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JOSÉ CIRLANIO SOUSA ALBUQUERQUE

HIDRÓLISE DA BIOMASSA DA MACROALGA MARINHA VERMELHA *Gracilaria birdiae* PARA OBTENÇÃO DE PRODUTOS DE INTERESSE INDUSTRIAL

FORTALEZA
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Área de concentração: Bioquímica vegetal
Orientadora: Prof.ª Dra. Norma Maria Barros Benevides.
Coorientadora: Profª Dra. Márjory Lima Holanda Araújo.

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Aprovada em 18/12/2019

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Não é pequeno serviço ajuntar o disperso, abreviar o largo, apartar o seleto, e fazer que facilmente se ache no capítulo de cada matéria o principal que a ela pertence...

Antônio de Sousa Macedo, 1606-1682, escritor português.

RESUMO

Os elevados teores de carboidratos hidrolisáveis na composição das macroalgas é a base para a obtenção de diversos produtos com importância industrial. No entanto, estudos são necessários para estabelecer as condições ideais de hidrólise e fermentação dos carboidratos desses organismos. O objetivo deste estudo foi avaliar os efeitos dos parâmetros concentração de ácido sulfúrico (SAC), concentração de biomassa algácea (GbBC) e o tempo de reação (RT) sobre a hidrólise da macroalga vermelha *Gracilaria birdiae*, visando o estabelecimento de condições ótimas para a obtenção de soluções ricas em monossacarídeos, ácidos orgânicos e compostos furânicos. A biomassa de *Gracilaria birdiae* rica em carboidratos (73,01 ± 1,03, %) foi hidrolisada a partir de um desenho experimental utilizando a ferramenta estatística Delineamento composto central rotacional aliado à Metodologia de superfície de resposta. Os resultados mostraram que cada variável independente teve influência distinta e significante sobre a reação. A obtenção de açúcares foi mais afetada pela concentração de biomassa algácea, enquanto, as concentrações de ácido sulfúrico tiveram mais efeitos sobre o 5-hidroximetilfufural e ácidos orgânicos. As condições mais adequadas para obtenção de glicose (28,56 ± 0,72 $g.L^{-1}$) e galactose (108,03 ± 1,32 $g.L^{-1}$) foram a 121 °C, com ácido sulfúrico 1,3 $mol.L^{-1}$, 841,59 $g.L^{-1}$ de biomassa de algas e tempo de 20 min. Para a celobiose (25,39 ± 0,43 $g.L^{-1}$), as condições foram ácido sulfúrico 0,6 $mol.L^{-1}$, 680 $g.L^{-1}$ de biomassa e 10 min Para 5-HMF (19,82 ± 0,43 $g.L^{-1}$), foram ácido sulfúrico 0,6 $mol.L^{-1}$, 680 $g.L^{-1}$ de biomassa e 30 min e para os ácidos levulínico (38,88 ± 0,58 $g.L^{-1}$) e fórmico (26,75 ± 0,54 $g.L^{-1}$) e, ácido sulfúrico 2,0 $mol.L^{-1}$, 680 $g.L^{-1}$ de biomassa e 30 min. Glicose e galactose nos hidrolisados da faixa ótima foram fermentados por uma cepa de levedura *Saccharomyces cerevisiae* aclimatada à galactose (*Sc*$_{Gal}$). O GbH3 (GbBC: 230 $g.L^{-1}$; SAC: 0,8M e RT 10 min) apresentou o melhor rendimento e produtividade de etanol cujos fatores de conversão ($g.g^{-1}$) foram $Y_{P/S\ gli}$ 0,843 ± 0,001, $Y_{P/S\ gal}$ 0,664 ± 0,010, Y_{EtOH} 1,427 gerando a concentração máxima de 7,82 ± 0,06 $g.L^{-1}$ de etanol. Assim sendo, os resultados obtidos neste estudo mostraram que os hidrolisados de *Gracilaria birdiae* obtidos sob condições otimizadas, constituem boas fontes de moléculas comercialmente importantes que podem ser purificadas, bem como soluções ricas em carboidratos fermentescíveis, as quais podem ser destinadas à produção de uma variedade de compostos com valor biotecnológico, inclusive etanol.

Palavras-chave: biomassa; monossacarídeos; ácidos orgânicos; 5-HMF; bioetanol.

ABSTRACT

The high content of hydrolyzable carbohydrates in the composition of macroalgae is the basis for obtaining several products of industrial importance. However, studies are needed to establish the ideal conditions for hydrolysis and fermentation of carbohydrates in these organisms. The objective of this study was to evaluate the effects of the parameters sulfuric acid concentration (SAC), algae biomass concentration (GbBC), and reaction time (RT) on the hydrolysis of the red macroalgae *Gracilaria birdiae*, aiming at the establishment of optimal conditions for the obtaining solutions rich in monosaccharides, organic acids, and furanic compounds. The biomass of *Gracilaria birdiae* rich in carbohydrates (73.01 ± 1.03, %) was hydrolyzed from an experimental design using the statistical tool Rotational central compound design combined with the Response surface methodology. The results showed that each independent variable had a distinct and significant influence on the reaction. Sugar production was more affected by the concentration of algae biomass, while sulfuric acid concentrations had more effects on 5-hydroxymethylfurfural and organic acids. The most suitable conditions for obtaining glucose (28.56 ± 0.72 $g.L^{-1}$) and galactose (108.03 ± 1.32 $g.L^{-1}$) were at 121 °C, with 1.3 $mol.L^{-1}$ sulfuric acid 1,841.59 $g.L^{-1}$ of algae biomass and time of 20 min. For cellobiose (25.39 ± 0.43 $g.L^{-1}$), the conditions were 0.6 $mol.L^{-1}$ sulfuric acid, 680 $g.L^{-1}$ of biomass and 10 min for 5-HMF (19.82 ± 0, 43 $g.L^{-1}$), were 0.6 $mol.L^{-1}$ sulfuric acid, 680 $g.L^{-1}$ of biomass and 30 min and for levulinic (38.88 ± 0.58 $g.L^{-1}$) and formic acids (26.75 ± 0.54 $g.L^{-1}$) and sulfuric acid 2.0 $mol.L^{-1}$, 680 $g.L^{-1}$ of biomass and 30 min. Glucose and galactose in the hydrolysates of the optimal range were fermented by a yeast strain *Saccharomyces cerevisiae* acclimated to galactose (*Sc*$_{Gal}$). GbH3 (GbBC: 230 $g.L^{-1}$; SAC: 0.8 M and RT 10 min) showed the best ethanol yield and productivity whose conversion factors ($g.g^{-1}$) were $Y_{P/S\ gli}$ 0.843 ± 0,001, $Y_{P/S\ gal}$ 0.664 ± 0.010, Y_{EtOH} 1.427 generating the maximum concentration of 7.82 ± 0.06 $g.L^{-1}$ of ethanol. Therefore, the results obtained in this study showed that the hydrolysates of *Gracilaria birdiae* obtained under optimized conditions, constitute good sources of commercially important molecules that can be purified, as well as solutions rich in fermentable carbohydrates, which can be used to produce a variety of compounds with biotechnological value, including ethanol.

Keywords: biomass; monosaccharides; organic acids; 5-HMF; bioethanol.

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LISTA DE ABREVIATURAS E SIGLAS

5-HMF	5-hidroximetilfurfural
ADP	Adenosina Difosfato
APAFG	Associação de Produtores de Algas de Flexeiras e Guajiru
ATP	Adenosina Trifosfato
BB	Box-Behnken
CE	Comissão Europeia
CO_2	Dióxido de Carbono
DCCR	Delineamento Composto Central Rotacional
FAO	Organização das Nações Unidas para a Agricultura e Alimentação
GAL	Genes metabólicos da Galactose
H^+	Íons Hidrogênio
Kg	Quilograma
Mbd	Milhões de Barris de petróleo por Dia
MSR	Metodologia de Superfície de Resposta
Opep	Organização dos países exportadores de petróleo
P_I	Fosfato Inorgânico

SUMÁRIO

1. INTRODUÇÃO

Nas últimas décadas, problemas relacionados à progressão acelerada da população mundial, insegurança energética e as preocupações com as alterações climáticas globais intensificaram as buscas pelo desenvolvimento de combustíveis e produtos químicos baseados em matérias-primas sustentáveis e menos poluidoras (ASHOKKUMAR et al., 2017; YUAN et al., 2017; AMAMOU et al., 2018).

Diante desse cenário, as macroalgas marinhas despertaram grande interesse como fonte de matéria-prima para a obtenção de tais compostos, por apresentarem uma série de características singulares atraentes (SHOBANA et al., 2017). Primeiramente, haja visto que o mar é o local de produção das macroalgas, não há competição por terras agricultáveis, além de seu cultivo dispensar o uso de insumos agrícolas e agrotóxicos (CESÁRIO et al., 2018). Adicionalmente, as macroalgas apresentam elevados teores de carboidratos e a ausência de lignina facilita os processos de despolimerização (AMAMOU et al., 2018).

As macroalgas vermelhas (Rhodophyta) apresentam os maiores teores de carboidratos (polissacarídeos) (GHADIRYANFAR et al., 2016; AMAMOU et al., 2018). *Gracilaria* constitui o gênero mais importante de algas vermelhas, sendo encontrada em todas as regiões tropicais e subtropicais do mundo (TORRES et al., 2019). Nesse gênero, destaca-se a espécie *Gracilaria birdiae*, abundante em toda a costa nordeste do Brasil (Fidelis et al. 2014; Fernandes et al., 2017; Dyah et al., 2017).

O elevado teor de carboidratos nas macroalgas é a base para a obtenção de diversos compostos químicos comercialmente importantes, porém, tratamentos hidrolíticos são necessários para liberar os monossacarídeos (YUN et al., 2015). Entre os vários métodos, a hidrólise ácida destaca-se por ser um método simples, rápido e econômico bastante utilizado para hidrolisar biomassas (SUKWONG et al., 2018). O ágar e a celulose presentes na parede celular das macroalgas agarófitas, ao serem hidrolisados, liberam os monômeros galactose e glicose, respectivamente, que podem ser fermentados para a obtenção de uma variedade de outros compostos por via química ou biotecnológica (YUN et al., 2016; SUKWONG et al., 2018). Na literatura, estudos mostram a aplicação dos carboidratos de macroalgas para obtenção de diversos compostos químicos, como: ácido lático (Hwang et al., 2011), hidrogênio, ácido butírico, etanol (Mutripah et al., 2014; Xia et al., 2015) e butanol (Hou et al., 2017).

Os principais fatores que influenciam o processo de hidrólise de biomassas são: concentração de biomassa, concentração de ácido, tempo e temperatura da reação (KIM et al., 2015). Contudo, dependendo das condições de hidrólise, compostos como: ácido fórmico, ácido acético, ácido levulínico, furfural e 5-HMF podem ser obtidos (JEONG et al., 2015). Portanto, determinar as condições ótimas para a obtenção de cada produto reacional evita desperdícios de biomassa, reagentes e tempo (KIM et al., 2015; YUN et al., 2015). Entre os vários métodos, a Metodologia Delineamento Composto Central Rotacional (DCCR) aliado à Metodologia de Superfície de Resposta (MSR) são ferramentas eficazes na otimização de processos complexos, permitindo a interpretação da influência de todas as variáveis simultaneamente (XU et al., 2018).

Portanto, o presente estudo visa avaliar os efeitos dos parâmetros concentração de biomassa algácea, concentração de ácido sulfúrico e o tempo de reação sobre a hidrólise da macroalga *G. birdiae*, a fim de obter condições otimizadas para obtenção de produtos de interesse industrial.

1.2. Revisão de Literatura

1.2.1. As macroalgas

As macroalgas abrangem um grupo heterogêneo de organismos eucarióticos fotossintéticos, avasculares, apresentando talo em vez de raízes, caules e folhas (JUNG et al., 2013; ALENCAR, 2016). Elas apresentam tamanhos variáveis, algumas espécies podem ultrapassar 70 m de comprimento, como exemplo, os gigantes *kelps* (LIMA, 2016). São predominantemente aquáticas, sendo encontradas em mares e corpos de água doce (SARAVANAN et al., 2018). Todavia, algumas espécies são capazes de habitar ambientes com condições adversas, como lagos eutrofizados e em águas com variações extremas de temperatura e salinidade (GANESAN; THIRUPPATHI; JHA, 2006).

As macroalgas representam a base da cadeia alimentar nos oceanos, servindo como fonte de alimentos para muitas espécies de organismos marinhos (ROBIN et al., 2017). Além disso, elas são responsáveis por realizar boa parte da atividade fotossintética global (CESÁRIO et al., 2018). Sob condições ideais, as macroalgas utilizam a energia da luz solar para fixar o carbono inorgânico da atmosfera na forma de carboidratos e lipídios (MICHALAK, 2018). A composição química e o grau de crescimento das macroalgas podem ser influenciados por diversos fatores, como: tipo de hábitat, luminosidade, temperatura, salinidade, disponibilidade de nutrientes, poluição e até mesmo o movimento das águas (RODRIGUES et al., 2015; REEN et al., 2018).

Por apresentar origem polifilética, as macroalgas marinhas estão classificadas em dois reinos diferentes. As algas verdes e vermelhas pertencem ao Reino Plantae, já as macroalgas pardas estão incluídas no Reino Chromista (LIN; QIN, 2014). A classificação delas baseia-se em aspectos bioquímicos (pigmentos acessórios, polissacarídeos de reserva, etc.), organização celular, filogenia molecular, ciclo de vida, morfologia e ecologia (VIDOTTI E ROLLEMBERG, 2004). Com base nesses aspectos, as macroalgas foram classificadas em três grandes filos, sendo que, as macroalgas verdes pertencem ao filo Chlorophyta, as vermelhas estão agrupadas no filo Rhodophyta e as pardas estão inseridas no filo Ochrophyta (ROBIN et al., 2017). O filo Chlorophyta possui aproximadamente 7.000 espécies e apresentam clorofilas a e b, assim como, as plantas terrestres (ADENIYI; AZIMOV; BURLUKA, 2018). Já o filo Rhodophyta possui aproximadamente 6.000 espécies, distribuídas em mais de 600 gêneros, sendo encontradas principalmente em ambientes

marinhos tropicais (WEI; QUARTERMAN; JIN, 2013a). A coloração avermelhada das macroalgas vermelhas é proveniente dos pigmentos ficocianina e ficoeritrina (SUKWONG et al., 2019). O grupo das macroalgas pardas (Ochrophyta) apresenta aproximadamente 2.000 espécies, os pigmentos fotossintéticos são as clorofilas a e c, b-caroteno e xantofilas (CHEN et al., 2010; JUNG et al., 2013).

1.2.2. Composição química das macroalgas

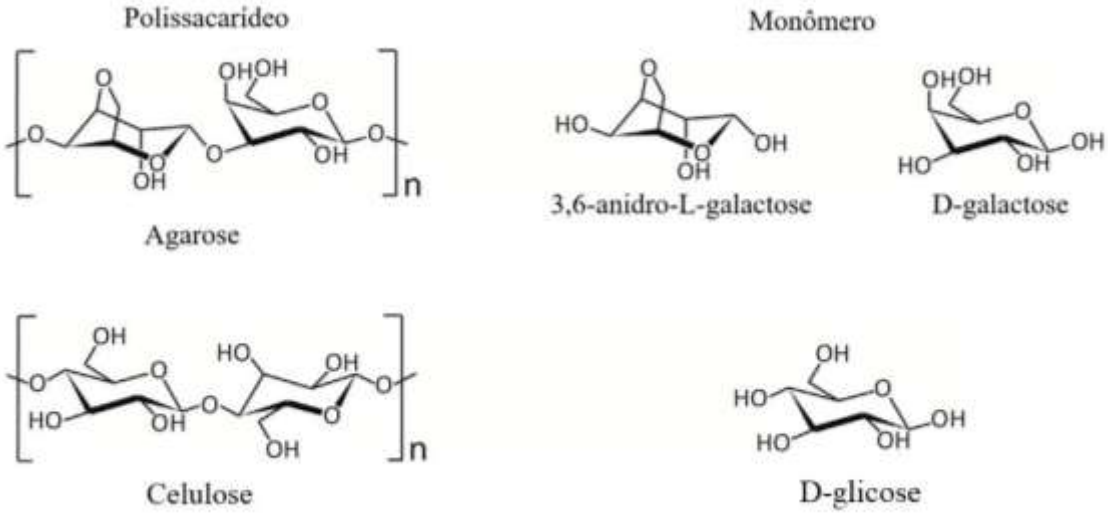
A composição química das macroalgas (Tabela 1) é significativamente diferente da composição das plantas terrestres (CESÁRIO et al., 2018). As macroalgas possuem menores teores de carbono, hidrogênio e oxigênio e maiores teores de nitrogênio e enxofre do que os da biomassa lignocelulósica (GHADIRYANFAR et al., 2016). Os teores e os tipos de carboidratos, proteínas e lipídeos constituintes variam entre as espécies de macroalgas (YUN et al., 2016; GHOSH et al., 2019). As macroalgas vermelhas apresentam teores de carboidratos superiores aos das macroalgas verdes e marrons (KAWAI E MURATA, 2016). Nas macroalgas verdes, os principais polissacarídeos constituintes são o amido e celulose, compostos por monômeros de glicose. Nas macroalgas pardas, estão presentes os carboidratos laminarina, manitol, alginato, fucoidanas e celulose, formados por glicose e manitol (SUDHAKAR et al., 2017). Já as macroalgas vermelhas podem ser agarófitas ou carragenófitas com base na presença de ágar, carragenana e celulose, que ao serem hidrolisados, liberam monômeros de glicose e galactose (YUN et al., 2016; RODRÍGUEZ SÁNCHEZ et al., 2019). A composição química das macroalgas é apresentada na Tabela 1.

