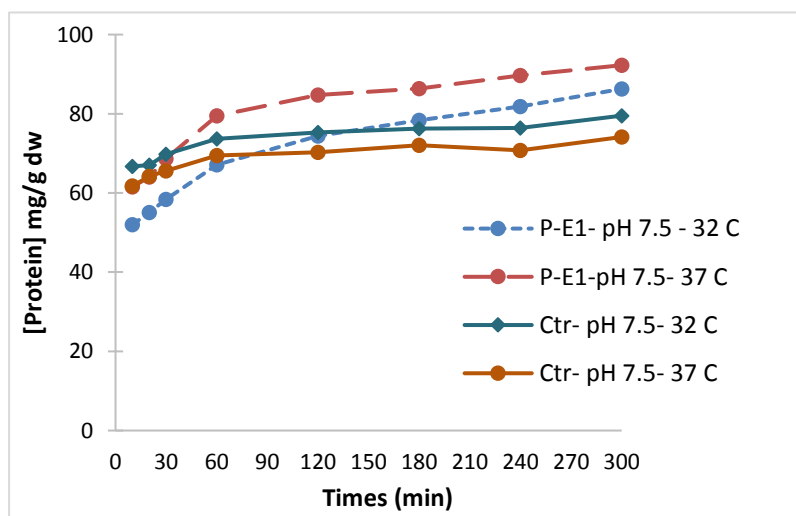
3.2. Protein release efficiency

The released quantities of soluble proteins from operating temperature 32 °C were also higher with enzyme cocktail than with only single enzyme (Figure III-1B). Among the three enzymes, cellulase and enzyme cocktail yielded significantly higher values compared to protease. Maximum recovery was obtained from enzyme cocktail at 80.57 mg/g dw, followed by cellulase at ~60 mg/g dw compared to the control conditions value of 12 mg/g dw. The use of protease also positively affected soluble protein yield, albeit half the value of cellulase at ~30 mg/g dw. The difference between extracted protein from cellulase and enzyme cocktail did not differ much although total enzyme content in enzyme cocktail was twice cellulase. It is worth noting that the presence of protease in enzyme cocktail did not boost the recovery of protein by a large margin. In this regard, cellulase showed an interesting insight into the optimization of protein release in the function of enzyme availability.

Effect of temperature investigated from protease addition

The results of protein released with protease addition under two different operating temperatures were presented in Figure III-2. Results showed that the addition of protease did increase the protein liberation compared to the control condition (without enzyme). Between the two operating temperatures, 92 mg/g dw of protein was obtained at 37 °C while 86 mg/g dw algae was obtained at 32 °C from the final point (5 h) of enzyme-assisted extraction. This result could be suggested that there was no different by a large margin between the two temperature regarding the release of protein. In this regard, applying protease at 32 °C could be used (the high release efficiency could be ensured) and it was beneficial from economic aspect.

It is worth noticed that protease was known to catalyze the peptides bonds for a high release of protein (Vandenberghe et al., 2020). Thus, it could be possible that the released protein was affected by protease activity. Interestingly, the obtained protein concentration in this current study showed that from the beginning (10 min) until the final point (5 h), the released concentration was increasing. From this observation, this result could suggest that the protease may not have a significant degrading effect regarding the quantitative measurement under the experimental conditions of our study. If it is the case, it means that the proteins could be hydrolyzed by the protease, but we cannot observe the hydrolysis because we use a global protein analysis. It is not excluded that the amino acid composition could be affected by the kinetics of protein release by proteases. Further analysis of the nature of the amino acids is necessary, but was not addressed in this study.

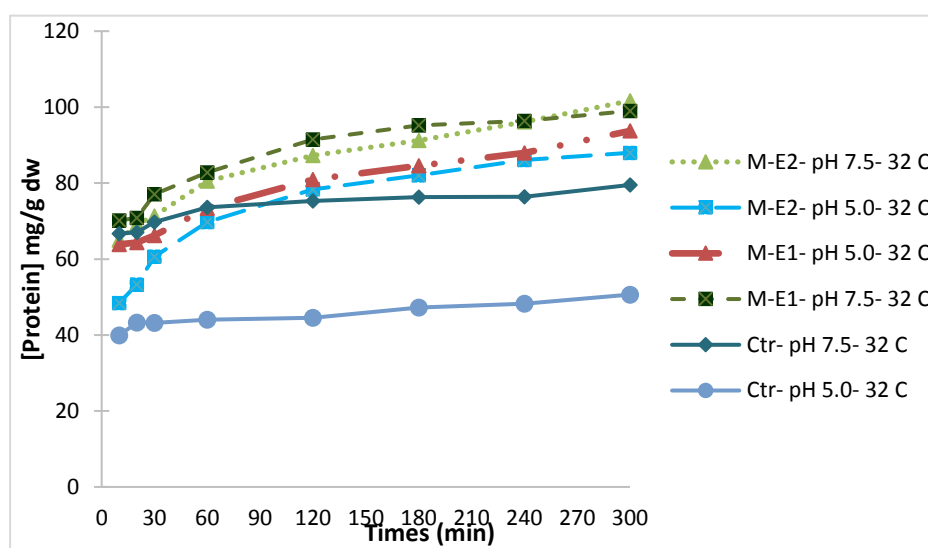


P-E1: Protease at 47 mg/g dw algae (Acetate buffer 50 mM, pH 7.5)
Ctr: control condition (without enzyme addition)

Figure III- 2: Soluble protein released from freeze-dried biomass under protease addition

Effect of pH and enzyme dose investigated from enzyme cocktail addition

The release of protein from enzyme cocktail at 32 °C under different pH and enzyme concentrations was summarized in Figure III-3. Regarding the effect of pH, it showed that using buffer acetate 50 mM with pH 7.5 could produce higher protein release efficiency than pH 5.0. Regarding the investigation on enzyme concentration, it was revealed that 47 mg/g dw algae (E1) of mixture cellulase and protease could produce similar results compared to the two fold concentration 94 m/g dw algae (E2). Under pH 7.5, the release of protein was 99 and 101 mg/g dw algae from E1 and E2 respectively. In this regard, enzyme dose of enzyme mixture could be reduced by half (of each enzyme) which is beneficial for total costs. The concentration of proteins also increased from the beginning to the final point which showed that protein was probably not significantly degraded regarding with protease activity as explained in above section.



M-E1: Enzyme cocktail at 47 mg/g dw algae
M-E2: Enzyme cocktail at 94 mg/g dw algae
Ctr: control condition (without enzyme addition)
Results were obtained with operating temperature 32 °C

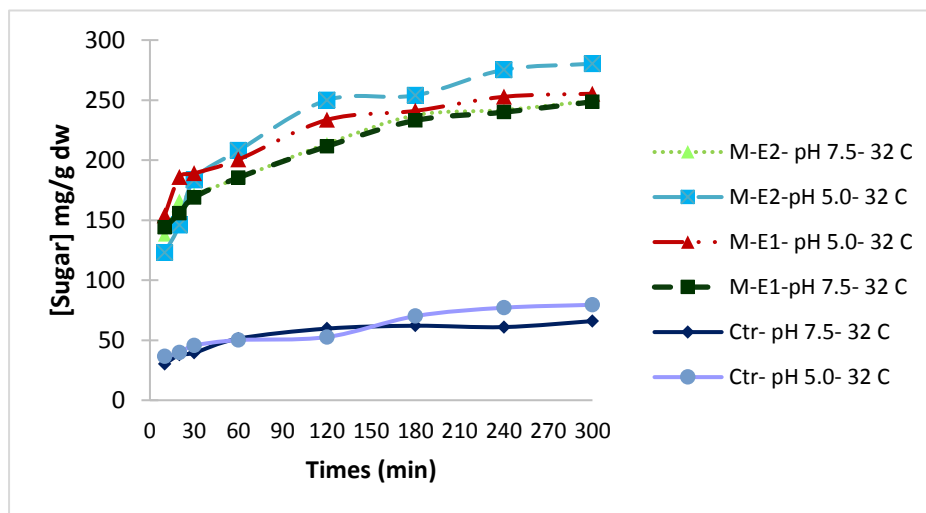
Figure III- 3: Soluble protein released from freeze-dried biomass under enzyme cocktail addition

3.3. Sugar release efficiency

For soluble sugars released at operating temperature 32 °C (Figure III-1C), similar to the results on protein, cellulase, and enzyme cocktail presented a significant increase compared to the control (~23 mg/g dw). Protease did not affect the recovery yield of soluble sugars. On the other hand, the highest sugar concentration was obtained with the enzyme cocktail (236 mg/g dw) followed by cellulase at 80 mg/g dw. The difference between both enzymes is clear as enzyme cocktail resulted in 3 times the value obtained with cellulase.

Effect of pH and enzyme dose investigated from enzyme cocktail addition

The results of sugar time-release from enzyme cocktail (32 °C) was presented in Figure III-4. It could be seen that the release of sugar work best under pH 5.0 (the best pH for cellulase) while pH 7.5 (more compatible with protease) was better for the release of protein from freeze-dried biomass. The concentration of enzyme showed clearly that the more enzyme (cellulase) provided, the higher sugar was obtained. In this regard, enzyme cocktail E2 which contains twice cellulase dose than enzyme cocktail E1, provided the highest sugar liberation 280 mg/g dw algae at the final point (5 h) of the extraction.



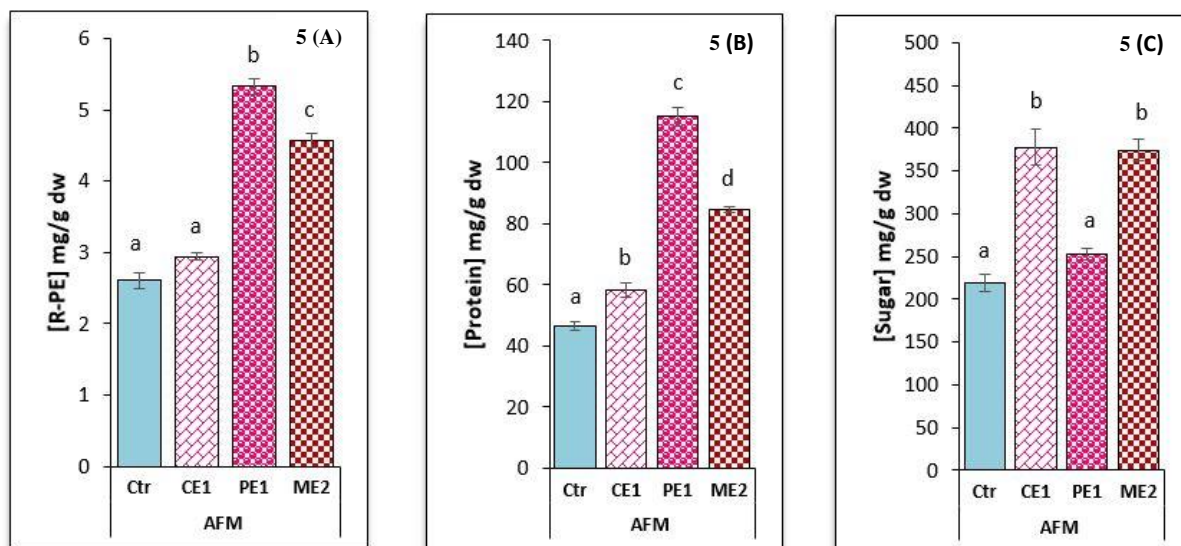
M-E1: Enzyme cocktail at 47 mg/g dw algae
M-E2: Enzyme cocktail at 94 mg/g dw algae
Ctr: control condition (without enzyme addition)
Results were obtained from operating temperature 32 °C

Figure III- 4: Soluble sugar released from freeze-dried biomass under enzyme cocktail addition

4. Results of enzyme-assisted extraction on fresh biomass

4.1. R-PE release efficiency

After working with freeze-dried biomass, enzyme-assisted extraction was performed on fresh biomass. Operating temperature was maintained at 32 °C. R-PE liberation from fresh biomass varied from 2.80 to 5.50 mg/g dw (Figure III-5A). The addition of protease and enzyme cocktail resulted in a significantly higher R-PE release efficiency compared to the control (absence of enzyme) ($p < 0.05$). In contrast to the freeze-dried sample, the highest liberation was obtained from protease ~5.50 mg/g dw by comparison with enzyme cocktail ~4.5 mg/g dw against the baseline ~3 mg/g dw. Interestingly, enzyme cocktail did not induce more R-PE than single enzyme albeit it contains twice the enzyme quantity. The synergy effect between enzymes did not seem to occur for the liberation of R-PE on fresh biomass. Moreover, the addition of cellulase did not generate more R-PE release compared to the control.



