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**PRODUÇÃO DE BEBIDA TIPO KOMBUCHA E CELULOSE BACTERIANA UTILI-
ZANDO SUBPRODUTO DA ACEROLA COMO MATÉRIA-PRIMA**

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2020

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PRODUÇÃO DE BEBIDA TIPO KOMBUCHA E CELULOSE BACTERIANA UTILIZANDO SUBPRODUTO DA ACEROLA COMO MATÉRIA-PRIMA

Dissertação submetida ao Programa de Engenharia de Alimentos da Universidade Federal de Santa Catarina para a obtenção do título de mestre em Engenharia de Alimentos.

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O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de mestre em Engenharia de Alimentos.

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Florianópolis, 2020

Dedico...

... Aos meus avós, dona Lourdes e Inácio e as minhas irmãs e sobrinha, Jaine, Laura e Maria Helena.

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RESUMO

Kombucha é uma bebida adocicada à base de chá fermentado. Normalmente, o chá preto ou chá verde (*Camellia sinensis*) é utilizado como base para a produção de kombucha, e uma cultura/associação simbiótica de bactérias e leveduras (SCOBY) é responsável pelo processo de fermentação. Além da bebida, ocorre a formação de celulose bacteriana durante a fermentação, a qual tem sido pouco estudada em termos de reutilização. Neste trabalho, o subproduto da acerola (1, 3 e 5% m/v) foi utilizado como nova matéria-prima para a produção de bebida tipo kombucha fermentada durante 15 dias a 30 °C. A identificação dos microrganismos presentes no SCOBY apresentou bactérias ácido acéticas (*Komagataeibacter rhaeticus*, *K. xylinus* e *K. hansenii*) e leveduras (*Brettanomyces bruxellensis* e *Zygosaccharomyces bisporus*). As maiores produções de ácido acético (16,3 g/L) e celulose bacteriana (4,0 g/L) foram alcançadas com 5% de subproduto. A concentração final de etanol foi semelhante em todas as amostras, aproximadamente 9,5 g/L, mas foi alcançada em 12, 9 e 6 dias, respectivamente, sugerindo que o metabolismo da levedura foi acelerado na presença de maiores quantidades de subproduto de acerola. A celulose bacteriana (BC) apresentou membranas pouco porosas e mais densas, com estrutura altamente cristalina (96,4%), também nas amostras contendo maior quantidade de subproduto de acerola. A partir do resultado obtido da produção de celulose, foi realizado o isolamento do principal microrganismo produtor de celulose bacteriana da kombucha e novamente fermentado com meio contendo 5% do subproduto de acerola (AC). Este foi suplementado com 20 g/L de glicose e comparado com o meio tradicional Hestrin-Schram (HS). Após 12 dias de fermentação (30 °C), a produção de celulose foi igual a 2.91 g/L e 2.26 g/L para os meios HS e AC, respectivamente. Ambas as celuloses produzidas apresentaram similaridades quanto às propriedades físico-químicas e morfológicas, entretanto, a amostra fermentada pelo meio AC apresentou maior cristalinidade (90.8%). Com isso, o subproduto de acerola apresentou potencial tanto para produção de bebida tipo kombucha quanto para a produção de celulose, sendo ainda necessária a otimização das condições de fermentação para atingir maiores concentrações de celulose bacteriana.

Palavras-chave: Fermentação, ácido acético, etanol, polifenóis, atividade antioxidante.

ABSTRACT

Kombucha is a sweet fermented tea-based drink. Usually, black tea or green tea (*Camellia sinensis*) is used as the base of kombucha production, and a symbiotic culture/association of bacteria and yeasts (SCOBY) is responsible for fermentation process. SCOBY (bacterial cellulose formed during fermentation) has been poorly studied in terms of reuse. In this work, acerola byproduct (1, 3 and 5% w/v) was used as a new raw material for the production of kombucha-like and fermentation lasted for 15 days at 30 °C. The SCOBY presented acetic acid bacteria (*Komagataeibacter rhaeticus*, *xylinus* and *hansenii*) and some yeasts (*Brettanomyces bruxellensis*, and *Zygosaccharomyces bisporus*). The highest productions of acetic acid (16.3 g/L) and bacterial cellulose (4.0 g/L) were achieved with 5% byproduct. The final concentration of ethanol was similar in all samples, approximately 9.5 g/L, but it was achieved at 12, 9 and 6 days, respectively, suggesting that yeast metabolism was accelerated in the presence of higher amounts of acerola byproduct. Bacterial cellulose (BC) presented less porous and denser membranes were obtained with a highly crystalline structure (96.4%), also in higher amounts of acerola byproduct. From the result obtained from the production of cellulose, the main microorganism that produced bacterial cellulose from kombucha was isolated and again fermented with medium containing 5% of the acerola byproduct (AC), as a source of nitrogen. It was supplemented with 20 g/L of glucose and compared with traditional Hestrin-Schram (HS) medium. After 12 days of fermentation (30 °C), cellulose production was 2.91 g/L and 2.26 g/L for HS and AC media, respectively. Both samples were similar in terms of cellulose characterization, however, the sample fermented by the AC medium showed greater crystallinity (90.8%). As a result, the by-product of acerola showed potential both for the production of kombucha-type drinks and for the production of cellulose, which still requires optimization of the process in order to acquire greater yield,

Keywords: Fermentation. Acetic Acid. Ethanol. Polyphenols. Antioxidant activity.

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LISTA DE ABREVIATURAS E SIGLAS

SCOBY - Cultura simbiótica de bactérias e leveduras

MAPA - Ministério da Agricultura, Pecuária e Abastecimento

CAGR - Taxa de crescimento anual composta

LDL-C - Lipoproteína de baixa densidade – colesterol

DSL - Ácido D-sacárico-1,4-lactona

UDPGlc - Difosfoglucoose de uridina

DPPH – 2,2-diphenyl-1-picrylhydrazyl

CFT – Compostos fenólicos totais

HPLC - High-Performance Liquid Chromatography

IR - refractive index

GAE – Gallic acid

TPC – Total phenolic content

AA – Ascorbic Acid

FTIR - Fourier Transform Infrared Spectroscopic

XDR - X-ray Diffraction

ANOVA – Analysis of variance

AC1, AC3 e AC5 – Bebida tipo kombucha com extrato do subproduto da acerola nas concentrações de 1, 3 e 5 % (m/v), respectivamente

CFU – Colony forming unity

BC – Bacterial cellulose

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CAPÍTULO 1

1 INTRODUÇÃO

A kombucha atualmente é popular em todo o mundo, sendo uma bebida fermentada que têm origem na Ásia há mais de 2 milênios de anos. (JAYABALAN et al., 2007). A cultura simbiótica de bactérias e leveduras (SCOBY) é responsável pelo processo de fermentação, sendo o chá verde ou preto (*Camellia sinensis*) utilizado como extrato para fermentação da kombucha, resultando em uma bebida refrescante, agridoce e levemente carbonatada. As características da kombucha podem variar de acordo com vários fatores, como o tipo de chá ou a matéria-prima, os microrganismos presentes no SCOBY e o tempo de fermentação e temperatura (CHAKRAVORTY et al., 2016; JAYABALAN, et al., 2014).

Uma ampla gama de metabólitos, como compostos bioativos interessantes, vem de interações bem-sucedidas entre bactérias e espécies de leveduras, incluindo aquelas resultantes da produção de bebidas fermentadas (VILLAREAL-SOTO et al., 2020). No caso da kombucha, vários são os metabólitos presentes na bebida, dentre eles podemos citar: alguns minerais como manganês, ferro, cobre e zinco, além de algumas vitaminas solúveis em água (B1, B2, B6, B12 e C). Além disso, a bebida é composta por açúcares que levam à formação de ácidos orgânicos e também auxiliam na biossíntese da celulose. Etanol, dióxido de carbono, ácidos acético, láctico, glucurônico e cítrico também são produzidos por meio da fermentação. A bebida também tem um grande número de polifenóis, como catequinas e epicatequina (BAUER-PETROVSKA & PETRUSHEVSKA-TOZI, 2000; JAYABALAN, et al., 2007; MALBASA et al., 2011; NEFFE-SKOCIŃSKA et al., 2017; GAGGIA et al., 2019).

Por ser uma bebida carbonatada, a kombucha tende a ser um possível substituto aos refrigerantes e de acordo com o Expert Market Research (2020), tende a crescer cerca de 20% nos próximos 5 anos. No Brasil não tem sido diferente, várias empresas surgiram nos últimos anos com uma grande quantidade de sabores para a kombucha. Portanto, além do chá verde ou preto, atualmente têm-se estudado outras matérias-primas para a produção de kombucha, a fim de aumentar as variedades e também entender os metabólitos formados durante a fermentação.

De acordo com Emiljanowicz and Malinowska-Pańczyk et al. (2019), ainda há uma vasta quantidade de matérias-primas que não foram testadas para a produção de kombucha, como por exemplo o bagaço de fruta resultante do processamento dos frutos (10-35% do total).

Estes subprodutos, geralmente possuem compostos valiosos para a alimentação humana, como vitaminas, minerais, polifenóis e fibras.

Uma alternativa a ser considerada é a utilização do subproduto proveniente do processamento de acerola, uma vez que este possui uma vasta quantidade de compostos bioativos (Marques et al., 2016, 2018; Rezende et al., 2017) e também pode impulsionar a resposta imunológica humana, por exemplo, contribuindo para as propriedades promotoras da saúde já associadas a kombucha tradicional. Portanto, a primeira etapa do trabalho é a utilização do subproduto de acerola para a produção de uma bebida tipo kombucha, a fim de se obter uma bebida com características promotoras de saúde tanto quanto à bebida tradicional.

Além da bebida, a celulose (produzida por bactérias ácido acéticas) é outro produto importante da fermentação da kombucha. É composta por fibrilas de celulose pura, possui alta cristalinidade e termoestabilidade além de ter grande capacidade de retenção de água (DIMA et al., 2017; EMILJANOWICZ & MALINOWSKA-PANCZYK, 2019). O rendimento da celulose pode variar de acordo com as condições de fermentação e as espécies de bactérias produtoras presentes na SCOBY (GOH et al., 2012). Ainda na primeira parte do trabalho, a celulose bacteriana foi avaliada quanto ao seu rendimento e suas propriedades morfológicas e físico-químicas.

Os resultados obtidos para a produção e caracterização da celulose bacteriana foram interessantes na produção de kombucha, uma vez que a concentração de compostos bioativos pode ter acelerado o metabolismo dos microrganismos. Portanto, na segunda etapa do trabalho, optou-se por utilizar esse meio novamente para a produção da celulose e para isso primeiramente foi realizado o isolamento da cepa produtora. Após o isolamento, o extrato obtido do resíduo de acerola foi utilizado como meio para a produção da celulose.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Avaliar a cinética de fermentação de bebida tipo kombucha e produção de celulose bacteriana utilizando subproduto de acerola.

1.1.2 Objetivos Específicos

Como citado anteriormente, o trabalho foi dividido em duas etapas, portanto os objetivos específicos são destacados para cada etapa do trabalho.

Na etapa 1 tem-se os seguintes objetivos específicos:

- avaliar o efeito das concentrações do subproduto de acerola através da avaliação cinética da fermentação da kombucha;
- determinar a concentração de compostos bioativos e atividade antioxidante durante a fermentação;
- caracterizar a celulose bacteriana proveniente da fermentação.

Na etapa 2 destacam-se os seguintes objetivos específicos:

- isolar a bactéria produtora de celulose do consórcio de microrganismos da kombucha;
- avaliar a cinética de produção da celulose bacteriana com a cepa isolada em meio tradicional (HS) e meio com subproduto de acerola através do pH e rendimento da celulose;
- caracterizar a celulose bacteriana proveniente da fermentação.

CAPÍTULO 2

2 REVISÃO BIBLIOGRÁFICA

2.1 DEFINIÇÃO DA BEBIDA FERMENTADA

A kombucha é de origem asiática, sendo uma bebida doce fermentada à base de chá. Geralmente, utiliza-se para a produção da kombucha chá preto ou chá verde como base. É adicionado ao chá açucarado uma associação simbiótica de bactérias e leveduras, geralmente acomodadas numa matriz de celulose sintetizada por bactérias acéticas, sendo estas responsáveis pelo processo fermentativo (GREENWALT; STEINKRAUS; LEDFORD, 2000; SANTOS, 2016; DUTTA; PAUL, 2019).

De acordo com a Instrução Normativa nº 41, de 17 de setembro de 2019 do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), a kombucha é uma bebida fermentada, sendo obtida por respiração aeróbia e fermentação anaeróbia do mosto obtido pela infusão ou extrato de *Camellia sinensis* e açúcares por cultura simbiótica de bactérias e leveduras microbiologicamente ativas (BRASIL, 2019).

Ainda de acordo com a mesma Instrução Normativa, os ingredientes obrigatórios para elaboração da kombucha são: água potável, infusão ou extrato de *Camellia sinensis*, açúcares e cultura simbiótica de bactérias e leveduras (SCOPY). Como ingredientes opcionais, têm-se a infusão de espécies vegetais em água, ou seus extratos, frutas, vegetais, melado ou açúcares de origem vegetal, entre outros (BRASIL, 2019).

O resultado da fermentação é uma bebida refrescante, agridoce e ligeiramente carbonatada. Geralmente utiliza-se o tempo de fermentação de 7 a 15 dias, pois se este prolongar-se, tende a desenvolver um sabor ácido intenso (similar ao vinagre). As características da kombucha variam muito, pois dependem de diversos fatores, tais como o tipo de chá ou extrato utilizado como base, os microrganismos presentes no SCOPY e ainda o tempo de fermentação (DUTTA; GACHHUI, 2007; SANTOS, 2016).

No ano de 2019, de acordo com Expert Market Research (2020), o mercado da kombucha atingiu a marca de US\$ 1,85 bilhão, crescendo rapidamente a uma taxa de crescimento anual composta (CAGR) de 23% no período de 2015-2019. Ainda de acordo com a empresa, este mercado possivelmente atingirá a marca de US\$ 5,6 bilhões em 2025, com uma taxa (CAGR) de 20,6% prevista para o período de 2020-2025.

2.2 BENEFÍCIOS A SAÚDE

A kombucha é composta por alguns minerais como manganês, ferro, níquel, cobre e zinco e ainda por vitaminas hidrossolúveis (B₁, B₂, B₆, B₁₂ e C), sendo estes componentes provenientes do chá. Além disso, devido a vasta quantidade de microrganismos, há a formação de vários componentes durante a fermentação da kombucha. Os açúcares adicionados como fonte de carbono levam a formação de ácidos orgânicos como ácido acético, láctico, glucorônico e glucônico, além da produção de etanol e dióxido de carbono. A kombucha também é caracterizada pela formação de uma película na superfície da bebida identificada como celulose bacteriana. A bebida também possui grande quantidade de polifenóis, tais como as catequinas e epicatequinas derivadas do chá utilizado na preparação da bebida (BAUER-PETROVSKA; PETRUSHEVSKA-TOZI, 2000; CHEN; LIU, 2000; JAYABALAN; MARIMUTHU; SWAMINATHAN, 2007).

O consumo da kombucha vem sendo associado a inúmeros benefícios à saúde, principalmente devido a propriedades terapêuticas e promotoras de saúde, como: anti-inflamatório, anticâncer, anti-hipertensivo, antidiabético, hepatoprotetor e antimicrobiano (DUTTA; PAUL, 2019). Isso fez com que a bebida gerasse interesse popular, entretanto na época, esses efeitos não haviam sido comprovados cientificamente (GREENWALT; STEINKRAUS; LEDFORD, 2000). A composição da kombucha assim como a sua funcionalidade ganhou muito recentemente sua devida popularidade científica e comercial (DUTTA; PAUL, 2019).