Tabela 1. Composição química das macroalgas marinhas

Componente	Macroalgas marinhas		
	Verdes	Vermelhas	Pardas
Carboidratos	30-60 %	30-50 %	20-30 %
Polissacarídeos	Ulvana, Amido, celulose, manana	Ágar, carragenana, celulose	Laminarina, alginato, manitol, fucoidana, celulose
Monossacarídeos	Glicose, manose, Ramnose, xilose, galactose, ácido urônico, ácido glicurônico	Glicose, galactose	Glicose, galactose, xilose, fucose, ácido urônico, ácido glicurônico
Proteínas	10-20 %	6-15 %	10-15 %
Lipídios	1-3 %	0,5-1,5 %	1-2 %
Cinzas	13-22 %	5-15 %	14-28 %

Fonte: TRIVEDI; GUPTA; SALT, 2015

O ágar presente nas macroalgas vermelhas é composto por agarose, um polissacarídeo que consiste em unidades dissacarídicas repetidas compostas por D-galactose e 3,6-anidro-α-L-galactose, enquanto a celulose é composta por unidades de D-glicose (CASTRO et al., 2017; RODRÍGUEZ SÁNCHEZ et al., 2019). A estrutura da agarose e celulose são apresentadas na Figura 1.

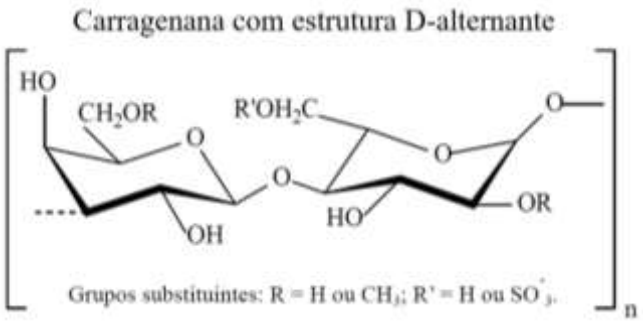


Fonte imagem: YUN et al., 2015

Figura 1. Estrutura da agarose e celulose e seus respectivos monômeros constituintes.

A carragenana (Figura 2) é composta por dímeros de galactose sulfatada (D-galactose e 3,6-anidro-galactose), com teor de éster sulfato variando de 15 a 40% (YUN;

CHOI; KIM, 2015). As carragenanas possuem diversas aplicações na indústria alimentícia e farmacêutica por suas propriedades gelificantes, além de serem agentes de espessamento ou estabilização (OLIVEIRA, 2014).



Fonte imagem: ANDRADE, 2016

Figura 2. Carragenana com estrutura D-alternante.

1.2.3. O gênero Gracilaria

Entre as macroalgas vermelhas, destacam-se as do gênero *Gracilaria* abrangendo mais de 100 espécies, distribuídas em todas as regiões tropicais e subtropicais do mundo (YANG et al., 2006). Elas apresentam talo cilíndrico ou achatado, filamentoso ou pseudoparenquimatoso, com comprimento variando de 0,1 a 5 metros, com coloração entre algumas tonalidades de vermelho, mas também com algumas variantes de cor verde (PLASTINO E OLIVEIRA, 1999). O gênero *Gracilaria* é considerado o mais importante como fonte de matéria-prima para a produção de ágar, sendo responsável por mais de 80% de todo o ágar produzido mundialmente (FERNANDES et al., 2017; DYAH et al., 2017; MEINITA et al., 2018). Nesse gênero, destaca-se a espécie *Gracilaria birdiae*, mostrada na Figura 3. No Brasil, a espécie é abundante em toda a costa nordeste e explorada economicamente para a obtenção de ágar e outros bioprodutos (FIDELIS et al., 2014; MACIEL et al., 2008). A exploração comercial de *Gracilaria spp*. no litoral brasileiro começou na década de 1960, com a colheita direta de algas em leitos naturais. No entanto, na década de 1970, o cultivo de espécies selecionadas de agarófitas foi proposto visando conter a exploração intensiva e descontrolada dos leitos naturais (AYRES-OSTROCK et al., 2016). Na região nordeste do Brasil, *G. birdiae* tem sido cultivada em condições de campo em diversos

locais, especialmente na Praia do Rio do Fogo no estado do Rio Grande do Norte (FIDELIS et al., 2014) e na praia de Flecheiras na cidade Trairi no Ceará.

Reino: Plantae
Divisão: Rhodophyta
Classe: Florideophyceae
Ordem: Gracilariales
Família: Gracilariaceae
Gênero: *Gracilaria*
Espécie: *Gracilaria birdiae*

Fonte - Autor

Figura 3. Exemplar de *Gracilaria birdiae* coletada em estruturas agrícolas na Praia de Flecheiras - Trairi, Ceará, Brasil.

A maricultura artesanal de espécies de *Gracilaria* para produção de ágar, alimentos e cosméticos tem colaborado com o desenvolvimento econômico e social em comunidades pesqueiras (ESTEVAM et al., 2017). Atualmente, *G. birdiae* é a espécie mais cultivada comercialmente por membros da Associação de Produtores de algas de Flexeiras e Guajiru (APAFG) no município de Trairi no Ceará. O cultivo da referida espécie no estado foi aprovado em 1997 e dois anos depois foi financiado o primeiro projeto-piloto para o cultivo no mar. Atualmente, existe um sistema de maricultura bem definido utilizando a técnica de *long line*. Entre as diversas características naturais, *G. birdiae* apresenta composição rica em carboidratos, os quais podem ser hidrolisados para obtenção de diversos bioprodutos.

1.2.4. Aspectos ambientais, econômicos e sociais das macroalgas

As macroalgas marinhas representam uma enorme biodiversidade de organismos, configurando-se um vasto recurso que pode ser explorado para a produção de diversos compostos químicos economicamente importantes (MICHALAK, 2018). Elas já possuem

diversas aplicações na indústria alimentícia, farmacêutica, cosmética e mais recentemente, despertaram grande atenção como matéria-prima renovável potencial para a produção de biocombustíveis (PHANG, 2018; TORRES; KRAAN; DOMÍNGUEZ, 2019). O potencial das macroalgas como matéria-prima para a produção de biocombustíveis e outros compostos químicos é estimulado por uma série de vantagens ambientais, econômicas e sociais diante das matérias-primas convencionais (KAWAI E MURATA, 2016; NGUYEN et al., 2017).

As macroalgas são importantes fontes de fitocolóides como, o ágar, carragenana, alginato e fucoidana (CAO et al., 2019). A biomassa seca de algumas espécies de macroalgas apresenta até 75% de carboidratos (HOU et al., 2017; AMAMOU et al., 2018). Esses carboidratos são moléculas poliméricas compostas por longas cadeias de unidades monossacarídicas unidas por ligações glicosídicas, que ao serem hidrolisadas, liberam monossacarídeos ou oligossacarídeos que podem ser fermentados em etanol em processos subsequentes (XU et al., 2018b). O elevado teor de carboidratos na composição das macroalgas é a base para a produção de combustíveis e outros compostos químicos (PHANG, 2018; CAO et al., 2019).

Além disso, as macroalgas apresentam elevadas taxas de crescimento, não competem por terras agricultáveis, podem ser cultivadas em águas residuais, dispensam o uso de sistemas de irrigação, pesticidas e insumos agrícolas (AMAMOU et al., 2018). A ausência de lignina em sua estrutura facilita os processos de despolimerização (SARAVANAN et al., 2018). A eficiência fotossintética nas plantas terrestres é apenas 2,2%, enquanto que nas macroalgas a eficiência fotossintética varia de 6 a 8% (GHADIRYANFAR et al., 2016). Portanto, a elevada produção de biomassa algácea ocorre a partir da remoção do carbono da atmosfera (PHANG, 2018). De acordo com trabalhos publicados, o cultivo de uma tonelada de algas seca absorve aproximadamente 960 kg de CO_2 da atmosfera (GHADIRYANFAR et al., 2016). Em sistemas de maricultura, as macroalgas reduzem a proliferação de algas nocivas, diminuem a eutrofização e acidificação da água, ajudando a manter os sistemas de maricultura mais saudáveis (CASTRO et al., 2017; TABASSUM; XIA; MURPHY, 2017). Dessa forma, a melhoria da qualidade da água possibilita a integração da produção de algas com a piscicultura, podendo oferecer alguns benefícios socioeconômicos, como fonte de emprego e produção local de alimentos em comunidades costeiras (ROBIN et al., 2017).

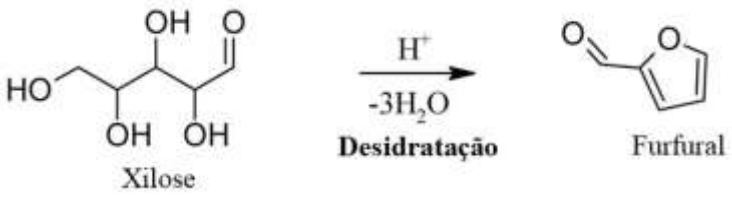
1.2.5. Hidrólise dos carboidratos de macroalgas

Os polímeros de carboidratos constituintes das macroalgas, podem ser hidrolisados para gerar monossacarídeos e outros compostos químicos (SUDHAKAR et al., 2017; PHANG, 2018; SUKWONG et al., 2018; XU et al., 2018). No entanto, o processo de conversão de qualquer biomassa em bioprodutos requer tratamentos para alterar a estrutura celular e tornar os polissacarídeos acessíveis aos processos subsequentes (ALALWAN; ALMINSHID; ALJAAFARI, 2019). A produção de açúcares fermentescíveis, a partir da biomassa algácea, exige métodos hidrolíticos que promovam a redução da cristalinidade da celulose e a solubilização das estruturas recalcitrantes da parede celular das macroalgas (YUN; CHOI; KIM, 2015).

Nas últimas décadas, inúmeras tecnologias envolvendo métodos físicos, químicos e biológicos vem sendo estudadas visando a obtenção de altos rendimentos de açúcares fermentescíveis (LYU et al., 2018; ALALWAN; ALMINSHID; ALJAAFARI, 2019). Entre os vários métodos, dois são comumente utilizados: as hidrólises ácidas e as enzimáticas (PHANG, 2018). Os processos envolvendo as hidrólises enzimáticas são desfavorecidos devido ao alto custo e a alta especificidade das enzimas, que são específicas para cada tipo de carboidrato (TEH et al., 2017). Já a hidrólise ácida caracteriza-se por ser um método simples, rápido e econômico para hidrolisar a biomassa de macroalgas em monossacarídeos (SUKWONG et al., 2018). A hidrólise ácida tem sido amplamente testada em equipamentos em escala de bancada, configurando-se como um método favorável para aplicações industriais (YUN et al., 2016).

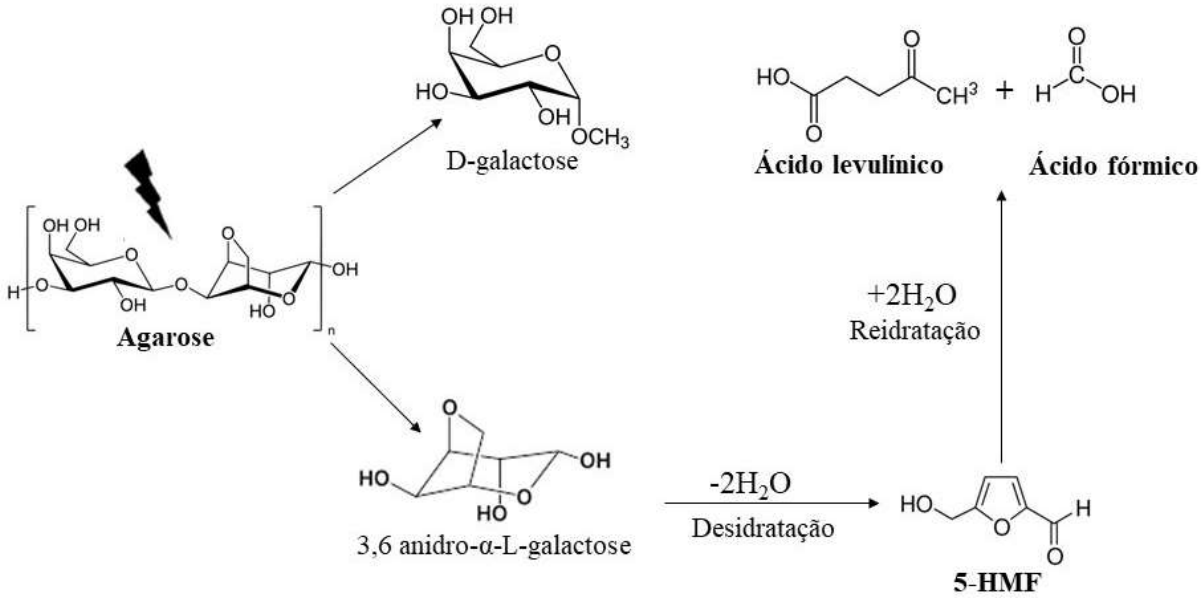
Parâmetros como o tipo de ácido (catalisador), concentração de ácido, concentração do substrato, temperatura e tempo de reação, afetam diretamente a produtividade de monossacarídeos por hidrólise ácida (KIM et al., 2015; MICHALAK, 2018). Vários tipos de ácidos (diluídos ou concentrados) vem sendo utilizados como agentes catalizadores para hidrolisar biomassas, como H_2SO_4, HCl e H_3PO_3 (DAWEI et al., 2011; MEINITA et al., 2012). Entre eles, o ácido sulfúrico diluído tem sido amplamente utilizado em processos de hidrólise. Ele possui íons H^+ extra que aumentam a acidez do meio, interrompendo a rede de ligações de hidrogênio intra e intercadeias dos polissacarídeos, clivando as ligações 1,3-glicosídicas (TEH et al., 2017). Dessa forma, o ácido afeta a integridade estrutural dos polissacarídeos e libera oligômeros ou carboidratos simples (AGBOR et al., 2011). No entanto, esse processo exige mais estudos, principalmente porque o

substrato está em fase sólida e o catalisador está em fase líquida, sugerindo investigações relacionadas a área de contato sólido-líquido e à difusão do líquido no sólido, para o sucesso da hidrólise (KIM et al., 2015). Os monômeros de glicose e galactose obtidos por hidrólise da biomassa de algas vermelhas podem ser convertidos em vários produtos químicos importantes, como compostos furânicos e ácidos orgânicos (SUKWONG et al., 2018). Em condições severas de hidrólise ácida, a desidratação de hexoses gera 5-hidroximetilfurfural (5-HMF). Já a desidratação de pentoses produz furfural (BINDER et al., 2010). Além disso, reações secundárias de hidratação do 5-HMF em condições drásticas de hidrólise ácida, geram os ácidos levulínico e fórmico (CAO et al., 2019). As vias de formação do furfural e 5-HMF a partir da desidratação de pentoses e hexoses são mostradas nas Figuras 4, 5 e 6.



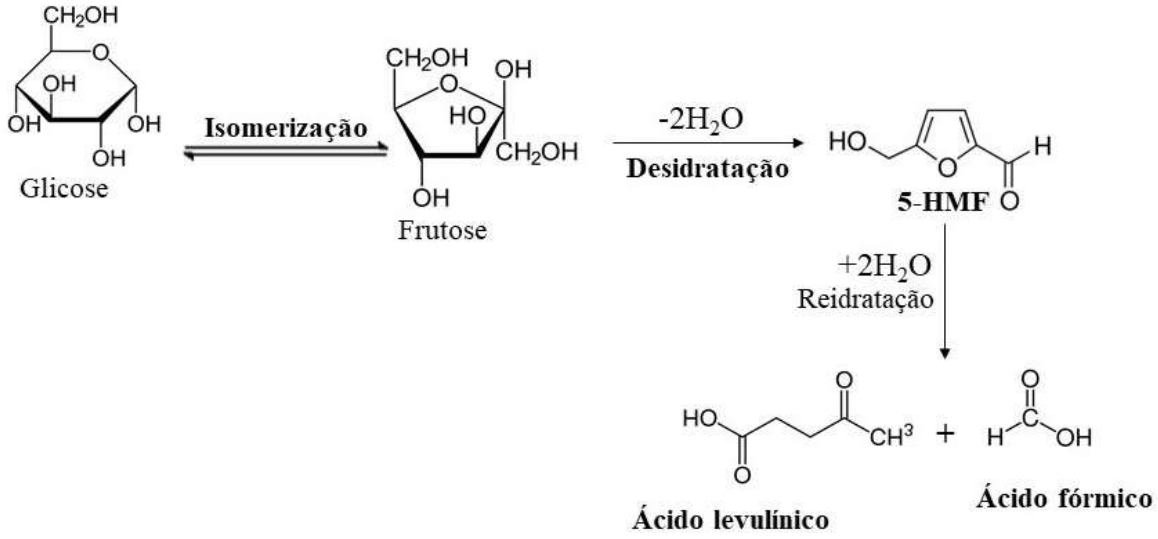
Fonte imagem: DANON; MARCOTULLIO; JONG, 2014

Figura 4: Via de formação do furfural a partir da xilose (pentose).



Fonte imagem: Adaptada de OH et al., 2015

Figura 5: Via de formação de galactose, 5-HMF, ácido levulínico e ácido fórmico a partir da agarose



Fonte imagem: Adaptada de ZHANG et al., 2017.

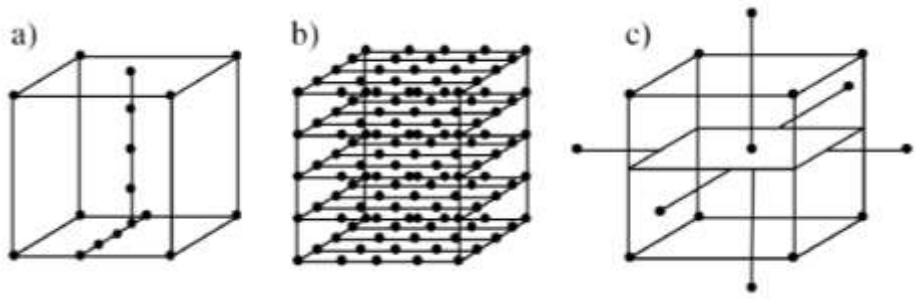
Figura 6: Via de formação do 5-HMF, ácido levulínico e ácido fórmico, a partir da molécula de glicose.