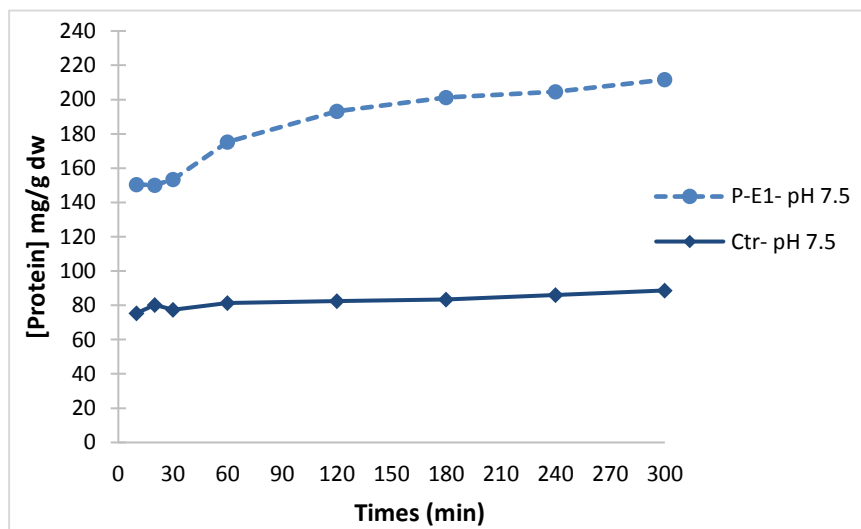
Ctr: control condition (without enzyme addition)
CE1: Cellulase at 47 mg/g dw algae (Acetate buffer 50 mM, pH 5)
PE1: Protease at 47 mg/g dw algae (Acetate buffer 50 mM, pH 7.5)
ME2: Enzyme cocktail (cellulase and protease) at 94 mg/g dw (Acetate buffer 50 mM, pH 5)
Results were obtained at operating temperature 32 °C

Figure III- 5 : Soluble contents released from fresh biomass (A) R-PE, (B) protein, and (C) sugar

4.2. Protein release efficiency

The recovery of soluble protein at 32 °C with the addition of enzyme (single or cocktail) showed a significant increase compared to the control as indicated in Figure III-5B. Similar to R-PE, protease yielded the highest liberation (~115 mg/g dw) followed by enzyme cocktail (~84 mg/g dw), and cellulase (~58 mg/g dw). Following the release of R-PE and protein, the effect of protease was significantly higher among others enzymes on fresh biomass while it was less effective when using with dried biomass. In this regard, protease seems to be more compatible and gains more access to the broken cell walls of fresh biomass. This would allow more accessibility to the intracellular components within EAE.

As shown in Figure III-6, the investigation of protein time-release was conducted under protease addition. Results showed that the protein concentration increased from the beginning to final point of extraction. With the same observation as indicate from freeze-dried biomass, it could suggest that protease may not have a significant degrading effect on the released protein with regards to quantitative measurements in this study. The released proteins could be hydrolyzed by the protease, but we cannot observe the hydrolysis because we use a global protein analysis. It is not omitted that the amino acid composition could be affected by the kinetics of protein release by proteases. Further investigation relying on the nature of the amino acids is necessary, but was not addressed in this study.

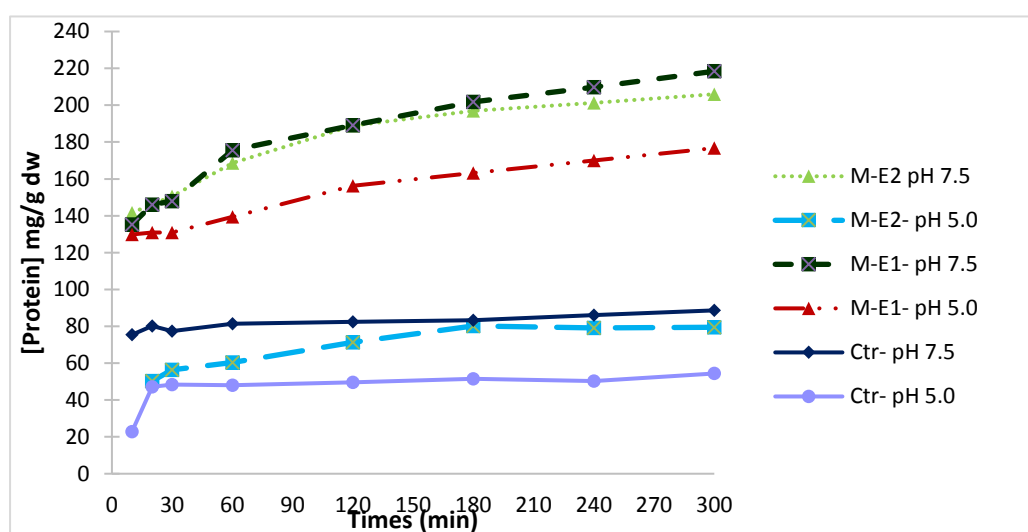


P-E1: Protease at 47 mg/g dw algae (Acetate buffer 50 mM, pH 7.5)
Ctr: control condition (without enzyme addition)

Figure III- 6: Soluble protein released from fresh biomass under protease addition

Effect of pH and enzyme dose investigated from enzyme cocktail addition

Regarding the effect of pH, enzyme cocktail (32 °C) provided higher protein release efficiency when using buffer acetate at pH 7.5 rather than pH 5.0. Under pH 7.5 (Figure III-7), enzyme concentration, either 47 (E1) or 94 (E2) mg/g dw algae produced high release of soluble protein, releasing around 218 and 205 mg/g dw algae respectively. Therefore, enzyme dose for enzyme mixture could reduce by half (from two enzymes), which positively reduce the operating cost. Besides, the results showed that at pH 5.0 enzyme dose 94 mg/g dw produced significantly less protein than enzyme dose 47 mg/g dw. This result could suggest that more release of sugar might probably lower the release of protein from fresh biomass. As presented in the literature, the low protein yields extracted from algae was due to the entrapment of certain protein in the cell wall together with extracellular polysaccharides matrix (Kadam et al., 2017; Kazir et al., 2019; Maehre et al., 2016). The high viscosity of polysaccharides exert in water and the ionic interaction between cell wall and protein also known to affect the extractability of the protein (Harrysson et al., 2018). Furthermore, the amount of soluble fiber was related to protein digestibility and therefore prevent bioavailability (MacArtain et al., 2007).



M-E1: Enzyme cocktail at 47 mg/g dw algae
M-E2: Enzyme cocktail at 94 mg/g dw algae
Ctr: control condition (without enzyme addition)
Results were obtained with operating temperature 32 °C

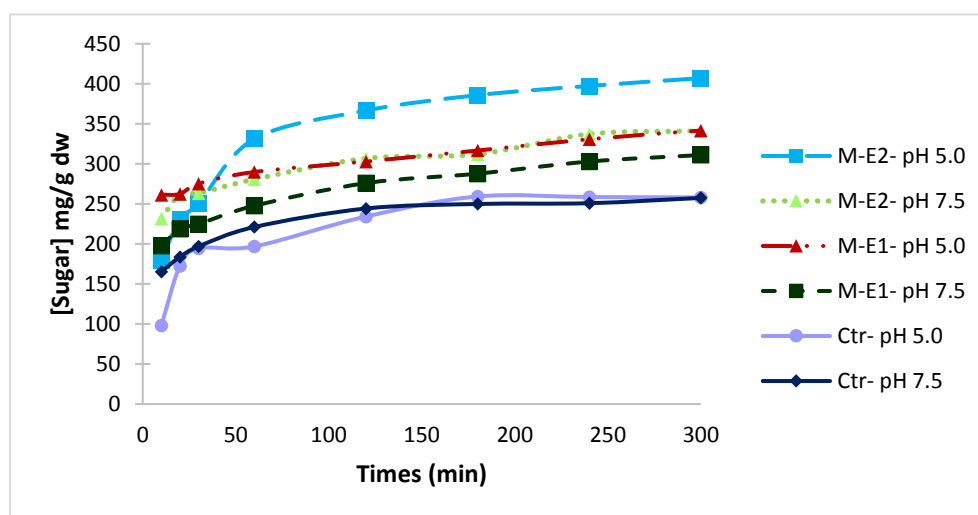
Figure III- 7: Soluble protein released from fresh biomass under enzyme cocktail addition

4.3. Sugar release efficiency

The quantities of soluble sugar released at 32 °C in the presence of cellulase and enzyme cocktail were significantly increased when compared to the control condition (Figure III-5C). In contrast to the results of R-PE and protein, the presence of protease was less effective on the release of sugar (~252 mg/g dw), without significant difference compared to the baseline (~218 mg/g dw). Similar liberation (~ 374 mg/g dw) was obtained with the presence of cellulase and enzyme cocktail. These results were not in accordance to those obtained with freeze-dried biomass, where enzyme cocktail resulted in three times higher sugar release than cellulase. Synergetic relationships between enzymes once again did not occur which could boost the extractability of soluble contents on fresh biomass.

Effect of pH and enzyme dose investigated from enzyme cocktail addition

The use of enzyme cocktail at 32 °C for the release of sugar worked better under buffer acetate at pH 5.0 (Figure III-8) with nearly 400 mg/g dw of sugar was obtained after 5 h extraction. In the meantime, it was observed that the more enzyme dose (cellulase) was applied, the more sugar will release. Therefore, among the enzyme concentration investigated in the current study, higher sugar was obtained under enzyme dose 94 mg/g dw algae (E2) which contain twice dose of cellulase than enzyme dose 47 mg/g dw (E1).



M-E1: Enzyme cocktail at 47 mg/g dw algae
M-E2: Enzyme cocktail at 94 mg/g dw algae
Ctr: control condition (without enzyme addition)
Results were obtained from operating temperature 32 °C

Figure III- 8: Soluble protein released from fresh biomass under enzyme cocktail addition

5. Discussion

5.1. Extraction yields

The use of carbohydrases and protease on enzyme assisted-extraction did improve the release yields of protein, neutral sugar, reducing sugar, and polyphenols from others red algae such as *Solieria chordalis*, *Palmaria palmata*, and *Grateloupia turuturu* (Denis et al., 2009a; Hardouin et al., 2014; Kulshreshtha et al., 2015). The comparison between our results with the literatures was summarized in Table III-2. Endo-peptidase was shown to give the highest protein release (15.20 %) from *Solieria chordalis* (carrageenophyte) rather than endo-protease, cellulase, xylanase, β -glucanase, and arabanase (Hardouin et al., 2014). The study of Kulshreshtha et al., 2015 showed that Novozyme-cellulase allowed the highest protein recovery from red algae carrageenophyte *C. crispus* (7.1 %) among others enzymes (β -glucanase, ultaflo, and neutrase). According to the study of Nguyen et al. (2017), the yield of protein released at optimal condition was reported at 10.31 % (per total content) under cellulase addition from freeze-dried *G.gracilis* biomass. Comparing to Nguyen et al. (2017), our results showed that protein yield increased three and two times respectively when using enzyme cocktail (32 %) and cellulase (24.18 %) on freeze-dried biomass. The improvement was probably related to the grinding method which could be more efficient in our study. Furthermore, our results revealed that under enzyme addition, the release of protein improved by 6.7 (enzyme cocktail) and 5 (cellulase) times higher compared to control (absence of enzyme) condition. This improvement was higher than the value reported by Fleurence et al. (1995), as only 3 times was improved in comparison to the control when enzyme mixture of agarase and cellulase was used on *G.gracilis* for the release of protein. In this regard, our results confirmed the efficiency of using carbohydrase and protease enzyme for the release of biomolecule from algal biomass. The release efficiency depends on enzyme type which could vary according to the biomass species and operating conditions as well as the analysis methods.

Table III- 2: Comparison the release of protein from different enzymes and red algae

Algae	Enzyme	Protein Extraction yields	References
<i>Gracilaria gracilis</i>	Enzyme cocktail (Cellulase and protease)	32 %	Current study
	Cellulase	24.18 %	
<i>Gracilaria gracilis</i>	Cellulase	10.31 %	(Nguyen, 2017)
<i>Gracilaria gracilis</i>	Enzyme mixture agarase and cellulase	Protein yields improved 3 times than control condition	(Fleurence et al., 1995b)
<i>Solieria chordalis</i> (carrageenophyte)	Endo-peptidase	15.20 %	(Hardouin et al., 2014)
<i>Chondrus crispus</i> (carrageenophyte)	Novozyme-cellulase	7.1 %	(Kulshreshtha et al., 2015)

5.2. Influence of biomass treatment

The maximum extraction yields (per total content) of protein and sugar from dried biomass were 32 % and 54 %, respectively with enzyme cocktail. Meanwhile, the yields from fresh biomass were higher at 46 % of protein (from protease addition) and 85 % of sugar (with cellulase addition). Therefore, single-enzymes seemed to work better for EAE from fresh biomass, while an enzyme mixture may be needed to handle dried biomass.

It could be seen that biomass treatment plays an important role prior to the extraction methods. As shown in the current study, both types of biomass required different enzymes for a better release of target compounds. This was clearly due to the different levels of cell wall degradation, altering enzyme attacks toward the biomass. After grinded with liquid nitrogen, the grinded freeze-dried biomass (Figure III-9A) was observed around 1-3 mm and the cell surface remained firm. Meanwhile, the grinded fresh biomass sized a bit smaller, reporting around 1-2 mm (Figure III-9B). In addition, the cell of fresh biomass after grinding was already hydrated and seemed to be more soften (due to the intracellular water). These could allow fresh biomass rediluted with small amount of water within the extraction, giving more access of enzyme into the cell structure which favors the release of soluble contents. From this phenomenon, an enzyme is more productive as it can probably reach easily to catalyze the appropriate substrates within the grinded fresh biomass. The size of substrates (specific area) alongside temperature, pH system, enzyme concentration, and extraction time are known as critical factors for the extraction process (Nadar et al., 2018). Although freeze-drying is a well-

known process used to facilitate the extraction process on algal biomass (Denis et al., 2009b; Dumay et al., 2013; Munier et al., 2015; Nguyen, 2017), our study suggests that working on fresh biomass yielded better results.

In this study, the recovery of soluble components increased by 43 % (protein) and 57 % (sugar) better than freeze-dried biomass upon the application of the proper grinding method on fresh biomass prior to the EAE itself. The grinding method conducted on fresh biomass in our study was therefore effective. Given these results, our hypothesis could suggest that the cell wall degradation of fresh biomass was superior to that of freeze-dried biomass and drying process could lead to additional barriers for the release. This could allow a better access between the enzyme and the substrate for a better release of the soluble contents. The grinding of the algae was always the same and we did not look at its effect on the exchange surfaces and the release of compounds of interest. Further investigations are suggested to take into consideration this aspect for a better comprehension.