Embora estudos com humanos ainda não tenham sido realizados, há relatos de pesquisas utilizando animais que comprovam os efeitos benéficos da kombucha. Como observado no estudo de Aloulou et al. (2012), no qual induziram toxicidade nas células pancreáticas de ratos pela dieta com Aloxan. A inibição da atividade da alfa-amilase pancreática foi observada, sendo esta uma das abordagens terapêuticas comumente usadas para o controle e prevenção da hiperglicemia pós-prandial em pacientes diabéticos não dependentes de insulina, reduzindo a captação de glicose liberada. Com isso, a bebida pode ser considerada uma alternativa para aplicação futura como um complemento funcional para o tratamento e prevenção do diabetes.

Yang et al. (2009) avaliaram efeitos hipolipidêmicos em ratos. O estudo demonstrou que a bebida diminui o colesterol total sérico e o LDL-C (lipoproteína de baixa densidade -

colesterol), melhorando significativamente o status antioxidante no soro e reduzindo o número de gotículas de gordura nos ratos.

Estudos realizados com ratos por Kabiri, Setorki e Darabi (2013) induziram estes a ter dano hepático pela tioacetamida (toxina relacionada à fibrose hepática). Os resultados demonstraram que a bebida diminuiu os danos hepáticos e isso se deve à ação protetora dos componentes polifenólicos, que protegeram o fígado contra a formação de radicais livres. Wang et al. (2014) também relatou efeitos hepatoprotetores, atribuídos à presença de DSL (ácido D-sacárico-1,4-lactona) produzidos por *Gluconacetobacter* sp. A4, em ratos submetidos a dietas com kombucha, evidenciando a possível prevenção da bebida contra doenças hepáticas.

Deghrigue et al. (2013) avaliaram as propriedades antiproliferativas da kombucha, preparadas com chá preto ou verde, em duas linhagens celulares de câncer humano (A549, carcinoma de células pulmonares e Hep-2, célula epidermóide). O chá verde teve um efeito citotóxico maior, inibindo 50% em concentrações de 200 a 250 µg/mL nas linhagens celulares A549 e Hep-2, respectivamente. Em contrapartida, o chá preto mostrou uma atividade citotóxica moderada, sendo necessário 386 µg/mL para inibir 50% do crescimento celular, tendo efeito somente nas células Hep-2.

Embora estes trabalhos demonstrem resultados promissores quanto aos efeitos promotores de saúde da kombucha, ainda faltam informações quanto aos efeitos causados em humanos. Segundo Morales (2020), ensaios *in vivo* estão sendo realizados e tendem a ser finalizados em 2021. Ainda de acordo com o autor, são necessárias análises de bioacessibilidade e biodisponibilidade. Assim como modulação da microbiota, além disso são necessários isolamento dos compostos bioativos e também dos microrganismos, a fim de validar os efeitos biológicos descritos pelas técnicas *in vitro*.

2.3 PREPARAÇÃO DA KOMBUCHA

Atualmente, a kombucha ainda é produzida em pequena escala, principalmente por meio de processos de fermentação natural. À medida que a demanda do consumidor por esta bebida vem aumentando, mais a indústria precisa de informações confiáveis e detalhadas sobre o processo de fermentação da kombucha (LAUREYS; BRITTON; DE CLIPPELEER, 2020). Coton et al. (2017), a fim de atender essa demanda, elaboraram kombucha de chá verde e preto em escala industrial (1000 L) e analisaram a composição química e microbiológica da bebida durante 10 dias de fermentação.

O processo de elaboração da kombucha ocorre inicialmente pela infusão do chá (geralmente 0,5% m/v) durante 5-10 min a 100 °C. A seguir é adicionado o substrato (açúcar) sendo o mesmo dissolvido na bebida ainda quente. Após chegar a temperatura ambiente é então adicionado o SCOBY (4% m/v) e uma parte da kombucha preparada previamente (10% v/v), sendo estes considerados como inóculo. Então, a bebida é fermentada por cerca de 7-15 dias em temperaturas de 25-30 °C. Após, a película formada na superfície é retirada, a porção líquida é filtrada e a bebida está pronta para o consumo (WATAWANA et al., 2015). É possível também realizar uma segunda fermentação, na qual geralmente adiciona-se 50% da kombucha anteriormente fermentada (7-15 dias) e 50% de suco de fruta, em recipiente fechado (para produção de gás) e fermentado por 2-4 dias. Na Tabela 2.1 estão expostos os chás utilizados e os parâmetros de tempo e temperatura comumente encontrados na literatura.

Tabela 2.1. Condições de tempo e temperatura usadas para a fermentação da kombucha preparada com *Camelia sinensis* (chá verde e chá preto).

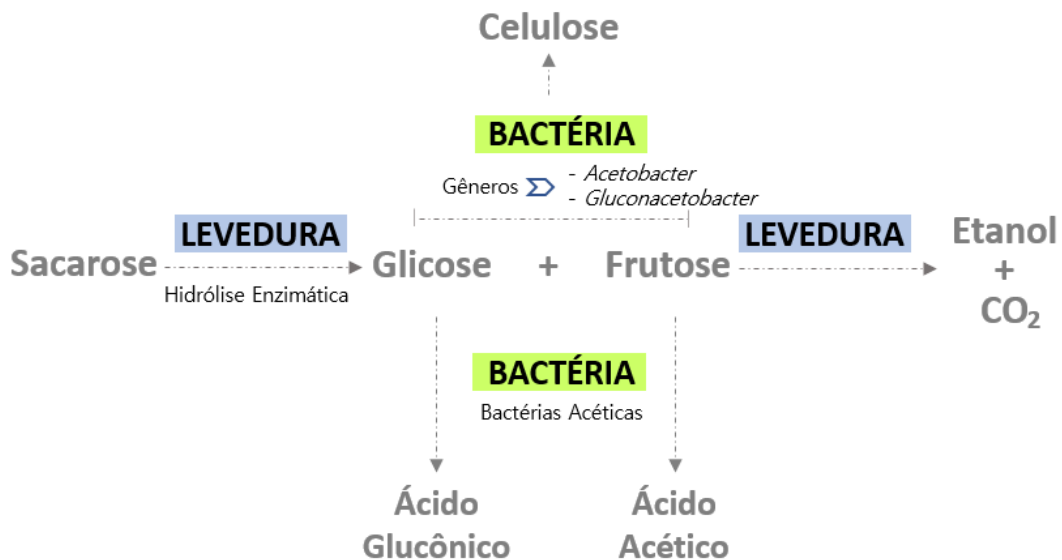
Extrato	Tempo	Temperatura	Referência
Chá preto	21 dias	28 °C ± 2 °C	Bhattacharya et al. (2016)
	14 dias	28 °C ± 2 °C	Cvetković et al. (2008)
	8 dias	30 °C ± 3 °C	Goh et al. (2012)
	10 dias	22 e 30 °C	Loncar et al. (2014)
	21 dias	Ambiente	Miranda et al. (2016)
	14 dias	24 °C ± 3 °C	Muhialdin et al. (2019)
	15 dias	30 °C	Chu & Chen (2006)
	21 dias	25 °C	Villarreal-Soto et al. (2019)
Chá verde e Chá preto	14 dias	27 °C ± 1 °C	Gaggia et al. (2019)
	15 dias	Ambiente	Kaewkod et al. (2019)
	10 dias	25 °C	Cardoso et al. (2020)
	18 dias	24 °C ± 3 °C	Jayabalan et al. (2007)
	60 dias	Ambiente	Vohra et al. (2019)

2.4 COMPOSIÇÃO DA KOMBUCHA

De acordo com Jayabalan et al. (2014), são vários os fatores que influenciam na composição microbiana da kombucha, geralmente a composição microbiana da cultura do chá de kombucha varia de uma cultura para outra e depende da localização geográfica, do clima, as espécies locais de bactérias e leveduras e a origem do inóculo. Já a composição química (polifenóis, vitaminas, entre outros) e os metabólitos como ácidos orgânicos, etanol, gás carbônico formados na kombucha são dependentes da fonte do inóculo, concentração de substrato, tipo e concentração de matéria-prima para elaboração do extrato, tempo e temperatura de fermentação (VILLARREAL-SOTO et al., 2018).

Durante o processo de fermentação da kombucha, as diferentes espécies de leveduras e bactérias atuam em paralelo. Entretanto, produzem dois produtos finais distintos: o líquido fermentado e o biofilme (celulose). Inicialmente na fermentação, as leveduras são responsáveis pela hidrólise da sacarose em glicose e frutose, além da produção de etanol. Já as bactérias acéticas produzem o ácido acético, e também ácidos glucônico e glucurônico, conforme é apresentado na Figura 2.1 (VILLARREAL-SOTO et al., 2018).

Figura 2.1. Atividade metabólica principal durante a fermentação da kombucha.

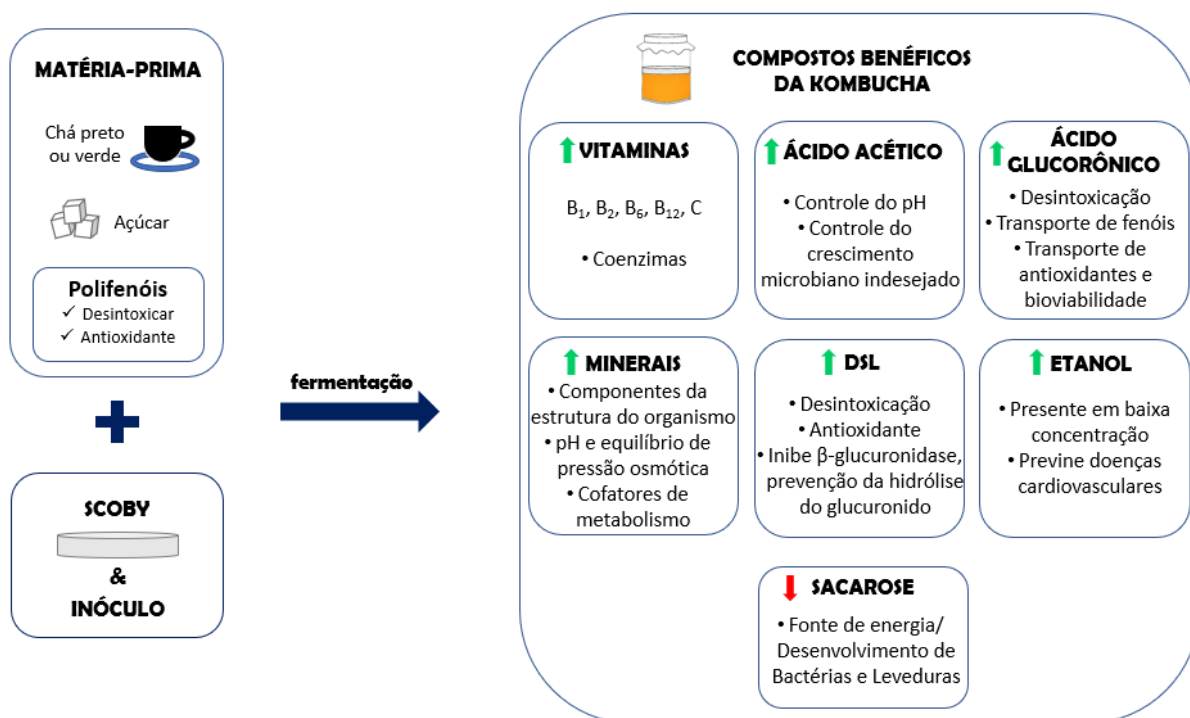


Fonte: Adaptado de Markov et al. (2003)

Durante a fermentação, as espécies de microrganismos podem excretar produtos metabólicos que estimulam ou inibem o crescimento de outras espécies. Em consequência disso, ainda se tem muitas lacunas e obstáculos na compreensão da fermentação da kombucha, devido principalmente à diversidade e complexidade das comunidades microbianas, sendo que

algumas podem participar em paralelo, enquanto outras agem de maneira sequencial com uma evolução dominante durante a fermentação (CHAKRAVORTY et al., 2016). A Figura 2.2 apresenta os principais compostos que estão presentes na kombucha originados durante a fermentação.

Figura 2.2. Principais compostos presentes na Kombucha e sua função biológica.



Fonte: Adaptado de Leal et al. (2018).

2.5 FATORES QUE INFLUENCIAM A FERMENTAÇÃO

Como citado anteriormente, a composição química da kombucha assim como os metabólitos formados durante a fermentação são dependentes de vários fatores: microrganismos, concentração e tipo de substrato e matéria-prima para elaboração do extrato, tempo e temperatura de fermentação (VILLARREAL-SOTO et al., 2018).

2.5.1 Microrganismos

O SCOBY é o consórcio simbiótico de bactérias e leveduras, que inclui cepas microbianas anaeróbicas e aeróbicas, podendo estas estarem incorporadas em uma membrana de celulose ou dispersos no líquido (DIMA et al., 2017; UȚOIU et al., 2018). De acordo com

Nikolaev e Plakunov (2007) durante a fermentação os microrganismos produzem um biofilme celulósico espesso na interface ar-líquido. A celulose é formada sobre a superfície da bebida em condições favoráveis de temperatura (cerca de 30 °C) e oxigenação, se espalhando por toda a superfície do chá (DUTTA; GACHHUI, 2007). A celulose contendo os microrganismos impregnados (SCOBY mãe) é geralmente utilizada como inóculo para iniciar uma fermentação, onde ocorre a formação de uma nova película (SCOBY filho).

Segundo Villarreal-soto et al. (2018), várias são as espécies de leveduras presentes na fermentação da kombucha, sendo as principais delas apresentadas na Quadro 2.1. Na kombucha, o principal efeito das leveduras é a produção de invertases que atuam na hidrólise da sacarose em frutose e glicose, sendo esse efeito o desencadeamento de várias outras reações (MAY et al., 2019). As leveduras também são responsáveis pela produção de gás carbônico e etanol. Entretanto, o aumento do etanol pode ocasionar a inibição do crescimento de leveduras quando associado ao baixo pH e a presença de ácido acético (PAMPULHA; LOUREIRO-DIAS, 2000; GRAVES et al., 2006; AGUILAR USCANGA; DÉLIA; STREHAIANO, 2003).

Quadro 2.1. Leveduras presentes na cultura da kombucha.

Levedura	Principais características
<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> ➤ Alta tolerância ao etanol ➤ Fermentação rápida ➤ Insensibilidade à temperatura e concentração de substrato
<i>Schizosaccharomyces pombe</i>	<ul style="list-style-type: none"> ➤ Alto poder fermentativo ➤ Capacidade de converter ácido málico em etanol ➤ Liberação de grandes quantidades de polissacarídeos
<i>Brettanomyces bruxellensis</i>	<ul style="list-style-type: none"> ➤ Alta resistência ao estresse osmótico e etanol ➤ Maior eficiência que a <i>Saccharomyces cerevisiae</i> para otimizar as fontes de nitrogênio disponíveis ➤ Produção de altas concentrações de ácido acético em condições aeróbicas (STEENSELS et al., 2015)
<i>Zygosaccharomyces rouxii</i>	<ul style="list-style-type: none"> ➤ Altamente osmotolerante e halotolerante ➤ Combate melhor estresse com açúcar e sal que <i>S. Cerevisiae</i>

Fonte: Adaptado de Villarreal-Soto et al. (2018).

Com relação as bactérias, as dominantes na cultura da kombucha são as ácido acéticas, sendo essas aeróbicas responsáveis pela formação de ácido acético, além disso, estas também

são responsáveis pela produção do ácido gluconico e glucorônico. Essas bactérias, ao contrário das leveduras, necessitam de grandes quantidades de oxigênio para seu crescimento e atividade (VILLARREAL-SOTO et al., 2018).