Glicose e galactose são metabolizados por microrganismos em processos fermentativos para produzir diversos compostos químicos, como: etanol (HARGREAVES et al., 2013), butanol (HOU et al., 2017), ácido lático (HWANG et al., 2011), hidrogênio e ácido butírico (MUTRIPAH et al., 2014). O 5-HMF foi identificado como uma molécula intermediária potencial que pode ser transformada em vários compostos químicos valiosos, incluindo produtos farmacêuticos, solventes, resinas, fungicidas e combustíveis (WANG; A. BROWN; CHEN, 2018). O ácido fórmico é amplamente utilizado nas indústrias química, agrícola, têxtil, farmacêutica e de borracha, além disso, é facilmente degradado em hidrogênio à temperatura ambiente por conversão catalítica (NIU et al., 2015). Já o ácido acético tem muitas aplicações nas indústrias química, de alimentos, polímeros e de materiais eletrônicos (BAUMANN; WESTERMANN, 2016).

No entanto, a obtenção desses compostos a partir de macroalgas é fortemente influenciada pelos parâmetros: concentração de biomassa, concentração do catalizador (ácido), temperatura e tempo de processo. Assim sendo, determinar as condições para a geração de cada produto da hidrólise evita desperdícios de biomassa, reagentes, tempo, dentre outros (KIM et al., 2015; YUN et al., 2015).

1.2.6. Otimização da hidrólise de biomassas

Dentre várias metodologias estatísticas que são aplicadas ao estudo da hidrólise de biomassas, a metodologia Delineamento Composto Central Rotacional (DCCR) aliado à Metodologia de Superfície de Resposta (MSR) são ferramentas efetivas que permitem uma melhor organização e interpretação da influência das variáveis de um processo simultâneo, evitando o empirismo das técnicas de tentativa e erro (XU et al., 2018). Estas ferramentas podem ser usadas em diferentes modelos de delineamento de experimentos, como: Box-Behnken (BB), Fatorial, "d-optimal" e DCCR (GHAZANFARI; KASHEFI; JAAFARI, 2016). De acordo com Haaland (1989), três caminhos podem ser adotados para a resolução de um problema experimental (CASTRO, 2016), mostrados na Figura 7.



Fonte imagem: CASTRO, 2016

Figura 7: Modelos experimentais para o estudo de 3 parâmetros. a) "One-at-a-time", b) Matriz completa e c) Delineamento Composto Central Rotacional.

1. O "one-at-a-time" ou "um fator por vez" (Figura 7a) é o procedimento experimental mais difundido e utiliza uma variação independente de parâmetros ou variáveis. Nesse procedimento, um parâmetro é estudado em diferentes condições e os demais são fixados. Essa metodologia, embora mais utilizada, é bastante ineficiente, pois não considera interação entre os parâmetros avaliados e não explora completamente a região de interesse.
2. O segundo investiga, em uma matriz completa (Figura 7b), a combinação de todos os fatores estudados para a busca das melhores condições experimentais. Esse modelo explora toda a região de interesse, porém, é necessário um grande número de experimentos para a sua realização. Por exemplo, no estudo de três parâmetros, são necessários 125 experimentos para explorar todas as combinações dos cinco fatores.
3. O DCCR (Figura 7c) investiga a resolução de um problema experimental com um menor número de experimentos e explora toda a região de interesse. Pelo exemplo mostrado, para o

estudo de três parâmetros são necessários apenas 17 experimentos, sendo possível calcular, ainda, o erro experimental.

A relação matemática entre variáveis independentes e variáveis de resposta é dada por uma equação polinomial quadrática, apresentada pela equação 1.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j$$

Equação (1)

Onde Y_i é a resposta prevista, β_0 é o coeficiente constante, X_iX_j são as variáveis independentes que influenciam as variáveis de resposta, Y; β_i é o coeficiente linear em relação a i, β_{ii} é o coeficiente quadrático em relação a i e β_{ij} é o coeficiente de interação em relação a ij, k é o número de fatores.

A metodologia de DCCR aliada à MSR têm sido amplamente utilizadas para otimizar vários processos bioquímicos (XU et al., 2018). CASTRO, 2016 aplicou estas metodologias para otimizar a hidrólise ácida da macroalga marinha vermelha *Solieria filiformis* e obtiveram a concentração máxima de 174,06 $g.L^{-1}$ de monossacarídeos totais cuja concentração foi superior às obtidas por vários estudos descritos na literatura. Portanto, a otimização de processos para a obtenção de bioprodutos a partir de matérias-primas renováveis são estratégias importantes para a segurança energética mundial frente ao aquecimento global.

1.2.7. A crise energética mundial e o desenvolvimento dos biocombustíveis

A energia desempenha um papel fundamental para o desenvolvimento econômico dos países (AWAN; KHAN, 2014). Entre as diversas fontes de energias, destacam-se os combustíveis, os quais são responsáveis pelo fornecimento de aproximadamente 88% de toda a energia consumida no mundo (REEN et al., 2018). Grande parte dessa energia é derivada do petróleo, carvão vegetal e gás natural (ASHOKKUMAR et al., 2017). Estima-se que em 2050, a população mundial atingirá 9,4 bilhões de pessoas e a demanda global de energia será 40,8 terawatts (TW) (TABASSUM; XIA; MURPHY, 2017). Em 2007, havia aproximadamente

806 milhões de automóveis e caminhões leves no mundo e estima-se um aumento para 1,3 bilhões até 2030 e 2 bilhões em 2050 (BHARATHIRAJA et al., 2015).

Além da potencial insustentabilidade, a queima de combustíveis fósseis lança na atmosfera elevadas taxas de gases de efeito estufa, que agravam os problemas ambientais relacionados ao aquecimento global, além de piorar a qualidade do ar nos grandes centros urbanos (REEN et al., 2018; ALALWAN; ALMINSHID; ALJAAFARI, 2019). Portanto, a progressão acelerada da população mundial aliada ao rápido crescimento econômico liderado pela industrialização geraram altas demandas de combustíveis e outros compostos químicos (ALFONSÍN; MACEIRAS; GUTIÉRREZ, 2019). Dessa forma, tornou-se desafiador para a sociedade do século XXI suprir a crescente demanda de energia para os setores de transporte, aquecimento e processos industriais de forma sustentável (ROCHA, 2010).

Visando a redução da emissão de gases poluentes da atmosfera, em 2005, entrou em vigor o Tratado de Quioto cujos objetivos forçavam os países industrializados a reduzir a emissão de gases poluentes (SOUZA, 2010). Em 2009, a Comissão Europeia (Diretiva 2009/28/CE) propôs uma redução da emissão de gases de efeito estufa a um nível de 10% até 2020 (TABASSUM; XIA; MURPHY, 2017). Portanto, os problemas relacionados à insegurança energética, preocupações ambientais e legislação globais, incentivaram a busca por desenvolvimento de combustíveis e compostos químicos baseados em matérias-primas sustentáveis e menos poluidoras (ASHOKKUMAR et al., 2017; HOU et al., 2017; KOSTAS et al., 2019).

Diante desses desafios, surgiram os biocombustíveis, apresentando diversas vantagens em relação aos combustíveis convencionais (SUGANYA et al., 2016). Eles são renováveis, sustentáveis, biodegradáveis, menos poluidores do meio ambiente e facilmente aplicáveis aos motores de combustão (TABASSUM et al., 2017). Os biocombustíveis são substâncias líquidas ou gasosas, entre eles destacam-se o bioetanol, biodíesel, biometano, hidrogênio e hidrocarbonetos, produzidos por processos de fermentação, transesterificação, gaseificação, entre outros (ALALWAN; ALMINSHID; ALJAAFARI, 2019). Esses compostos podem ser produzidos a partir de uma variedade de biomassas, incluindo algumas culturas de plantas, resíduos agroindustriais, organismos aquáticos, etc. (PHANG, 2018; ARNOLD; TAINTER; STRUMSKY, 2019).

De acordo com a matéria-prima utilizada, a produção de biocombustíveis é classificada como sendo de primeira e segunda geração, e estudos estão sendo realizados visando o desenvolvimento e a viabilidade dos biocombustíveis de terceira geração

(TABASSUM; XIA; MURPHY, 2017; HEBBALE; BHARGAVI; RAMACHANDRA, 2019). Os biocombustíveis de primeira geração são produzidos a partir de matérias-primas convencionais, como: cana-de-açúcar, milho, soja, compostas por sacarose, amido e óleos, respectivamente (TABASSUM et al., 2017). Já os biocombustíveis de segunda geração são produzidos a partir de resíduos agroindustriais ou matérias-primas lignocelulósicas dedicadas a esse fim, como: caules de milho e trigo, palha, grama e lascas de madeira, etc. (MATHIMANI; PUGAZHENDHI, 2019).

Entre os biocombustíveis, o bioetanol é atualmente o mais utilizado no mercado global, configurando uma das formas mais modernas de gerar energia a partir de biomassas (REEN et al., 2018). O etanol como combustível apresenta queima mais limpa, além dos gases gerados na sua combustão serem menos tóxicos em relação aos combustíveis fósseis (SUKWONG et al., 2018). Os Estados Unidos e o Brasil são os maiores produtores mundiais de etanol, produzindo anualmente mais de 95 bilhões de litros de etanol, representando 85% da produção mundial (REEN et al., 2018; ALALWAN; ALMINSHID; ALJAAFARI, 2019). Mais de 60 países já estão buscando promover o etanol como combustível convencional (REEN et al., 2018). No Brasil, o projeto "Proálcool" foi lançado ainda em 1975 com a finalidade de reduzir a dependência do país em relação às importações de petróleo, produzindo etanol a partir da cana-de-açúcar. Em 1980, a produção de veículos leves movidos por etanol atingiu 95% de toda a frota produzida no país. No século XXI, o uso do etanol foi intensificado motivado pelos altos preços do petróleo no mercado internacional, desenvolvimento da tecnologia “flexfuel” e por ser menos poluente do meio ambiente (LOPES et al., 2016). Recentemente, a gasolina passou a ter uma mistura de 27% de etanol e está projetada a utilização de misturas de até 40% (LOPES et al., 2016). O aumento do percentual de etanol na gasolina aumentou a eficiência da combustão interna dos motores e melhorou a qualidade do ar nos grandes centros urbanos (REEN et al., 2018).

O bioetanol produzido em escala industrial é classificado como sendo de primeira geração, obtido principalmente a partir da cana-de-açúcar, milho e beterraba (GHADIRYANFAR et al., 2016). O desvio de culturas agrícolas alimentares para a produção de biocombustíveis tem gerado questões éticas sérias diante da crescente demanda por alimentos no mundo, além de necessitar do fornecimento de grandes quantidades de água e terras agricultáveis (BHARATHIRAJA et al., 2015). Entretanto, dos problemas relacionados aos biocombustíveis de primeira geração, a produção dos biocombustíveis de segunda geração vem sendo investigada, porém, sua produção ainda não é rentável devido algumas barreiras

técnicas (GHADIRYANFAR et al., 2016; SUDHAKAR et al., 2017). O alto grau de recalcitrância causado pela presença de lignina e hemicelulose dificulta o acesso de reagentes e catalisadores. A resistência da matéria-prima à bioconversão e a ausência de um sistema de coleta da matéria-prima são os principais fatores limitantes da produção dos biocombustíveis de segunda geração (TABASSUM; XIA; MURPHY, 2017).

Diante desse cenário, a busca pelo desenvolvimento dos biocombustíveis de terceira geração foi intensificada (ARNOLD; TAINTER; STRUMSKY, 2019). Eles são produzidos a partir de micro ou macroalgas, as quais representam a mais recente fonte de matéria-prima renovável e economicamente sustentável para a produção de biocombustíveis e outros compostos químicos (SUGANYA et al., 2016). As macroalgas apresentam uma série de vantagens diante das fontes de biomassas convencionais. O potencial das macroalgas como fonte de matéria-prima para a produção de bioetanol é mostrado na Tabela 2.

Tabela 2 – Rendimento de produção, carboidratos hidrolisados e potencial de geração de bioetanol entre algumas matérias-primas convencionais e as macroalgas

	Trigo	Milho	Beterraba	Cana de açúcar	Macroalgas
Rendimento médio de produção mundial (kg ha^{-1} ano^{-1})	2.800	4.815	47.070	68.260	730.000
Peso seco de carboidrato hidrolisado (kg ha^{-1} ano^{-1})	1.560	3.100	8.825	11.600	40.150
Volume potencial de bioetanol (L ha^{-1} ano^{-1})	1.010	2.010	5.150	6.756	23.400

Fonte: (BORINES; LEON; MCHENRY, 2011)

Portanto, os biocombustíveis de terceira geração encontram-se em grande fase de desenvolvimento e já há na literatura relatos dos biocombustíveis de quarta geração. Estes últimos foram postos com o intuito de reduzir ou eliminar etapas do processamento final dos mesmos e sua produção envolve a utilização de organismos geneticamente modificados (ALALWAN; ALMINSHID; ALJAAFARI, 2019). No entanto, a produção de biocombustíveis a partir da biomassa algácea envolve a fermentação dos carboidratos. Porém, os carboidratos presentes na estrutura de macroalgas não estão livremente disponíveis aos microrganismos, mas na forma de polissacarídeos e exigem tratamentos hidrolíticos para clivar as ligações glicosídicas e liberar os monossacarídeos que podem ser subsequentemente fermentados (YUN; CHOI; KIM, 2015).

1.2.8. Fermentação alcoólica dos carboidratos de macroalgas

A fermentação alcoólica é o processo bioquímico mais utilizado para a produção de etanol (HOU et al., 2017). Esse processo pode ser realizado por vários microrganismos, como as leveduras: *Saccharomyces cerevisiae* (*S. cerevisiae*), *Brettanomyces custersii* e bactérias como *Zymomonas mobilis* (PARK et al., 2014). Entre os microrganismos, *S. cerevisiae* é a espécie mais explorada industrialmente para a produção de etanol (SUNWOO et al., 2017; SARAVANAN et al., 2018). É um microrganismo anaeróbico facultativo apresentando-se normalmente na forma unicelular, tipicamente esférica ou oval, não filamentosa com 2 a 8 micrometros de diâmetro (GUIDINI, 2013). O grande sucesso da aplicação dessa levedura deve-se a algumas características importantes, como: tolerância a baixos valores de pH, altas concentrações de açúcar e etanol, alta competitividade perante contaminação por bactérias ou outras leveduras e a resistência a inibidores de fermentação (NGUYEN et al., 2017).

O processo de fermentação alcoólica à base de sacarose contidas nas plantas já apresenta tecnologia bem estabelecida. Porém, este processo pode ser realizado a partir de qualquer biomassa contendo carboidratos que possam ser degradados em monossacarídeos fermentescíveis (CASTRO et al., 2017b). Tradicionalmente, o bioetanol vem sendo produzido a partir das biomassas de primeira geração, como amido ou açúcares da cana-de-açúcar, trigo e milho (HEBBALE; BHARGAVI; RAMACHANDRA, 2019).

A principal rota metabólica envolvida na fermentação alcoólica é a oxidação da molécula de glicose pela via glicolítica. Nessa via, cada molécula de glicose é oxidada produzindo duas moléculas de piruvato (Figura 9). Em condições anaeróbicas, o piruvato é descarboxilado em uma reação irreversível catalisada pela enzima piruvato descarboxilase formando acetaldeído (VAN MARIS et al., 2006). Este último sofre redução por ação da enzima álcool desidrogenase, formando etanol (Figura 9). A equação geral da reação da fermentação alcoólica a partir da oxidação da molécula de glicose é mostrada na equação 2.

$$\text{Glicose} + 2\text{ADP} + 2\text{P}_\text{I} \rightarrow 2\ \text{etanol} + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O} \qquad \text{Equação (2)}$$

Nos últimos anos, vários estudos foram publicados reportando o potencial das macroalgas como matéria-prima para a produção de biocombustíveis e outros produtos químicos economicamente importantes. Há na literatura estudos publicados relatando a

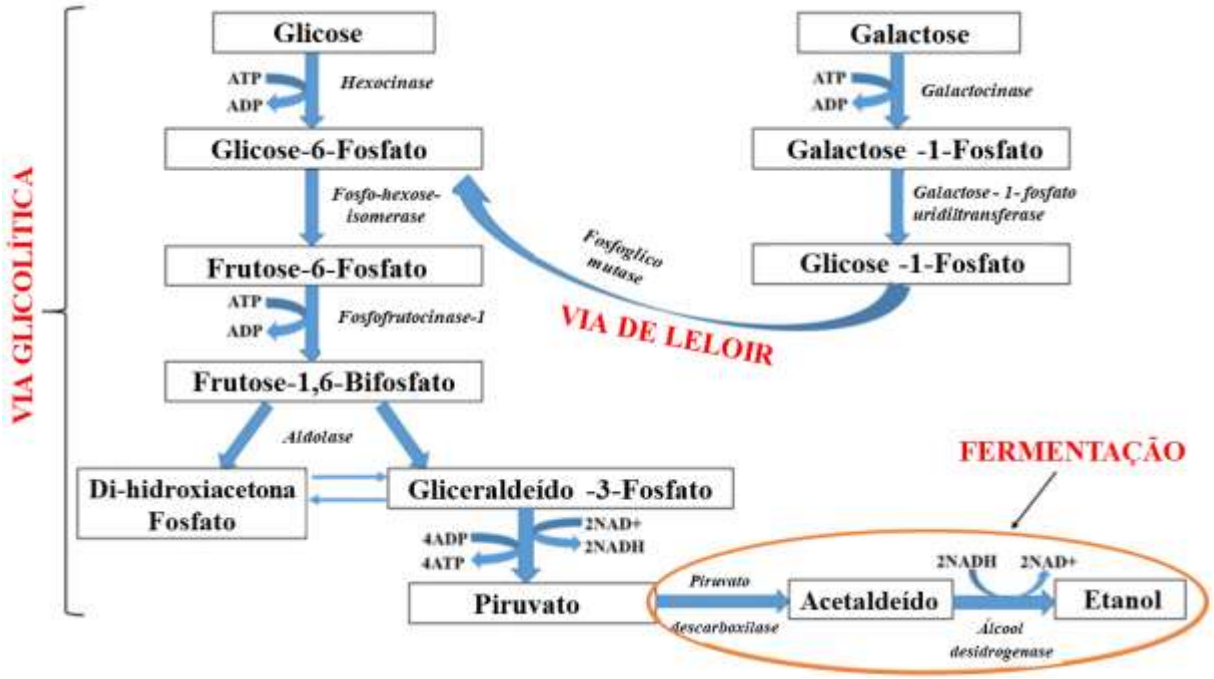
aplicações dos carboidratos de macroalgas em processos fermentativos para produção de ácido lático (HWANG et al., 2011), hidrogênio, ácido butírico (MUTRIPAH et al., 2014; XIA et al., 2015) e butanol (HOU et al., 2017). O potencial de uma variedade de macroalgas também foi examinado e demonstrado para a produção de bioetanol, incluindo representantes das macroalgas vermelhas (*Gracilaria salicornia, Gelidium amansii, Gelidium elegans*, *Kappaphycus alvarezii*), macroalgas verdes (*Ulva pertusa*, *Ulva lactuca*) e macroalgas marrons (*Laminaria hyperborea*, *Alaria crassifólia*, *Laminaria japonica*, *Sargassum fulvellum*, *Saccharina latissima*, *Undaria pinnatifida*) e entre outras espécies (GHADIRYANFAR et al., 2016; AMAMOU et al., 2018).