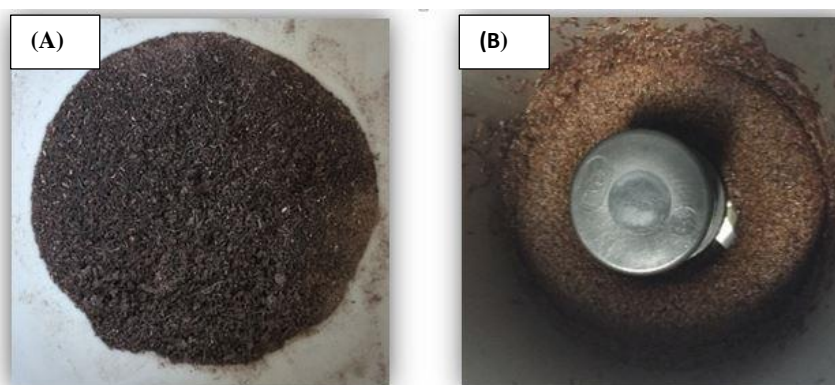


Figure III- 9: Grinded biomass (A) Freeze-dried, (B) Fresh

5.2. Enzyme cocktail: effect of pH and enzyme concentration

This study applied enzyme cocktail which is the mixture between cellulase and protease for the release of soluble contents from biomass algal. The two enzymes presented different catalyze activity. Cellulase is known to catalyze the polysaccharides and therefore generated the release of small oligosaccharides (Fernandes and Carvalho, 2017; Nadar et al., 2018). Meanwhile, protease is an enzyme which target the peptides bonds in protein. Based on different catalyze activity, each enzyme works best at different pH conditions (5.0 for cellulase

and 7.5 for protease). The combination of the two enzymes at a fix operating temperature 32 °C under different pH and concentrations showed that the soluble contents (either protein or sugar) determined the operating pH condition of enzyme cocktail.

Between the two pH (5.0 and 7.5) investigated, results from both type of biomass (freeze-dried and fresh) showed that protein gave higher release in the buffer pH 7.5 while sugar released higher with the buffer pH 5.0. From this result, it showed that more release of protein might come from high protease activity (pH 7.5) and vice versa for the release of sugar (pH 5.0 for cellulase). It could be noticed that between the two enzymes, one will operate better than other according to the provided buffer pH. When enzyme was operated at different optimal pH, it is logically that the enzyme activity would be reduced and less efficient comparing to the activity operated at the optimal pH condition.

Regarding the enzyme concentration, both biomass (freeze-dried and fresh) showed that under pH 7.5, protein released from enzyme dose 47 mg/g dw could produce same release efficiency as two-fold dose (94 mg/g dw). These results were promising and therefore reduce of enzyme cost could be expected. Nevertheless, the release of sugar had a strong relationship with cellulase concentration. The higher the concentration, the higher release of sugar will occur. Therefore, enzyme cocktail 94 mg/g dw algae with contain two times cellulase than 47 mg/g dw produced the highest sugar in the study. Algal cell wall is known with abundant of polysaccharides. In this regard, cellulase dose (47 mg/g dw) was needed in order to increase the liberation of abundant algal sugar from their cell wall. This concentration was also confirmed with the study of Nguyen (2017).

5.3. Influence of enzymes on freeze-dried biomass

Enzymes are known to be able to work synergistically. Based on the broken cell of freeze-dried biomass, the mixing of two enzymes allows for a simultaneous attack on polysaccharides (cellulase) and protein (protease) within the algal cell wall, resulting in a better breakdown of this cell-environment barrier. Cellulase and protease are usually employed to solubilize the plant cell wall for the release of intracellular biomolecules (Nadar et al., 2018). Fleurence et al., 1995 recovered three-fold the quantity of protein from *G. gracilis* by using the enzyme mixture of agarase and cellulase. The yield of R-PE extracted from *Gelidium pusillum* was shown to improve by 26 % with the enzyme consortia (agarase, cellulase, and xylanase) (Mittal and Raghavarao, 2018). Thus, the synergy between enzymes presented a strong effect on the degradation of red seaweed. In this study, it is shown that enzyme cocktail (protease and

cellulase) applied in acetate buffer (50 mM, pH 5.0) is more efficient for the extraction of soluble contents for freeze-dried biomass than single enzyme. In this case, the complementary effect of both enzymes allows a better alteration of the cellular walls of dried biomass.

Under cellulase addition (47 mg/g dw algae at 32 °C), Nguyen (2017) obtained the release of soluble R-PE and protein from freeze-dried *G. gracilis* biomass with a maximum yield of 15.75 mg/g dw and 29.30 mg/g dw respectively. In comparison to this study, the maximum yield of R-PE was lower albeit using an enzyme cocktail. However, the yields of protein were increased by 2.7 times and 2 times using enzyme cocktail and cellulase, respectively. Looking at the two different results from the same biomass and preparation methods, it appears that the efficiency of extraction has surely been affected by the compositions of biomass that vary according to the harvest season, location, tide, etc. The analysis method may also explain the different results of the extraction. The hypothesis is well supported when looking at the discrepancies for biochemical contents of our biomass and literatures, as discussed in Table III-1 above.

Between the two enzymes in this study, cellulase (in acetate buffer 50 mM, pH 5.0) is theoretically more promising since it attacks polysaccharides which are the major component of the algal cell walls. Most of cellulase attacks randomly the internal sites of the amorphous region of the polysaccharide chains, resulting in the generation of small oligosaccharides and facilitate the release of entrapped molecules (Fernandes and Carvalho, 2017; Nadar et al., 2018). Furthermore, cellulase was reported to provoke microcracks on the surface of the cell wall, which led to increased extractability of soluble compounds (Nguyen, 2017). Prior enzyme-assisted extraction studies on *Chondrus crispus* (red algae) and *Codium fragile* (green algae) also revealed that commercial carbohydrase enzyme (Novozyme-cellulase, β -glucanase, and ultaflo) was much more efficient than commercial protease (neutrane) for the release of protein and sugar (Kulshreshtha et al., 2015).

The effect of protease (in acetate buffer 50 mM, pH 7.5) on dried *G. gracilis* biomass for extraction of soluble compounds was less effective for both protein and sugar as shown in the current results. The release of protein and sugar were reported to be lower compared to cellulase (in acetate buffer 50 mM, pH 5.0) and enzyme cocktail. The limitation possibly came from its action mechanism that differs from cellulase (in acetate buffer 50 mM, pH 5.0). Facing with freeze-dried biomass, protease seems to only destabilize the algal cell wall, thus allowing only small quantities of either sugar or protein to be liberated.

5.4. Influence of enzymes on fresh biomass

When using cellulase (16.50 mg/g dw) at 32 °C with fresh biomass, Nguyen (2017) obtained the recovery of soluble R-PE and protein at ~ 4.62 mg/g dw and 9 mg/g dw, respectively. In this study, enzyme concentration was 3 times higher, yet only small improvements were made on the yield of R-PE extraction. On the other hand, the recovery of protein increased significantly by 6 times (cellulase), 9 times (enzyme cocktail), and 12 times (protease).

It is worth noted that the release of R-PE and protein from fresh biomass was higher in the presence of protease. Enzyme cocktail (in acetate buffer 50 mM, pH 5.0), which contains both protease and cellulase should logically yield better results (complementary effect between enzymes) than protease (in acetate buffer 50 mM, pH 7.5) alone. However, we observed the opposite in the current finding. The combination between enzymes did not seem to boost the extractability of soluble contents on fresh biomass. This limitation might come from the nature of substrate, referring to the fresh biomass which enzyme cocktail may not be compatible. In the enzyme assay, it is noteworthy that the state, purity, and stability of the substrate are recognized as very important factors. Enzyme, single or mixture, have a defined affinity to the substrate according to its physiological function (Bisswanger, 2014). Adding to the nature of biomass, the competition between enzymes itself could probably occur, limiting the efficiency of enzyme cocktail during the extraction process. The cellulase activity might provoke more release of small oligosaccharides which could probably slow down the attack of protease for the release of protein from fresh biomass. This phenomenon hinders obtaining better protein and R-PE yields from enzyme cocktail than protease alone. In addition, enzyme cocktail didn't boost the release of sugar more than cellulase alone. This pushed into a confirmation that enzyme consortia (cocktail) was less effective for fresh biomass than single enzyme. Therefore, enzyme applied in EAE with fresh biomass depended on target components, either protease (protein and R-PE) or cellulase (sugar).

6. Conclusions

The addition of enzyme at most suitable conditions and proportions was demonstrated to enhance the extractability of the biomolecule components from algal biomass. The type of enzyme depends on biomass status and target components. Enzyme cocktail at 32 °C released better protein under pH 7.5 with two times less of enzyme concentration (47 mg/g dw algae). On the other hand, using enzyme cocktail for the release of sugar required more of cellulase. Therefore, the operating condition at 32 °C and pH 5.0 with enzyme dose 94 mg/g dw algae was more compatible for the high release of sugar from both type of biomass (freeze-dried and fresh). Our study showed that complementary effect (synergy) between enzymes (enzyme cocktail) was suitable when using freeze-dried biomass while single enzyme (protease or cellulase) handled better with fresh biomass.

This study shows that biomass treatment is a primordial step which will determine the degree and selectivity prior to the extraction process. The extraction was improved by 43 % and 57 % of protein and sugar liberation from fresh biomass compared to dried biomass. From these results, it is suggested that working on fresh biomass upon applying the proper grinding method could yield better results than freeze-dried biomass. Therefore, the wet extraction is encouraging which is more beneficial (drying step elimination, single enzyme needed) for an up-scaling of industrial implementation. Further, the qualitative analysis of extracted liquid, as well as others polysaccharidase-type enzymes, should investigate to explore the release of other intracellular biomolecules. Last but not least, the explanation regarding the level of cell wall degradation should investigate. This aspect would provide more understanding related to the degradation mechanism on each type of biomass.

Chapter IV:

Extrusion coupled with enzymatic hydrolysis for the extraction of hydrosoluble compounds from the red algae *Gracilaria gracilis*

Summary

This chapter focused on the enzymatic extrusion on fresh algae *G.gracilis* for the release of soluble R-PE, sugar, and protein. The experimental design was conducted with response surface methodology using Box-Behnken design. In this study, three variables were investigated including biomass flow rate, enzyme concentration, and screw speed. Enzyme cellulase was used and a constant operating temperature 32 °C was applied during the extrusion process. Results revealed that biomass flow rate and enzyme concentration presented significant effect on the release of protein and sugar respectively. Meanwhile, both biomass flow rate and enzyme concentration were shown to have the significant effect on the release of R-PE. Furthermore, the response surface models from the obtained results were adequate for all responses. Predicted optimal condition (maximal release of together R-PE, protein, and sugar) was suggested with biomass flow rate at 1 kg/h, enzyme concentration at 4.45 %/ dw algae, and screw speed at 350 rpm. The predicted optimal condition was experimentally verified. Results confirmed the release efficiency of protein and sugar between predicted and observed values. Finally, extraction yields were compared between extrusion alone and extrusion with (rapid) maceration as well as with enzymatic hydrolysis (batch without extrusion, 5 h). Results showed that extrusion with maceration was more promising as the released yields were better than extrusion alone. Meanwhile, it is worth noted that extrusion with maceration could generate high extraction yields which were comparable to those obtained from enzymatic hydrolysis (batch).

1. Introduction and objective

Macroalgae made up 27 % of global production from aquaculture. This resource has a long history in people's daily life i.e. food, medicine, fertilizer as well as animal feedstock (Bezerra et al., 2018; P. Torres et al., 2019; Wells et al., 2017). They are prized for their nutritional benefits and are rich in carbohydrates, proteins, vitamins, minerals as well as bio-active components (Beaulieu et al., 2015; Chiboub et al., 2017; Zhu et al., 2017). Hence, they are considered as a potential caloric source for future global food requirements (Kumar et al., 2015). *Gracilaria* genus from *Rhodophyta* spread all around the world from tropical to temperate latitude (Iyer et al., 2004; P. Torres et al., 2019). It is known for the high content in polysaccharides which are reported up to 62-63 % of biomass dry weight (Rioux et al., 2017). Furthermore, this genus is rich in R-phycoerythrin with a content that can be up to 50 % w/w of total protein (Pimentel et al., 2019). R-phycoerythrin is regarded with a high market value and is widely used for immunology, cell biology, flow cytometry, cosmetics, and food as natural dye (Bedoux et al., 2014; Dumay et al., 2013; Munier et al., 2014).

Extrusion is a continuous process that consists of several treatments including mixing, shearing, heating, cooling, shaping, partial drying, forming or transforming a material within a screw or barrel system (Arora et al., 2020; Makoure et al., 2019a; Vauchel et al., 2008). Extrusion has been used since the mid-1930 and became one of the most ideal food processing for different kind of products such as breakfast, cereals, ready-to-eat snack, etc. (Makoure et al., 2019b; Singh et al., 2018). Reactive extrusion is an advancement of extrusion since there are chemical or biological reactions inside apparatus in addition to extrusion (Choulak, 2004). The first development of reactive extrusion dated in 1980 with the modification of synthetic polymer. Later, it was quickly spread and applied in various areas such as polymerization, grafting, cross-linking, etc (Xie et al., 2006). Reactive extrusion was shown to enhance extracting yields and products properties as well as time and consumables consumption (Vauchel et al., 2008). Nevertheless, it is regarded as an interesting alternative choice toward the batch process (Baron et al., 2010). Among reactive extrusion, the concept of enzymatic reactive extrusion is recently proposed in order to generalize the use of extruder as enzymatic reactor in the wider fields (E. Xu et al., 2020).