A produção da celulose geralmente ocorre por bactérias da família *Acetobacteraceae*, principalmente bactérias do gênero *Komagataeibacter*, e geralmente linhagens das espécies *Komagataeibacter xylinus* e *Komagataeibacter hansenii* (anteriormente conhecidas como *Gluconacetobacter xylinus* e *Gluconacetobacter hansenii*) (SEMJONOVSKA et al., 2017). Outras bactérias encontradas na kombucha são as ácido lácticas, responsáveis principalmente pela produção de ácido láctico. Cotton et al. (2017) verificou as seguintes bactérias ácido lácticas presentes na kombucha: *Lactobacillus satsumensis*, *Lactobacillus nagelii* e *Oenococcus oeni*.

De acordo com May et al. (2019), as bactérias podem inibir possíveis competidores devido à acidificação do meio, e quando a fonte de carbono estiver em pequenas quantidades, bactérias acéticas podem utilizar o etanol com fonte de carbono.

2.5.2 Substrato

A fonte tradicional de carbono para a fermentação de Kombucha é a sacarose. Ao adicionarmos outro açúcar (lactose, glicose ou frutose) pode-se levar a uma influência distinta na formação de etanol e ácido láctico, entretanto possuir ainda assim um impacto menor no sabor do chá fermentado (REISS, 1994).

Vohra et al. (2019) utilizou diferentes fontes de carbono (açúcar tradicional, açúcar mascavo e mel) para fermentação de kombucha de chá verde e preto. Os autores obtiveram melhor resultado de atividade antioxidante para a kombucha de chá verde com açúcar tradicional (84% para o radical DPPH no sétimo dia de fermentação) e para atividade antimicrobiana para a kombucha de chá preto com açúcar tradicional.

Malbaša, Lončar e Djurić (2008) elaboraram kombucha de chá preto com açúcar tradicional e com melaço. Os autores obtiveram maior produção de ácido acético e menores valores de pH para o kombucha com açúcar como fonte de carbono. Já com melaço, a kombucha apresentou maior teor de ácido láctico.

Goh et al. (2012) utilizaram várias concentrações de sacarose afim de identificar qual seria a concentração ideal para um maior rendimento na produção da celulose pela kombucha. Os resultados que apresentaram melhor resposta foram as concentrações de 50 g/L, 70 g/L e 90

g/L, produzindo um rendimento de 47,9%, 44% e 66,7% de celulose bacteriana, respectivamente. Concentrações de sacarose acima de 90 g/L apresentaram um decréscimo significativo na produção da celulose.

2.5.3 pH

Sabe-se que o pH da kombucha diminui durante o processo de fermentação, e isso se deve à formação de ácidos orgânicos durante esse processo (SIEVERS et al., 1995). É um importante parâmetro para as fermentações, uma vez que este é relacionado à segurança contra crescimento de microrganismos patogênicos (pH < 4,2 estes não se desenvolvem) e as mudanças estruturais dos compostos fitoquímicos que podem influenciar a atividade antioxidante (HUR et al., 2014).

De acordo com Jayabalan et al. (2008), o pH da kombucha tende a baixar rapidamente nos primeiros dias de fermentação, tendendo a estabilizar devido a efeitos tamponantes causados pelos ácidos. Malbasa et al. (2011) explica que isso se deve ao fato de que ocorre um efeito tampão causado pela síntese de ácidos orgânicos fracos, que interagem com componentes minerais do chá.

2.5.4 Tempo e Temperatura

A fermentação da kombucha pode variar de 7 a 60 dias e as atividades biológicas podem aumentar durante esse processo. Entretanto os melhores resultados foram obtidos em uma média de 15 dias (CHU; CHEN, 2006; VILLARREAL-SOTO et al., 2018). Geralmente, os valores de temperatura da fermentação de kombucha variam entre 22 °C e 30 °C e manter a temperatura ideal durante toda a fermentação resulta em um melhor crescimento microbiano e atividade enzimática, portanto, os benefícios da fermentação são aprimorados (VILLARREAL-SOTO et al., 2018).

Vários trabalhos relataram aumento significativo da atividade antioxidante durante a fermentação da kombucha, sendo verificado por Sun, Li e Chen (2015) em 10 dias, por Ayed, Ben Abid e Hamdi (2017) em 12 dias e por Villarreal-Soto et al. (2019) em 21 dias de fermentação. Logo, o tempo de fermentação da kombucha pode ser favorável para o aumento da atividade antioxidante da bebida.

Neffe-Skocińska et al. (2017) avaliaram em 10 dias diferentes temperaturas (20, 25 e 30 °C) para a fermentação da kombucha obtendo notas sensoriais similares para todas as amostras, sendo um pouco superior para a amostra a 25 °C. Com relação ao consumo de açúcar, a amostra que apresentou maior consumo foi a de 30 °C. Já para a produção de ácido acético, glucorônico e cítrico, a maior produção foi a 25 °C. Entretanto, para os ácidos quínico, málico e cítrico não houve diferença significativa entre as temperaturas. Com isso, pode-se observar que a temperatura é um fator que pode ocasionar mudanças na composição química da kombucha.

2.6 EXTRATOS ALTERNATIVOS AO CHÁ

Atualmente, têm-se estudado vários extratos alternativos ao chá para a elaboração da kombucha (EMILJANOWICZ & MALINOWSKA-PANCZYK, 2019), isso se deve principalmente à grande notoriedade que o produto vem ganhando nos últimos anos, assim necessitando de novas alternativas para atender uma ampla demanda dos novos consumidores. Têm-se estudado a aplicação de extrato utilizando suco de frutas, como Goji berry, fruta-cobra, mirtilo, framboesa e também alguns tipos de sucos, como suco de uva, suco de cacto pêra, suco concentrado de cenoura e suco de grama de trigo. Além desses, também foi utilizado o leite, café, folhas de mostarda africana e chá de Rooibos como extrato. No Quadro 2.2, estão expostas as matérias-primas alternativas ao chá já usadas na fermentação da Kombucha, assim como os principais resultados obtidos pelos autores.

A acerola é consirada uma “superfruta” devido ao seu conteúdo de vitamina C e polifenóis (PRAKASH; BASKARAN, 2018). Esses compostos presentes na acerola têm sido associados a algumas atividades biológicas relacionadas à redução do estresse oxidativo (ALVAREZ-SUAREZ et al., 2017; KLOSTERHOFF et al., 2018). Assim como a acerola, os subprodutos provenientes da indústria de processamento do fruto apresentam também grande quantidade de compostos bioativos, sendo quantificados por análises do teor de compostos fenólicos, atividade antioxidante e vitamina C por vários autores (BRAGA et al., 2011; PEREIRA et al., 2013; ALVES, 2019).

Quadro 2.2. Estudos de fermentação da Kombucha com matérias-primas alternativas.

Matéria-prima	Principais resultados	Referência
<i>Goji Berry</i> vermelho e preto	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante e diminuição no teor de compostos fenólicos totais para <i>Goji Berry</i> vermelho e estabilidade para <i>Goji Berry</i> preto ➤ Preferência sensorial para a Kombucha de <i>Goji Berry</i> preto 	Abuduaibifu & Tamer (2019)
Suco de uva	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante e compostos fenólicos totais (CFT) ➤ Boa aceitação para amostras com fermentação de até 6 dias 	Ayed, Abid e Hamdi (2017)
Chá de Rooibos	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante ➤ Diminuição no teor de compostos fenólicos totais 	Gaggia et al. (2019)
Leite	<ul style="list-style-type: none"> ➤ Apresentou alta atividade antioxidante durante a estocagem ➤ Boa aceitação sensorial para aparência, sabor e impressão global 	Hrnjez et al. (2014)
Folhas de mostarda africana	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante e CFT ➤ Diminuição da anti-xantina oxidase e a ação citotóxica das folhas 	Rahmani et al. (2019)
Suco de grama de trigo (<i>wheatgrass</i>)	<ul style="list-style-type: none"> ➤ Aumento no teor de flavonóides totais e CFT 	Sun, Li & Chen (2015)
Suco de cacto pêra	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante e CFT ➤ Apresentou atividade antimicrobiana contra cepas patogênicas 	Ayed & Hamdi (2015)
Suco concentrado de cenoura preta, louro-cereja, mirtilo e framboesa vermelha	<ul style="list-style-type: none"> ➤ Aumento do CFT e atividade antioxidante quando comparados aos substratos não cultivados e aumento da atividade antioxidante durante a fermentação ➤ A Kombucha de louro-cereja obteve as melhores pontuações na análise sensorial 	Ulusoy & Tamer (2019)
Café	<ul style="list-style-type: none"> ➤ Aumento no teor de CFT e estabilidade da atividade antioxidante durante a fermentação 	Watawana et al. (2015)
Fruta-cobra (<i>Salak</i>)	<ul style="list-style-type: none"> ➤ Aumento da atividade antioxidante, CFT, flavonoides totais e taninos totais 	Zubaidah et al. (2019)

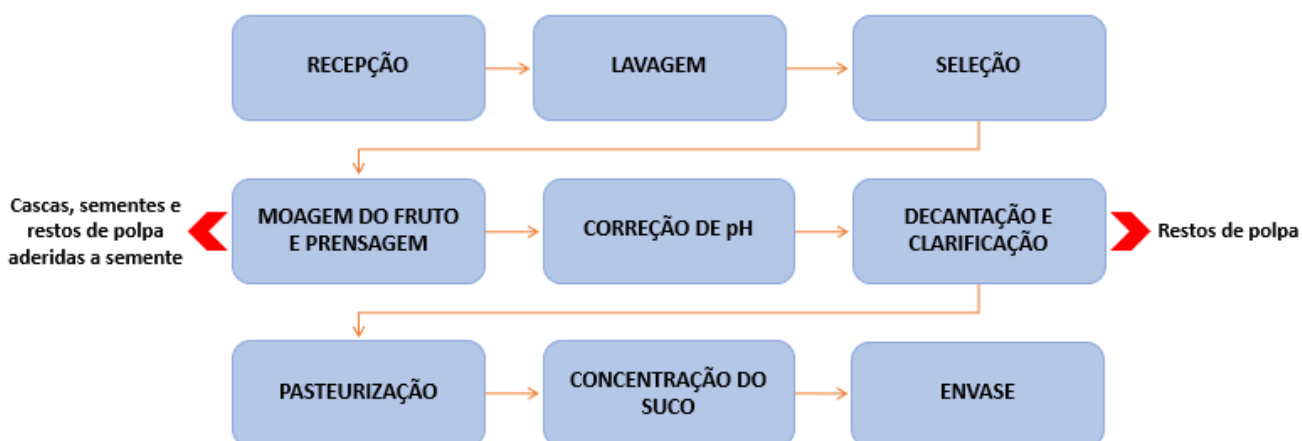
2.7 RESÍDUO DA ACEROLA PARA PRODUÇÃO DA KOMBUCHA

No país, as indústrias produzem resíduos que poderiam ter uma finalidade mais interessante para a sociedade e o meio ambiente. Muitos frutos são processados para fabricação de sucos naturais, doces em conserva, geleias, polpas e extratos, os quais geram resíduos, que geralmente são descartados e que poderiam ser utilizados para minimizar o desperdício de alimentos (KOBORI; JORGE, 2005).

Posteriormente ao processamento, as frutas geram subprodutos, sendo que estes muitas vezes não possuem um destino específico na empresa. Estes tornam-se contaminantes ambientais e, conseqüentemente, geram custos operacionais à indústria, pois estas necessitam de tratamento para o descarte (INFANTE et al., 2013).

A acerola é um dos frutos dos quais geram grandes quantidades de resíduos, representando entre 15 e 41% do volume total de acerola produzida (VASCONCELOS, 2002). Como dito anteriormente, estes subprodutos provenientes da indústria de processamento do fruto apresentam também grande quantidade de compostos bioativos (BRAGA et al., 2011; PEREIRA et al., 2013; ALVES, 2019), sendo este um fator importante para o reaproveitamento deste. Durante o processamento do suco clarificado de acerola (Figura 2.3), este possui duas etapas nas quais há a produção de subprodutos.

Figura 2.3. Diagrama esquemático do processo de produção do suco de fruta clarificado.



Fonte: Adaptado de Cianci et al. (2005).

Alguns trabalhos utilizaram estes subprodutos de acerola para diferentes aplicações, sendo estas expostas no Quadro 2.3. A produção de kombucha não foi relatada até o momento.

Quadro 2.3. Trabalhos evidenciando alternativas para utilização do subproduto de acerola.

Objetivo	Principais resultados	Referência
Utilizar a farinha do subproduto de acerola na elaboração de biscoitos tipo <i>cookies</i> .	O cookie formulado com adição de farinha do subproduto de acerola apresentou teor de carotenoides e vitamina C, embora a aceitação sensorial tenha sido inferior a amostra padrão (cookie somente com farinha de trigo)	Aquino et al. (2010)
Suplementar tilápias com extrato de subproduto de acerola para aumentar a capacidade antioxidante dos filés	A atividade antioxidante dos filés de tilápia foi superior para aqueles alimentados com o suplemento	Carbonera et al. (2014)
Avaliar o efeito da secagem por ar quente nos compostos fenólicos	Houve redução na concentração de fenólicos, carotenoides, antocianinas, ácido ascórbico e atividade antioxidante. No entanto, a concentração de bioativos remanescente caracterizou o resíduo como um potencial ingrediente alimentar	Nóbrega et al. (2014)
Avaliar o efeito de farinhas de subprodutos agroindustriais de acerola (semente e bagaço), substituindo a aveia no desenvolvimento de barras de cereais	A aceitação sensorial superior para amostra com substituição parcial da aveia pela semente do subproduto da acerola. Além disso, as amostras suplementadas apresentaram vitamina C na sua composição e teor superior de CFT quando comparada a padrão	Marques et al. (2015)
Avaliar a encapsulação dos extratos de compostos bioativos da polpa e subproduto agroindustrial de acerola por pulverização e liofilização, e determinar sua composição físico-química	A eficiência da microencapsulação foi superior a 50% para compostos fenólicos e flavonóides totais; além disso, também foram observadas atividades antioxidantes elevadas. Em geral, pós em spray e liofilizados têm melhores características físico-químicas.	Rezende et al. (2018)

Neste trabalho, o resíduo utilizado foi a fração obtida do processo de decantação e clarificação do suco, também conhecido como borra ou retentado. Esse subproduto, caracterizado por restos de polpa da acerola, pode ser reaproveitado como matéria-prima para a elaboração de novos produtos (PENHA et al., 2001). Portanto, uma bebida como a kombucha preparada com subproduto de acerola pode ajudar a aumentar a resposta imune humana,

por exemplo, contribuindo para as propriedades promotoras de saúde já associadas à bebida tradicional.

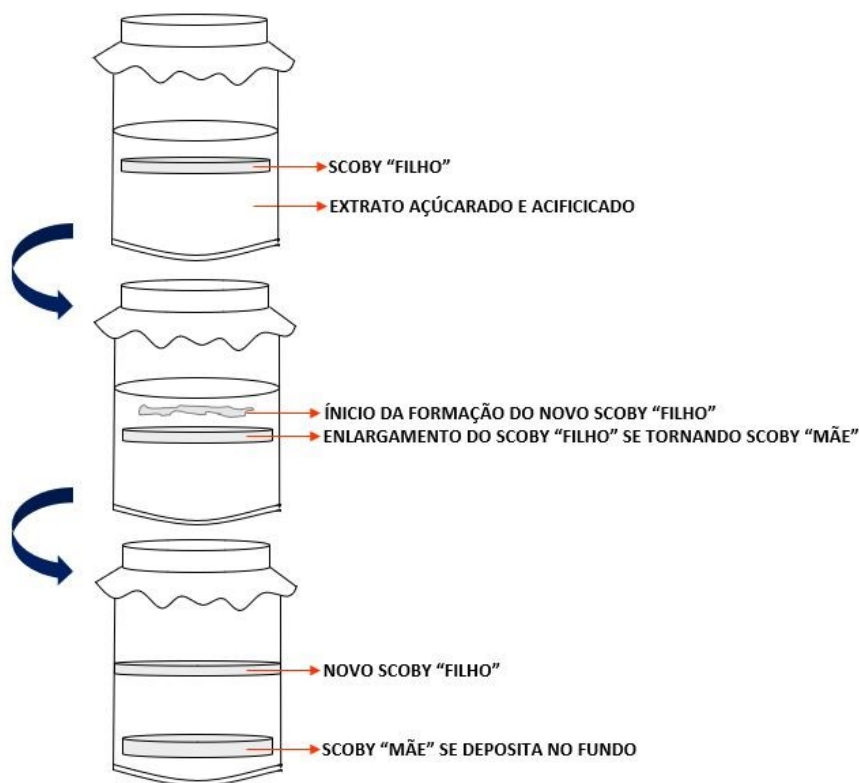
Além da bebida, a celulose bacteriana (bc) é um produto secundário da fermentação, ainda pouco explorado, mas que possui um vasto campo de aplicação. Portanto, a produção deste torna-se também um aspecto a ser considerado. Espera-se que a utilização do subproduto de acerola, assim como no caso do chá, continue a produzir uma quantidade considerável de celulose.