Entre as algas, destacam-se as macroalgas vermelhas, caracterizadas por apresentar elevados teores de carboidratos facilmente hidrolisáveis: ágar, carragenana, amido e cellulose (CASTRO et al., 2017; NGUYEN et al., 2017). A hidrólise desses polissacarídeos gera dois monossacarídeos principais, glicose e galactose, proveniente da celulose e galactanas, respectivamente, os quais podem ser fermentados subsequentemente (DAVE et al., 2019). Após a hidrólise dos polissacarídeos, os monossacarídeos são fermentados e o produto final é recuperado (MEINITA; HONG; JEONG, 2012).

No entanto, algumas barreiras técnicas relacionados aos processos de hidrólise e fermentação dos carboidratos de macroalgas precisam ser superadas para viabilizar a produção de etanol de terceira geração em escala industrial (AMAMOU et al., 2018). Condições drásticas de hidrólise é um fator limitante, pois causam a degradação dos carboidratos produzindo compostos indesejados que podem atuar como interferentes do processo fermentativo (JEONG et al., 2015). Os principais inibidores de fermentação são agrupados em três categorias: os derivados fenólicos, os ácidos orgânicos (ácidos acético e fórmico) e os derivados furânicos (furfural e 5-HMF) (YUN; CHOI; KIM, 2015). No meio fermentativo, esses inibidores retardam o crescimento celular do microrganismo reduzindo a produtividade do etanol (SUKWONG et al., 2018).

Os hidrolisados de macroalgas vermelhas apresentam maior concentração de galactose do que glicose. Porém, durante o processo fermentativo *S. cerevisiae* metaboliza preferencialmente glicose, sendo que, a galactose é consumida mais lentamente porque precisa ser convertida à glicose 6-fosfato pela via de Leloir para depois seguir pela via glicolítica (LEE et al., 2015). Este efeito é chamado repressão da glicose, na qual os genes metabólicos da galactose (GAL) são reprimidos na presença de glicose, reduzindo a

produtividade do etanol (SUNWOO et al., 2019). O metabolismo da glicose e galactose em condições anaeróbicas até a formação do etanol é mostrada na Figura 9.



Fonte imagem: Adaptada de DAVE et al., 2019

Figura 8. Rotas metabólicas envolvidas no processo de oxidação da molécula de glicose e galactose em condições anaeróbicas.

Todavia, estudos publicados recentemente mostraram que cepas de leveduras podem ser melhoradas por aclimatação em meios contendo altas concentrações de galactose. Dessa forma, é possível construir mutantes robustos para o consumo eficiente da galactose, aumentando a produtividade de etanol (CASTRO et al., 2017; NGUYEN et al., 2017; SUKWONG et al., 2018). Estudos recentes realizados com hidrolisados de macroalgas vermelhas aplicando cepas da levedura *S. cerevisiae* previamente aclimatadas a altas concentrações de galactose mostraram a conversão de até 80% dos monossacarídeos, glicose e galactose, em etanol (HESSAMI et al., 2018). Portanto, nos últimos anos várias biotecnologias usando microrganismos vem sendo desenvolvidas para viabilizar o uso da biomassa algácea para a produção de bioetanol e outros produtos químicos em escala industrial (KAWAI E MURATA, 2016; REEN et al., 2018).

2. OBJETIVOS

2.1. Objetivo Geral

- Otimizar o processo de hidrólise ácida da macroalga marinha vermelha *Gracilaria birdiae*, para a obtenção de produtos de interesse industrial e etanol.

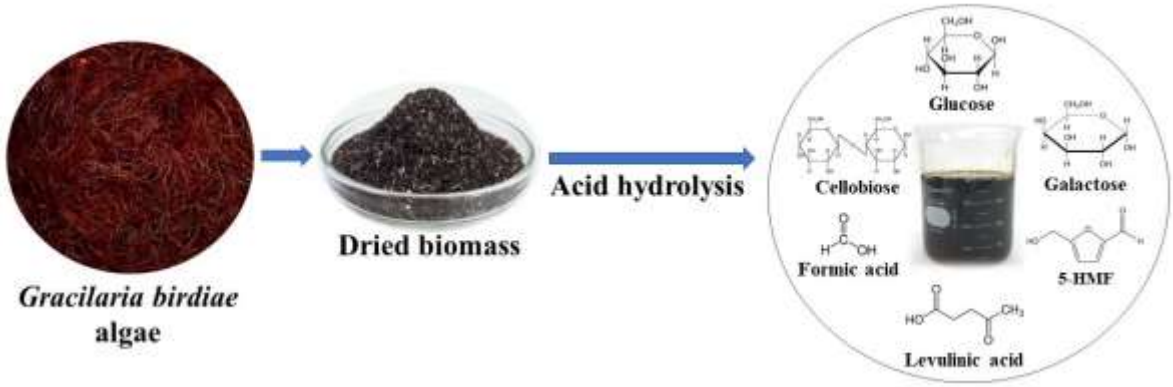
2.2. Objetivos Específicos

- Determinar a composição aproximada da biomassa seca de *G. birdiae*;
- Avaliar os efeitos isolados e combinados dos parâmetros concentração de biomassa, concentração de ácido sulfúrico e tempo reacional na hidrólise da biomassa de *G. birdiae*;
- Caracterizar a composição de monossacarídeos, ácidos orgânicos e compostos furânicos do hidrolisado de *G. birdiae*;
- Definir as condições ótimas de hidrólise que maximizam a geração de monossacarídeos, ácidos orgânicos e compostos furânicos aplicando MSR;
- Determinar a eficiência de hidrólise e o volume de hidrolisado recuperado (%) após a hidrólise da biomassa de *G. birdiae*;
- Realizar a hidrólise ácida da biomassa de *G. birdiae* em escala aumentada afim de obter meios de culturas ricos em carboidratos fermentescíveis;
- Avaliar os efeitos do tratamento com pó de carvão ativado sobre a remoção do 5-HMF presente no hidrolisado de *G. birdiae*;
- Avaliar o crescimento e os parâmetros fermentativos de *S. cerevisiae* em diferentes meios de culturas obtidos a partir de diferentes condições de hidrólise da biomassa de *G. birdiae*.

3. ARTIGO 1 DA TESE

José Cirlanio Sousa Albuquerque, Márjory Lima Holanda Araújo, Maria Valderez Ponte Rocha, Bartolomeu Warlene Silva de Souza, George Meredite Cunha de Castro, Edna Maria Silva Cordeiro, Jouciane de Sousa Silva e Norma Maria Barros Benevides. Acid hydrolysis conditions for the production of fine chemicals from *Gracilaria birdiae* alga biomass. **Algal Research,** 53 (2021) 102139, 2211-9264. https://doi.org/10.1016/j.algal.2020.102139.

Graphical abstract



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Acid hydrolysis conditions for the production of fine chemicals from *Gracilaria birdiae* alga biomass

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ABSTRACT

Gracilaria birdiae is a tropical agarophytes species of red seaweed that is rich in carbohydrates and presents a great potential for mariculture and economic use. However, it is still understudied as a potential renewable resource. In this study, the hydrolysis conditions of *G. birdiae* algal biomass, reaction time, and sulfuric acid concentration for obtaining monosaccharides, organic acids, and furanic compounds were optimized. Glucose and galactose concentrations were found to be more affected by algal biomass concentration, while the production of 5-hydroxymethylfurfural (5-HMF) and organic acid were most affected by sulfuric acid concentration. The most suitable conditions to produce glucose (28.56 ± 0.72 $g{\cdot}L^{-1}$) and galactose (108.03 ± 1.32 $g{\cdot}L^{-1}$) were at 121 °C, with sulfuric acid 1.3 $mol{\cdot}L^{-1}$, 841.59 $g{\cdot}L^{-1}$ of algal biomass and time of 20 min. For cellobiose (25.39 ± 0.43 $g{\cdot}L^{-1}$), the condition was sulfuric acid 0.6 $mol{\cdot}L^{-1}$, 680 $g{\cdot}L^{-1}$ of biomass, and 10 min. For 5-HMF (19.82 ± 0.43 $g{\cdot}L^{-1}$), it was sulfuric acid 0.6 $mol{\cdot}L^{-1}$, 680 $g{\cdot}L^{-1}$ of biomass and 30 min. Finally, for levulinic (38.88 ± 0.58 $g{\cdot}L^{-1}$) and formic acids (26.75 ± 0.54 $g{\cdot}L^{-1}$) and, the best conditions were sulfuric acid 2.0 $mol{\cdot}L^{-1}$, 680 $g{\cdot}L^{-1}$ of biomass and 30 min. Thus being, *Gracilaria birdiae* is a promising renewable resource to produce fine chemicals and fermentable compounds by optimizing its acid hydrolysis conditions.

1. Introduction

Considerable research has been focusing on the development of technologies based on sustainable and environmentally-friendly raw materials [1,2]. In this context, lignocellulosic biomass has been considering a potential candidate to produce biofuels and other chemicals, but the high recalcitrance caused by the presence of lignin in its composition requires high temperatures, pressures, and acid concentrations during acid hydrolysis processes [3,4]. These drastic conditions have caused a low yield of interesting products, such as monosaccharides [5–7]. On the other hand, seaweeds have attracted the attention of researchers as promising alternative biomass for the production of biofuels [7] because contain a high concentration of easily hydrolyzable carbohydrates, they are fast-growing and can be cultivated in wastewater and at sea, sparing the use of agricultural lands [8,9]. The world seaweed production was 32,886,200 tons in 2018, with the red seaweed representing 52,2% of this total. Within the group, the *Gracilaria* genus was the second most harvested in the world [10]. *Gracilaria*, one of the largest tropical agarophytes, has seen various applications, from human feeding to the extraction of several products, including agar [11]. In this scenario, the knowledge of the chemical composition of seaweed is crucial for their employment as feedstocks in biochemical production [12]. Moreover, the polysaccharides found in this seaweed are unique and they can be hydrolysed for producing a range of chemicals and chemical intermediates with established markets. However, such studies are still limited [13].

The absence of lignin in the cellular structure of seaweeds facilitates polysaccharide depolymerization and consequently, the generation of furanic compounds, organics acids, and monosaccharides, which can be used as substrates in bioprocesses to produce other fine chemicals and

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biofuels [14]. Among various platform chemicals, 5-hydroxymethylfurfural (5-HMF), levulinic acid, and formic acid have high potential in the synthesis of versatile chemicals and materials [15]. The production of 5-HMF, organic acids, potassium, nitrogen, sulfur-rich mother liquor (KNS-ML), and monosaccharides from polysaccharides of brown and red seaweeds have been reported in the recent years [12,16–25]. Some red seaweed species that are commercially important include *G. dura, G. verrucosa* and *Kappaphycus alvarezii.*

Gracilaria birdiae can be highlighted as a tropical species of red seaweed that has shown great potential for mariculture and other economic uses [26], but it is still understudied. The sulfated polysaccharide from *G. birdiae* seaweed is composed of galactose (65.4%) and methyl 6-O-methyl-galactose derivatives (9.2%), as well as 3-O- and 4-O-methyl-galactose (0.33%) in smaller amounts. The polysaccharide also presents a high content of 3,6-anhydrogalactose (25.1%) and a sulfate content of 8.4% [27].

The different reactional conditions, such as the type and concentration of acids and substrates, and reaction time and temperature have been optimizing to the generation of high contents of fine chemicals [28,29]. Glucose and galactose can be metabolized by microorganisms in fermentation processes to produce biofuels and other fine chemical compounds such as ethanol [30], butanol [9], butyric acid, and hydrogen [31,32]. 5-HMF is an intermediate molecule used to produce several specialty chemicals including pharmaceutical products, solvents, resins, fungicides, pigments, and fuels [33].

Formic acid is widely used in agricultural, textile, pharmaceutical, and rubber industries [31], and levulinic acid is an ideal platform chemical that can be utilized to produce several biochemicals including succinic acid, resins, polymers, herbicides, pharmaceuticals, and flavoring agents, solvents, plasticizers, anti-freeze agents and biofuels/oxygenated fuel additives [32]. However, few studies have established the conditions of acid hydrolysis for the generation of fine compounds from seaweed.

Therefore, the objective of the present study was to evaluate the influence of the parameters of sulfuric acid and algal biomass concentration as well as the reaction time on the production of glucose, galactose, cellobiose, 5-HMF, levulinic and formic acids *via* acid hydrolysis of the red seaweed *G. birdiae*. This is the first work reporting on the production fine chemicals from *G. birdiae*, including data on the recovered hydrolysate volumes and hydrolysis efficiency.

2. Materials and methods

2.1. Seaweed biomass

The seaweed *G. birdiae* was harvested from farming structures located in Flecheiras beach, Trairi, Ceará, Northwest Brazil (03° 13′ 06″ S 39° 16′ 47″ W). They were packed in plastic bags and transported to the Laboratory of Carbohydrates and Lectins (CARBOLEC) of the Department of Biochemistry and Molecular Biology of the Federal University of Ceará (UFC). In the laboratory, the seaweed was washed with running tap water to remove the salt, contaminant organisms, and epiphytes. The *G. birdiae* biomass was dried at 25 °C, cut, and milled in a knife mill grinder. The milled powder was then sieved through an 80-mesh sieve (<0.18 mm) and stored at room temperature (25 °C).

2.2. Proximate composition of G. birdiae seaweed

Crude protein was determined by the micro-Kjeldahl method system, using a conversion factor of 6.25 [34]. The contents of lipid, ash, and moisture were determined according to the Association of Official Analytical Chemists (AOAC, 2000). Crude lipids were extracted from the dried *G. birdiae* biomass using a Soxhlet extractor (Tecnal TE-044, São Paulo, Brazil) at 80 °C for 4 h using acetone as a solvent. The ash content was determined after incineration in a muffle furnace at 550 °C for 4 h. Moisture was determined after drying at 105 ± 5 °C in a drying oven (Icamo, mod. 2, Rio de Janeiro, Brazil). Carbohydrate content was determined by weight difference with the following correlation: Carbohydrate (%) = [100% − total percentage of lipid, protein, ash, and moisture], according to the methodology used by Castro et al. [35]. The assays were done in triplicate and the results were shown as the average mean ± standard deviation.

2.3. Acid hydrolysis of G. birdiae biomass

The effects of sulfuric acid concentration (SAC, X_1, 0.12–2.47 mol·L^{-1}), *G. birdiae* biomass concentration (GbBC, X_2, 38.40–841.59 g·L^{-1}) and reaction time (RT, X_3, 3.26–36.73 min) on the production of cellobiose, glucose, galactose, 5-HMF, levulinic acid and formic acid were evaluated. A 2^3 Central Composite Rotational Design (CCRD), with 17 assays (8 factorial points, 6 axial points, and 3 replicate in the central point) was performed. Each factor in the design was studied at five different levels (−α, −1, 0, +1 and +α). The minimum and maximum range of the variables were defined by preliminary assays (data not shown) and from other reports in the literature. The minimum and maximum range of the variables investigated along with the experimental plan and the values in the actual and coded forms are shown in Table 1.

The experimental data sets in the CCRD experiments were used to fit the second-order Equation (Eq. 1) by Response Surface Methodology, for each response variable, in which Y represent the response variables (Cellobiose; Glucose; Galactose; 5-HMF; Levulinic acid; Formic acid; Recovered hydrolysate volume and, Hydrolysis efficiency).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where β_0 is the constant coefficient; X_iX_j are the independent variables that influence the response variables Y; β_i is the linear coefficient relative to *I*; β_{ii} is the quadratic coefficient relative to *I*; β_{ij} is the interaction coefficient relative to *ij*; *k* is the number of factors. The quality of the fit of the polynomial model equation was expressed as the coefficient of determination (R^2).

The hydrolysis reactions of the dry biomass were carried out in 25-mL Erlenmeyer flasks containing 10 mL of sulfuric acid solution (Synth, São Paulo, Brazil) in an autoclave (Phoenix Luferco, São Paulo, Brazil), at 121 °C. The optimal temperature for hydrolysis was reached 8 min after the incubation of the reaction medium in the autoclave, and the timing of hydrolysis was only started when this temperature (121 °C) had been reached. Sulfuric acid concentrations, algal biomass concentrations, and reaction times were set according to the values dictated by the experimental design (Table 2). When the hydrolysis time was reached, each sample (in triplicates) was cooled down for 1 h to room temperature. The samples were filtered using a nylon membrane followed by filtration in a 0.45 μm filter. The Recovered Hydrolysate Volume (RHV) was expressed in percentage, according to Eq. (2):

$$\text{RHV}\ (\%) = \frac{V_f}{V_s} \times 100 \quad (2)$$

In which V_f is the volume recovered after filtration and *Vs* is the

Table 1
Actual and coded values of the independent variables used in the experimental design for the optimization of the hydrolysis of *G. birdiae* biomass.

Independent variables	Unit	Coded and actual levels				
		α	−1	0	+1	+α
SAC	(mol·L^{-1})	0.12	0.60	1.30	2.00	2.47
GbBC	(g·L^{-1})	38.40	200.00	440.00	680.00	841.59
RT	(min)	3.26	10.00	20.00	30.00	36.73

SAC: Sulfuric Acid Concentration; GbBC: *G. birdiae* Biomass Concentration; RT: Reaction Time.