Operating parameters and mechanical design of the extruder are responsible for a good performance of extrusion process and vary one to another according to the nature of the material. Parameters such as biomass/solvent ratio, screw speed, flow rates, enzyme

concentration, temperature, screw configuration as well as die diameter are often investigated and optimized for various extractions, notably for oil and starch extractions (Amalia Kartika et al., 2010; Hirth et al., 2014). Several researches have shown positive effect of extrusion compared to traditional extraction methods. Reactive extrusion yielded better than batch extraction (discontinuous process) for the alkaline extraction of alginates from brown macroalgae *Laminaria digitata* (Vauchel et al., 2008). The oil extraction from fish by-product also proved that enzymatic extrusion induced higher yields in comparison to batch process (Makoure et al., 2019a). Taking this into account, reactive/enzymatic extrusion could provide more opportunities to enhance the release of biomolecules from marine biomass.

To our knowledge, the use of enzymatic extrusion for macroalgae bio-refinery is still limited. No report of optimization regarding enzymatic extrusion with twin-screw extruder from *Gracilaria gracilis* biomass was yet conducted. Therefore, this current study aimed to conduct the enzymatic extrusion on *Gracilaria gracilis* in the presence of enzyme (cellulase) and optimized the extrusion parameters using experimental design with response surface method. Three parameters including sample (biomass) flow rate and enzyme concentration as well as screw speed were investigated. The hydrosoluble contents of sugar (notably polysaccharides), protein, and R-phycoerythrin released in aqueous medium were determined.

2. Biochemical contents of raw materials

The biochemical compositions of *G. gracilis* biomass in this study were given in Table IV-1. The content of carbohydrate and protein was equal to 34.08 % dw and 30.5 % dw respectively. Following the literature, the amount of protein in *G. gracilis* varies around 16-31 % dw meanwhile the carbohydrate content varies from 18-34 % dw (CEVA, 2015; Francavilla et al., 2013a; Mensi, 2019; Rodrigues et al., 2015; Sfriso et al., 1994). Therefore, our results are in accordance with others previous investigations. On the other hand, ash content in our biomass was equal to 2.80 % dw (biomass dry weight) which was lower in comparison to other works where ash contents ranged from 17 to 24 % dw (CEVA, 2015; Rodrigues et al., 2015). Beside these principal contents, *G. gracilis* contained low lipid content (1-3 % dw) and the remainder of the contents were vitamin, mineral, and polyphenol compounds. Environmental factors such as season, geographical location, climate, water quality, tide, etc are the major factors for the variation of these biochemical contents of biomass.

Table IV- 1: Biochemical components of *G. gracilis* raw biomass

Compositions	%
Dry weight*	12.72 ± 0.26
Water*	87.28 ± 0.20
Ash**	2.80 ± 0.03
Total Carbohydrate**	34.08 ± 2.53
Total Protein**	30.5 ± 0.90
Soluble R-Phycoerythrin**	0.70 ± 0.12

*: Contents are expressed per fresh weight

** : Contents are expressed per dry weight

3. Analysis of experimental design (enzymatic extrusion without maceration)

3.1. Summary of responses results

Results from Box-Behnken design of 15 experiments conditions of enzymatic extrusion were summarized in Table IV-2. These experimental data were used to calculate the coefficients of the second-order polynomial equation as well as the agreement between experimental and predicted values. Results of fitting model to the data from ANOVA analysis were given in Table IV-3.

The release yields of R-PE varied from 44 to 61 %/ dw algae. The best R-PE liberation was obtained during experiments 1, 3, 5, and 7. The highest release of R-PE (61.86 %) was observed from experiment 7 (flow rate 1 kg/h, enzyme 2.85 %, and screw speed 350 rpm). Meanwhile, protein liberation ranged from 11 to 15 %/ dw algae. Experiments (3, 7, and 8) provided the best protein yields and the maximal release was 15.14 % from experiment 7. Regarding the release of sugar, it was shown that high enzyme concentration gave high sugar liberation (more osidic bonds were broken) (Dumay et al., 2013). High release of sugar was found from experiments 3, 7, 8, and 12 while the highest release was from experiment 3 (flow rate 1 kg/h, enzyme 4.7 %, and screw speed 225 rpm).

Table IV- 2: Variables and Response results

Exp	Variables			Responses		
	Flow rate kg/h	Enzyme concentration %	Screw speed rpm	R-PE (%)	Sugar (%)	Protein (%)
1	1 (-1)	1 (-1)	225 (0)	60.57 ± 5.41	25.45 ± 0.29	13.37 ± 1.86
2	5 (+1)	1 (-1)	225 (0)	51.04 ± 2.05	24.61 ± 0.35	13.40 ± 1.35
3	1 (-1)	4.7 (+1)	225 (0)	61.43 ± 2.07	28.53 ± 2.70	13.13 ± 1.52
4	5 (+1)	4.7 (+1)	225 (0)	55.07 ± 4.66	27.58 ± 1.17	12.15 ± 2.46
5	1 (-1)	2.85 (0)	100 (-1)	61.57 ± 1.70	26.99 ± 2.83	14.34 ± 1.62
6	5 (+1)	2.85 (0)	100 (-1)	48.44 ± 2.12	25.19 ± 0.88	12.97 ± 1.39
7	1 (-1)	2.85 (0)	350 (+1)	61.86 ± 1.64	27.61 ± 3.55	15.14 ± 1.14
8	5 (+1)	2.85 (0)	350 (+1)	53.99 ± 2.65	27.95 ± 1.39	13.91 ± 1.29
9	3 (0)	1 (-1)	100 (-1)	46.62 ± 1.73	22.18 ± 0.98	13.07 ± 0.76
10	3 (0)	4.7 (+1)	100 (-1)	49.30 ± 2.85	26.20 ± 1.31	11.88 ± 1.30
11	3 (0)	1 (-1)	350 (+1)	44.12 ± 2.07	20.62 ± 0.83	11.24 ± 1.79
12	3 (0)	4.7 (+1)	350 (+1)	54.36 ± 1.60	27.19 ± 1.16	13.38 ± 0.28
13	3 (0)	2.85 (0)	225 (0)	50.37 ± 1.50	27.03 ± 1.64	12.74 ± 0.45
14	3 (0)	2.85 (0)	225 (0)	54.64 ± 2.91	25.24 ± 2.54	13.29 ± 0.91
15	3 (0)	2.85 (0)	225 (0)	54.62 ± 3.63	25.60 ± 1.52	12.56 ± 1.74

Results were presented as mean ± standard deviation

3.2. Evaluation of RSM model

Base on the obtained results, ANOVA analysis (Table IV-3) showed that the fitting regression models in all responses variables were highly adequate ($p < 0.05$) and no significant model error (lack of it) was detected. Furthermore, the correlation coefficient (R^2) of each response was superior to 0.8 which corresponded to a good prediction model for chemical data (Lundstedt et al., 1998; Sugiono et al., 2019a). In this regard, the response surface models developed from the obtained results were adequate.

Table IV- 3: ANOVA analysis for regression model of soluble sugar, protein, and R-PE responses

	DF	SS	MS (variance)	F	p-value	SD
R-PE (%), R² 0.923						
Regression	7	412.08	58.86	12.05	0.002	7.67
Residual	7	34.19	4.88			2.21
Lack of Fit (Model error)	5	22.09	4.41	0.73	0.664	2.10
Pure error (Replicate error)	2	12.09	6.04			2.45
Total	15	43970.5	2931.37			
Sugar (%), R² 0.829						
Regression	5	54.44	10.88	8.72	0.003	3.29
Residual	9	11.23	1.24			1.11
Lack of Fit (Model error)	7	9.43	1.34	1.50	0.456	1.16
Pure error (Replicate error)	2	1.79	0.89			0.94
Total	15	10100.4	673.36			
Protein (%), R² 0.806						
Regression	6	10.41	1.73	5.53	0.015	1.31
Residual	8	2.50	0.31			0.56
Lack of Fit (Model error)	6	2.21	0.36	2.55	0.308	0.60
Pure error (Replicate error)	2	0.28	0.14			0.38
Total	15	2588.91	172.59			

DF: Degree of freedom, SS: Sum square, MS: Mean square, F: Fisher calculated, p-value: probability, SD: Standard deviation

3.3. Regression equations of responses

Once the developed model was validated, the significance level of each variable coefficients in the polynomial equation was investigated in order to understand the effect of each variable on the responses. Tables IV-4 summarized the variables coefficient and their significant level for the three selected responses (R-PE, sugar, and protein). The developed second-order equations were found as below:

$$\text{Sugar (\%)} = 25.49 - 0.40 X_1 + 2.08 X_2 + 0.35 X_3 + 1.79 X_1 * X_1 - 1.09 X_2 * X_2 - 0.75 X_3 * X_3 - 0.02 X_1 * X_2 + 0.53 X_1 * X_3 + 0.63 X_2 * X_3$$

$$\text{Protein (\%)} = 13.05 - 0.44 X_1 - 0.06 X_2 + 0.17 X_3 + 0.89 X_1 * X_1 - 0.79 X_2 * X_2 + 0.30 X_3 * X_3 - 0.25 X_1 * X_2 + 0.03 X_1 * X_3 + 0.83 X_2 * X_3$$

$$\text{R-PE (\%)} = 53.21 - 4.61 X_1 + 2.22 X_2 + 1.05 X_3 + 5.84 X_1 * X_1 - 2.02 X_2 * X_2 - 2.58 X_3 * X_3 + 0.79 X_1 * X_2 + 1.31 X_1 * X_3 + 1.89 X_2 * X_3$$

3.4. Linear and interaction effect between factors and responses

ANOVA analysis (Tables IV-4) showed that both linear variables X1 (biomass flow rate) and X2 (enzyme concentration) had significant effect on the release of R-PE. On the other hand, only X1 and X2 were found to affect the release of protein and sugar respectively. Interestingly, screw speed (X3) did not seem to have any significant effect on the release of soluble R-PE, protein, and sugar. However, this variable interacted with other variables (X2*X3 on the release of protein) and gave significant effect. The quadratic term X1*X1 was shown to affect significantly on the release of the three responses. From these results, it showed that all variables affected the release of soluble contents and each variable affected differently according to the target responses. Flow rate (X1) and Enzyme concentration (X3) were the most influencing ($p < 0.01$) on the release of R-PE and sugar respectively.

Significant effects

The coefficient plot of significant variables and interactions on each response were illustrated in Figure IV-1. It is worth noticed that flow rate affected negatively on all responses, showing that it was inversely proportion to the responses value. In this regards, low flow rate is required for increasing the responses values. Enzyme concentration and screw speed, on the other hand affected positively on all responses. Therefore, high enzyme concentration and high screw speed were needed to obtain the high responses values.

Table IV- 4A: Coefficient of each parameters for R-PE response

R-PE	Coeff. SC	Std. Err.	p-value	Conf. int(±)
Constant	53.21	1.27	1.19005e-09	3.01
Flow rate (X1)	-4.61	0.78	0.0005	1.84
Enzyme concentration (X2)	2.22	0.78	0.024	1.84
Screw speed (X3)	1.05	0.78	0.220	1.84
Flo*Flo (X1*X1)	5.84	1.15	0.001	2.71
Enzy*Enzy (X2*X2)	-2.02	1.15	0.121	2.71
Scr*Scr (X3*X3)	-2.58	1.15	0.059	2.71
Flo*Enzy (X1 *X2)	0.79	1.11	0.50	2.86
Flo*Scr (X1*X3)	1.31	1.11	0.29	2.86
Enzy*Scr (X2*X3)	1.89	1.10	0.130	2.61

Std. Err. Standard Error, p-value: probability, Conf. Int (±) : Confidence intervals

Table IV-4B: Coefficient of each parameters for Sugar response

Sugar	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	25.49	0.53	4.06823e-12	1.21
Flow rate (X1)	-0.40	0.39	0.330	0.89
Enzyme concentration (X2)	2.08	0.39	0.0005	0.89
Screw speed (X3)	0.35	0.39	0.396	0.89
Flo*Flo (X1*X1)	1.795	0.57	0.012	1.31
Enzy*Enzy (X2*X2)	-1.09	0.57	0.092	1.31
Scr*Scr (X3*X3)	-0.75	0.58	0.25	1.50
Flo*Enzy (X1*X2)	-0.02	0.56	0.96	1.44
Flo*Scr (X1*X3)	0.53	0.56	0.38	1.44
Enzy*Scr (X2*X3)	0.63	0.56	0.30	1.44

Std. Err. Standard Error, p-value: probability, Conf. Int (±) : Confidence intervals

Table IV-4C: Coefficient of each parameters for Protein response

Protein	Coeff. SC	Std. Err.	P	Conf. int(±)
Constant	13.05	0.26	3.60927e-11	0.62
Flow rate (X1)	-0.44	0.198	0.055	0.45
Enzyme concentration (X2)	-0.06	0.198	0.741	0.45
Screw speed (X3)	0.17	0.198	0.399	0.45
Flo*Flo (X1*X1)	0.89	0.290	0.014	0.67
Enzy*Enzy (X2*X2)	-0.79	0.290	0.025	0.67
Scr*Scr (X3*X3)	0.30	0.321	0.388	0.826
Flo*Enzy (X1*X2)	-0.25	0.308	0.450	0.794
Flo*Scr (X1*X3)	0.03	0.308	0.914	0.794
Enzy*Scr (X2*X3)	0.83	0.280	0.017	0.64

Std. Err. Standard Error, p-value: probability, Conf. Int (±) : Confidence intervals