2.8 PRODUÇÃO DE CELULOSE BACTERIANA

A celulose bacteriana produzida em fermentações de kombucha ainda é um componente pouco explorado, apesar do biofilme produzido pelas bactérias do ácido acético poder representar outro importante produto da fermentação da kombucha. O aumento na produção de kombucha pode gerar uma grande quantidade de celulose bacteriana que ainda não foi amplamente utilizada, apesar das possibilidades (DAI et al., 2017; DOMSKIENE et al., 2019). Alguns trabalhos já exploraram o uso dessa celulose como material para supercapacitores (DAI et al., 2017), sorção de metais (MAMISAHEBEI et al., 2007), e também como matéria-prima para a produção de moda sustentável (DOMSKIENE et al., 2019).

Embora exista uma variação de acordo com aspectos ambientais e geográficos, as principais cepas bacterianas produtoras de celulose identificadas no SCOBY, geralmente são *Acetobacter xylinoides*, *Acetobacter xylinus*, *Acetobacter aceti*, *Acetobacter pasteurianus* (FU et al., 2014), e *Gluconacetobacter sacchari* (TROVATTI et al., 2011). A Figura 2.4 demonstra o processo de formação da celulose durante a fermentação da kombucha, na qual o SCOBY filho é proveniente de uma kombucha previamente fermentada, utilizando-se de 2,5-5% (m/v). Durante a fermentação, ocorre o alargamento do SCOBY filho, o qual se torna o SCOBY mãe. Por fim, o novo SCOBY filho é formado na superfície, e parte deste será utilizado subsequentemente para uma próxima fermentação.

Figura 2.4. Demonstração passo a passo da formação do scoby “filho” a partir de um SCOBY “mãe” durante a fermentação da kombucha.



Fonte: Adaptado de Dutta & Paul (2019).

De acordo com Jayabalan, Malbaša e Muthuswamy (2016), a síntese de celulose envolve a síntese de difosfoglicose de uridina (UDPGlc) pela pirofosforilase de UDPGlc, posteriormente sendo polimerizadas pela enzima celulose sintase. A conversão de glicose em UDPGlc possui mais duas etapas que convertem as moléculas iniciais de glicose, primeiro em glicose-6-fosfato e depois em glicose-1-fosfato. Também é possível produzir celulose utilizando frutose por sua conversão em glicose-6-fosfato.

Composta por fibrilas de celulose pura, a celulose bacteriana da kombucha tem grande capacidade de retenção de água, alta cristalinidade e termoestabilidade, semelhante à celulose bacteriana produzida em meio sintético (Dima et al., 2017; Emiljanowicz e Malinowska-Pańczyk, 2019). Rendimentos de celulose interessantes podem ser obtidos de acordo com as condições de fermentação e as espécies de bactérias produtoras presentes em SCOBY (Goh et al., 2012). A produção de celulose em meio tradicional (HS), geralmente traz melhores resultados em quantidade de tempo menor que a kombucha (Tabela 2.2). Pode-se observar na Tabela 2.2 que os melhores resultados de produção são utilizando o meio HS

(4,4-8,7 g/L), embora os demais meios também apresentaram produção considerável (1,24-4,0 g/L), uma vez que estes meios têm custo inferior se comparado com o meio tradicional.

A celulose produzida durante a fermentação da kombucha é considerada um produto secundário, entretanto, o custo de produção da mesma é inferior quando comparado ao meio tradicional (HS). Portanto, a kombucha passa a ser uma alternativa potencial para a produção de celulose, sendo necessária a caracterização desta para aumentar seu possível campo de aplicação.

Tabela 2.2. Produção de celulose bacteriana utilizando distintos meios de fermentação.

Cepa	Meio	BC (g/L)	Referência
<i>K. rhaeticus</i> TJPU03	HS	8.3 (10 dias)	He et al. (2020)
<i>K. rhaeticus</i>	HS + goma de caju (50% da fonte de carbono)	6.0 (7 dias)	Pacheco et al. (2017)
<i>K. saccharivorans</i> BC1	Efluente de destilaria bruto	1.24 (8 dias)	Gayathri e Srinikethan (2019)
<i>K. rhaeticus</i> P 1463	HS	4.4 (5 dias)	Semjonovs et al. (2017)
<i>K. rhaeticus</i>	Extrato de levedura, glicose, melão de cana e etanol	4.0 (5 dias)	Machado et al. (2018)
<i>K. rhaeticus</i> PG2	HS + 3% (m/v) glicerol	≈8.7 (10 dias)	Thorat e Dastager (2018)
<i>G. medellinensis</i>	Bioproduto de abacaxi e cana de açúcar	3.24 (13 dias)	Algar et al. (2015)
<i>K. rhaeticus</i> AF-1	HS	6.7 (4 dias)	Machado et al. (2016)
<i>G. medellinensis</i> ID13488	Bioporoduto de maçã e cana de açúcar	2.5 (14 dias)	Urbina et al. (2017)

A utilização do subproduto da acerola torna-se interessante tanto para a produção da bebida tipo kombucha, quanto para a produção de celulose bacteriana, uma vez que a sua composição bioativa tende a trazer aspectos positivos para ambos produtos da fermentação. Além disso, a utilização deste subproduto é uma alternativa viável quanto ao seu baixo custo, pois seria um substituto aos chás tradicionais e também como um meio alternativo ao tradicional (apresenta elevado custo) para produção da celulose.

CAPÍTULO 3

3 ARTIGO I: PRODUÇÃO DE BEBIDA TIPO KOMBUCHA E CELULOSE BACTERIANA UTILIZANDO SUBPRODUTO DE ACEROLA COMO MATÉRIA-PRIMA

PRODUCTION OF KOMBUCHA-LIKE BEVERAGE AND BACTERIAL CELLULOSE BY ACEROLA BYPRODUCT AS RAW MATERIAL

Artigo publicado: ANEXO A

ABSTRACT

In this study, acerola byproduct was used as a new raw material for the production of kombucha-like beverage and bacterial cellulose. The amount of acerola byproduct (1, 3 and 5% w/v) in the preparation of the beverage affected the product formation. The final concentration of ethanol was similar in all samples, approximately 9.5 g/L, but it was achieved at 12, 9 and 6 days, respectively, suggesting that yeast metabolism was accelerated in the presence of higher amounts of acerola byproduct. In addition, the highest productions of acetic acid (16.3 g/L) and bacterial cellulose (4.0 g/L) were achieved with 5% byproduct. Less porous and denser membranes were obtained with a highly crystalline structure (96.4%), also in higher amounts of acerola byproduct. These results suggest that the increase in the content of polyphenols and vitamin C, naturally present in the acerola byproducts, increased the concentration of acetic acid, and improved the environment for bacterial cellulose production.

Keywords: fermentation; acetic acid; ethanol; polyphenols; antioxidant activity

3.1 INTRODUCTION

Kombucha is a fermented tea drink of Asian origin, commonly prepared with black or green tea (Jayabalan, Marimuthu, and Swaminathan, 2007). A symbiotic culture of bacteria and yeasts (SCOBY) is responsible for the fermentation process, resulting in a refreshing, bittersweet, lightly carbonated drink. The characteristics of kombucha can vary greatly with several factors, such as the type of tea or the raw material, the microorganisms present in SCOBY, and the fermentation time and temperature (Chakravorty et al., 2016; Jayabalan et al., 2014). Typically, the fermentation time is 7 to 15 days. Extended periods can yield a drink with an intense acid flavor (similar to vinegar). In addition to acetic acid, different components during the fermentation of kombucha are produced, due to the presence of various strains of bacteria and yeast. Ethanol, acetic, lactic, and glucuronic acids are the most common products found in kombucha fermentation. The final drink also has a large quantity of tea-derived polyphenols (Chakravorty et al., 2016; Jayabalan, Marimuthu, and Swaminathan, 2007). Another important product of kombucha fermentation is the biofilm produced by acetic acid bacteria. It consists of pure cellulose fibrils, has great water retention capacity, high crystallinity, and thermostability (Emiljanowicz and Malinowska-Pańczyk, 2019). The cellulose yield varies according to the fermentation conditions and the species of producing bacteria present in SCOBY (Goh et al., 2012).

Currently, some studies have presented alternative raw materials for the fermentation of kombucha-like beverages, such as milk (Hrnjez et al., 2014); coffee (Watawana, Jayawardena, and Waisundara, 2015); cactus pear juice (Ayed and Hamdi, 2015); grape juice (Ayed, Ben Abid, and Hamdi, 2017); snake fruit (Zubaidah et al., 2018); rooibos tea (Gaggia et al., 2019); black and red Goji Berry (Abuduaibifu and Tamer, 2019); African mustard leaves (Rahmani et al., 2019); and black carrot juice, cherry laurel, blackthorn, and red raspberry (Ulusoy and Tamer, 2019). The use of alternative raw materials can promote the development of new beverages with novel functional properties due to their diverse composition, mainly focused on polyphenols (Emiljanowicz and Malinowska-Pańczyk, 2019).

An alternative to be considered is the use of industrial byproducts from fruit processing, such as acerola. Acerola is considered a "superfruit" due to the content of vitamin C and polyphenols (Prakash and Baskaran, 2018). The compounds present in acerola have been associated with some biological activities related to the reduction of oxidative stress (Alvarez-Suarez et al., 2017; Klosterhoff et al., 2018). Like acerola fruit, its byproduct also

has important polyphenolic compounds, which contribute to a high antioxidant activity (Marques et al., 2016; Marques et al., 2018; Rezende et al., 2017). Therefore, a drink like a kombucha prepared with acerola byproduct can help to boost the human immune response, for example, contributing to the health-promoting properties already associated with traditional kombucha.

In this work, acerola byproduct (from the juice clarification step) was used as raw material for kombucha-like fermentation. It is important to highlight that the final product doesn't have green or black tea, as the traditional kombucha and for this reason it was called kombucha-like beverage. To the best of our knowledge, this report is the first study that details the production of kombucha-like beverage with acerola byproduct. The fermentation kinetic profile was evaluated for 15 days in samples prepared with 1, 3 and 5% (w/v) acerola byproduct. The consumption of sugars, pH variation, total phenolic content, and products (ethanol, acetic acid and cellulose) were monitored during the fermentation period. In addition, the physicochemical and morphological structures of bacterial cellulose were characterized.

3.2 MATERIAL AND METHODS

3.2.1 Materials

The main materials used in this study are listed below: Glucose (Sigma-Aldrich, St. Louis, USA); Fructose (Sigma-Aldrich, St. Louis, USA); Glacial acetic acid PA (Lafan, Várzea Paulista, Brazil); Ethyl alcohol (Ethanol) PA (Neon, Suzano, Brazil); Folin-Ciocalteu (Sigma-Aldrich, St. Louis, USA); DPPH: 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St. Louis, USA), Ascorbic acid (Vetec, Rio de Janeiro, Brazil); PDA: Agar Potato Dextrose (Himedia, Mumbai, Índia); Ampicillin (Sigma-Aldrich, St. Louis, USA); Mannitol (Vetec, Rio de Janeiro, Brazil); Yeast extract (KASVI, São José dos Pinhais, Brazil); Peptone Bacteriological (Himedia, Mumbai, Índia); Agar Medium (KASVI, São José dos Pinhais, Brazil); Cycloheximide (Sigma-Aldrich, St. Louis, USA).

3.2.2 Kombucha culture and inoculum preparation

The starter culture or SCOBY was obtained from a local source in Florianópolis (Brazil) and maintained in sugared green tea. It was called "mother" kombucha. The inoculum was prepared by infusing green tea (0.5% w/v) at 100 °C for 10 min. The tea was filtered under sterile conditions and 35 g/L of glucose plus 35 g/L of fructose was added. Then, 10%

(v/v) of liquid broth and 4% (w/v) of biofilm from the mother kombucha were added and maintained for 10 days at 30 °C.

The main strains identified in the inoculum were: *Komagataeibacter rhaeticus*, *Bacillus megaterium*, *Bacillus aryabhattai*, *Bacillus flexus*, and *Bacillus simplex* as bacteria; and *Brettanomyces bruxellensis* and *Zygosaccharomyces bisporus* as yeasts. The identification of microorganisms was performed by high-throughput sequencing technologies using MiSeq Sequencing System (Illumina Inc., USA) by Neopropecta Microbiome Technologies (Florianópolis, Brazil).

3.2.3 Acerola byproduct preparation

Acerola byproduct, from juice clarification step, was supplied by a juice producing industry from Ceará, Brazil. The byproduct, basically consisting of residual pulp, was dried in a vacuum oven at 40 °C for 48 h, and then milled in a knife-mill (1.0 mm).

Extracts rich in acerola compounds were prepared by hydrothermal extraction in an autoclave at 121 °C for 15 min. The extractions were carried out in concentrations of acerola byproduct of 1, 3 and 5% (w/v on dry basis) to yield extracts with varied total phenolic content. The extracts were filtered under sterile conditions and 70 g/L of the substrate (glucose and fructose 1:1), and 10% (v/v) inoculum were added. At least, 720 mL of mixture was prepared for each acerola concentration and distributed in falcon tubes containing 40 mL. Triplicates were prepared and each replicate was fully harvested at 0, 3, 6, 9, 12 and 15 days of fermentation (30 °C).

3.2.4 Characterization of the kombucha-like beverage

3.2.4.1 pH Measurement

The pH value was measured in triplicate during fermentation with a pHmeter K39-2014B (Kasvi, São José dos Pinhais, Brazil).

3.2.4.2 Determination of sugars, acetic acid and ethanol

The concentration of sugars (glucose and fructose), acetic acid and ethanol were determined by High-Performance Liquid Chromatography (HPLC) using the Shimadzu Prominence LC-20A (Shimadzu, Tokyo, Japan) chromatograph with a Bio-rad Aminex HPX-87H

column. The samples were previously centrifuged at 8160 g for 5 min, filtered (0.22 μm), and transferred to the vials. Injection of 10 μL of sample was eluted with 5 mmol/L Na_2SO_4 at 0.5 mL/min flow rate. The oven temperature was maintained at 35 $^\circ\text{C}$ and the run time was 30 min. Concentrations were determined with a refractive index (IR) detector and calculated from standard glucose, fructose, acetic acid and ethanol curves.

3.2.4.3 *Total phenolic content and antioxidant activity*

The evaluation of total phenolic content (TPC) and antioxidant activity (2,2-diphenyl-1-picrylhydrazyl - DPPH scavenging capacity) were performed based on the colorimetric methods described by Singleton et al. (1999) and Brand-Williams et al. (1995), respectively, with some modifications. Gallic acid (GAE) and Trolox were used as standards. TPC was expressed as mg GAE/L and the antioxidant activity as mmol Trolox/L.