Table 2
The approximate composition of seaweeds of the genus *Gracilaria*. Data shown are the mean ± SD, $n = 3$.

Species	Carbohydrate (%)	Protein (%)	Lipid (%)	Ash (%)	Moisture (%)	Reference
Gracilaria birdiae	73.01 ± 1.03	8.03 ± 0.42	0.46 ± 0.06	6.05 ± 0.51	12.45 ± 0.62	Current work
Gracilaria sp	76.6	16.0	1.2	6.1	–	[34]
Gracilaria gigas	64.7	12.6	1.3	19.6	–	[37]
Gracilaria cervicornis	63.1	19.7	0.4	10.5	14.6	[38]
Gracilaria verrucosa	60.8	9.8	0.8	13.8	–	[37]
Gracilaria gracilis	46.6	20.2	0.6	24.8	8.0	[39]
Gracilaria grassa	42.0	5.1	1.3	43.2	7.4	[40]
Gracilaria foliferas	41.3	–	0.3	13.7	–	[37]
Gracilaria cornea	36.3	5.4	–	29.0	–	[38]
Gracilaria salicornia	26.9	–	0.4	10.4	–	[41]

(−) Not determined.

initial volume of the acid solution.

Hydrolysis efficiency (HE%) of the total carbohydrates of *G. birdiae* was determined by the relationship between the cellobiose (P_1), glucose (P_2) and galactose (P_3) total contents in the hydrolysate and the total carbohydrate content (TC_{Gb}) in the dried algal biomass, according to Eq. (3). All analyses were performed in triplicate.

$$HE(\%) = \frac{(P_1 + P_2 + P_3)}{TG_{Gb}} \times 100 \quad (3)$$

2.4. Analytical methods

The concentrations of carbohydrate (cellobiose, glucose and galactose), organic acids (levulinic acid and formic acid) and furanic compounds (5-HMF and furfural) in the hydrolysates were determined by a High-Performance Liquid Chromatography apparatus (HPLC, Walters, Milford, MA, USA) equipped with refractive index and UV absorbance detectors (Waters 2414), and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Sulfuric acid (5.0 mM) diluted in MilliQ grade water was used as a mobile phase at 0.5 mL·min^{-1} flow rate, 65 °C, and a total running time of 60 min, according to Rodrigues et al. [36]. The carbohydrates, furanic compounds, and organic acids were determined and quantified from the standard curves developed using standard reagents [Carbohydrates: CAR10 Lot: SLBJ8685V; Furfural Lot: SHBD3699V; 5-HMF Lot: BCBM2548V (Sigma-Aldrich); and Organic Acids Lot: LC09525 (Supelco) and Levulinic acid: 41474 (Supelco)].

2.5. Statistical analysis

The assays of the CCRD were performed randomly, and the data evaluated by STATISTICA 12.0 software (Statsoft Inc., Tulsa, OK, USA). The software also generated response surface graphs to show the isolated and the combined effects of the independent variables as well as the optimal conditions for monosaccharide, organic acid, and furanic compound production. The quality of fit of the models was expressed by the coefficient of determination (R^2), and the statistical significance was determined by an *F* test (Analysis of Variance - ANOVA). Validation of the statistical models was performed based on the values predicted by the quadratic model as a reference for the values obtained for monosaccharides (glucose and galactose). Three independent assays were performed applying the parameters SAC 1.3 mol·L^{-1}, GbBC 841.59 g·L^{-1} and RT 20 min to validate the mathematical models.

3. Results and discussion

3.1. Proximate composition of G. birdiae seaweed

The approximate centesimal composition of *G. birdiae* and those of other seaweeds species of the *Gracilaria* genus are shown in Table 2.

The dried biomass of *G. birdiae* presented 73.01 ± 1.03% total carbohydrates, 8.03 ± 0.42% proteins, 0.46 ± 0.06% lipids, 6.05 ± 0.51% ash and 12.45 ± 0.62% moisture. Compared to the data for other Gracilaria species, the carbohydrate content of *G. birdiae* is the most abundant, whereas lipid was the least abundant. Nevertheless, since *G. birdiae* presented a very high carbohydrate content, it represents a promising renewable source of biomass from where various compounds of industrial interest can be obtained by chemical and biotechnological routes.

The protein content of *G. birdiae* (8.03 ± 0.42%) was similar to that of *G. verrucosa*, and lower than those of *Gracilaria sp*., *G. gigas*, *G. cervicornis*, and *G. gracilis*. These results indicate a great variation in the protein content (5.1 to 20.2% dry algae mass) of the *Gracilaria* genus of seaweed species.

The ash content was low, but there is also a great variation among species, which range from 6.05 ± 0.51% for *G. birdiae* to 43.2% for *G. grassa* [42]. Disparities regarding the biochemical composition of macroalgae are mainly associated with seasonal variations and the life stage of these organisms [35].

Environmental parameters such as water temperature, salinity, light and nutrients can inhibit or stimulate the biosynthesis of several compounds causing differences in algae composition [42].

The high carbohydrate content (73.01 ± 1.03%) present in *G. birdiae* dried biomass, and the fact that this species has potential for cultivation in mariculture systems in tropical seas [38], makes it an attractive source of raw material to recover several chemical compounds by acid hydrolyses, such as monosaccharides, organic acids and furanic compounds, which may then be purified or used for the production of a variety of others fine chemicals by biotechnological pathways.

3.2. Acid hydrolysis of G. birdiae biomass

3.2.1. Fine-chemical yields and hydrolysate recovery volume

The cellobiose, glucose, galactose, 5-HMF, levulinic and formic acids concentrations determined in the acid hydrolysate from *G. birdiae* seaweed by CCRD matrix are shown in Table 3.

The highest concentrations for cellobiose (25.39 ± 0.43 g·L^{-1}), in assay 2 (SAC: 0.6 mol·L^{-1}, GbBC: 680 g·L^{-1}; RT: 10 min); for glucose (28.56 ± 0.72 g·L^{-1}) and g·L^{-1}; galactose (108.03 ± 1.32 g·L^{-1}) were achieved in assay 13 (SAC: 1.3 mol·L^{-1}, GbBC: 841.59 g·L^{-1}; RT: 20 min); and for 5-HMF (19.82 ± 0.43 g·L^{-1}), in assay 7 (SAC: 0.6 mol·L^{-1}, GbBC: 680; RT: 30 min). Under these conditions, the yields (based total carbohydrates) were 5.1% for cellobiose, 4.6% for glucose, 17.6% for galactose, 3.9% for 5-HMF, 7.8% for levulinic acid and 5.4% for formic acid.

The cellobiose concentration observed in this study was higher than that found for the *Solieria filiformis* hydrolysate (2.0 g·L^{-1}). The disaccharide is quickly converted into monosaccharides when in acidic media, and there are only a few reports regarding cellobiose production from seaweed in the literature.

The maximum glucose and galactose concentrations obtained by acid hydrolysis (H_2SO_4) of the macroalga *Kappaphycus alvarezii* were

Table 3
Experimental matrix with the values (levels) of the independent variables evaluated in the Central Composite Rotational Design for the generation of cellobiose, glucose, galactose, 5-hydroxymethylfurfural, levulinic acid, formic acid, and the recovered volume from the acid hydrolysis of *G. birdiae*. Data shown are the mean ± SD, $n = 3$.

Independent variables				Carbohydrates			5-HMF ($g{\cdot}L^{-1}$)	Organic acids		RHV (%)
Assay	SAC ($mol{\cdot}L^{-1}$)	GbBC ($g{\cdot}L^{-1}$)	RT (min)	Cellobiose ($g{\cdot}L^{-1}$)	Glucose ($g{\cdot}L^{-1}$)	Galactose ($g{\cdot}L^{-1}$)		Levulinic ($g{\cdot}L^{-1}$)	Formic ($g{\cdot}L^{-1}$)	
1	2.00	200.00	30.00	5.54 ± 0.27	10.27 ± 0.36	42.58 ± 0.74	0.24 ± 0.08	19.63 ± 0.37	9.92 ± 0.37	91.0 ± 0.62
2	0.60	680.00	10.00	25.39 ± 0.43	17.49 ± 0.46	74.08 ± 0.88	15.70 ± 0.41	4.99 ± 0.25	4.21 ± 0.22	83.0 ± 0.71
3	1.30	38.40	20.00	0.00 ± 0.0	2.00 ± 0.17	8.80 ± 0.23	0.38 ± 0.01	3.65 ± 0.22	1.56 ± 0.15	96.0 ± 0.56
4	0.60	200.00	10.00	6.51 ± 0.19	8.62 ± 0.34	39.95 ± 0.64	9.28 ± 0.32	3.56 ± 0.23	1.77 ± 0.13	82.0 ± 0.70
5	1.30	440.00	20.00	18.32 ± 0.37	19.73 ± 0.46	78.36 ± 0.82	8.29 ± 0.33	21.71 ± 0.42	13.57 ± 0.39	87.0 ± 0.83
6	1.30	440.00	36.73	16.97 ± 0.31	18.35 ± 0.42	74.96 ± 0.79	2.39 ± 0.18	28.70 ± 0.47	15.42 ± 0.41	87.0 ± 0.81
7	0.60	680.00	30.00	19.00 ± 0.41	23.11 ± 0.52	90.34 ± 0.91	19.82 ± 0.43	14.58 ± 0.43	9.39 ± 0.34	83.0 ± 0.73
8	0.12	440.00	20.00	0.00 ± 0.0	5.60 ± 0.23	25.54 ± 0.53	8.75 ± 0.33	0.14 ± 0.02	4.51 ± 0.26	79.0 ± 0.76
9	1.30	440.00	3.26	11.79 ± 0.33	19.14 ± 0.38	79.32 ± 0.83	12.95 ± 0.39	11.95 ± 0.36	7.83 ± 0.33	82.0 ± 0.78
10	2.00	200.00	10.00	6.89 ± 0.24	11.34 ± 0.28	46.14 ± 0.55	1.49 ± 0.11	17.45 ± 0.41	8.67 ± 0.31	90.0 ± 0.55
11	2.00	680.00	30.00	17.95 ± 0.39	26.62 ± 0.62	100.37 ± 0.98	1.93 ± 0.13	38.88 ± 0.58	26.75 ± 0.54	78.0 ± 0.69
12	1.30	440.00	20.00	11.26 ± 0.25	19.53 ± 0.54	78.93 ± 0.86	10.96 ± 0.36	19.79 ± 0.42	12.60 ± 0.41	87.0 ± 0.78
13	1.30	841.59	20.00	20.13 ± 0.37	28.56 ± 0.72	108.03 ± 1.32	11.25 ± 0.34	24.11 ± 0.52	15.30 ± 0.38	68.0 ± 0.92
14	1.30	440.00	20.00	11.66 ± 0.31	20.07 ± 0.66	79.55 ± 0.85	9.76 ± 0.38	21.02 ± 0.45	12.77 ± 0.36	85.0 ± 0.78
15	2.47	440.00	20.00	20.47 ± 0.41	19.28 ± 0.55	75.43 ± 0.82	1.02 ± 0.09	30.22 ± 0.61	19.95 ± 0.44	92.0 ± 0.53
16	2.00	680.00	10.00	16.82 ± 0.36	25.88 ± 0.61	97.12 ± 0.91	4.90 ± 0.23	30.33 ± 0.57	19.23 ± 0.41	75.0 ± 0.66
17	0.60	200.00	30.00	3.13 ± 0.18	10.29 ± 0.32	43.37 ± 0.69	6.65 ± 0.29	11.11 ± 0.33	7.30 ± 0.35	91.0 ± 0.65

SAC: Sulfuric Acid Concentration; GbBC: *G. birdiae* Biomass Concentration; RT: Reaction Time; 5-HMF: 5-hydroxymethylfurfural; RHV: Recovered hydrolysate volume; Furfural was not detected in *G. birdiae* hydrolysates.

0.89 $g{\cdot}L^{-1}$ and 23.87 $g{\cdot}L^{-1}$, respectively, using 10% (*w/v*) algal biomass hydrolysate with 0.2 $mol{\cdot}L^{-1}$ H_2SO_4 at 130 °C for 15 min [43]. The maximum galactose concentration obtained by acid hydrolysis of the red macroalga *Gelidium amansii* was 25.50 $g{\cdot}L^{-1}$, using 8% (*w/v*) algal biomass hydrolysed with 91 mM H_2SO_4 at 121 °C for 45 min [44].

The acid hydrolysates of the red macroalga *Gelidium latifolium* generated 2.40 $g{\cdot}L^{-1}$ and 34.40 $g{\cdot}L^{-1}$ of glucose and galactose, respectively, when 12% (*w/v*) biomass was exposed to 0.2 $mol{\cdot}L^{-1}$ H_2SO_4 for 15 min at 130 °C [41].

Acid hydrolysis of the *Solieria filiformis* macroalgae generated a maximum concentration of 18.10 $g{\cdot}L^{-1}$ of monosaccharides (glucose + galactose) in the hydrolysates when the hydrolysis parameters were 7% (w/v) algal biomass, 0.5 $mol{\cdot}L^{-1}$ H_2SO_4, 121 °C and 20 min [35]. The maximum concentration of 38.45 $g{\cdot}L^{-1}$ of reducing sugars was detected in the acid hydrolysate of the *K. alvarezii* macroalgae at 10% (w/v) algal biomass, 0.2 $mol{\cdot}L^{-1}$ H_2SO_4, 130 °C and 15 min [43].

In another study, acid hydrolysis of the *Eucheuma denticulatum* macroalgae generated 51.47 $g{\cdot}L^{-1}$ of reducing sugars using 20% (*w/v*) algal biomass, 0.1 $mol{\cdot}L^{-1}$ H_2SO_4, 10 min and 160 °C [45].

In the hydrolysates of the *G. amansii* macroalgae 7.0 $g{\cdot}L^{-1}$ of 5-HMF was detected when the hydrolysis parameters were 10% (w/v) of algal biomass, 94 mM H_2SO_4, 121 °C and 60 min [46]. Thus being, the maximum concentration of 5-HMF (19.82 ± 0.43 $g{\cdot}L^{-1}$) obtained in the *G. birdiae* hydrolysate (Assay 7) was higher than the previously-reported concentrations. Furfural has not been detected in *G. birdiae* hydrolysates.

The highest concentrations of levulinic acid (38.88 ± 0.54 $g{\cdot}L^{-1}$) and formic acid (26.75 ± 0.54 $g{\cdot}L^{-1}$) were achieved in assay 11, (SAC: 2.0 $mol{\cdot}L^{-1}$, GbBC: 680 $g{\cdot}L^{-1}$; RT: 30 min), under conditions that correspond to the three high levels (+1) of each of the independent variables. On the other hand, the lowest concentrations of these compounds were obtained in assay 8 (SAC: 0.12 $mol{\cdot}L^{-1}$, GbBC: 440 $g{\cdot}L^{-1}$; RT: 20 min) and 3 (SAC: 1.3 $mol{\cdot}L^{-1}$, GbBC: 38.40 $g{\cdot}L^{-1}$; RT: 20 min), respectively. The results revealed that the severe acid hydrolysis conditions of *G. birdiae* used in this study promoted the high generation of organic acids (formic and levulinic acid). Wu et al. [47] reported that 12% (*w/v*) *Pterocladiella capillacea* biomass exposed to 12% (*v/v*) H_2SO_4 at 121 °C for 15 min maximized the formic acid concentration (6.2 $g{\cdot}L^{-1}$), which was lower than the values achieved in the present study.

The highest and lowest percentages of recovered hydrolysate volumes were achieved in assay 3 (SAC: 1.3 $mol{\cdot}L^{-1}$, GbBC: 38.40 $g{\cdot}L^{-1}$; RT: 20 min) and assay 13 (SAC: 1.3 $mol{\cdot}L^{-1}$, GbBC: 841.59 $g{\cdot}L^{-1}$; RT: 20 min), respectively. These results suggest that the high proportion of algal biomass reduced the volume of recovered hydrolysate and hydrolysis efficiency (data not shown), but did not inhibit the hydrolysis reaction of hydrocolloids.

In this study, the concentrations of algal biomass evaluated (3.8 at 84.1%, *w/v*) were higher than those previously reported (7.0 at 20.0%, w/v). Also, the highest concentrations of sugars and 5-HMF were observed under the hydrolysis conditions tested in this work. This can be explained by the simpler structural composition of these seaweeds which, due to the lack of lignin and hemicellulose, allows for conditions of weaker acid concentrations, lower temperatures, and reaction times. Moreover, the high content of polysaccharides (73.1%), the homogeneity of the galactan from *G. birdiae*, and the easily hydrolysable cellulose found in the cell walls of red seaweeds form hexoses epimers (glucose and galactose), and 3,6-anhydrogalactose. These monosaccharides can lead to an easier conversion and obtainment of fine chemicals that are purer than those obtained from lignocellulosic materials.

3.2.2. *Effect of independent variables on fine chemical generation, hydrolysate recovered volume and hydrolysis efficiency*

The effect of each independent variable on the production of the fine chemicals, such as cellobiose, monosaccharides, 5-HMF, levulinic acid, formic acid, as well as the volume of hydrolysate recovered and hydrolysis efficiency from *G. birdiae* algal biomass is shown in Fig. 1.

The results show that only GbBC (L) showed a significant and positive effect on cellobiose generation. This disaccharide comes from the hydrolysis of the cellulose present in the algal biomass, which in the acidic medium, can be hydrolysed, producing glucose [48].

GbBC (L) and SAC (L) were the most significant variables, showing the highest positive effects on the glucose and galactose formation, while increases in SAC (Q) and GbBC (Q) levels cause accentuated decrease of these compounds. The negative effects of SAC (Q) can be explained by the acid degradation of the glucose and galactose generated to furanic compounds and organic acids [49]. As for the negative effect of GbBC (Q) on glucose and galactose generation, it is suggested that the high water retention capacity of hydrocolloids present in dry algal biomass may make these molecules unavailable for the hydrolysis reaction.