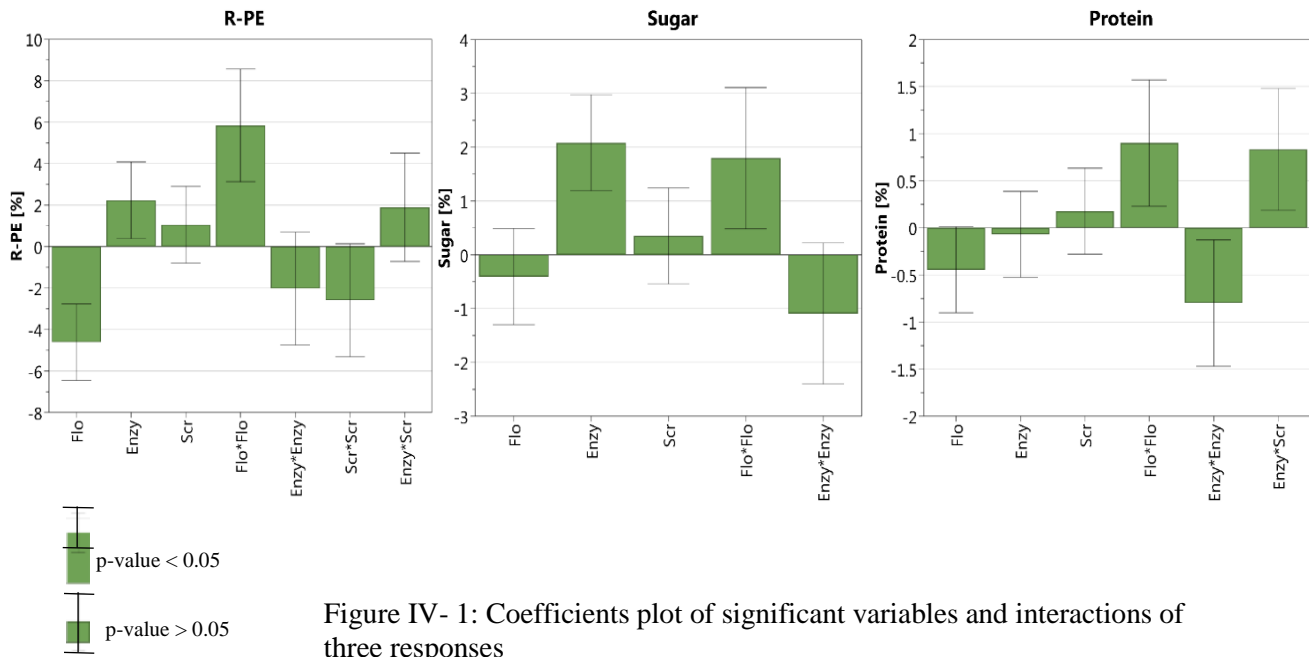


Figure IV- 1: Coefficients plot of significant variables and interactions of three responses

3.5. Observed vs predicted

The observed and predicted values from the obtained data and developed model were presented in Figure IV-2. The good fit between observed and predicted value should logically locate as close as possible to the orthogonal straight line. Modde 12.1 (software) suggested that the perfect fit was obtained with the value of DF (degree of freedom) of residues < 3 (Sartorius Stedim Data Analytics, 2017). As shown in the current results, the DF of residues of R-PE, protein, and sugar from the current results were 7, 8, and 9 respectively (Table IV-2). In this regard, less fit (between the predicted and observed values) was observed and may come from biomass variation as well as instrumental error. However, the regression model developed presented acceptable correlation coefficient ($R^2 > 0.80$) and ANOVA analysis did confirm that the model was significant. Based on this observation, the developed regression model could be applicable while more tests are needed for further improvements.

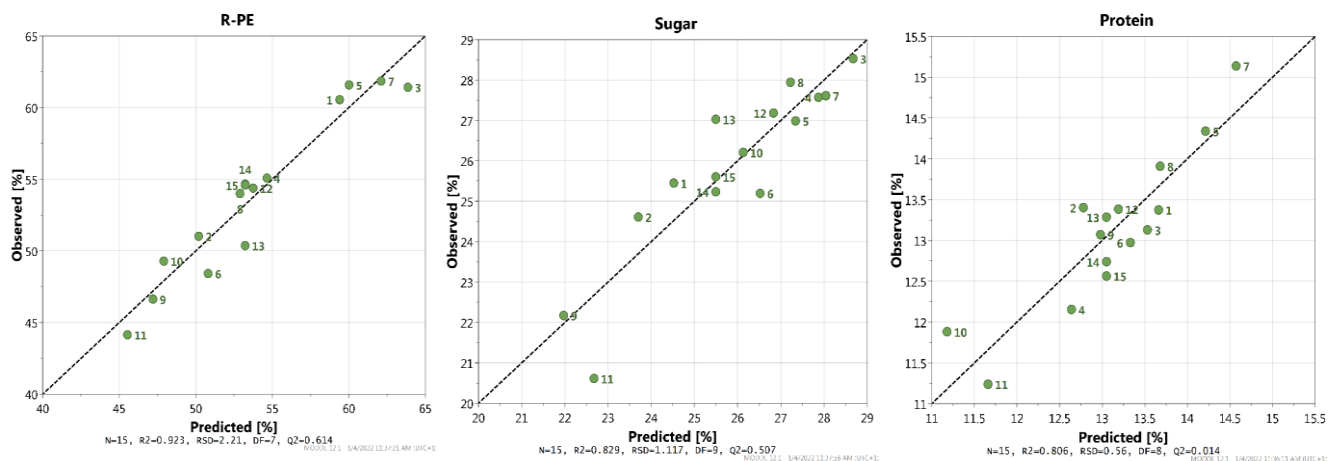
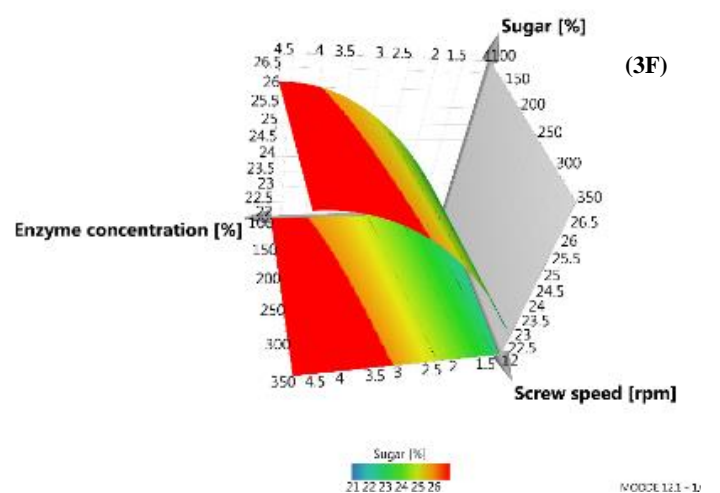
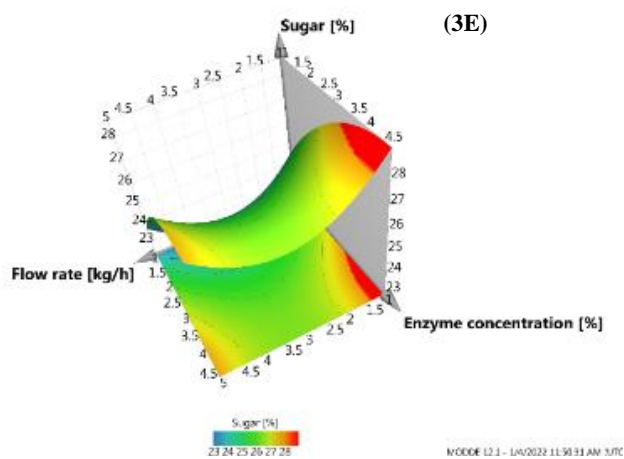
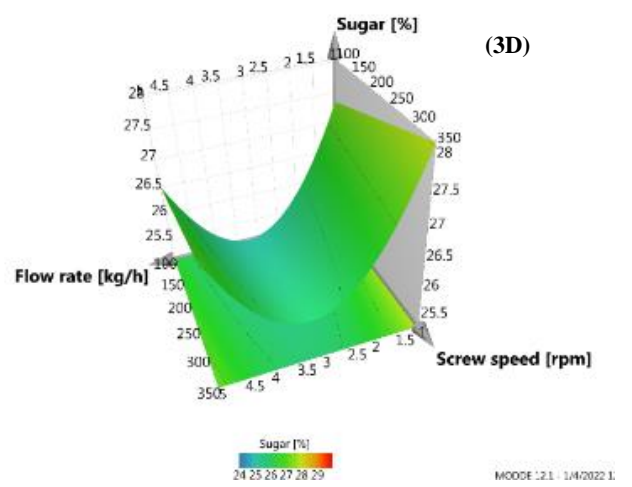
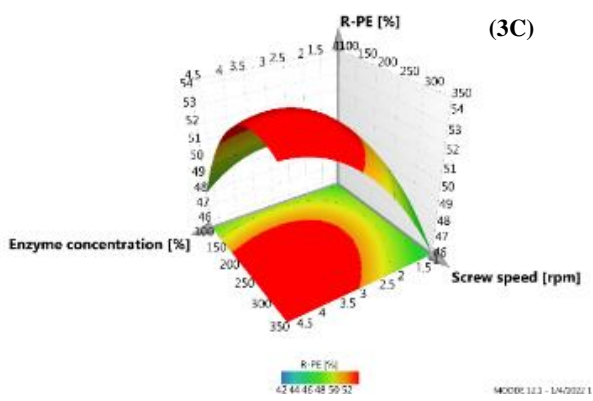
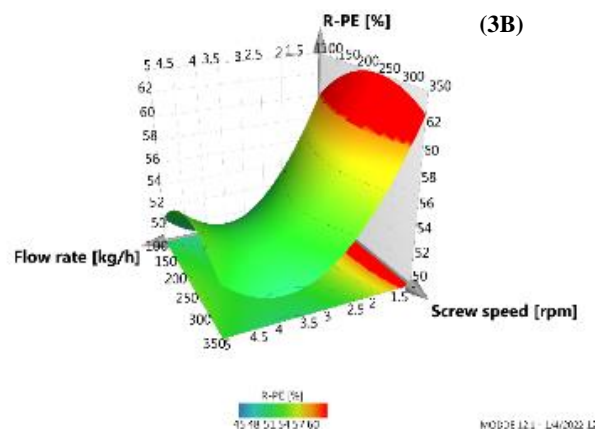
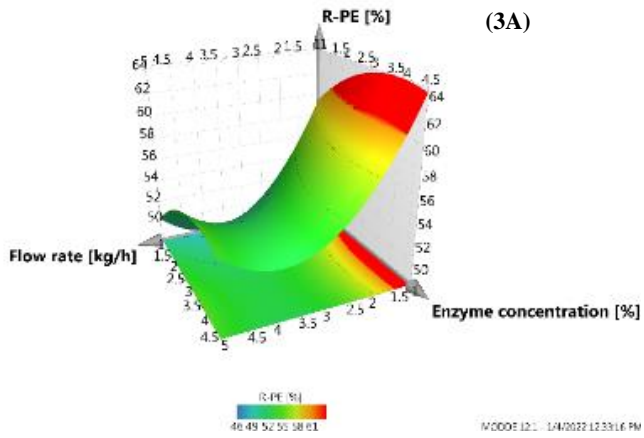


Figure IV- 2: Observed vs predicted plots of soluble R-PE, Sugar, and Protein response

3.6. Response surface representation

The graphs of response surfaces were shown in Figure IV-3. This 3D graphical representation gave better understanding and illustrated the effect of two variables (X1&X2, X2&X3, and X1&X3) on the predicted responses. Meanwhile the other one variable was kept at basal level. As observed in Figure IV-3 A, B, and C, the release of sugar from the developed model showed that low flow rate, high enzyme concentration together with high screw speed tented to give high release of sugar from the developed regression model. The release of R-PE (Figure IV-3D, E, and F) illustrated the same observation, showing that low flow rate with high enzyme concentration and screw speed could generate more yields of R-PE. Regarding the release of protein (Figure IV-3G, H, and I), the model's response surface plot showed that low flow rate, high enzyme concentration, and high screw speed also tented to produce better extraction yields of protein from the obtained developed model. In this regard, all predicted responses (soluble sugar, protein, and R-PE) were the highest when low biomass flow rate (X1), high enzyme concentration (X2), and high screw speed (X3) were operated.