3.2.4.4 *Ascorbic Acid (Vitamin C)*

Ascorbic acid was quantified according to Selimović and Selimovic (2011), reacting the sample with 0.005 mol/L oxalic acid. The absorbance was read at 266 nm using quartz cuvettes. The results were obtained through a calibration curve previously constructed with ascorbic acid and the results expressed as mg/L.

3.2.4.5 *Microbiological analysis*

Plate counting method was used for the total counts of yeasts and acetic acid bacteria during the fermentation (Khosravi et al., 2019). Potato Dextrose Agar medium was used for total counting of yeasts, with the addition of 200 mg/L of ampicillin to inhibit bacteria growth. The plates were incubated for 3 days at 30 $^\circ\text{C}$. Yeast Peptone Mannitol Agar medium (5 g/L yeast extract, 25 g/L mannitol, 3 g/L peptone, and 15 g/L agar medium) was used for the total count of acetic acid bacteria, containing 500 mg/L cycloheximide to inhibit yeast growth. The plates were incubated at 25 $^\circ\text{C}$ for 5-7 days.

3.2.5 **Cellulose characterization**

The cellulose samples were purified to remove impurities and attached microorganisms. The purification was conducted by immersing the cellulose in a 0.1 mol/L NaOH for at least 24 h in an oven at 50 $^\circ\text{C}$. The membranes were washed with distilled water until

neutral pH, frozen for 24 h, and lyophilized model Liotop 101 (Liobras, São Carlos, Brazil) for 48 h. The cellulose concentration was expressed as grams of dry weight per liter of medium (g/L). The morphological and physicochemical properties of dry cellulose were analyzed.

3.2.5.1 *Morphological analysis*

The morphological characteristics of cellulose were investigated using Scanning Electron Microscopy JSM 6390LV (JEOL, Tokyo, Japan). The lyophilized membranes were distributed on carbon tapes on the surface of stubs and then coated with gold. The images were obtained with a tungsten electron source, a secondary electron detector, and an accelerated voltage of 10 kV.

3.2.5.2 *Fourier Transform Infrared Spectroscopic (FTIR)*

Fourier transform infrared spectra of lyophilized cellulose were recorded in an Cary 600 Series (Agilent Technologies, St. Clara, United States), in the wavelength range of 4000 to 500 cm^{-1} , with a 4 cm^{-1} resolution and accumulation of 16 scans in attenuated total reflectance (ATR) mode.

3.2.5.3 *X-ray Diffraction (XRD)*

The crystallinity was determined by X-ray diffractometry (XRD) MiniFlex600 (Rigaku, Tokyo, Japan), using $\text{CuK}\alpha$ radiation, a voltage of 40 kV, filament emission of 1.5 mA. Each sample was scanned from 5° to 50° 2θ range with a scan speed of 0.05°/step. The crystallinity was calculated according to Mohammadkazemi et al. (2015), as shown in Eq. (1)

$$\text{Crystallinity (\%)} = \left(\frac{S_c}{S_t} \right) \times 100 \quad (1)$$

where S_c is the sum of the net area, and S_t is the sum of the total area.

3.2.6 Statistical analysis

Statistical analysis was conducted by Past software. The results were evaluated by analysis of variance (ANOVA) and the significant differences were determined using Tukey's Test at a probability level of less than 5% ($p < 0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1 Kinetic evaluation of pH

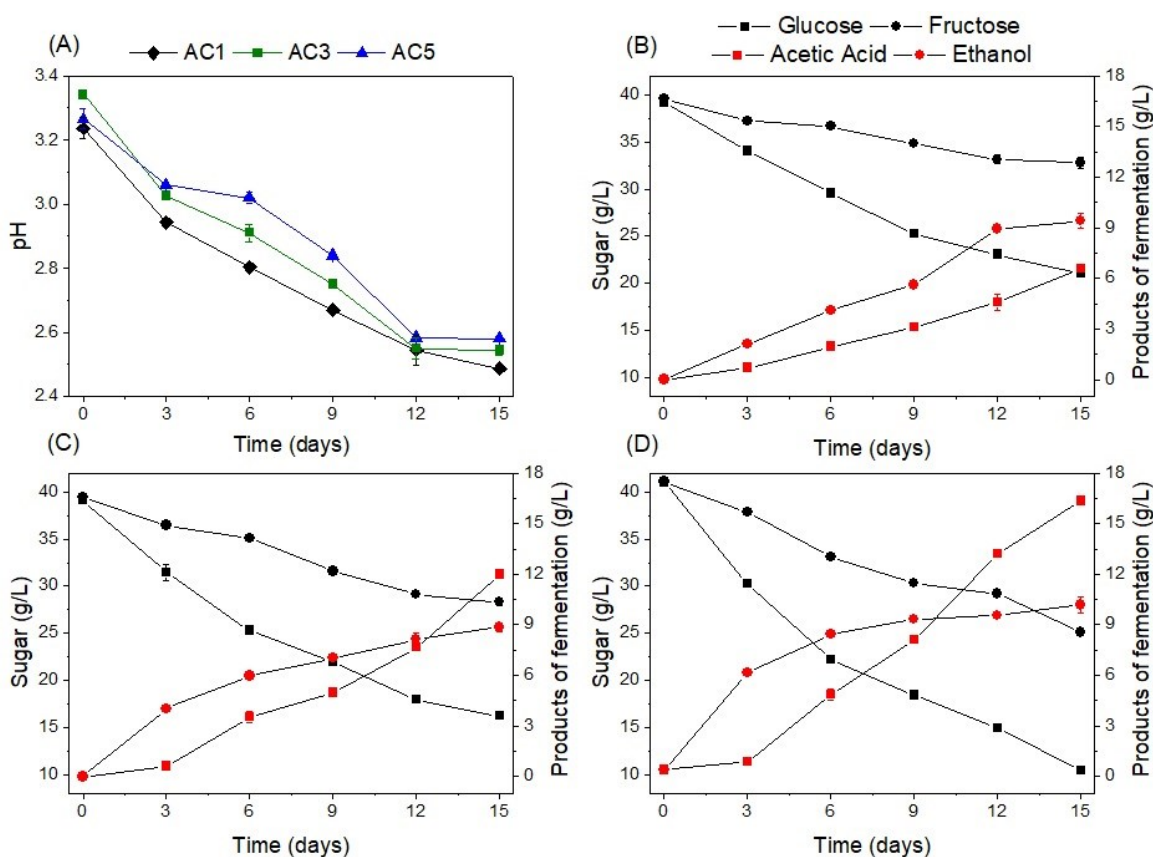
The pH is an important parameter in fermentative processes, as it ensures safety against the growth of pathogenic microorganisms ($pH < 4.2$) and prevents structural changes of antioxidant compounds (Hur et al., 2014). The initial pH was 3.24, 3.34, and 3.27 in the beverages prepared with 1% (AC1), 3% (AC3), and 5% (AC5) acerola byproduct, respectively. The low initial pH obtained is due to the pH of acerola fruit (3.04). All samples showed a similar behavior of pH reduction until the 12th day of cultivation and remained practically constant (Fig. 3.1A). After 15 days, the pH was reduced to 2.49, 2.54, and 2.58 in AC1, AC3, and AC5, respectively. This drop is mainly caused by the production of organic acids. A similar result was observed for grape after 8 days (Ayed et al., 2017), green tea and black tea kombucha after 9 days (Jayabalan et al., 2007), and pear cactus juice after 6 days of fermentation (Ayed and Hamdi, 2015). In our research, in addition to the drop in pH, we evaluated the production of acetic acid (Fig. 3.1B, 3.1C, 3.1D). Even with the highest concentration of acetic acid in AC5, the final pH of the kombucha-like beverages was similar. According to (Malbaša et al., 2011), the weak organic acids produced during fermentation promotes a buffering effect on the system, avoiding further reductions in pH.

3.3.2 Sugar consumption and product formation

Fig. 3.1B-D shows the profile of sugar consumption. Using a mixture of glucose and fructose (1:1) as substrate, it was possible to observe a clear preference for the consumption of glucose in all samples (Figure 3.1B-D). It is important to highlight that distinct community of microorganism can be found in kombucha. Among yeasts, several species were already identified, such as *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces*, *Kluyveromyces*, and *Candida* (Jayabalan et al., 2014; Emiljanowicz and Malinowska-Pańczyk, 2019; Chakravorty et al., 2016). In our study, *Brettanomyces bruxellensis* and *Zygosaccharomyces bisporus* were found as the predominant strains. It is known that yeasts have a higher

preference for glucose, when glucose and fructose are available in the medium cultivation, as showed for *Brettanomyces bruxellensis* (Leite et al., 2013), *Saccharomyces cerevisiae* (Guillaume et al., 2007) and *Kluyveromyces marxianus* (Fonseca et al., 2013) resulting in a significant amount of fructose at the end of fermentation. Despite this, sugar consumption shows a linear increase with the increase of acerola byproduct. The consumption of 56.6% of total sugars (74.5% glucose and 38.8% fructose) was observed in AC5 kombucha, 43.4% (58.5% glucose and 28.2% fructose) in AC3, and finally, 31.6% (49.2% glucose and 17.1% fructose) in AC1, after 15 days. Gaggia et al. (2019) demonstrated that 41.1% and 38.7% of total sugar was consumed in black and green tea fermentation, respectively, after 14 days. Zubaidah et al. (2018) observed that approximately 40-50% of total sugar was consumed in the kombucha-like fermentation of snake fruits after 14 days.

Figure 3.1. Kinetic profile of pH (A), reducing sugar consumption, acetic acid and ethanol production in kombucha-like beverage containing 1% (AC1) (B), 3% (AC3) (C), and 5% (AC5) (D) of acerola byproduct.



The final concentration of ethanol was similar for all samples, approximately 9.4 ± 0.44 , 8.84 ± 0.23 , and 10.18 ± 0.50 g/L for AC1, AC3, and AC5, respectively. However, the

production rate was higher in AC5 kombucha. After 6 days, the ethanol concentration reached 8.44 g/L and showed a slight increase to 10.18 g/L in 15 days. In addition, it is observed that ethanol production was inhibited when the concentration of acetic acid achieved approximately 5 g/L. This was observed in the AC1 sample (Fig. 1B) after 12 days, which achieved 4.57 g/L of acetic acid and 8.95 g/L of ethanol. The same behavior was observed in samples AC3 and AC5, but at 9 and 6 days, respectively. The inhibitory effect on ethanol production due to the presence of acetic acid associated with low pH was also reported by *Brettanomyces bruxellensis* (Aguilar Uscanga et al., 2003), yeast found in kombucha also by other authors (Jayabalan et al., 2014; Chakravorty et al., 2016).

Acetic acid is the main organic acid produced by the acetic acid bacteria present in kombucha. Among bacteria, *Komagataeibacter* and *Gluconobacter* strains are generally the genera found in the liquid and biofilm of kombucha, as shown by Chakravorty et al. (2016); in our study, *Komagataeibacter rhaeticus* was the strain identified as acetic acid bacteria. The production rate of acetic acid was higher from 3rd day, and the final concentrations of 6.58 ± 0.01 , 11.98 ± 0.03 , and 16.35 ± 0.01 g/L varied according to the amount of acerola byproduct added. Jayabalan et al. (2007) found 9.51 g/L of acetic acid for green tea kombucha, Kaewkod et al. (2019) obtained between 10.42 and 11.15 g/L of acetic acid for green, black and oolong tea kombuchas, both in 15 days of fermentation. Chakravorty et al. (2016) achieved 16.57 g/L of acetic acid in 21 days of fermentation and reported that ethanol was consumed during the fermentation by acetic acid bacteria to produce acetic acid. The oxidation of ethanol by acetic acid bacteria is common in kombucha fermentation, as many strains have this ability. In this work, analyzing the behavior of AC5 sample, we can see that ethanol was not consumed, possibly due to the higher amount of sugar available. Villareal-Soto et al. (2019) observed that ethanol oxidation occurred only after, approximately, 80% of sugar consumption. In this study, the maximum sugar consumption observed in AC5 was 56.6%; in fact, 35.5 g/L of total sugar was still available in the cultivation medium.

In general, substrate consumption and product formation depend on the consortium of the microorganism, but also the conditions and medium of fermentation. The increase in the amount of acerola byproduct in the fermentation medium released a higher content of compounds such as phenolic (Fig. 3.2A), which appears to have the power to boost the metabolic activity of the microorganisms.

3.3.3 Phenolic content and antioxidant activity

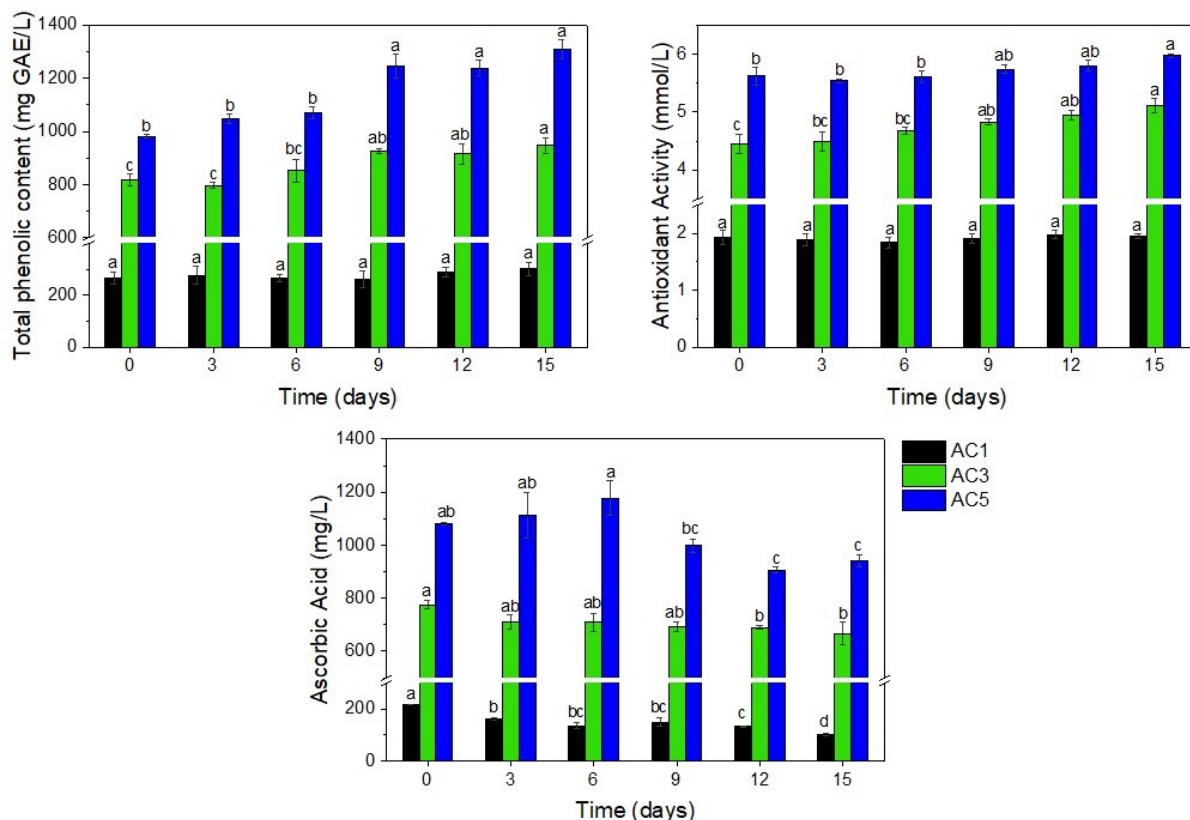
Kombucha is known to be beneficial for health, mainly due to its phenolic content and its ability to act as an antioxidant (Chakravorty et al., 2016). The total phenolic content (TPC) at initial AC1, AC3, and AC5 (Fig. 3.2A) samples was 264.7, 817.6, and 982.6 mg GAE/L, respectively. During the fermentation, samples AC3 and AC5 showed a significant increase ($p < 0.05$) of 15.9% and 33.2% in the concentration of total phenolic compounds, increasing the TPC values to 947.6 and 1309.0 mg GAE/L after 15 days, respectively. The low pH of kombucha is beneficial to avoid the chemical degradation of polyphenols since they are generally stable at acidic pH (Kwon et al., 2014). Jayabalan et al. (2007) verified the increase in the total catechins in green and black tea kombuchas after 9 days of fermentation and attributed to the biotransformation of epigallocatechin gallate in its isomers epicatechin gallate and epicatechin. The increase is related to the release of enzymes by the consortium of microorganisms in an acidic environment, causing the hydrolysis of polyphenols into smaller molecules (Jayabalan et al., 2008).