The SAC (L) had a negative effect on the 5-HMF formation and was

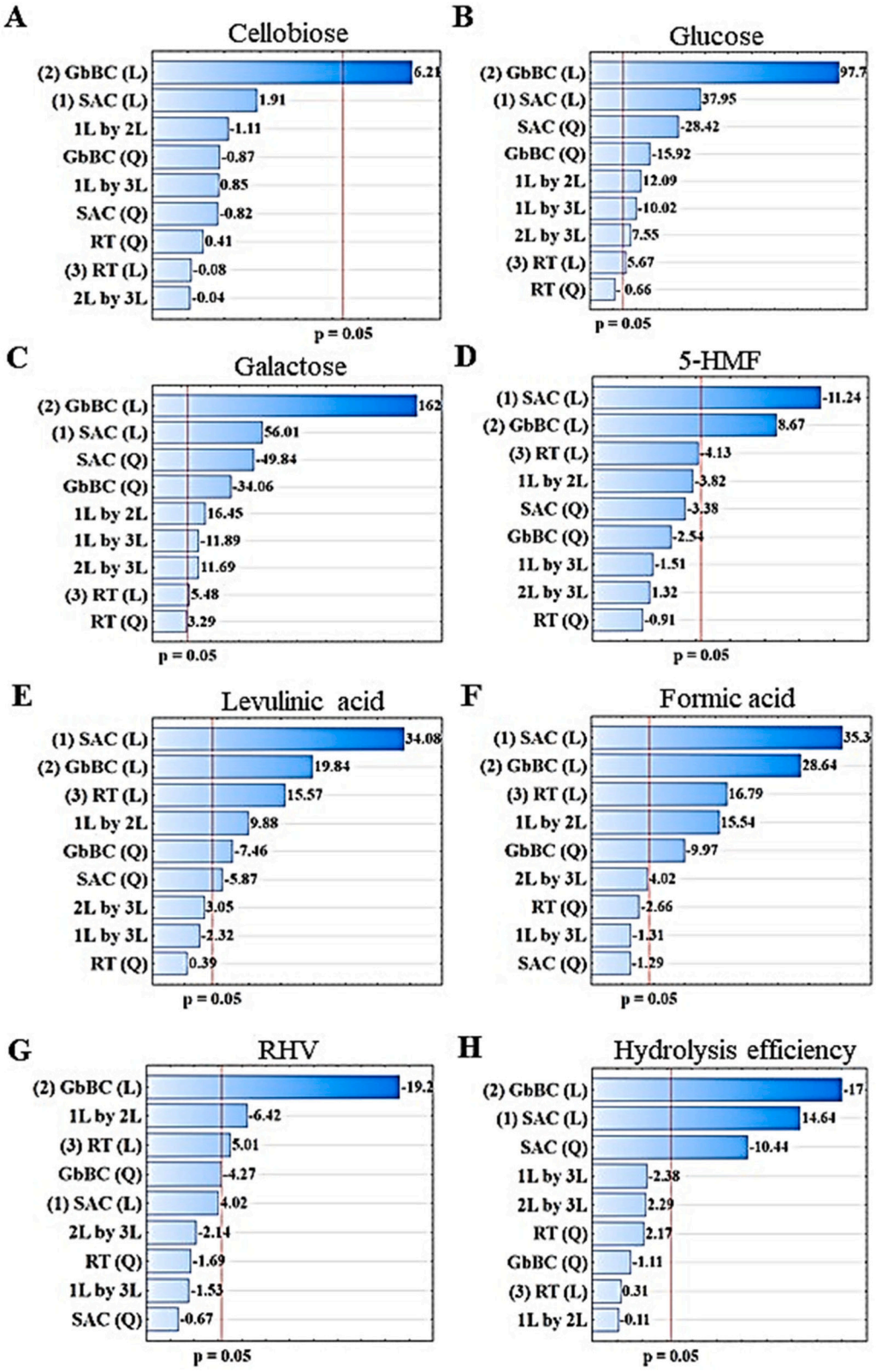


Fig. 1. Pareto diagrams representing the isolated effects and the reciprocal interactions of the independent variables of sulfuric acid concentration (SAC), *G. birdiae* biomass concentration (GbBC) and reaction time (RT) in the obtainment of the fine chemicals (A) glucose; (B) galactose; (C) cellobiose; (D) 5-HMF (5-hydroxymethylfurfural); (E) formic acid; (F) levulinic acid. (G) recovered hydrolysate volume and (H) hydrolysis efficiency of *G. birdiae*. (L) represents the linear effect and (Q) represents the quadratic effect of each independent variable. 1 L by 2 L: interaction between the linear effect of SAC and the linear effect of GbBC; 1 L by 3 L: interaction between the linear effect of SAC and the linear effect of RT; 2 L by 3 L: interaction between the linear effect of GbBC and the linear effect of RT. The estimated effects of the independent variables were evaluated at a confidence interval of 95%.

the most significant variable. GbBC (L) affected positively and significantly. The negative effect of SAC (L) suggests the acid conversion of 5-HMF to formic acid and levulinic acid by rehydration [50].

The GbBC (L), SAC (L), and RT (L) variables had positive and significant effects on formic and levulinic acids formation, while an isolated increase in the GbBC (Q) variable had negative effect in formic acid generation. The hydrolysis conditions with the most GbBC, SAC and RT levels increased the concentrations of formic and levulinic acids. These results suggest that the increase in concentrations of these acids comes from the degradation of glucose, galactose, and 5-HMF.

The GbBC (L) caused a negative effect on the volume of the recovered hydrolysate. No hydrolysate showed gel formation. A high degree of

hydrolysis of the hydrocolloids present in the algal biomass promotes a loss of viscosity of the hydrolysate, as well as decreases the water retention of algal biomass. Then, the recovery of a larger volume of hydrolysate by filtration step was possible. Another phenomenon that can be considered is the mass transfer: since there is more dry mass and less liquid phase, acid diffusion through the algae, as well as inside the algae cells, can be negatively affected, decreasing hydrolysis efficiency.

Due to the lower hydrolysis levels, more mass will retain water, so the recovered volume is smaller.

Similarly, the efficiency of hydrolysis was impaired by the linear increase of GbBC since the hydrocolloids of the dry algal biomass retain water from the environment. SAC (L) had a positive effect on glucose, cellobiose and galactose formation from polysaccharides, cellulose and agar, but SAC (Q) had negative effect because promotes the 5-HMF and formic and levulinic acids formation from monosaccharides.

As shown in the results above, at 121 °C, the condition of high algal biomass concentration, middle SAC and middle RT was appropriate for the generation of glucose and galactose. The formation of cellobiose occurred in the lowest SAC and RT, and at a high GbBC. 5-HMF was properly produced under the condition of low catalyst concentration, high GbBC and longer reaction time. Finally, the levulinic and formic acids were formed in the most severe hydrolysis conditions used in this study.

The Analysis of Variance (ANOVA) with all statistical data for each variable evaluated was presented as supplementary data (Table 1S). According to an analysis of variance (ANOVA), the model equations for glucose, galactose, recovered hydrolysate volume and hydrolysis efficiency need adjustment. R^2 values for glucose, galactose, levulinic acid and formic acid were equal to or greater than 0.95 (95%). The variables cellobiose, 5-HMF, recovered hydrolysate volume, and hydrolysis efficiency presented R^2 values below 0.95. For biological processes, it is accepted that the values of R^2 above 0.80 in a 95% confidence interval are considered good [51].

Thus, the predictive response model equations with the significant variables, which represent the glucose, galactose, cellobiose, 5-HMF, formic acid and levulinic acid formation by acid hydrolysis from *G. birdiae* are showed in Table 4.

3.2.3. Optimization of fine chemical production

Given the mathematical models obtained for each response variable, the optimized conditions for the generation of total monosaccharides, reducing sugars, cellobiose, 5-HMF, and organic acids through the acid hydrolysis of *G. birdiae* are shown in Fig. 2.

Cellobiose generation was maximized when GbBC and SAC reached concentrations greater than 600 $g{\cdot}L^{-1}$ and 0.5 $mol{\cdot}L^{-1}$, respectively. Under these conditions, the cellobiose concentration was greater than 20 $g{\cdot}L^{-1}$ (Fig. 2A).

Within the reaction time tested (20 min), the cellobiose concentration was not significantly affected by the increase of the catalyst concentration. However, under high sulfuric acid concentrations, cellobiose production was reduced.

Fig. 2B shows that 30.0 $g{\cdot}L^{-1}$ of glucose was formed when GbBC was greater than 680 $g{\cdot}L^{-1}$ combined with SAC greater than 1.0 $mol{\cdot}L^{-1}$. The galactose formation was formed in similar conditions, but the concentration can reach over 100 $g{\cdot}L^{-1}$ (Fig. 2C).

High GbBC and reduced SAC maximized the production of 5-HMF. Conditions with GbBC greater than 680 $g{\cdot}L^{-1}$ and SAC between 0 and 0.5 $mol{\cdot}L^{-1}$ at 121 °C and 20 min generated 5-HMF concentrations greater than 18 $g{\cdot}L^{-1}$, but in this condition the 5-HMF concentration is quickly reduced with the increase of sulfuric acid concentration (Fig. 2D). The acid hydrolysis of *G. birdiae* generated hexoses, that by dehydration is formed 5-HMF, that by hydration it is converted into formic and levulinic acids [32].

Severe acid hydrolysis conditions cause carbohydrate degradation to generate organic acids, furanic and phenolic compounds [49]. Similar results were obtained by Teh et al. [52] when performing the acid hydrolysis of the red macroalgae *Eucheuma denticulatum*.

Hydrolysis conditions with high GbBC and SAC maximized the formic and levulinic acids formation. GbBC and SAC greater than 600 $g{\cdot}L^{-1}$ and 2.0 $mol{\cdot}L^{-1}$ generated concentrations greater than 25 $g{\cdot}L^{-1}$ and 40 $g{\cdot}L^{-1}$ formic and levulinic acids, respectively (Fig. 2E and F).

The optimal condition for the recovery of hydrolysate is in a range of GbBC less than 300 $g{\cdot}L^{-1}$ combined with a SAC greater than 1.0 $mol{\cdot}L^{-1}$. Under these conditions, the recovery of hydrolysate is greater than 90% and there are important fine chemicals for purification or fermentation. The optimal region of hydrolysis efficiency is in a range of GbBC combinations of less than 250 $g{\cdot}L^{-1}$ and SAC between 1.0 and 2.5 $mol{\cdot}L^{-1}$ when the hydrolysis efficiency was maximized (> 40%).

Therefore, according to this study, the optimized hydrolysis conditions for *G. birdiae* biomass for cellobiose, hexoses (glucose and galactose), 5-HMF and levulinic and formic acids formation, corroborate with the reaction mechanisms for the conversion of hexose sugars and 3,6-anhydrogalactose to levulinic acid reported by Assary et al. [53] and Oh et al. [50].

In this scenario, the optimized conditions for levulinic acid formation from *G. birdiae* could reach higher concentrations than those observed in assay 11 (SAC: 2.0 $mol{\cdot}L^{-1}$, GbBC: 680 $g{\cdot}L^{-1}$; RT: 30 min), with an unconverted hexose amount of 127 $g{\cdot}L^{-1}$.

The glucose (28.42 ± 0.36 $g{\cdot}L^{-1}$) and galactose (110 ± 0.69 $g{\cdot}L^{-1}$) concentrations obtained experimentally (at 841.59 $g{\cdot}L^{-1}$ GbBC and 1.3 $mol{\cdot}L^{-1}$ H_2SO_4 for 20 min.), were similar to the values found for glucose (28.56 ± 0.72 $g{\cdot}L^{-1}$) and galactose (108.03 ± 1.32 $g{\cdot}L^{-1}$) obtained from the CCRD, when the same parameters were employed.

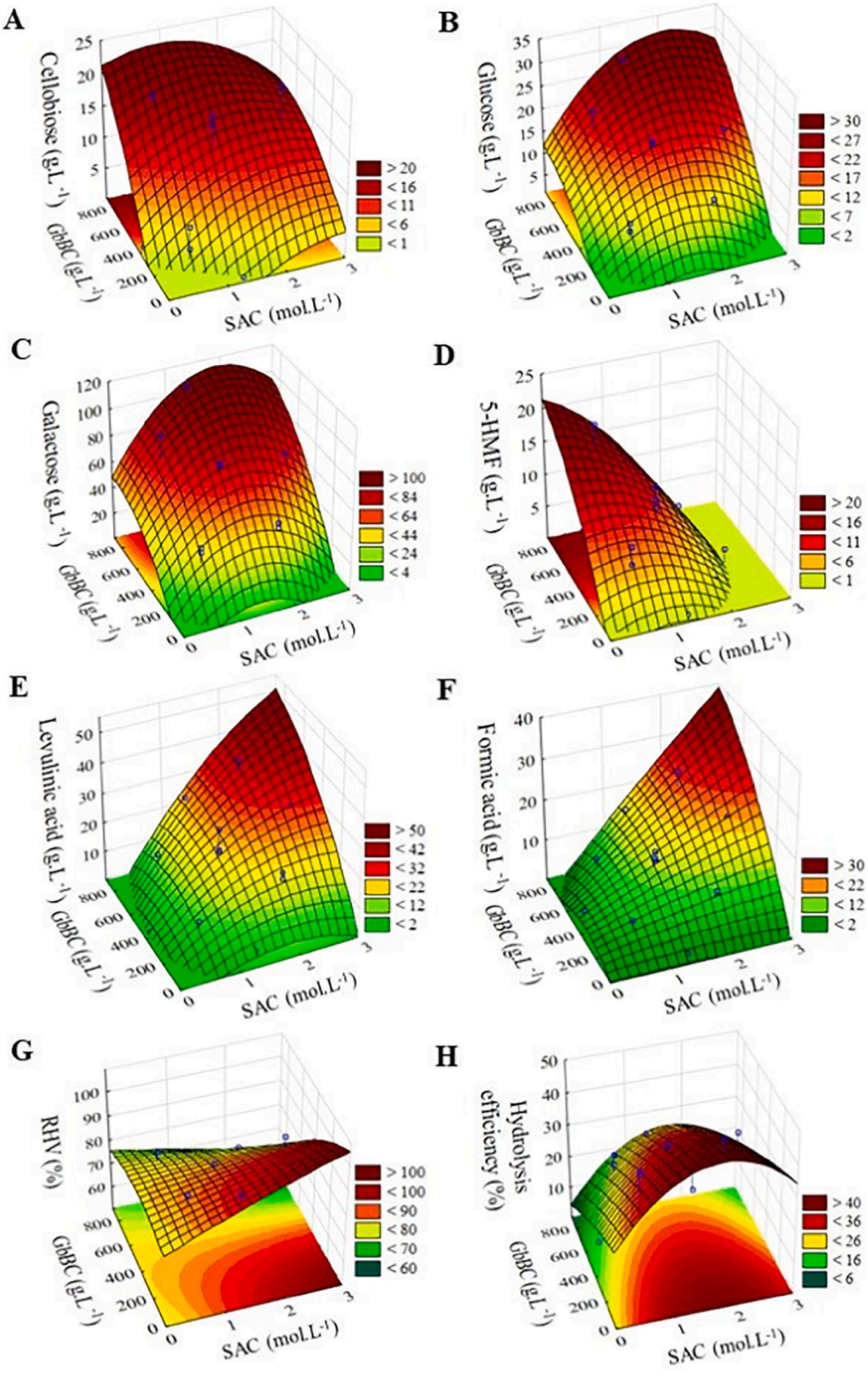
Given the similarity between the values obtained in the isolated trials and the values obtained in the CCRD, the reproducibility of the models was successfully confirmed. Therefore, these results indicate that the mathematical model is suitable for evaluating the obtainment of these compounds in face of variations of SAC, GbBC, and RT.

With the present study, it was shown that the dry biomass of the red seaweed *G. birdiae* can be easily hydrolysed using mild acid hydrolysis conditions that promotes the production of several economically relevant chemicals.

Table 4
Predictive response model equations, with the significant terms of the quadratic models, for fine chemicals production by acid hydrolysis from *G. birdiae* algal biomass.

Predictive response model equations	*p*-Value regression	p-Value lack of adjustment	R^2
$Y_1 = -8.1005 + 0.056X_2$	0.12611	0.30791	0.75
$Y_2 = -8.489 + 15.332X_1 - 4.5033X_1^2 + 0.034X_2 - 0.00002X_2^2 + 0.108X_3 - 0.0068X_1X_2 - 0.1361X_1X_3 + 0.0003X_2X_3$	0.00056	0.00548	0.96
$Y_3 = -24.027 + 56.217X_1 - 17.450X_1^2 + 0.1556X_2 - 0.0001X_2^2 - 0.132X_3 + 0.0059X_3^2 + 0.0206X_1X_2 - 0.3570X_1X_3 + 0.001X_2X_3$	0.00080	0.00294	0.95
$Y_4 = -1.63 + 0.79X_1 + 0.037X_2$	0.02938	0.11107	0.98
$Y_5 = -13.76 + 1.48X_1 - 0.03X_1^2 + 0.02X_2 - 0.0001X_2^2 + 0.002X_1X_2$	<0.00001	0.18092	0.98
$Y_6 = -4.37 + 0.127X_1 + 0.012X_2 - 0.00003X_2^2 + 0.31X_3 + 0.0017X_1X_2$	<0.00001	0.16392	0.98

X_1, X_2, and X_3 represent the independent variables Sulfuric acid concentration (M), *G. birdiae* biomass concentration ($g{\cdot}L^{-1}$) and Reaction time (min), respectively. Y represents the values of the response variables ($g{\cdot}L^{-1}$): (Y_1) Cellobiose; (Y_2) Glucose; (Y_3) Galactose; (Y_4) 5-hydroxymethylfurfural; (Y_5) Formic acid and (Y_6) Levulinic acid. Coefficient of determination (R^2).



(caption on next page)

Fig. 2. Response surfaces plot representing the effects of the sulfuric acid concentration (SAC), *G. birdiae* biomass concentration (GbBC) and Reaction time (RT) on the production of fine chemicals by acid hydrolysis from *G. birdiae*. Reaction time was set at the midpoint 20 min, at 121 °C. (A) Glucose; (B) Galactose; (C) Cellobiose; (D) 5-HMF; (E) Formic acid; (F) Levulinic acid; (G) Recovered hydrolysate volume (RHV); (H) Hydrolysis efficiency. The estimated effects of the independent variables were evaluated at a confidence interval of 95%.