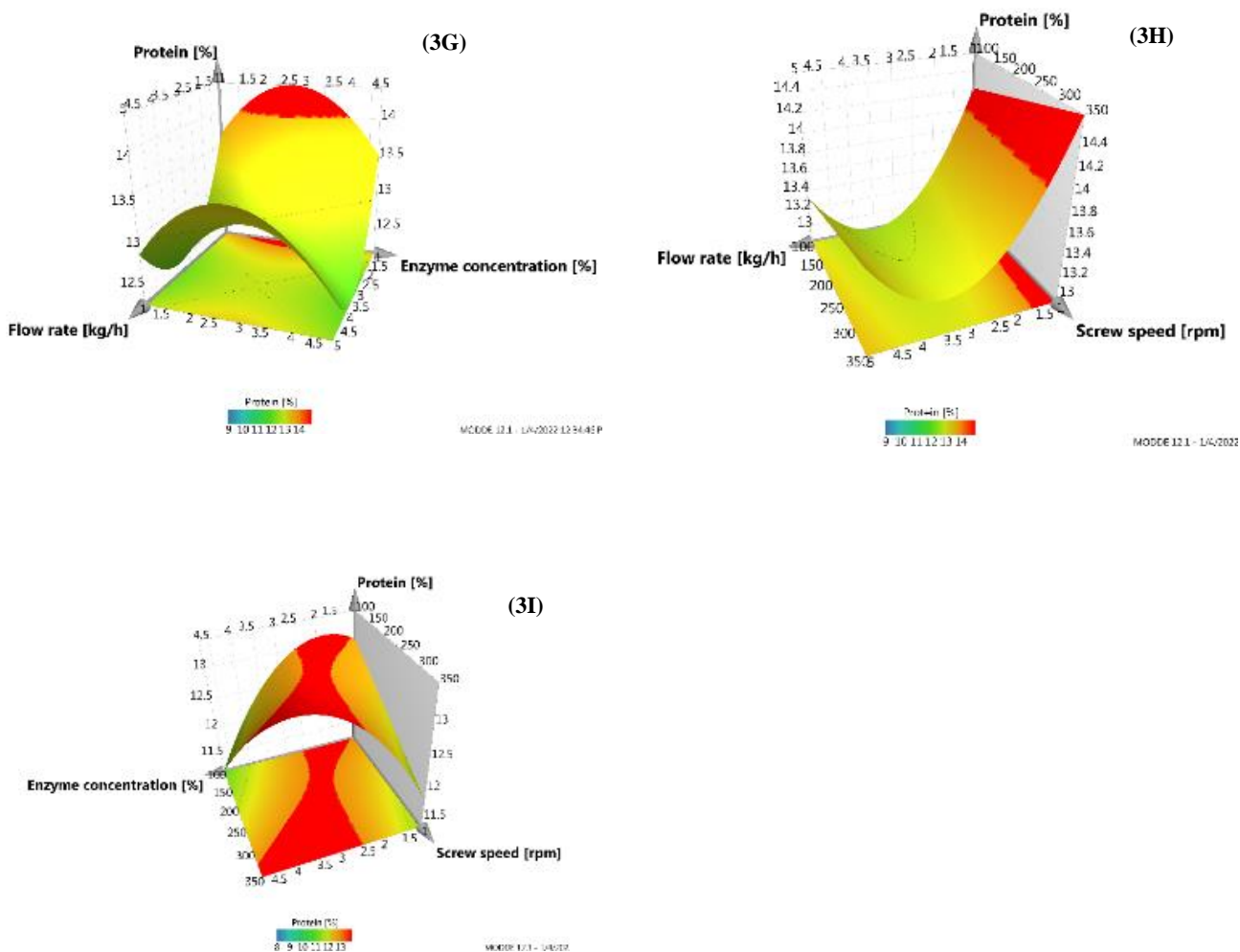


Figure IV- 3 : Response surface plot on the release of soluble R-PE (A-C), sugar (D-F), and protein (G-I)

3.7. Influence of extrusion variables

From the obtained results (Table IV-2), it is worth noted that low sample flow rate gave high release yields of all responses. Reducing sample flow rate was known to increase the residence time which allowed biomass to stay longer in the extruder (Baron et al., 2010). Thus, it allows sufficient contact time between biomass and enzyme, inducing enhancement of the extraction yields. Vauchel (2007) obtained a good extraction yields at sample flow rate 1 kg/h when extracting alginate by extrusion from brown algae *Laminaria digitata*. A good balance between biomass and solvent amount is known as one of the important parameters for the extrusion process driving. In case of high dilution of biomass/solvent mixture, low shear stress

can appear and biomass cannot be fully destructured. However, when the mixture is not diluted enough, biomass accumulation can take place and can create a plug which could stop the operation (Vauchel, 2007).

Besides working with low sample flow rate, the release of sugar, and R-PE was affected significantly by enzyme concentration in this study. The extraction yield was greatly improved when enzyme concentration was increased. It could be noted that the more enzyme applied, the more degradation of small polysaccharides on algal cell wall, resulting more release of sugar and others intracellular molecules (Fernandes and Carvalho, 2017; Nadar et al., 2018).

Screw speed seemed to have less effect on the release of soluble contents in the current study (Table IV-2). Our results showed that high liberation was obtained mostly with screw speed at high level (350 rpm). Increasing screw speed was known to provoke shear, elevate thermal stress, and high pressure on the material (Hirth et al., 2014; Sugiono et al., 2019a). The accessibility of enzyme to the substrate should logically increase under high screw speed, resulting to more liberation. On the other hand, several researches have shown that high screw speed provoked the rapid residence time of the material in the extruder (Amalia Kartika et al., 2005; Baron et al., 2010), which resulted not enough interaction time between enzyme/substrate for the release of biomolecule from biomass. It could be seen that screw speed exerted two effects which contrasted each other, one was responsible for the accessibility in the mixture system while the other one affected the residence time. These effects made it difficult to predict its effect on nutritional status of food (Bhattacharya et al., 1988). Both effects were known important for the extraction, hence these could explain the less effect of this variable within this experimental design.

3.8. Optimal point and verification

The prediction of optimal condition of enzymatic extrusion was obtained at sample flow rate 1 kg/h, enzyme concentration 4.45 %/ dw algae, and screw speed 350 rpm. The desirability of this prediction was 0.98. The desirability equals to 1 means that the prediction has high validity level (Sugiono et al., 2019a). The results of predicted and observed responses from this optimal condition was summarized in Table IV-5. The results of verification showed that the predicted values of sugar and protein was in close agreement with the observed values and was found not significant different ($p > 0.05$). However, the observed R-PE yields was two times lower than the prediction. The low release of R-PE may come from several possibilities. First, the release R-PE might entrap on the pellet during the separation (centrifugation) process. In

addition, biomass itself could generate the variation. Biomass was collected largely at the same zone, however they may have different habitat conditions (temperature, submersion time, attach in sand or rock, etc) which could alter the release efficiency of R-PE. Even the release yield of R-PE was not as predicted, the verification validated for protein and sugar liberation. Therefore, the predicted optimal condition could be applicable. Further works were recommended in order to ensure the validity of R-PE liberation.

It is worth noticed that some conditions (experiments 3 and 7) in Table IV-2 gave high release of some components than the predicted optimal condition. However, the main focus of this study was to obtain together the maximal release of all responses (R-PE, sugar, and protein). In this regard, the optimal condition was selected with the predicted variables to achieve the maximal release efficiency.

Table IV- 5: The released yields from observed and predicted optimal condition

		Extrusion condition			Released yields		
	Exp	Flow rate kg/h	Enzyme concentration %	Screw speed rpm	R-PE %	Sugar %	Protein %
Observed	Opt cond.	1	4.45	350	35.97 ± 7.87 ^a	29.23 ± 4.75 ^c	16.38 ± 3.55 ^d
Predicted (Desirability 0.98)	Opt cond.	1.00003	4.45	350	62.17 ^b	28.67 ^c	14.97 ^d

4. Comparison between enzymatic extrusion (with and without maceration) and enzymatic hydrolysis (batch without extrusion)

4.1. Extrusion with and without enzyme

Extrusion without enzyme (control condition) at optimal condition (highest release of target compounds) was investigated on fresh biomass for a better understating of enzyme addition. The obtained results were summarized in Table IV-6. Results showed that enzyme addition did improved the released yields better than control condition. The release efficiency increased by 55.71 %, 48.30 %, and 40.84 % of R-PE, sugar, and protein respectively from enzyme addition compared to the control. In this regard, the thermomechanical effect generated from extrusion together with enzyme activity could make the improvement of release yields in a short time. The results could confirm the advantage of applying enzyme with extrusion where the thermomechanical and enzymatic activity could operate at the same time to produce high yields.

Table IV- 6: The released yields from extrusion with and without enzyme at optimal condition

Exp	Extrusion condition			Released yields		
	Flow rate kg/h	Enzyme concentration %	Screw speed rpm	R-PE %	Sugar %	Protein %
Control	1	-	350	23.10 ± 4.35 ^a	19.71 ± 1.00 ^c	11.63 ± 1.91 ^e
Opt cond.	1	4.45	350	35.97 ± 7.87 ^b	29.23 ± 4.75 ^d	16.38 ± 3.55 ^f

4.2. Enzymatic extrusion with and without maceration

The release yields at optimal condition of soluble R-PE, sugar, and protein from extrusion (E) alone, extrusion with maceration (EM), and enzymatic hydrolysis alone (EH: Batch conducted 5 h without extrusion) were presented in Figure IV-4.

Between enzymatic extrusion alone and enzymatic extrusion with maceration, results showed that the rapid maceration (10 min at 4 °C to minimize the enzyme activity) after extrusion was beneficial and help to improve the release efficiency. The release of R-PE, sugar, and protein increased by 1.5, 1.3, and 1.3 times respectively when comparing to enzymatic extrusion alone. As shown in Figure IV-4, extrusion with maceration could release R-PE yield 56 % /dw algae per total content. The protein yield was reported 21 % / dw algae while sugar release efficiency was reported around 40 % / dw algae per total content. Basing from these results, it could suggest that the entrapment (during the first separation process) of the release R-PE, sugar, and protein after extrusion could liberate thanks to the maceration process. It could be noted that extrusion was efficient and able to produce more condense media between algae and enzyme for a better release of biomolecule. In this regard, high release efficiency could obtain with respect to the rapid time consuming and less solvent was needed.

4.3. Comparison between enzymatic extrusion without maceration and enzymatic hydrolysis (batch without extrusion)

When comparing enzymatic extrusion alone and enzymatic hydrolysis alone (batch without extrusion), it showed that extrusion alone produced about two times less of R-PE and sugar yields compared to enzymatic hydrolysis (Figure IV-4). Interestingly, the release of protein was similar (16-18 %/dw algae) between these two processes. In this regard, conducting extrusion could be beneficial, in particular for the release of protein. In term of productivity,

as shown in Table IV-7, results from enzymatic extrusion for the three target components were much higher in comparison to those obtained from enzymatic hydrolysis (Batch). It is worth noticed that in a very short time (5 min), extrusion could produce 100 (R-PE), 181 (Protein), and 85 (Sugar) times higher of R-PE than batch extraction. Beside, among the three components, the productivity of sugar was the highest which was probably related to the applied enzyme (cellulase catalyzes polysaccharides and favors the release of sugar) (Fernandes and Carvalho, 2017; Nadar et al., 2018). Therefore, extrusion offers as a promising extraction technique toward the enzymatic hydrolysis (batch) with respect to industrial scale-up, short time consuming, and high productivity of the product.

Table IV- 7: Productivity of soluble R-PE, protein, and sugar by different techniques

Technique	Times (min)	Productivity (kg/l/h)		
		R-PE	Protein	Sugar
Hydrolysis enzymatic (Batch)	300	$24.10^{-6} \pm 2.1 e^{-6}$	$33.10^{-5} \pm 1.72 e^{-5}$	$13.10^{-4} \pm 8.11 e^{-8}$
Enzymatic Extrusion	5	$28.10^{-4} \pm 6. 10^{-4}$	0.06 ± 0.01	0.11 ± 0.02

Results were calculated at optimal conditions (enzyme concentration 4.45 % dw algae)

4.4. Comparison between enzymatic extrusion with maceration and enzymatic hydrolysis (batch without extrusion)

The extrusion followed by maceration with pure water gave promising results and presented great interest toward the enzymatic hydrolysis alone. The release of R-PE from extrusion followed by maceration was reported at 56 %/ dw algae which was similar to the release obtained from enzymatic hydrolysis (batch without extrusion, 5 h). The protein yields 21 %/ dw algae was slightly higher comparing to enzymatic hydrolysis alone (18 %/ dw algae). Nevertheless, the release of sugar (60 %/ dw algae) remained the highest with enzymatic hydrolysis while extrusion with maceration released around 40 % / dw algae of sugar.

Basing from these results, enzymatic extrusion followed by maceration enhanced extraction yields and reduced greatly time consuming (from 5 h with enzymatic hydrolysis alone to less than 30 min with enzymatic extrusion with maceration). Furthermore, adding with the high level of shearing and mixing induced by the twin-screw extruder as well as the enzyme action allowed to probably cause microporous and degrade the algal cell-wall. This phenomenon boosted mixing and permeation of solvent between biomass/solvent mixture, resulting to a better release of algal biomolecules (Gatt et al., 2019; Li et al., 2020; Vauchel et

al., 2008). Based on this output, it could suggest that extrusion technique could be beneficial on algal extraction and more works are needed (i.e. screw profile) to improve the process on each and every material (biomass) as well as the target components.

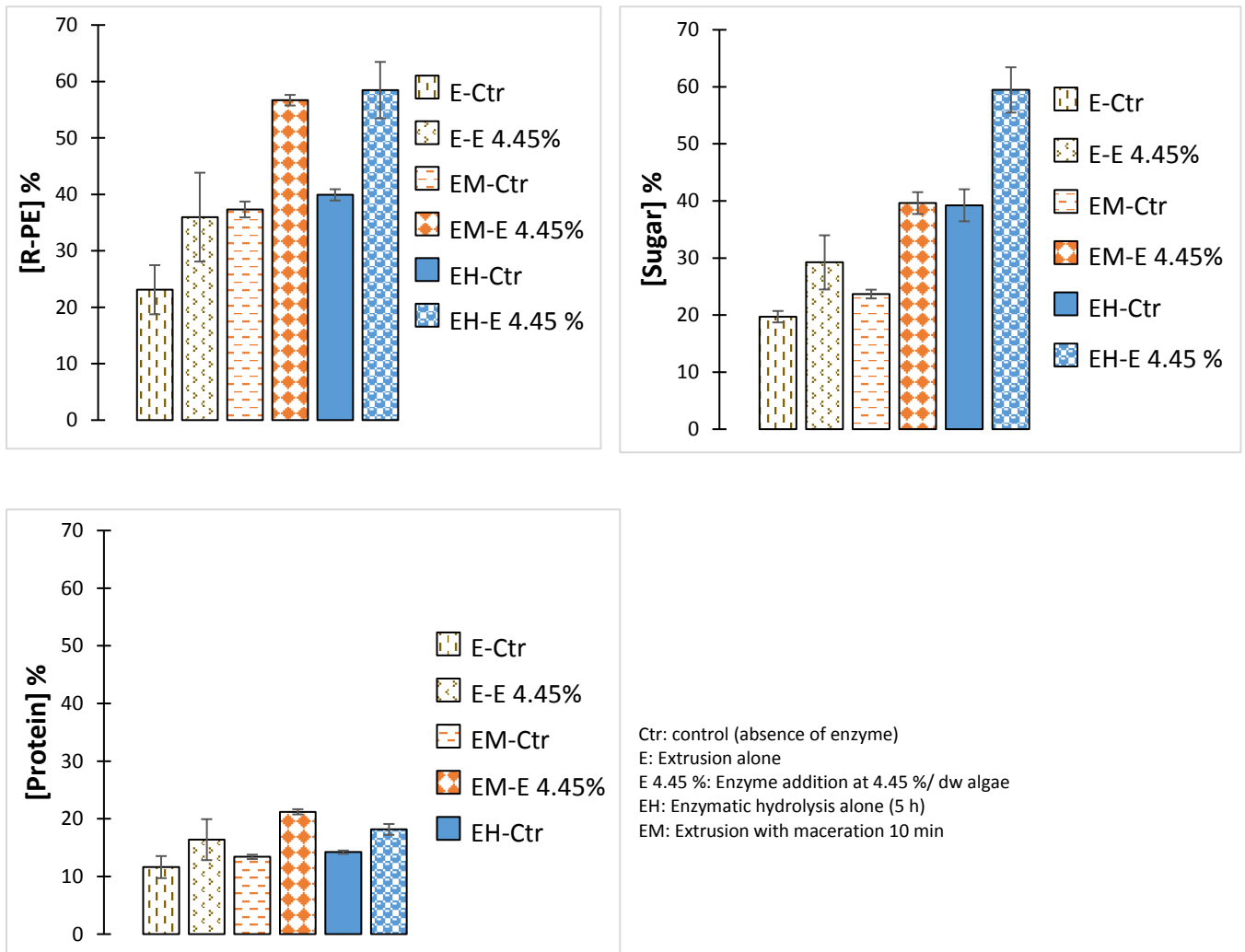


Figure IV- 4: Released yields from enzymatic extrusion alone, enzymatic extrusion with maceration, and enzymatic hydrolysis (Batch) alone of soluble R-PE (A), sugar (B), and protein (C)