Fig. 3.2B shows a significant increase ($p < 0.05$) in antioxidant activity for samples AC3 and AC5, while AC1 remained constant during 15 days of fermentation. The increase in antioxidant activity was 14.8% (5.12 mmol/L) and 6.6% (5.98 mmol/L) for AC3 and AC5, respectively. Ayed and Hamdi (2015) showed an 18% increase in antioxidant activity in pear cactus juice kombucha in 12 days of fermentation. Zubaidah et al. (2018) reported a 7.9% increase in antioxidant activity in snake fruit kombucha after 14 days of fermentation, and Khosravi et al. (2019) achieved a 7.0% increase for black tea kombucha in 15 days. According to Jayabalan et al. (2014), the increase in the antioxidant capacity of kombucha depends on several factors, such as the type of substrate, fermentation time, and kombucha microbiota, which is mainly responsible for determining the nature of the metabolites formed during fermentation.

Vitamin C content was also assessed during the fermentation (Fig. 3.2C). All samples showed a significant decrease ($p < 0.05$) in the ascorbic acid content from the 1st to 15th day of fermentation, 52.76, 13.83, and 12.82% for AC1, AC3, and AC5, respectively. The highest concentrations of acerola byproduct, again, presented the best results, preserving more vitamin C in the final product. The main factors influencing ascorbic acid degradation include temperature, oxygen, water activity, pH, and presence of metal ion catalysis (Uddin et

al., 2001). In the case of kombucha-like fermentation, the exposure to temperature (30 °C during 15 days) and the presence of oxygen can be the causes of vitamin C loss.

Figure 3.2. Variation of (A) total phenolic content, (B) antioxidant activity and (C) ascorbic acid concentration in kombucha-like beverage containing 1% (AC1), 3% (AC3), and 5% (AC5) of acerola byproduct. Different letters indicate significant differences by Tukey test ($p < 0.05$).



3.3.4 Microbial concentration of acetic acid bacteria and yeasts

Table 3.1 shows the microbial concentrations obtained for acetic acid bacteria and yeasts in 0, 9, and 15 days of fermentation. As already observed in black tea kombucha by Goh et al. (2012), the amount of yeasts is higher than the number of acetic acid bacteria. There was a significant increase in the concentration of acetic acid bacteria and yeast, verified on the 9th day. The microbial concentration could be compared with the typical phases of the growth curve. In AC1 and AC3, the values between 9 and 15 days did not differ statistically ($p < 0.05$), which probably indicates that yeasts and acetic acid bacteria were in stationary phases. In AC5, due to the different values ($p < 0.05$), we can infer that the yeast reached the death phase, corroborating to the stationary concentration of ethanol after 6 days. The decrease in the number of acetic acid bacteria in AC5 can be caused by acid shock,

verified by Jayabalan et al. (2007) after 9 days of fermentation and by Zubaidah et al. (2018) after 7 days of fermentation due to the high amounts of acetic acid. However, the concentration of acetic acid continues to increase, indicating that the acetic acid bacteria still show metabolic activity.

Table 3.1. Microbial concentrations (CFU/mL) of acetic acid bacteria and yeasts in kombucha-like beverage containing 1% (AC1), 3% (AC3), and 5% (AC5) of acerola by-product.

Day	Acetic Acid Bacteria (CFU/mL)		
	AC1	AC3	AC5
0	25.50 ± 4.50 ^{aB} (x 10 ²)	35.00 ± 8.00 ^{aB} (x 10 ²)	37.50 ± 3.50 ^{aC} (x 10 ²)
9	19.55 ± 7.45 ^{bA} (x 10 ³)	23.55 ± 2.55 ^{bA} (x 10 ³)	64.00 ± 6,00 ^{aA} (x 10 ³)
15	19.05 ± 1.95 ^{aA} (x 10 ³)	23.80 ± 3.20 ^{aA} (x 10 ³)	24.05 ± 2,95 ^{aB} (x 10 ³)
Day	Yeast (CFU/mL)		
	AC1	AC3	AC5
0	24.10 ± 3.90 ^{aB} (x 10 ³)	16.05 ± 2.95 ^{aB} (x 10 ³)	13.05 ± 1.95 ^{aB} (x 10 ³)
9	23.05 ± 3.95 ^{cA} (x 10 ⁴)	67.50 ± 2.50 ^{bA} (x 10 ⁴)	53.00 ± 8.00 ^{aA} (x 10 ⁵)
15	20.50 ± 3.45 ^{bA} (x 10 ⁴)	55.50 ± 2.50 ^{aA} (x 10 ⁴)	24.50 ± 1.50 ^{bC} (x 10 ⁴)

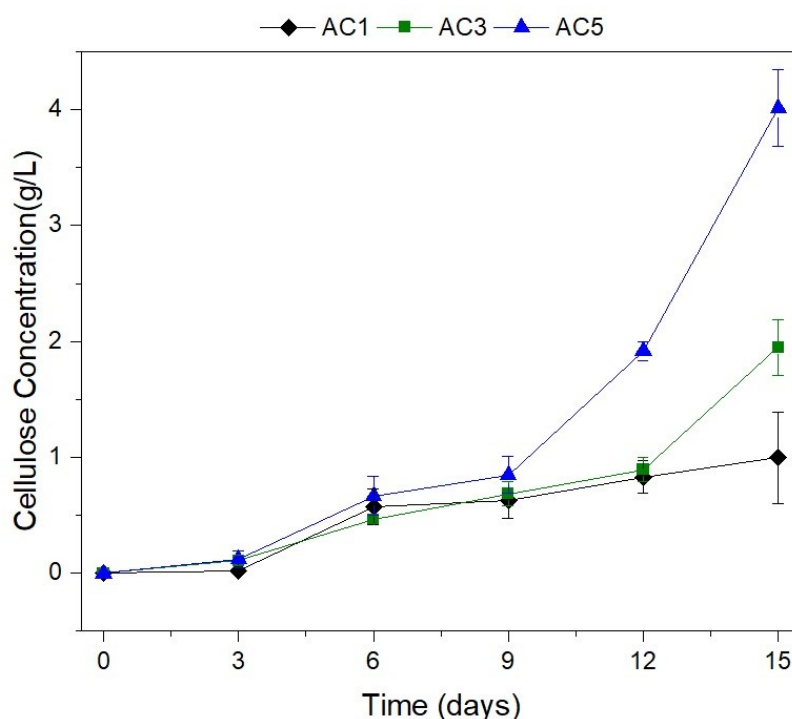
Mean ± standard deviation of two determinations. Lowercase letters in the row and uppercase letters in the column followed by different letters indicate significant differences by Tukey test ($p < 0.05$).

3.3.5 Cellulose concentration

Fig. 3.3 shows the kinetics of cellulose production during fermentation. Until the 9th day, a small increase in cellulose production was observed, and no differences ($p < 0.05$) were identified between the samples. After 9 days, AC5 showed a large increase in the cellulose concentration, while AC3 showed an increased production only on the last day of fermentation. AC1 showed the lowest cellulose concentration resulting in 1.00 g/L after 15 days. The concentrations obtained in AC5 and AC3 samples were 4.01 and 1.95 g/L, respectively. Few studies have reported the cellulose concentration obtained from kombucha or kombucha-like fermentation. In tea kombucha, it was reported 3.5 g/L (Soh and Lee, 2002), 2.15 g/L (Lee and Kim, 2000), and approximately 0.9 g/L (Villarreal-Soto et al., 2019) of cellulose concentrations at 12, 18 and 21 days of fermentation, respectively.

The higher cellulose concentration obtained in AC5 may be related to the phenolic content and vitamin C found in the acerola byproducts. Keshk (2014) evidenced an increase in cellulose production by the *Gluconacetobacter xylinus* by adding 0.5% ascorbic acid in the culture medium. In this work, the increase in the acerola byproduct in the preparation of fermentation medium resulted in higher contents of polyphenol and vitamin C, which could improve the metabolic rate of cellulose-producing bacteria.

Figure 3.3. Variation of cellulose concentration in kombucha-like beverage containing 1% (AC1), 3% (AC3), and 5% (AC5) of acerola byproduct.



It is important to highlight that *K. rhaeticus*, the strain identified in the SCOBY used in this work, was already cited as the largest producer of cellulose compared to *G. xylinus* (Machado et al. 2016; He 2020). *K. rhaeticus* also prefers glucose to fructose for cellulose production (Thorat and Dastager et al., 2018; He et al., 2020), which is in agreement with the results observed in the sugar consumption profile.

3.3.6 Morphological characteristics of cellulose

The morphology of the cellulose membranes was evaluated. The surface in contact with the liquid (Fig. 3.4 A-C) showed more interfibrillar space making it more porous, while the surface in contact with air (Fig. 3.4 D-F) showed a denser structure, similar to the morphological structure observed in cellulose produced by *Gluconacetobacter xylinus* (Neera, Ramana, and Batra, 2015). In addition, the structure became denser with the increase in the

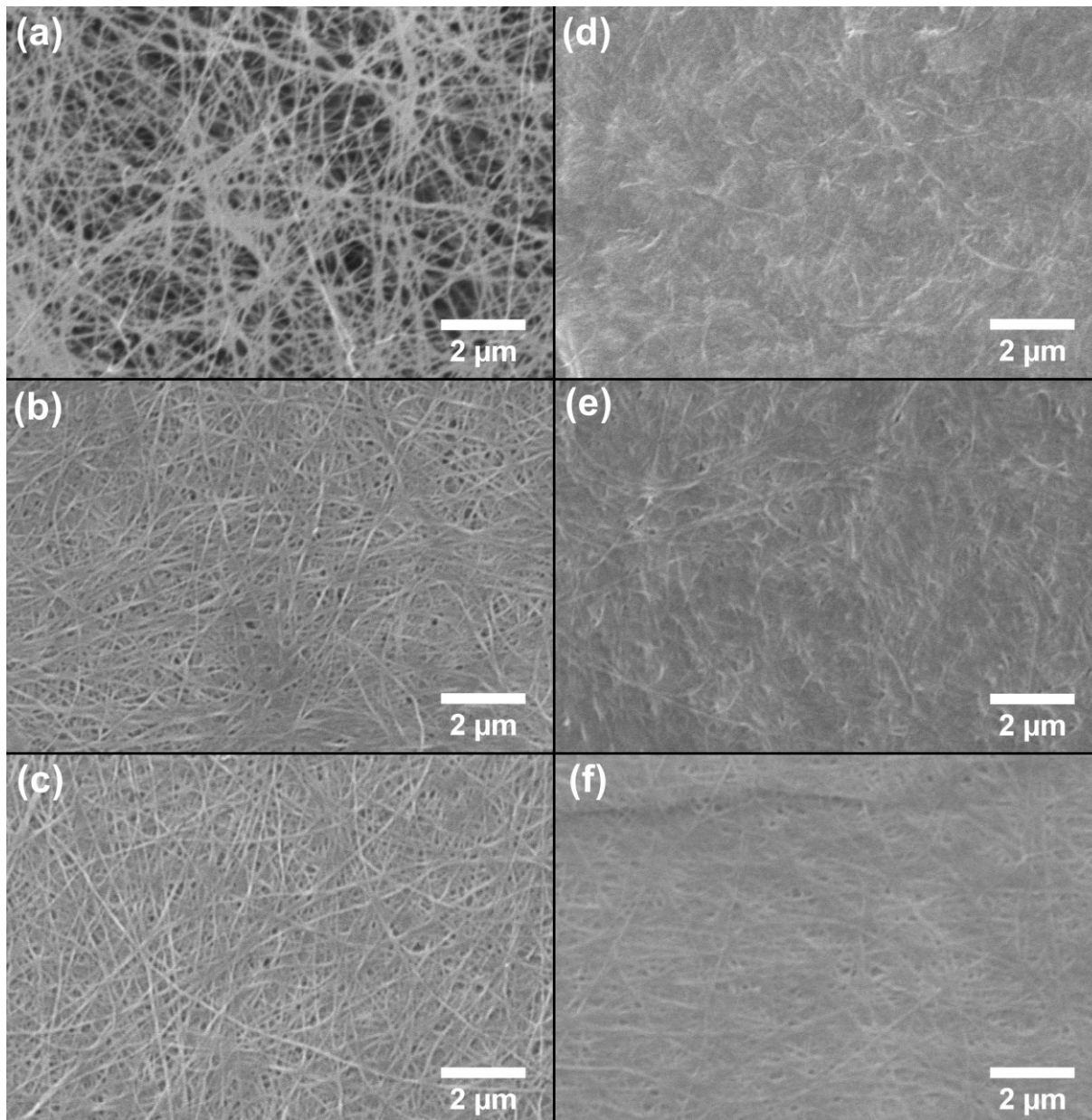
concentration of acerola byproduct. This result corroborates the cellulose concentration. The culture medium with the highest cellulose concentration resulted in a higher number of entangled fibrils, less interfibrillar spaces, and a superior network structure. These characteristics are mainly desired for applications in the biomedical field (Huang et al., 2014).

3.3.7 Physicochemical properties of cellulose

FTIR spectra were similar for the three samples (Fig. 3.5A) and similar to the chemical structure found in cellulose produced by typical producing bacteria *K. rhaeticus* (Machado et al., 2016; He et al., 2020). The analysis showed typical bands of bacterial cellulose, such as OH- transmittance close to 3350 cm^{-1} (A), CH stretch of groups CH_2 , and CH_3 close to 2900 cm^{-1} (B). Bands close to 1630 cm^{-1} (C) and 1410 cm^{-1} (D) are related to glucose carbonyl group (C=O), and CH_2 bending and C–OH in plane bending. The region between 1340 (E) and 1200 cm^{-1} are attributed to the presence of crystalline regions in the cellulose structure.

X-ray diffraction was used to assess the degree of crystallinity of the celluloses. All samples showed 3 diffraction peaks (Fig. 3.5B) characteristic of cellulose type I, close to $2\theta = 15^\circ$ (triclinic structure (I_α) = 110 and monoclinic structure (I_β) = 100), 17° ($I_\alpha = 010$ e $I_\beta = 110$) and $22,6^\circ$ ($I_\alpha = 110$ e $I_\beta = 200$) (Machado et al., 2016; He et al., 2020). The crystallinity of the membranes produced by the variation of acerola in the culture medium (AC1, AC3, and AC5) resulted in 81.4%, 95.3%, and 96.7%, respectively. High purity and crystallinity are important characteristics to improve the mechanical properties of bacterial cellulose (Huang et al., 2014).