Thus, these results demonstrate the enormous potential of *G. birdiae* as a raw renewable material to produce fine chemicals for biotechnological and industrial applications. Also, the results are a starting point for determining optimal conditions for obtaining fine chemicals considering other aspects such as downstream processes, cost, equipment depreciation, effluent and waste treatment, safety, biorefinery, among others.

4. Conclusion

The macroalgae *G. birdiae*, with potential for cultivation in the mariculture system, presented a biochemical composition with high carbohydrate content and dry biomass that is easily hydrolysable in acid conditions, even in high biomass concentration. Predictive mathematical models and optimized hydrolysis conditions were established for the highest generation of monosaccharides (glucose and galactose), cellobiose, 5-HMF, levulinic and formic acids from *G. birdiae* biomass, at 121 °C, 20 min and sulfuric acid range of 0.1 at 2.5 mol·L^{-1}. The hydrolysates are good sources of commercially important fine chemicals that can be purified, as well as can be solutions rich in fermentable compounds. Therefore, the red seaweed *G. birdiae* is a renewable resource easily hydrolysable for fine chemicals production with high biotechnological, industrial and economic value.

The following are the supplementary data related to this article.

Table 1S. ANOVA for the obtainment of cellobiose, glucose, galactose, 5-HMF, levulinic acid, formic acid, recovered hydrolysate volume and hydrolysis efficiency.

Author contributions statement

José Cirlanio Sousa Albuquerque contributed to the conception and performance of the experiments, data analysis and interpretation, and writing of the manuscript. Márjory Lima Holanda Araújo contributed to the conception of the experiments, data analysis and interpretation and the revising of the manuscript. Maria Valderez Ponte Rocha contributed to the conception of the experiments and of revising the manuscript. Bartolomeu Warlene Silva de Souza contributed to the performance of the experiments. George Meredite Cunha de Castro contributed to the conception and performance of the experiments. Edna Maria Silva Cordeiro contributed to the performance of the experiments. Jouciane de Sousa Silva contributed to the performance of the experiments, analysis of the data. Norma Maria Barros Benevides contributed with funding acquisition, the conception of the experiments and revising the manuscript.

Declaration of competing interest

The authors and funding agencies have no conflicts of interest to declare. No conflicts, informed consent, human or animal rights applicable in this paper.

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4. ARTIGO 2 DA TESE

Fermentation of carbohydrates from a species of red seaweed abundant on the coast of northeastern Brazil

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ABSTRAC

Marine macroalgae are potential sources of raw materials for the production of biofuels. However, the enormous diversity of macroalgae and their chemical constituents requires further studies to make it possible to harness the chemical and biotechnological potential of these organisms. Given the above, the objective of this study was to carry out the acid hydrolysis of the red sea macroalgae *Gracilaria birdiae* to obtain media-rich fermentable carbohydrates for the production of ethanol. Hydrolysis was carried out in different concentrations of biomass (GbBC), sulfuric acid concentration (SAC), and reaction time (RT). Two different culture media, GbH2 and GbH3, obtained from monosaccharide-rich hydrolysates were subsequently fermented by a yeast strain Saccharomyces cerevisiae acclimated to galactose (Sc_{Gal}). Glucose and galactose were consumed in both fermentation media, however, GbH3 showed the best ethanol yield and productivity. The conversion factors ($g.g^{-1}$) in GbH3 were Y_{EtOH} 1,427, $Y_{P/S\ glu}$ 0.843 ± 0.001, $Y_{P/S\ gal}$ 0.664 ± 0.010, $Y_{P/S\ total}$ 0.371 ± 0.003 and the maximum concentration of ethanol obtained was 7.82 $g.L^{-1}$. Therefore, the biomass of *Gracilaria birdiae* was hydrolyzed under acidic conditions, making it possible to obtain culture media-rich in glucose and galactose that were subsequently fermented in ethanol when a yeast strain acclimated to galactose was used.

Keywords: Monosaccharides; *S. cerevisiae*; Acclimatization; Fermentative parameters; Bioethanol.

1. INTRODUCTION

Problems related to the unsustainability of fossil fuels and global climate issues have intensified the search for fuel development from sustainable and less polluting raw materials [1, 2]. In this context, biofuels arose, produced from renewable, sustainable raw materials and with cleaner burning compared to fossil fuels [3]. Bioethanol is the most widely used biofuel on the global market and can partially replace the use of fossil fuels [4,5]. First-generation bioethanol is produced from food raw materials, such as sugar cane, corn, beet, among others [6]. However, the shift from edible crops to commercial bioethanol production has raised serious ethical questions in the face of the growing need for food in the world [7,8]. Second-generation bioethanol is produced from lignocellulosic materials or agro-industrial waste [9]. However, the high degree of recalcitrance caused by the presence of lignin in these biomasses, in addition to the lack of a raw material collection network, constitutes the main limiting factors for industrial-scale production [10]. In this scenario, macroalgae emerged as a potential renewable raw material source for the production of third-generation bioethanol [11]. They have high levels of carbohydrates, high growth rates, and the absence of lignin in their structure, facilitating depolymerization processes [12]. Additionally, the sea as a place for the production of macroalgae does not compete for arable land, dispensing with the use of pesticides and agricultural inputs [13].

The macroalgae of the genus Gracilaria constitutes one of the most important red algae (Rhodophyta), being found in all tropical and subtropical regions of the world [14]. They have high levels of sulfated and neutral polysaccharides such as galactans and cellulose, which are easily hydrolyzable chemically and biochemically [15]. Hydrolysis of galactans and cellulose from red macroalgae generates two main monosaccharides, galactose, and glucose, respectively [16], which are substrates for several fermenting microorganisms [17]. Among the Gracilaria, the species *Gracilaria birdiae* (*G. birdiae*) stands out, abundant throughout the northeast coast of Brazil and with high potential for cultivation on the Brazilian and economic coast [18,19,20]. The microorganisms used for the production of ethanol, the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), is the most exploited species industrially [21,22].

However, fermentative tests carried out with red macroalgae hydrolysates showed that *S. cerevisiae* preferentially consumes glucose, while galactose is consumed secondarily after being converted into glucose-6-phosphate by the Leloir pathway [23,24]. This is because the galactose metabolic genes (GAL) are repressed in the presence of glucose [6].

Studies aimed at building robust microorganisms for the consumption of different monosaccharides present in the medium reported that the mutants showed less efficiency in glucose metabolism and prolonged acclimatization phase [25]. However, a study by Castro et al. 2017, showed that previous acclimatization of *S. cerevisiae* in culture media-rich in galactose reduced the repression of glucose, increasing the consumption of galactose and increasing the productivity of ethanol [26]. Therefore, the present study aimed to evaluate the potential of the red seaweed *G. birdiae* as a source of fermentable carbohydrates for the production of ethanol using a yeast strain acclimated to galactose.

2. MATERIAL AND METHODS

2.1. Algae biomass

Specimens of the *G. birdiae* macroalgae were harvested from cultivation structures installed on the beach of Flecheiras, Trairi-CE, Brazil (03° 13 '06 "S 39° 16' 47" W), maintained by the Flecheiras Algae Producers Association and Guajiru-APAFG. In the laboratory, most algae were carefully washed with running water to remove sand, salt, and fouling organisms. After drying at room temperature (25 °C) and 10% moisture, the algae were crushed using a knife mill (Skymsen®, Brazil). The resulting powder was standardized using granulometric sieves (<0.18 mm) and stored at 25 °C.

2.2. Acid hydrolysis of *G. birdiae*

Sulfuric acid (H_2SO_4) was used to hydrolyze the dry seaweed biomass and release the fermentable monosaccharides. Three hydrolysis conditions varying the concentrations of *G. birdiae* biomass (GbBC, 230 - 280 $g.L^{-1}$), sulfuric acid (SAC, 0.8 - 1.3 M), and reaction time (RT, 10 - 20 min) were established based on the study published by Albuquerque et al. 2021 [27], shown in Table 1.

Table 1. Hydrolysis conditions were used to obtain fermentable monosaccharides from the biomass of *G. birdiae*.

Hydrolysate	Hydrolysis parameters		
	GbBC ($g.L^{-1}$)	SAC (M)	RT (Min)
GbH1	280	1.3	20
GbH2	280	0.8	20
GbH3	230	0.8	10

GbH: *G. birdiae* Hydrolysate; SAC: GbBC: *G. birdiae* Biomass Concentration; Sulfuric Acid Concentration; RT: Reaction Time; (M): Molar; (min): Minutes.

The hydrolysis was carried out in 250 mL Schott flasks containing 100 mL of sulfuric acid solution (Synth, São Paulo, Brazil) in an autoclave (Phoenix Luferco, São Paulo, Brazil) with a temperature set at 121 °C. The average autoclave rise and fall times were 10 and 13 minutes, respectively. After hydrolysis, each reaction system was cooled for approximately 1 h at room temperature (25 °C). Then, the residues of the hydrolyzed biomass were separated from the liquid phase by filtration using a nylon membrane. The pH of the hydrolysates was adjusted to 5.5 using calcium hydroxide $Ca(OH)_2$. The solid precipitate generated after pH adjustment was removed by filtration on nylon fabric, followed by vacuum filtration (Tecnal Pump - TE 058I, Brazil) using sintered plate funnel and filter paper. The hydrolysates of *G. birdiae* were called GbH and the tests were carried out in triplicate.

2.3. Effect of activated carbon on the fermentable carbohydrates and 5-HMF of the hydrolysate of *G. birdiae*

The removal of GbH 5-HMF was carried out according to Hargreaves et al. (2013) [28]. GbH1 was treated with powdered activated carbon at concentrations of 10%, 15%, 20% and 25% (w/v). The tests were conducted in Erlenmeyer flasks (125 mL) containing 25 mL of *G. birdiae* hydrolysate. After adding the respective concentrations of activated carbon, each system was maintained for 1 hour in an orbital shaker (Tecnal TE-420, Brazil) under constant temperature and agitation at 30 °C and 200 rpm, respectively. Then, the activated carbon was removed from the liquid phase using vacuum filtration with filter paper followed by 0.45 µm syringe filters and the furfural-free liquid phase was obtained.

2.4. Activation of the microorganism and preparation of the inoculum

The yeast *S. cerevisiae* acclimatized to galactose (Sc_{Gal}) according to Castro et al. (2017) [26] was used in GbH alcoholic fermentation tests. Sc_{Gal} colonies were activated in solid culture medium YPG agar (1.5% agar, 10.0 $g.L^{-1}$ yeast extract, 10.0 $g.L^{-1}$ peptone, and 40.0 $g.L^{-1}$ galactose) for 48 h in a bacteriological incubator (Nova Instruments, Brazil) at 30 °C. For the inoculum preparation, the fastest-growing Sc_{Gal} colonies were removed from the solid medium and transferred to an Erlenmeyer flask (250 mL) containing 100 mL of the YPG liquid culture medium, pH 5.5. After incubation for 24 h under orbital shaking (Tecnal TE-420, Brazil) at 30 °C and 150 rpm, the inoculants were used in the alcoholic fermentation tests of *G. birdiae* hydrolysates.

2.5. Alcoholic fermentation of *G. birdiae* hydrolysate

The fermentative tests were carried out according to the methodology described by Castro et al. (2017) [26]. In this study, the furfural-free medium (GbH1) was not used due to the high loss of fermentable carbohydrates after treatment with activated carbon powder. Fermentation was carried out in Erlenmeyer flasks (125 mL) containing 50 mL of GbH2 and GbH3, supplemented with 10.0 $g.L^{-1}$ yeast extract and 10.0 $g.L^{-1}$ peptone. A volume of 5.0 mL of the Sc_{Gal} inoculum was transferred to both fermentation media. The resulting cell concentration at time zero (t_0) of the fermentation was 10^7 $CFU.mL^{-1}$. Then, incubated for 72 hours under constant orbital shaking at 150 rpm (Tecnal TE-420, Brazil) at 30 °C. Aliquots of 1 ml of the fermentation media were collected in a laminar flow chamber (Pachane-PA50, Brazil), periodically to analyze the levels of glucose, galactose, cellobiose, formic acid, levulinic acid, 5-HMF, and ethanol.

2.6. Chemical analysis

2.6.1. Content of carbohydrates, organic acids, furanic compounds, and ethanol

The levels of glucose, galactose, cellobiose, formic acid, levulinic acid, 5-HMF, and ethanol present in the hydrolysates and fermentation media were determined by HPLC (Walters, Milford, MA, USA) equipped with a gas detector. refractive index-RID (Waters 2414) and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Filtered and

degassed 5.0 mM sulfuric acid (H_2SO_4) was used as a mobile phase solution at a flow rate of 0.5 mL min^{-1}, 65 °C and a total operating time of 60 min according to Rodrigues et al. (2016) [29]. The compounds were determined and quantified based on the standard curves obtained from standard reagents. Carbohydrate Kit: CAR10 Batch: SLBJ8685V, Organic Acids Kit Batch: LC09525 (Supelco). Batch of Furfural: SHBD3699V, 5-(hydroxymethyl) furfural Batch: BCBM2548V (Sigma-Aldrich).

2.6.2. Fermentative parameters

Ethanol yield (Y_{EtOH}), monosaccharide to ethanol conversion factor ($Y_{P/S}$), and ethanol productivity (P_P) were calculated according to equations 1, 2, and 3, respectively.

$$Y_{EtOH}\ (g\ g^{-1}) = \frac{[EtOH]_{max}}{[Monosaccharides]_{ini}} \quad \text{(Eq. 1)}$$

$$Y_{P/S} = \frac{P_{max}}{S0 - S} \quad \text{(Eq. 2)}$$

$$P_P = \frac{P_{max}}{t_{fP}} \quad \text{(Eq. 3)}$$

Where $[EtOH]_{max}$ is the maximum concentration of ethanol reached during fermentation ($g.L^{-1}$) and $[monosaccharides]_{ini}$ is the initial total concentration of glucose + galactose ($g.L^{-1}$) at the start of fermentation. *Pmax* is the maximum concentration of ethanol produced during fermentation ($g.L^{-1}$), S_0 is the initial concentration of monosaccharides (glucose + galactose) ($g.L^{-1}$), *S* is the final concentration of monosaccharides (glucose + galactose) ($g.L^{-1}$) and *tfP* is the fermentation time (h) in which the maximum ethanol concentration was obtained.

2.6.3. *Sc_{Gal}* growth curve

The counting of viable *Sc_{Gal}* cells in plaque followed the methodology described by Thomas et al. (2015) [30]. Aliquots of 100 µL removed from the fermentation

media were diluted in a 0.9% NaCl solution in a serial form (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) in a 1.5 ml Eppendorf flask. Then, 20 µL aliquots were transferred to Petri dishes containing solid YPG agar culture medium, pH 5.5. The plates were incubated for 48 h at 30 °C in a bacteriological incubator (Nova Instruments, Brazil). Colony-forming units (CFU) were counted with the help of a colony counter (Phoenix Luferco - CP 600 Plus, Brazil). The number of CFU was determined according to equation 4.

$$\text{CFU. mL}^{-1} = \frac{\text{N x F}}{10^{-4}} \qquad \text{(eq. 4)}$$

Where N is the number of colonies read on the plate, F is the dilution factor of the solution, and 10^{-4} is the dilution that made it possible to count the colonies.

2.6.4. Statistical analysis

The hydrolysis and fermentation tests were performed in triplicate and the results were expressed as mean ± standard deviation (SD). The graphs were created with the aid of GraphPad Prism® Software version 5.01 (GraphPad, San Diego, USA).

3. RESULTS

3.1. Acid hydrolysis of *G. birdiae* biomass

The concentrations of monosaccharides, cellobiose, organic acids, and furanic compounds present in the hydrolysates of *G. birdiae* are shown in Table 2.

Table 2. Glucose, galactose, cellobiose, formic acid, acetic acid, and 5-HMF concentrations of *G. birdiae* hydrolysates.

Hydrolysate	Carbohydrates			Organic acids		Furanic compound
	Glucose ($g.L^{-1}$)	Galactose ($g.L^{-1}$)	Cellobiose ($g.L^{-1}$)	Formic ($g.L^{-1}$)	Levulinic ($g.L^{-1}$)	5-HMF ($g.L^{-1}$)
GbH1	6.31 ± 0.34	41.22 ± 1.07	4.24 ± 0.22	12.66 ± 0.58	9.96 ± 0.38	6.14 ± 025
GbH2	10.95 ± 0.41	50.16 ± 1.33	5.57 ± 0.19	11.08 ± 0.45	7.93 ± 0.29	7.86 ± 0.31
GbH3	8.72 ± 0.28	52.31 ± 1.28	6.83 ± 0.28	6.02 ± 0.39	5.62 ± 0.22	4.37 ±0.19

Furfural was not detected in the hydrolysates of *G. birdiae*. The results represent the mean ± standard deviation.

The highest concentrations of glucose (10.95 $g.L^{-1}$) and 5-HMF (7.86 $g.L^{-1}$) were obtained under hydrolysis condition 2 (GbH2), while the highest concentrations of galactose (52.31 $g.L^{-1}$) and cellobiose (6.83 $g.L^{-1}$) were obtained in condition 3 (GbH3). On the other hand, the highest concentrations of formic (12.66 $g.L^{-1}$) and levulinic (9.26 $g.L^{-1}$) acids were obtained in condition 1 of hydrolysis (GbH1). Galactose was the major component in the hydrolysates of *G. birdiae* and the presence of furfural was not detected.

3.2. Effect of activated carbon on the fermentable carbohydrates and 5-HMF of the hydrolysate of *G. birdiae*

The effect of treatment with activated carbon powder on the concentrations of glucose, galactose, and 5-HMF in the hydrolysates of *G. birdiae* is shown in Table 3.

Table 3. Effect of treatment with different concentrations of activated carbon powder on the concentrations of 5-HMF and monosaccharides of the GbH1 hydrolysate of *G. birdiae*.

Compound ($g.L^{-1}$)	Activated carbon concentration (%, w/v)				
	0 %	10 %	15 %	20 %	25 %
Glucose	6.31 ± 0.78	2.60 ± 0.35	0.02 ± 0.006	0.02 ± 0.003	0.02 ± 0.002
Galactose	41.22 ± 1.57	20.67 ± 0.87	0.17 ± 0.034	0.17 ± 0.001	0.12 ± 0.001
5-HMF	6.14 ± 0.36	1.39 ± 0.23	0.02 ± 0.002	0.01 ± 0.001	0.01 ± 0.001

5-HMF: 5-hydroxymethylfurfural; The results represent the mean ± standard deviation.