5. Conclusions

To conclude, the results presented in this chapter showed that biomass flow rate and enzyme concentration affected significantly on the release of soluble R-PE, sugar, and protein following the RSM analysis. The adequate developed regression models were elaborated. Within the experimental setup, the experiment conditions with low biomass flow rate with high enzyme concentration and high screw speed would give best release of the three target responses (R-PE, protein, and sugar). The predicted optimal condition was suggested at biomass flow rate 1 kg/h, enzyme concentration 4.45 %/ dw algae, and screw speed 350 rpm. The verification of this suggested condition validated the release of protein and sugar while the release of R-PE needed more investigations. Comparing with enzymatic hydrolysis alone (batch without extrusion), the yields of R-PE and sugar released from enzymatic extrusion was around two times lower. However, the yields of protein were at the same level with enzymatic hydrolysis alone. Besides, extrusion generated higher productivity than batch hydrolysis extraction. In addition, enzymatic extrusion followed by rapid maceration (pure water) significantly improved extraction yields (especially R-PE) with the same result as shown in enzymatic hydrolysis alone (batch).

Therefore, this study demonstrated that conducting enzymatic extrusion in a twin-screw extruder followed by rapid maceration can be regarded as a promising technique to enhance the extraction of biomolecules from fresh *G. gracilis* biomass for food application. This technique consumes less solvent, time, and enhances the extraction yields. Finally, it is necessary to continue the investigation on extrusion parameters for further optimization such as shear force, screw configuration, residence time as well as other type of enzymes.

General Conclusions and Perspectives

Among the various extraction techniques of algal biorefinery, enzyme-assisted extraction is promising in comparison to physical and chemical extractions. The catalytic activities of enzymes are demonstrated to soften the algal cell wall, enhancing the liberation of confined and inaccessible molecules from the biomass. It is worth mentioning that the extraction is conducted in mild conditions and absence of toxic solvents, convincing for the valorization in various applications such as food, pharmaceuticals, and health.

In overall regard, our works incorporated extrusion with enzyme addition as a novel concept for algal refinery extraction. The originality of the thesis work is the use of protease and cellulase to study the release of all the molecules that can be used to develop an algal biorefinery. The other original aspect is the implementation of enzymatic reactions in an intensified bioreactor, the bioextruder, in which the functions of mixing, shearing and bioreactions are carried out simultaneously. The examinations were carried out on both dried and fresh *G.gracilis* algal biomass while the main objective focused to maximize the release of hydrosoluble components (sugar, protein, and R-phycoerythrin), knowing as promising components in this algae. After optimizing each technique, our study archived the enhancement of extraction yields from both techniques rather than the literature. Therefore, our applied procedures as well as the use of extrusion could become an essential part in terms of biorefinery improvement.

From our results, it can be concluded that the addition of enzyme (single or cocktail) did significantly increase the release yields of biomolecules from the studied *Gracilaria gracilis* biomass. Meanwhile, the choice of enzyme and operating conditions were significantly depended on biomass status and biomass treatment as well as target components. The investigation of enzyme-assisted extraction in batch mode showed that freeze-dried biomass was more compatible with enzyme cocktail due to the complementary effect (synergy) between enzymes while single enzyme is better for fresh biomass. Furthermore, fresh biomass induced high release of biomolecules than freeze-dried biomass (43 % of protein and 57 % of sugar) when the appropriate enzyme in optimal conditions was used. Single enzyme either cellulase or protease enhanced the release of soluble sugar and protein respectively from fresh biomass. In this regard, wet extraction could be convincing for industrial implementation with respect to drying step and required enzyme limitation. When using enzyme cocktail, the better release

of protein was obtained at pH 7.5 with two times less of enzyme dose (47 mg/g dw algae). However, more cellulase dose was needed in order to produce high release of sugar. Enzyme cocktail (pH 5.0 and 94 mg/g dw algae) was more compatible for the best release of sugar from both *G. gracilis* (fresh and dried) biomass under the operational conditions in our study.

It is worth to mention that algal protein extraction is usually known as the by-products which is associated with the polysaccharides extraction. The abundance of polysaccharides in the structure of the algal cell wall as well as the entrapment of proteins in the algae polysaccharides made the release of proteins more difficult. This phenomenon caused insignificant release of protein. In this regard, the degradation of polysaccharides is essential prior to the improvement of protein extractability. Some studies reported more protein extraction yields under the effect of enzyme consortia (the mixture of carbohydrases). However, our results revealed that the protein extraction was improved under the use of single enzyme (protease) with fresh biomass. Furthermore, our results could suggest that no significant degradation of the release protein in term of quantitative measurement (due to protease activity) in the applied experimental set up. These results offered the promising outputs, showing that protease was efficient and suitable for protein extractability from *Gracilaria gracilis* biomass. This enzyme could probably useful for protein extraction on others algae.

Nevertheless, this study archived enzymatic extrusion on fresh *Gracilaria gracilis* biomass with twin-screw extruder using cellulase at 32 °C. The operating conditions were optimized and promising outputs were gained. It is important to mention that the combination effect of enzyme and extrusion (enzymatic extrusion) did enhance the release efficiency of algal biomolecules compared to extrusion without enzyme addition. The extrusion technique depends on various parameters including biomass status, with the possibility to control the process with independent parameters such as flowrates, rotation speed, and screw profiles. It should be noticed that flowrates affect the residence time (essential for the reaction inside the extruder) while rotation speed and screw profiles generate shear for biomass/solvent mixture during the extrusion. Furthermore, the extrusion independent variables are usually presented in wide range, making an easy adaptation to various nature of biomass and offering a better process control for industry great interest.

The response surface of three variables (sample flow rate, enzyme concentration, and screw speed) investigated in this study showed that low sample flow rate (1.0 kg/h) with high enzyme

concentration (4.45 % dw) as well as high screws speed (350 rpm) were the predicted optimal condition which gave the highest release of target components within the experimental design. The extraction yields from this condition were reported 35.97, 29.23, and 16.38 % dw of R-PE, sugar, and protein respectively. Additionally, extrusion followed by rapid maceration (in pure water) could enhance the release yields than extrusion alone. Extrusion with maceration improved 1.5 (R-PE), 1.3 (Sugar), and 1.3 (Protein) times higher than those results from extrusion alone. It is worth to mention that extrusion with maceration could produce similar results as enzymatic hydrolysis (Batch, 5 h), in particular the release of R-PE and protein. Meanwhile, the release of sugar from extrusion with maceration was still a bit lower (1.5 times) than those released from enzymatic hydrolysis alone. Some of the released soluble contents from enzymatic extrusion might be trapped in the extruded pellet. In this regard, conducting maceration helped to release these entrapments and provided better yields.

Significant gain in time and release efficiency made enzymatic extrusion more promising than extraction in batch, albeit this technique is relatively recent for algae. Thus, further investigations are necessary and other algae can also be tested. This technique could become an alternative approach for the upgrading of industrial algal biorefinery application.

Perspectives

The current outputs from this study archived the process optimization of enzyme-assisted extraction either in batch or extrusion, which improve the release of soluble contents from *Gracilaria gracilis* biomass. Regarding the obtained results, the addition of cellulase/protease was shown as a suitable enzyme for a better release of target components from fresh biomass. Nevertheless, it is important for further studies to continue the investigation, notably with other enzymes (agarase or galactase as example). These enzymes could be beneficial to enhance the degradation of agar (molecule composes with galactose units), knowing as cell-wall polysaccharides of *Gracilaria gracilis*. Furthermore, the presence of agarase in enzyme consortia together with cellulase and kappa-carrageenase was shown to improve the protein liberation from *Gracilaria gracilis*. In this regard, the use of agarase/galactase either alone or in combination with protease should be worth to investigate with both dried and fresh biomass.

In addition, the determined optimal conditions of enzymatic extrusion in this study was preliminary and the process needs more examinations. It should be kept in mind that conducting experimental design with another level on the three selected variables (for example increasing screw speed or enzyme dose) could be beneficial in order to obtain more release efficiency. Others

independent variables such as biomass/solvent ratio, screw profiles configuration, moisture content as well as other enzymes should be taken into account. Screw profile of the extruder is essential as it is responsible to produce shear force during the extrusion reaction. For our biomass, a change of screw profile in the sense to improve the shearing force is recommended. This would allow more broken cells and therefore more contact with enzyme in the solvent. This phenomenon could lead to upgrade the release efficiency of extrusion enzymatic.

This study operated extrusion directly on fresh *G.gracilis* biomass. Therefore, the moisture content of the raw material is not controlled and relatively high (around 90 %) with the presence of less solvent needed. It might be beneficial to conduct the extrusion with the controlled of moisture content (around 40 to 60 %) in order to observe the influence of moisture content on the release efficiency of algal biomass. Material with less moisture content is usually presented as dough form which could favor the cell destruction under the effect of extruder screw. The mechanic dehydration such as pressing, drying, or centrifugating are known for their abilities to remove water from biomass. Therefore, these techniques are worth to investigate notably concerning their economic costs. Freezing or thawing techniques are also recognized as interesting techniques to improve the extraction, since it could help to break the algal cell wall and therefore generate more release target components. Nevertheless, our experiment of enzymatic extrusion was conducted with the addition of cellulase. The use of other enzymes such protease/agarase/galactase as described above should examine for further extrusion investigations. Further comprehension on process performance could also integrate the determination in detail of specific mechanical energy (SME) as well as the residence time.

Likewise, the maceration step (after extrusion) needs more investigations regarding the kinetic of liberation (either for all targeted components or one in particular) or the change of solvent (with the presence of glycerol for an example) for a better understanding and control on the release efficiency. It may be worth to examine other techniques such as pulsed electric field, ultrasounds, or hydrolysis by lactose fermentation (instead of maceration) in which the extraction yields might be more beneficial. Pulse electric field is based on the pores formation on the cell membrane after the short electrical pulses were applied. The ultrasound extraction heightens mass transfer from the cavitation and bubble collapse, and results cell disruption. Hydrolysis by lactose fermentation with bacteria is demonstrated to increase the nutritional values of the extracted compounds such as vitamins, organic acids, etc. In this regard, more release of biomolecules and high protein extractability could be expected. In the meantime, the techno-economic analysis among the techniques is necessary with respect to industrial

implementation. It is worth mentioned that membrane filtration process can also be integrated, allowing to recover the interesting compounds separately from the extruded liquids. The separation is based on the difference in molecular weight. Thus, the recovered liquid after extrusion or maceration, could be concentrated in R-phycoerythrin or polysaccharides/protein as well as pre-purified for notably industrial interests such as dietary supplement. Finally, the investigation should take into consideration the pellet part with respect to complete the biorefinery concept.

Last but not least, bioactivities (antioxidant, antimicrobial, anti-inflammatory, etc) and characteristics (solubility, emulsifying, foaming properties) of extracted compounds should be investigated in order to fully valorize them in appropriate applications (food, cosmetic, research, etc.). As limited in this current study, the additional results such as sugar (polysaccharides) and protein (amino acid) profiles, rheology property as well as R-phycoerythrin purity were not addressed and need to be examined. This complement information is primordial for the evaluation of the extracted compounds itself as well as the extraction technique.

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Glossary

A

Amylopectin: a water-soluble polysaccharide and highly branched polymer of α -glucose units found in plants. It contains a larger number of glucose units (2000 to 200,000).

Anticomplementary: a substance that neutralizes the action of a complement by combining with it and preventing its union with an antibody.

Antithrombotic: activities to prevent the formation or presence of a blood clot within a blood vessel.

Antinutritional: natural or synthetic compounds that interfere with the absorption of nutrients.

Antibodies: blood proteins produced in response to counteract a specific antigen. Antibodies combine chemically with substances which the body recognizes as alien, such as bacteria, viruses, and foreign substances in the blood.

Apolar organic solvents: carbon-based substances capable of dissolving or dispersing one or more other substances. These solvents have low dielectric constants and are not miscible with water.

B

Bioactive compounds: compounds that has a positive health effect on a living organism, tissue or cell.

Biorefinery: the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, materials) and bioenergy (biofuels, power and/or heat).