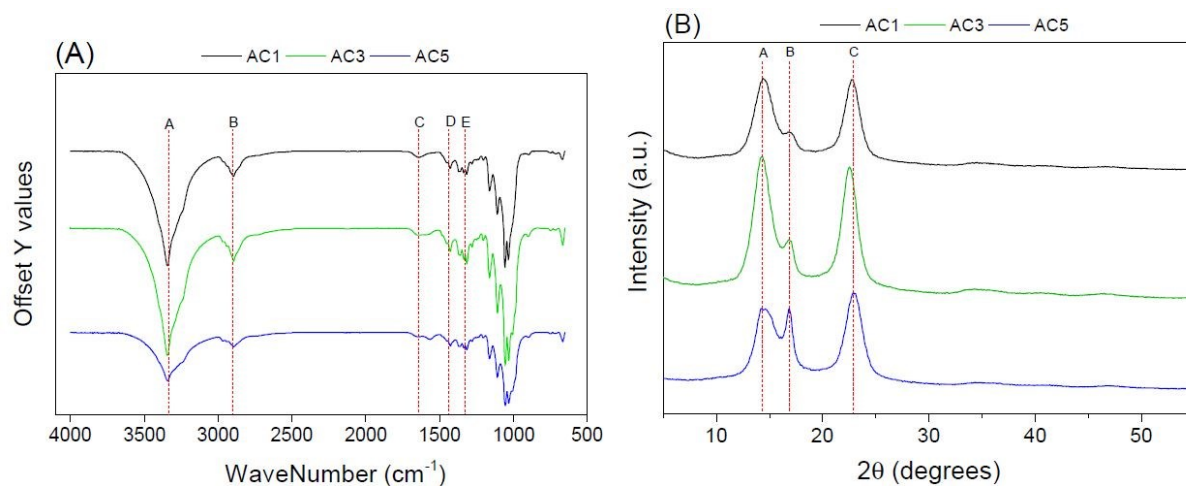
Figura 3.4. Scanning electron microscopy (10,000 x) of the bacterial cellulose surfaces: surface in contact with liquid (a) AC1, (b) AC3, (c) AC5, and surface in contact with air (d) AC1, (e) AC3, and (f) AC5.



The characterization results showed similar characteristics with bacterial cellulose produced by isolated bacteria and in synthetic culture medium, as previously described. These results are important because the medium used in the production process could be replaced or supplemented with acerola extracts, since one of the drawbacks of industrial production is the cost of synthetic media (Islam et al., 2017). This finding can improve the production and commercialization of bacterial cellulose, which is still little exploited. Few

companies, distributed in United States, Canada, Brazil and Poland, produce on an industrial scale, generally for biomedical applications (Portela et al., 2019).

Figure 3.5. FTIR (A) and DRX (B) of bacterial cells in kombuchas containing 1% (AC1), 3% (AC3), and 5% (AC5) of acerola byproduct.



3.4 CONCLUSIONS

The acerola byproduct proved to be a promising raw material for the production of kombucha-like beverage. By increasing its amount, the phenolic and vitamin C content also increased in the cultivation medium, affecting the fermentation kinetics. The content of bioactive compounds accelerated the metabolism of microorganisms, increasing the consumption of substrate, the production of ethanol, acetic acid, and bacterial cellulose. The physicochemical properties of cellulose were also influenced by the concentration of acerola byproduct, producing a more crystalline and dense structure. These results offer the opportunity to explore the acerola byproduct as raw material for kombucha, and also as cultivation medium for the production of bacterial cellulose.

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CAPÍTULO 4

4 ARTIGO II: SUBPRODUTO DE ACEROLA: UMA ALTERNATIVA PARA PRODUZIR CELULOSE BACTERIANA POR CEPA ISOLADA DA KOMBUCHA

ACEROLA BY-PRODUCT: AN ALTERNATIVE TO PRODUCE BACTERIAL CELLULOSE BY ISOLATED KOMBUCHA STRAIN

ABSTRACT

The production of bacterial cellulose has been explored in recent years because of its physico-chemical, morphological, and thermal characteristics. Thus, it has been possible to apply it in several areas, such as nanotechnological and biomedical applications, bioprocesses, food ingredients, and packaging material. An alternative for the production of bacterial cellulose is the use of alternative means (substituting as a source of carbon or nitrogen) to decrease its production cost. The by-products of fruit processing have been widely studied in terms of their application for the production of bacterial cellulose. Therefore, the objective of the work was to isolate the kombucha cellulose-producing strain and then to produce bacterial cellulose using this strain and acerola by-product (5% w/v), comparing with the traditional Hestrin-Schramm (HS) medium. The results reveal that the cellulose production was higher for the HS medium, reaching 2.91 g/L at the end of 12 days of fermentation, whereas, for the medium containing acerola by-product (AC), produced about 2.26 g/L. Regarding the characterization of cellulose, both were morphologically similar and also presented as type I cellulose. In addition, both indicated high crystallinity, the highest for the AC medium (90.8%).

Keywords: Fermentation, physico-chemical characterization, acetic acid bacteria, kombucha.

4.1 INTRODUCTION

The most abundant polymer on Earth is cellulose. Cellulose is a high molecular weight linear polymer that is composed of glucose units linked by (1–4) β -glycosidic linkages. The ordered crystalline arrangements are the result of hydroxyl groups that are involved in several intra- and intermolecular hydrogen bonds (Park et al., 2010; Petersen and Gatenholm, 2011; Ruka et al., 2013; Zhang et al., 2018). Its use has increased expressively due to its biodegradable, sustainable, and renewable characteristics, and to its fibrillar and potential nature as a reinforcement material in composites. Cellulose from plants has been widely used worldwide. However, bacterial cellulose (BC) may be an interesting alternative because of its properties such as purity and highly crystalline nanostructure (Ruka et al., 2013). Both have the same molecular formula but different network structures. BC is synthesized by gram-negative bacteria in a liquid sugar matrix.

The diverse biotechnological applications of BC are promising. BC is one of the products of the new era of Green Chemistry products and may be used in nanotechnological and biomedical applications, bioprocesses, food ingredients, and packaging material (Neera et al., 2015; Cacicedo et al., 2016; Azeredo et al., 2019). Also, the physical, chemical, or enzymatic treatments can lead to different properties of crystallinity, purity, surface reactivity, crystalline structure, morphology, among others, facilitating its use due to this great versatility (Machado et al., 2018).

The main cellulose-producing microorganism is *Komagataeibacter xylinus*. However, several other microorganisms have been reported in the literature regarding its cellulose production: *Komagataeibacter pasteurianus* and *Komagataeibacter lovaniensis* (Çoban and Biyik, 2011), *Komagataeibacter sacchari* (Gomes et al., 2013), *Komagataeibacter medellinensis* (Gayathri and Srinikethan, 2019), *Komagataeibacter saccharivorans* (Gayathri and Srinikethan, 2019), *Komagataeibacter hansenii* (Güzel and Akpınar, 2019), and *Komagataeibacter rhaeticus* (He et al., 2020). In the present work, the cellulose-producing strain was isolated from the kombucha.

BC production and its implementation are still considered high costly, which may be a challenge for industry. Thus, an alternative would be to replace the medium with a cheaper carbon/nitrogen source that would still obtain significant BC production (Azeredo et al., 2019; Güzel and Akpınar, 2019). Agro-industrial by-products have been revealed an excellent possibility for BC production due to low cost and large scale production, besides then

attractive characteristics for fermentation (Hussain et al., 2019). Some authors, obtained relevant results using agro-industrial byproducts, such as: 3.24 g/L of BC production using sugar cane juice and pineapple residues as carbon source (Algar et al., 2014), 2.67 g/L of BC production using discarded durian shell waste as carbon source (Luo et al., 2017), and 2.8 g/L of BC production using pineapple peel and sugar cane juice as a nitrogen source (Castro et al., 2011). In our previous work (Leonarski, 2021) extracts from acerola by-product fermented by kombucha consortium. Thus, high concentrations of cellulose were obtained (4.0 g/L) (Leonarski et al., 2021). It was found that the increase in the content of the by-product used to prepare the fermentation medium increased the production of bacterial cellulose due to the greater content of bioactive compounds that accelerate the microorganism metabolism. *Komagataeibacter rhaeticus* was identified as the main cellulose-producing bacteria from the kombucha consortium using high-throughput sequencing technologies (Leonarski et al., 2021).

Therefore, this work aims to isolate the kombucha cellulose-producing strain, perform its biochemical characterization, and then verify for the first time the production kinetics using the acerola by-product as a fermentation medium. In addition, the morphological and physico-chemical properties of the BC were evaluated.

4.2 MATERIAL AND METHODS

4.2.1 Materials

This study used the following materials: Glucose, Fructose, Cycloheximide, Ampicillin, Coomassie Brilliant blue, Bromocresol green, TEMED (N,N,N',N'-tetramethylethane-1,2-diamine) (Sigma-Aldrich, St. Louis, USA); Citric acid, Mannitol (Vetec, Rio de Janeiro, Brazil); PDA: Agar Potato Dextrose and Peptone Bacteriological (Himedia, Mumbai, Índia); Yeast extract, Peptone, Agar Medium and Tryptone (KASVI, São José dos Pinhais, Brazil); Congo Red (Metaquímica, Jaraguá do Sul, Brazil); Sodium acetate, Bromothymol blue, Calcium Carbonate, Disodium phosphate (Dinâmica, Indaiatuba, Brazil); Sodium lactat, Ethyl alcohol (Ethanol) PA (Neon, Suzano, Brazil). Acerola by-product was obted from juice clarification step, which was supplied by a juice producing industry from Ceará, Brazil. The by-product, basically consisting of residual pulp, was dried in a vacuum oven at 40 °C for 48 h, and then milled in a knife-mill (1.0 mm).

4.2.2 Methods

4.2.2.1 *Kombucha culture and bacteria isolation*

The SCOBY was obtained from a local source in Florianópolis (Brazil) and maintained in sugared green tea. The tea was filtered under sterile conditions, then was added 35 g/L of glucose plus 35 g/L of fructose, 10% (v/v) of liquid broth, and 4% (w/v) of biofilm. Fermentation was performed at 30 °C for 10 days.

4.2.2.2 *Acetic Acid Bacteria Isolation*

BC-producing strain was isolated from the kombucha consortium. Serial dilution was performed with 0.1 mL of kombucha tea and 0.9 mL of peptone (0.1%). Strains were grown on Luria Bertani (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 16 g/L agar medium) without the salt component at 30 °C for 5 days. Congo red (0.04 g/L) and coomassie brilliant blue (0.02 g/L) were added in the medium to visualize cellulose (Römmling and Lünsdorf, 2004).

Isolation was carried on HS medium (Hestrin and Schramm (1954): 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptones, 2.7 g/L disodium phosphate, 1.15 g/L citric acid, 15 g/L agar medium containing 500 mg/L cycloheximide to inhibit yeast growth. This procedure was consecutively repeated four times (30 °C for 7 days each).

After isolation, glucose-yeast extract-calcium carbonate (GYC) medium (50 g/L glucose, 10 g/L yeast extract, 5 g/L CaCO₃, 2 g/L agar medium) was used as a selective medium of acetic acid bacteria growth at 30 °C for 5-7 days (El-Salam, 2012).

4.2.2.3 *Biochemical Characterization of Acetic Acid Bacteria*

The Gram staining technique was conducted using a small drop of liquid medium culture onto a glass slide, covering with violet crystal for 30-60 s, washing with distilled water, adding lugol for 2 min, washing with water, bleaching with ketone alcohol, washing the slide again, covering with fuchsin for 30 seconds, and finally washing the slide with water and drying the slide on filter paper. The evaluation was performed on a microscope (Olympus CX21, Zhejiang, China) with a 100x objective lens.

Oxidase test was conducted with 50 µL of bacterial suspension deposited on a strip of filter paper (previously sterilized). Then, a drop of the aqueous solution of TEMED

(N,N,N',N'-tetramethylethane-1,2-diamine) 1% was deposited on the culture. If there is no color change, the test is negative (expected to *K. rhaeticus* strain), and if the color turns purple, it is positive.

A colony was deposited on a slide for the catalase test. Then, a drop of 3% (v/v) hydrogen peroxide was deposited on the strain. If bubbles appear, the test is positive (expected to *K. rhaeticus* strain). If there is no change, it is negative (Videira et al., 2007).

Oxidation of ethanol was performed using Carr medium (30 g/L yeast extract, 20 g/L agar, 0.02 g/L bromocresol green, and 2% (v/v) ethanol), incubated at 30 °C for 4-6 days (Maal et al., 2010; Mukadam et al., 2016).

Oxidation of acetate and lactate followed the method of Leifson (1953), with some modifications. The medium was composed of peptone 0.3%, yeast extract 0.2%, sodium acetate or sodium lactate 0.2%, bromothymol blue 0.002% and pH 6.5. Incubation was performed at 30 °C for 20 days. According to the author, the gram-negative strain must reveal an alkaline reaction, which makes the medium dark-blue.

4.2.2.4 *BC membrane production and purification*

In the preparation of pre-inoculum, a colony of the isolated strain was added to 10 mL of the HS medium remaining at 30 °C for 7 days. Afterward, 10% (v/v) of pre-inoculum was added in HS medium at 30 °C for 7 days. This solution was used in the subsequent steps.

The production of bacterial cellulose was performed in medium with acerola by-product extract prepared with 5% w/v and in HS medium as a control. Hydrothermal extraction was conducted at 121 °C for 15 minutes for the acerola by-product medium. The medium was filtered under sterile conditions, and 20 g/L of glucose solution was added (previously sterilized). HS medium was sterilized at 121 °C for 15 minutes. At room temperature, 10% (v/v) of inoculum was added in both media, which were distributed in a 6-well 10mL cell culture plate. The kinetics of cellulose growth (30 °C) was evaluated measuring the pH and dry weight until the 12th day at 2-day intervals. Purification of the membranes was performed by immersion in 0.1 M NaOH at 90 °C for about 1-2 h. The membranes were washed with distilled water at 50 °C for 24 hours and then washed every hour until the pH was neutral.

4.2.2.5 *pH measurement*

The pH value was measured in triplicate during fermentation with a pHmeter K39-2014B (Kasvi, São José dos Pinhais, Brazil).

4.2.2.6 *BC concentration*

After purification, BC membranes were frozen for 24 h, and lyophilized (Liotop 101, Liobras, São Carlos, Brazil) for 48 h. The cellulose concentration was expressed as grams of dry weight per liter of medium (g/L).

4.2.2.7 *Morphological and physicochemical analysis*

The morphological characteristics of cellulose were investigated using Scanning Electron Microscopy JSM 6390LV (JEOL, Tokyo, Japan). Fourier transform infrared spectra (FTIR) of lyophilized cellulose were recorded in a Cary 600 Series (Agilent Technologies, St. Clara, United States). The crystallinity was determined by X-ray diffractometry (XRD) MiniFlex600 (Rigaku, Tokyo, Japan. All methodologies are described by Leonarski et al. (2021).

The interplanar distances (d-spacing), crystallite size, and crystallinity were calculated according to Bragg's law Eq. (1), Scherrer's formula Eq. (2), and Eq. (3), respectively:

$$d - \text{spacing (nm)} = \left(\frac{\lambda}{2 \cdot \sin(\theta)} \right) \quad (1)$$

where θ is the angle between the plane and the diffracted and λ is the wavelength of the X-rays

$$\text{Crystallite size (nm)} = \left(\frac{0.9 \cdot \lambda}{FWHM \cdot \cos(\theta)} \right) \quad (2)$$

where FWHM is width of the peak at half the maximum height, θ is the Bragg's angle, and λ is the wavelength of the X-rays.

$$\text{Crystallinity (\%)} = \left(\frac{S_c}{S_t} \right) \times 100 \quad (3)$$

where S_c is the sum of the net area, and S_t is the sum of the total area.

4.2.2.8 Statistical analysis

Statistical analysis was conducted by Past software. The results were evaluated by analysis of variance (ANOVA), and the significant differences were determined using Tukey's Test at a probability level of less than 5% ($p < 0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 Biochemical characterization

The results of the biochemical characterization of isolated acetic acid bacteria are shown in Table 4.1. The isolation of acetic acid bacteria was confirmed by the halo formed by acidification of the GYC medium by the strain (Figure 4.1A). Acetic acid bacteria produce halo zone on GYC medium by the acid hydrolysis of CaCO_3 (Vashisht et al., 2019).