The treatment of GbH1 with 10% (w/v) of activated carbon reduced the concentration of 5-HMF by 83.78%. However, this same concentration of activated carbon caused losses of 58.80% and 49.86% of the levels of glucose and galactose, respectively. The concentrations of 15% (w/v), 20% (w/v) and 25% (w/v) of activated carbon reduced the concentrations of 5-HMF, glucose and galactose to values close to zero.

3.3. Fermentation of *G. birdiae* hydrolysate

The kinetics of ethanol production using the yeast Sc_{Gal} from the hydrolysates of *G. birdiae* is shown in Fig. 1.

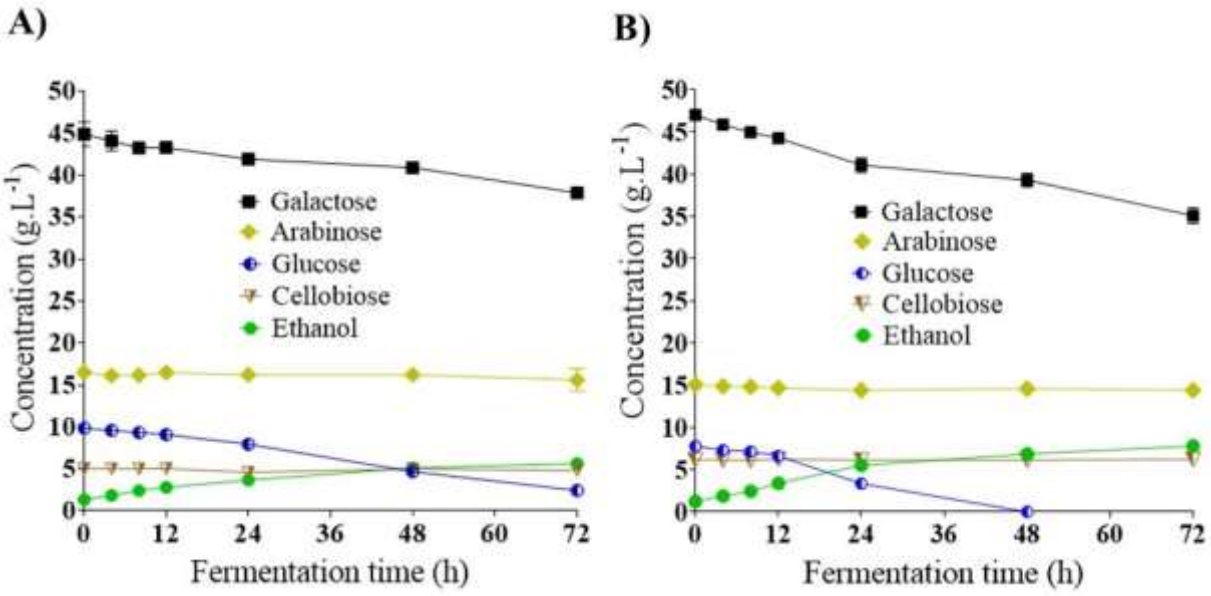

Fig 1. Ethanol production from *G. birdiae* hydrolysates by *S. cerevisiae* acclimated to galactose (*Sc*$_{Gal}$). a) Fermentation of GbH2 b) Fermentation of GbH3.

The yeast *Sc*$_{Gal}$ consumed both glucose and galactose as a carbon source, however, the consumption profile of these sugars, as well as the ethanol productivity were different in both culture media evaluated (Fig. 1 a, b).

In GbH2, the highest glucose consumption occurred from the time 24 h after the start of fermentation, while that of galactose occurred from the time 48 h, but was not fully consumed during 72 h of fermentation. The glucose concentration went from 9.86 g.L^{-1} to 2.36 g.L^{-1}, representing consumption of 76.06% (Fig 1a). On the other hand, the galactose concentration was reduced from 45.15 g.L^{-1} to 37.88 g.L^{-1}, representing the consumption of only 16.10% of the total galactose concentration (Fig 1a). Cellobiose concentrations remained constant in both fermentation media during the 72 h of fermentation (Fig 1 a, b). In GbH2, the maximum concentration of ethanol obtained was 5.65 g.L^{-1} (Fig. 1a).

In GbH3, the consumption profile of both glucose and galactose by *Sc*$_{Gal}$ was slightly increased (Fig. 1b). The consumption of both glucose and galactose started in the first hours of the fermentation process. All glucose (7.85 g.L^{-1}) present in the fermentation medium was consumed 48 h after fermentation, with the highest glucose consumption occurring between 12 and 24 h after the start of fermentation (Fig. 1b). On the other hand, galactose was partially consumed, with its initial concentration 47.08 g.L^{-1} reduced to 34.09 g.L^{-1} representing the consumption of 27.59% of galactose at the end of 72 h of fermentation

(Fig. 1b). In GbH3, the maximum concentration of ethanol obtained was 7.82 $g.L^{-1}$, 27.75% higher than in GbH2.

3.4. Growth of *Sc$_{Gal}$* in the hydrolysate of *G. birdiae*

The cell growth of the yeast *Sc$_{Gal}$* during fermentation in GbH2 and GbH3 is shown in Fig. 2.

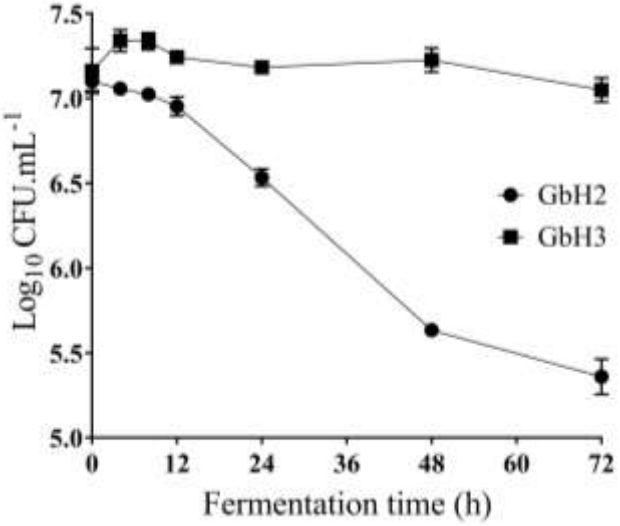

Fig 2. *Sc$_{Gal}$* growth curve during the fermentation of *G. birdiae* hydrolysate.

The growth profile of the *Sc$_{Gal}$* yeast was different in both fermentation media. In GbH2, the concentration of cells (CFU) began to decline from the beginning of the fermentation process, with the most pronounced decline being observed between 12 and 48 h after the start of fermentation. In GbH3, the concentration of *Sc$_{Gal}$* showed a moderate increase at the beginning of the fermentation, remaining constant between 12 and 48 h, however, a slight decline was observed after 48 h of fermentation.

3.5. Fermentative parameters

The productivity of ethanol in GbH2 and GbH3 is shown in Fig. 3.

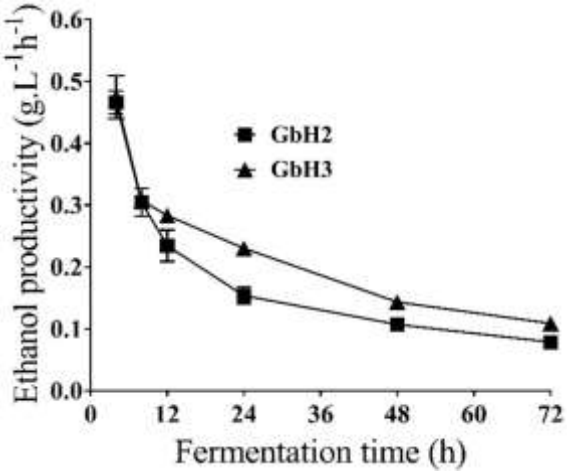

Fig 3. Productivity of ethanol ($g.L^{-1}h^{-1}$) by *Sc*$_{Gal}$ in GbH2 and GbH3 during 72 h of fermentation.

Ethanol productivity in both GbH2 and GbH3 was maximum in the initial 4 h of the fermentation process (Fig 3). During this period, ethanol productivity in both fermentation media was similar, with GbH2 productivity being 0.46 $g.L^{-1}h^{-1}$, while in GbH3 it was 0.47 $g.L^{-1}h^{-1}$. After 4 h of fermentation, ethanol productivity decreased in both fermentation media, however, in GbH3, productivity was higher at all times evaluated. The ethanol yield from glucose ($Y_{P/S\ glu}$), galactose ($Y_{P/S\ gal}$), total yield ($Y_{P/S\ gal}$), and the ethanol yield (Y_{EtOH}) in both fermentation media are shown in Table 4.

Table 4. Fermentation parameters of ethanol production from hydrolysates GbH2 and GbH3 from sea macroalgae *G. birdiae*.

Hydrolysates	$Y_{P/S\ glu}$ ($g.g^{-1}$)	$Y_{P/S\ gal}$ ($g.g^{-1}$)	$Y_{P/S\ total}$ ($g.g^{-1}$)	Y_{EtOH} ($g.g^{-1}$)
GbH2	0.580 ± 0.100	0.647 ± 0.210	0.303 ± 0.070	0.103 ± 0.013
GbH3	0.843 ± 0.001	0.664 ± 0.010	0.371 ± 0.003	0.143 ± 0.002

4. DISCUSSION

The hydrolysis of macroalgae of the genus Gracilaria for the generation of fermentable monosaccharides presented in this study was superior to those shown in the literature on the hydrolysis of some species of red algae. The acidic and enzymatic hydrolysis of the macroalgae *G. verrucosa* generated the maximum concentration of 39.6 $g.L^{-1}$ of total

monosaccharides at 8% (w/v) of biomass, 270 mM H_2SO_4, 121 °C and 60 min [25]. Castro et al. (2017) realizaram a hidrólise ácida da macroalga *Solieria filiformis* e obtiveram 18,10 $g.L^{-1}$ de monossacarídeos [26].

The acid hydrolysis of *G. gigas* generated concentrations of 18.54 $g.L^{-1}$ and 1.45 $g.L^{-1}$ of galactose and glucose, respectively, 10% (w/v) of algae biomass, H_2SO_4 0.2 M, 120 °C and 15 min [31]. The highest concentrations of fermentable monosaccharides obtained in the present study are mainly associated with high levels of carbohydrates (73.10%) in *G. birdiae* and the selection of hydrolysis conditions within an optimal range of hydrolysis, capable of degrading the polysaccharides without causing the conversion of monosaccharides into organic acids and furanic compounds.

The results presented showed that the milder hydrolysis condition (GbBC 230 $g.L^{-1}$; SAC 0.8 M and RT 10 min) contributed to the increase in glucose, galactose and cellobiose levels. In contrast, the most severe hydrolysis condition (GbBC 280 $g.L^{-1}$; SAC 1.3 M and RT 20 min) increased the levels of formic and levulinic acids. These results corroborate with several published studies on the hydrolysis of algae biomass, showing that the productivity of monosaccharides and the generation of fermentation inhibitors are directly affected by parameters such as the type and concentration of the catalyst, concentration of the substrate, temperature, and reaction time [32,33].

Generally, the more severe the hydrolysis conditions are, the lower concentrations of fermentable sugars and the higher concentrations of fermentation inhibitors are produced [32]. This is because acid hydrolysis with longer reaction times and higher acid concentrations degrades fermentable sugars into formic, levulinic acids, and furanic compounds such as 5-HMF and furfural [17]. In drastic conditions of hydrolysis (high GbBC, SAC, and RT) the dehydration of hexoses generates 5-HMF [34,35]. On the other hand, dehydration of pentose generates furfural [36]. 5-HMF and furfural are known to inhibit the action of the enzymes alcohol dehydrogenase, pyruvate dehydrogenase, and aldehyde dehydrogenase in microorganisms [36]. However, the presence of furfural was not detected in the *G. birdiae* hydrolysates since it presents galactans and cellulose as structural polysaccharides and starch as a reserve polysaccharide.

To promote the fermentation of vegetable hydrolysates, activated carbon powder has been used to remove 5-HMF and this treatment has also been applied to algae hydrolysates [17]. However, the treatment of *G. birdiae* hydrolysate with activated carbon powder carried out in the present study, proved to be an unviable method, since it caused the

levels of glucose and galactose monosaccharides. A similar result of the adsorption of fermentable carbohydrates in algae hydrolysates after treatments with activated carbon has already been reported in the literature. Meinita et al. (2012) treated the hydrolysate of the macroalga *K. alvarezii* with different concentrations of activated carbon and reported losses in the galactose concentration above 40% when 5% of activated carbon was added to the hydrolysate [34]. In another study, reduced concentrations of glucose and galactose were observed when the *G. amansii* hydrolysate was treated with concentrations greater than 5% (w/v) of activated carbon [17].

Therefore, monosaccharide consumption and ethanol productivity were higher in GbH3. These differences are mainly associated with the high concentrations of fermentation inhibitors in GbH2. Ra et al. (2013) reported slow uptake of glucose and galactose in the presence of concentrations of approximately 5 $g.L^{-1}$ of 5-HMF in red algae hydrolysates [35]. Among inhibitors, formic acid is a weaker inhibitor, however, concentrations greater than 10 $g.L^{-1}$ cause antagonistic effects on yeast growth [37,17]. Thus, the low consumption of glucose, galactose, and the low productivity of ethanol observed in GbH2 are also associated with Sc_{Gal} cell death, which started in the first hours of the fermentation process. The main causes of cell death are possibly associated with the high levels of fermentation inhibitors mentioned above. In anaerobiosis, the primary route of energy production is glycolysis, where 2 moles of ATPs are produced per mole of glucose together with a reduction of 2 moles of NAD^+ in the final balance until pyruvate generation. $NADH^+$ H^+ is re-oxidized in the final reaction of ethanol production and this coenzyme can be used again in glycolysis. Modig, Liden, and Taherzadeh (2002) studied the kinetics of furfural inhibition of the enzymes alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AlDH), and the pyruvate dehydrogenase complex (PDH) in vitro showing that the activities of PDH and AlDH are inhibited in more 90% at a concentration close to 2 mM, while ADH activity decreased by less than 20% at the same concentration. Furfural inhibition of ADH and AlDH activities could be well described by a competitive inhibition model, whereas PDH inhibition was better described as non-competitive. The inhibition of ADH was very similar to that caused by furfural by 5-hydroxymethylfurfural (5-HMF). However, 5-HMF did not inhibit both AlDH or PDH as severely as furfural, thus being able to relate the effect of these inhibitors on the general metabolism of S. cerevisiae, suggesting a critical role of these enzymes in the observed inhibition [38].

In the literature, researchers have reported the application of red macroalgae biomass for ethanol production. Wu et al. (2014) evaluated the fermentation process of carbohydrates from the red macroalgae Gracilaria sp., For 48 hours and the maximum concentration of ethanol generated was 4.72 $g.L^{-1}$ [32]. Castro et al. (2017) fermented the hydrolysate of the red macroalgae *S. filiformis* using a yeast strain acclimated to galactose and the maximum concentration generated during 30 h of fermentation was 4.9 $g.L^{-1}$ [26]. Meinita et al. (2018) fermented the carbohydrates of the red macroalgae *G. gigas* and obtained the maximum concentration of 3.56 $g.L^{-1}$ of ethanol [31]. In the present study, the concentration of ethanol (7.82 $g.L^{-1}$) obtained from the *G. birdiae* hydrolysate was higher than that found in the aforementioned studies, demonstrating that the *G. birdiae* macroalgae have potential as a promising raw material for ethanol production. In addition, it is important to highlight here the amount of galactose remaining at the end of the fermentation and which could increase the ethanol yield obtained from the hydrolysates of *G. birdiae*.

5. Conclusion

The results obtained in the present study showed that the biomass of the G. birdiae macroalgae presented a polysaccharide composition susceptible to acid hydrolysis, generating mainly galactose and glucose, which were converted into ethanol by the yeast *S. cerevisiae* acclimated to galactose. However, further studies should be conducted to promote the consumption of galactose in the environment by the microorganism to increase the yield of ethanol produced from the *G. birdiae* hydrolysate.

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5. CONSIDERAÇÕES FINAIS

A composição bioquímica da biomassa da macroalga marinha *G. birdiae* seca apresentou 73,01% de carboidratos que foram facilmente hidrolisados em condições brandas de hidrólise ácida. A aplicação da metodologia DCCR aliado à MSR possibilitou a obtenção de modelos matemáticos preditivos e o estabelecimento de condições ótimas de hidrólise para a obtenção de glicose, galactose, celobiose, 5-HMF, ácido fórmico e ácido acético. Os resultados obtidos mostraram que cada variável independente teve influência distinta e significativa sobre cada produto reacional. A obtenção de açucares foi mais afetada pela concentração de biomassa algácea, enquanto, as concentrações de ácido sulfúrico tiveram mais efeitos sobre o 5-HMF e ácidos orgânicos. As condições ótimas de hidrólise geraram as concentrações 28,56 $g.L^{-1}$, 108,03 $g.L^{-1}$, 25,39 $g.L^{-1}$, 19,82 $g.L^{-1}$, 26,75 $g.L^{-1}$, 38.88 $g.L^{-1}$ de glicose, galactose, celobiose, 5-HMF, ácido fórmico e ácido acético, respectivamente. Os resultados obtidos nos ensaios fermentativos mostraram que os carboidratos (glicose, galactose) presentes nos hidrolisados de *G. birdiae* foram utilizados como substratos por uma cepa de levedura aclimatada à galactose culminando na produção máxima de 7,82 $g.L^{-1}$ de etanol. Portanto, os resultados alcançados no presente estudo mostraram que os hidrolisados de *G. birdiae* obtidos a partir de condições otimizadas são considerados boas fontes de diversos compostos químicos de importância economicamente que podem ser purificados, assim como soluções ricas em carboidratos fermentescíveis que podem ser destinadas à produção de uma variedade de compostos por via química ou biotecnológica, inclusive etanol.

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