Brownian motion: the erratic random movement of microscopic particles in a fluid, as a result of continuous bombardment from molecules of the surrounding medium.

C

Centrioles: paired barrel-shaped organelles located in the cytoplasm of animal cells near the nuclear envelope and involved in the development of spindle fibers in cell division.

Cortex: an outer layer of tissue immediately below the epidermis of a stem or root.

Covalent force: a chemical bond that involves the sharing of electron pairs between atoms.

Cytoplasm: all of the material within a eukaryotic cell, enclosed by the cell membrane, except for the cell nucleus.

D

Die pressure: pressure at the exit of the extruder.

F

Fibers: dietary material containing substances such as cellulose, lignin, and pectin, which are resistant to the action of digestive enzymes.

G

Glycemic index: a rating system for foods containing carbohydrates. It represents the relative rise in the blood glucose level two hours after consuming that food.

Glycosidic bonds (glycosidic linkage): a type of covalent bond that joins a carbohydrate (sugar) molecule to another group, which may or may not be another carbohydrate.

Gossypol: a natural phenol derived from the cotton plant (genus *Gossypium*). It is a yellow pigment and a phenolic aldehyde that permeates cells and acts as an inhibitor for several dehydrogenase enzymes.

H

Hydrocolloid: polysaccharides of high molecular weight extracted from plants and algae or produced by microbial synthesis. This substance forms a gel in the presence of water.

I

Integrated multitrophic aquaculture: a system which provides the byproducts, including waste, from one aquatic species as inputs (fertilizers, food) for another.

Immunostimulating: a stimulation process of an immune response.

Immunosuppressive: partially or completely suppressing the immune response of an individual.

M

Motor torque: a specialized form of DC (direct current) electric motor which can operate indefinitely while stalled, without incurring damage. In this mode of operation, the motor will apply a steady torque (rotational equivalent of linear force) to the load.

Maillard reaction: a chemical reaction between amino acids and reducing sugars that gives browned food its distinctive flavor. The reaction is a form of non-enzymatic browning which typically proceeds rapidly from around 140 to 165 °C.

Q

Osmotic shocks: physiologic dysfunction caused by a sudden change in the solute concentration around a cell, which causes a rapid change in the movement of water across its cell membrane. Under conditions of high concentrations of either salts, substrates or any solute in the supernatant, water is drawn out of the cells through osmosis.

P

Polymerization: a process of reacting monomer molecules together in a chemical reaction to form polymer chains or three-dimensional networks.

Phycocolloides: special polysaccharides hydrocolloids produced by several seaweed species.

Photosynthetic pigments: a pigment that is present in chloroplasts or photosynthetic bacteria and captures the light energy necessary for photosynthesis.

Plastids: a membrane-bound organelle found in the cells of plants, algae, and some other eukaryotic organisms. Some examples are chloroplasts (used for photosynthesis), chromoplasts (used for pigment synthesis and storage), and leucoplasts (non-pigmented plastids that can sometimes differentiate).

Prebiotic effect: the effect of a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health.

Protoplast: the isolated cells produced by removing the surrounding cell wall either by mechanical means or by the use of cell wall degrading enzymes.

Protoplast protein: protein isolated from protoplast cells.

Phycobilisomes: light harvesting antennae protein complexes (up to 600 polypeptides) anchored to thylakoid membranes.

Prosthetic groups: non-protein components that attach mostly to proteins and assist the protein in various ways. When bound to proteins, prosthetic groups are called holoproteins.

R

Remedies: a medicine or treatment of a disease or injury.

S

Shear stress: force tending to cause deformation of a material by slippage in parallel to the imposed stress.

Shear force: unaligned forces pushing one part of a body in one specific direction, and another part of the body in the opposite direction.

Steroids: a biologically active organic compound with four rings arranged in a specific molecular configuration. Steroids have two principal biological functions: as important components of cell membranes which alter membrane fluidity; and as signaling molecules.

Syneresis: the extraction or expulsion of a liquid from a gel, as when serum drains from a contracting clot of blood.

Stokes shift: the difference (in energy, wavenumber or frequency units) between positions of the band maxima of the absorption and emission spectra (fluorescence and Raman spectroscopy) of the same electronic transition.

T

Thalli: a plant body that is not differentiated into stem and leaves and lacks true roots and a vascular system. Thalli are typical of algae, fungi, lichens, and some liverworts.

Thylakoids: membrane-bound compartments inside chloroplasts and cyanobacteria. They are the site of the light-dependent reactions of photosynthesis

V

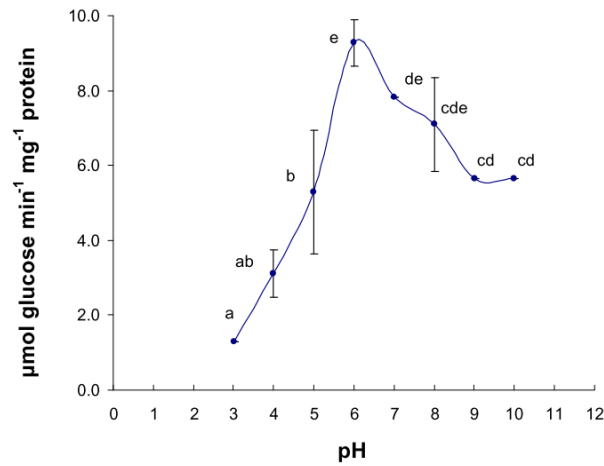
Vacuole: a space or vesicle within the cytoplasm of a cell, enclosed by a membrane and typically containing fluid.

Annex

Annex I

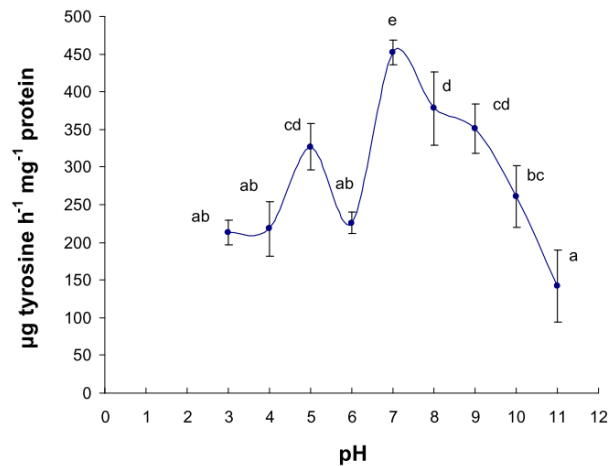
The effect of pH on Cellulase and Protease activity (Tizon et al., 2012)

Cellulase



Effect of pH on CM-cellulase activity. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 3.0-10.0) and 1.0 mL 0.25% CMC (w/v) was incubated for 15 min. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test)

Protease



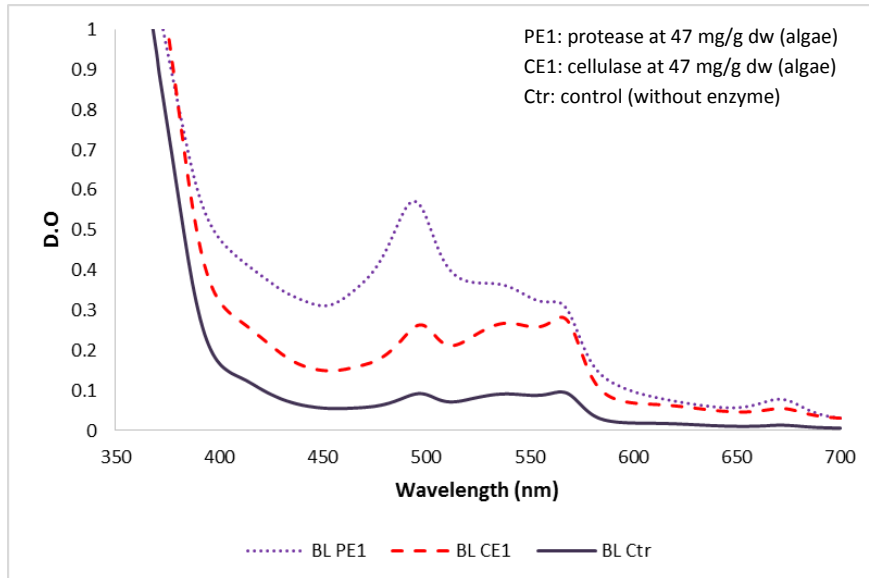
The assay mixture containing 0.5 mL enzyme extract, 1.5 mL buffer (pH 3.0-11.0) and 1.0 mL 1.0% casein (w/v) was incubated for 1 h. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test)

Tizon, R.U., Serrano, A.E., Traifalgar, R.F., 2012. Effects of pH on amylase, cellulase and protease of the Angelwing clam. *Eur. J. Exp. Biol.* 2, 2280–2285.

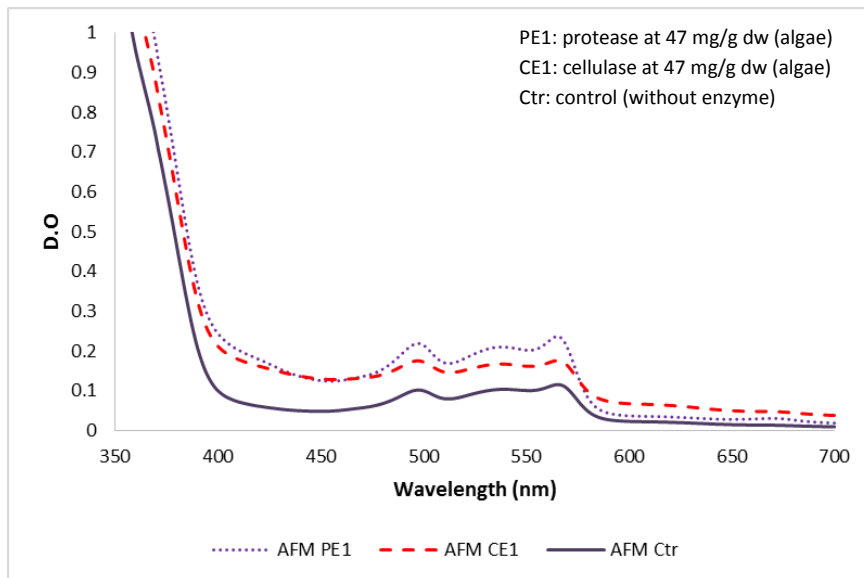
Annex II

R-PE spectrum from enzymatic hydrolysis with the addition of Cellulase, Protease and Cocktail enzyme

Freeze-dried biomass (BL)



Fresh biomass (AFM)



Titre : Etude de l'extrusion couplée à l'hydrolyse enzymatique pour l'extraction des composés hydrosolubles de l'algue rouge " *Gracilaria gracilis* "

Mots clés : Extrusion, Hydrolyse enzymatique, *Gracilaria gracilis*, Composés hydrosolubles

Résumé : L'algue rouge *G.gracilis* présente une teneur élevée en polysaccharides, protéines et autres composés bioactifs. L'extraction assistée par enzymes est une méthode de bioraffinage alternative aux procédés chimiques et mécaniques. La déstabilisation des membranes par l'activité enzymatique est connue pour libérer les composés confinés et inaccessibles, permettant ainsi d'améliorer les rendements d'extraction par rapport à des conditions modérées. L'extraction par hydrolyse enzymatique (Batch) et l'extrusion enzymatique (EE), ont été étudiées pour la libération des composés hydrosolubles de *G.gracilis*, notamment les protéines, les sucres et les R-phycoérythrine. Les résultats en Batch ont montré que le cocktail d'enzymes (mélange de

cellulase et protéase) fonctionnait mieux sur la biomasse lyophilisée alors qu'une seule enzyme fonctionne mieux sur la biomasse fraîche et les rendements étaient plus élevés avec celle-ci. En fonction de l'état de la biomasse, différentes enzymes peuvent être utilisées et cela impacte les rendements d'extraction. En outre, les résultats de l'EE avec la biomasse fraîche sont prometteurs. Les facteurs étudiés (débit d'alimentation, concentration d'enzyme et vitesse de la vis) affectent la libération des composés hydrosolubles. Le débit d'alimentation et la concentration d'enzyme sont les paramètres ayant le plus d'impact. Les rendements de l'EE suivis d'une macération rapide sont plus élevés comme les rendements en Batch. Ces techniques sont prometteuses pour le bioraffinage algale.

Title : Extrusion coupled with enzymatic hydrolysis for the extraction of hydrosoluble compounds of the red algae " *Gracilaria gracilis* "

Keywords : Extrusion, Enzymatic hydrolysis, *Gracilaria gracilis*, Hydrosoluble compounds

Abstract: Red algae *G.gracilis* presents high content of polysaccharides, proteins, and others bioactive compounds. Enzyme-assisted extraction has become an attractive alternative method compared to chemical and mechanical processes in respect to algae biorefinery. The disruption of tissue from enzyme activity is known to release more confined and inaccessible compounds, allowing more yields compared to moderate extraction conditions. The extraction with enzymatic hydrolysis (Batch) and extrusion enzymatic (EE), were investigated for the release of hydrosoluble compounds from *G.gracilis*, mainly protein, sugar, and R-phycoerythrin. Results from hydrolysis enzymatic showed that freeze-dried biomass worked better with enzyme cocktail (mixture of cellulase and protease) while

fresh biomass was more compatible with single enzyme depending on the target components. The recovery yields induced from fresh biomass were higher compared to dried biomass. The different of biomass status did required different enzyme type and affect the extraction yields. Furthermore, EE with fresh biomass gave promising results. The release of soluble contents depended on studied variables: sample flow rate, enzyme concentration, and screw speed while sample flow rate and enzyme concentration were the most significant variable. EE extraction followed by rapid maceration induced higher extraction yields similar to Batch extraction. Thus, wet extraction and EE could regard as promising approaches for an up-scaling algae biorefinery.