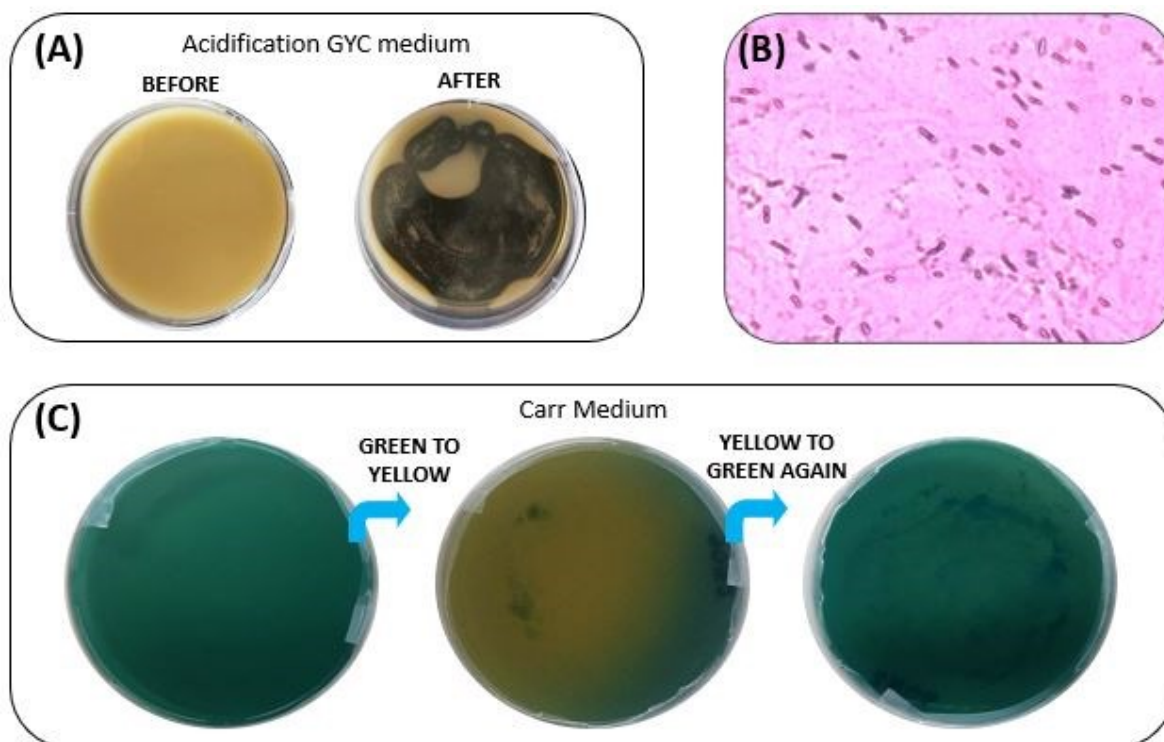
Table 4.1. Biochemical characterization of acetic acid bacteria isolated from kombucha.

Biochemical assay	Result
Gram stain	Gram negative
Catalase	+
Oxidase	-
Oxidation of acetate	+
Oxidation of lactate	+
Carr Medium	from green to yellow than green again

The strain was identified as gram-negative (Figure 4.1B), catalase positive, and oxidase negative. The same results were obtained by Semjonovs et al. (2017) isolating cellulose-producing bacteria from kombucha. According to Leifson (1953), gram-negative bacteria produce an alkaline reaction in a medium containing lactate or acetate due to oxidation. This was observed by increasing the pH and changing the color (dark blue) of the medium as the bacteria grew. These results classify the strain as *Acetobacter* genus (Mukadam et al., 2016; Thanh, 2019). The growth in Carr medium is generally used to differentiate strains of the genus *Acetobacter* from *Gluconobacter*. *Acetobacter* strains are capable of oxidizing ethanol to acetic acid and subsequently to CO_2 and H_2O through the tricarboxylic cycle both in acidic (pH 4.5) and neutral (pH 7.0) conditions. *Gluconobacter* strains have a non-functional tricarboxylic cycle, being unable to oxidize most organic acids (Kadere et al., 2008). Therefore, strains of the *Acetobacter* genus change the medium from green to yellow and back to green (Figure 4.1C), whereas those of the *Gluconobacter* genus change the medium from green to

yellow but do not return to green color. Therefore, the biochemical assays identified that the isolated bacteria were acetic acid and belonged to the genus *Acetobacter*.

Figure 4.1. (A) Growth of isolated strain in GYC medium, (B) Gram stain assay seen under the microscope (100x), (C) Growth of isolated *Acetobacter* strain in Carr Medium.

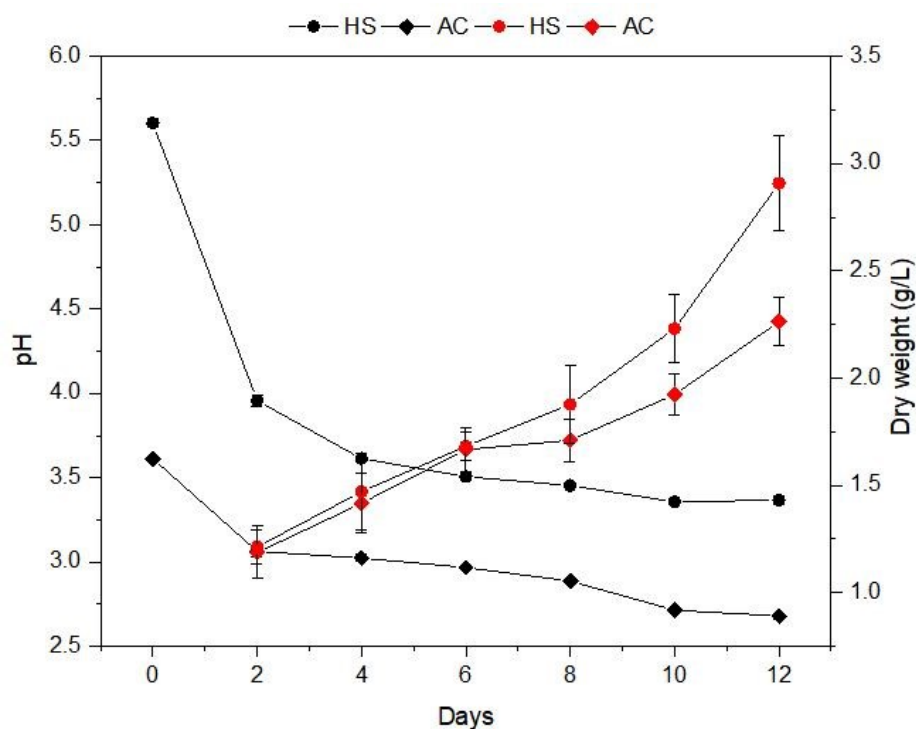


4.3.2 BC production

The pH variation was evaluated during the BC production. After inoculation, the pH of HS and AC medium was 5.6 and 3.6, respectively. The greatest pH drop occurred for both media in just 2 days of fermentation to 3.96 for the HS medium and 3.06 for the AC medium. The pH practically remained constant after the eighth day of fermentation for the HS medium and after 10 days of fermentation for the AC medium reaching 3.37 and 2.68, respectively. He et al. (2020), also verified a greater drop in the pH value in just two days of fermentation for *K. rhaeticus* strain in HS medium. This indicates the production of acids by the bacteria favored the BC production. However, the ideal pH may vary according to the carbon source (Lin et al., 2013). The author also verified that after 7 days the pH remained practically constant until the end of the fermentation, reaching around 3.6. Semjonovs et al.

(2017) produced cellulose using the *K. rhaeticus* strain, reaching pH of 4.53 and 4.23 in the end of fermentation using apple juice and cheese whey as substrates, respectively.

Figure 4.2. Kinetic profile of pH and bacterial cellulose concentration in HS and AC medium for 12 days at 30 °C.



The BC production in both media was the same until the sixth day of fermentation. Then, the production rate in HS medium increased, reaching the final concentration of 2.91 g/L, whereas the AC medium produced 2.26 g/L. The AC medium supplemented with glucose revealed considerable production when compared to the HS medium. There is a huge potential for using by-products from the agroindustry without complex pre-treatment and revealing similar or even superior production to the standard medium fermentation (Hussain et al., 2019). Algar et al. (2014), for example, evaluated the production of cellulose by *Gluconacetobacter medellinensis* using sugar cane juice and pineapple residues as sources of carbon and other nutrients, and obtained 3.24 g/L of BC in 13 days. Urbina et al. (2017), reached 2.5g/L in 14 days of fermentation using *G. medellinensis* ID13488, apple, and cane sugar by-products. These values were similar to those found in this work.

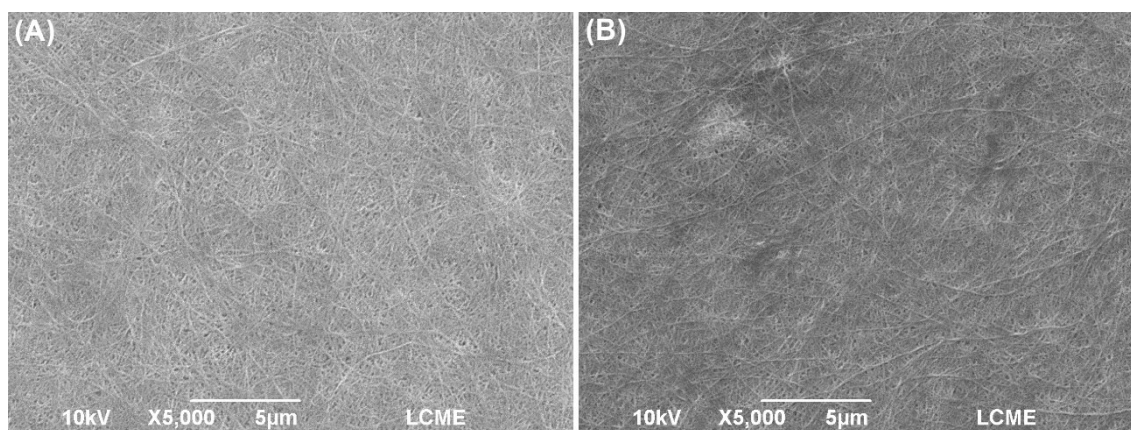
Machado et al. (2018) used *K. rhaeticus* to obtain cellulose, partially replacing glucose with sugarcane molasse in the HS medium, obtaining 4.0 g/L in 5 days. Pacheco et al. (2017) using the same bacteria produced 6.0 g/L of BC in 7 days of fermentation using HS

medium supplemented with cashew tree residues. The cellulose production reported by those authors was higher than that obtained in the study. However, both authors used the HS medium, commonly used in cellulose production, justifying this higher production.

4.3.3 Morphological and physicochemical properties of cellulose

BC morphology was verified by scanning electron microscopy (SEM). In our observations (Figure 4.3), both samples revealed a dense structure and fibrils uniform with almost nonexistent interfibrillar space. There was no difference in BC produced by HS or AC medium. A similar morphological structure was observed in BC produced by *Gluconacetobacter xylinus* (Ruka et al., 2013; Bandyopadhyay et al., 2018) and by *Komagataeibacter rhaeticus* (He et al., 2020).

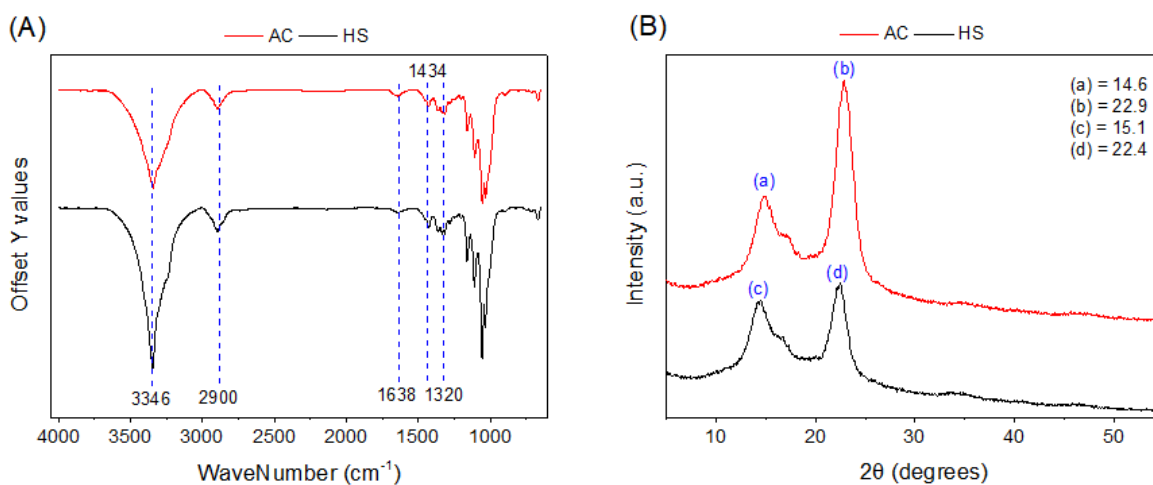
Figure 4.3. Scanning electron microscopy (10,000 x) of the BC by (A) HS, and (B) AC medium.



The physicochemical properties of BC were performed using FTIR spectra and DRX. Some authors have reported FTIR spectra similar to BC using different media, indicating that the polymer has a similar chemical structure (Algar et al., 2014; Pacheco et al., 2017; Machado et al., 2018), which is also observed in this work. FTIR spectra were illustrated in Figure 4A. Characteristic bands of bacterial cellulose were observed in 3346 cm^{-1} (-OH stretching), 2887 cm^{-1} (-CH stretching) and 1632 cm^{-1} corresponding to adsorbed water molecules. The 1433 cm^{-1} band is one of the main peaks, associated with symmetric (CH_2) bending vibration of cellulose I- α type. The -CH bending vibration, OH in-plane bending, and C-O stretching correspond to 1370 , 1320 , and 1060 cm^{-1} , respectively (Thorat and Dastager, 2018; Ashjarian and Sheybani, 2019; Illa et al., 2019). Therefore, there was no

mercerization by alkaline treatment. Bacterial or vegetal native cellulose are the type I, composed of two similar crystal allomorphs (I_α e I_β), which main structure is composed of glucose units. In plants, cellulose has a complex structure due to cellulose intercalated with hemicellulose, pectin, and lignin, which consists of two-chains monoclinic unit cells (I_β). However, BC is pure and composed of one-chain triclinic unit cells (I_α) (Dima et al., 2017; Illa et al., 2019).

Figure 4.4. FTIR (A) and DRX (B) of bacterial cellulose produced in HS and AC medium.



Cellulose was described as a two-phase combination: crystalline (ordered) and amorphous (less ordered) regions (Illa et al., 2019). The changes in BC morphology as a result of chemical and mechanical treatments can be observed by two parameters provided by DRX analysis: crystalline peaks angle and variation of interplanar distances (Dima et al., 2017). In Figure 4B, we can see that both BC reveal crystalline peaks around 15° and 22.5° , which is similar to the pattern of cellulose I and confirm the data obtained by the FTIR analysis. The values of Full Width at Half Maximum (FWHM), interplanar distances (d-spacing), crystallites size, and crystallinity degree were illustrated in Table 2. The values of FWHM and d-spacing were close for the HS and AC medium when calculated at the same crystallinity peak. Grande et al. (2009) found a value of 1.93° for the FWHM at the peak close to 15° , a lower value compared to those reported in the study. While for point 22.5° (Lee et al., 2011), they found 1.71° for pure bacterial cellulose, a value close to that found in this work for both samples. Grande et al. (2009) also presented values similar to those reported in this work for the interplanar space (d-spacing) between the crystallites, 0.61 and 0.39 nm at $2\theta = 14.5$ and 22.6 , respectively.

Table 4.2. Full width at half maximum (FWHM), interplanar distances (d-spacing), crystallites size, and crystallinity degree of BC by HS and AC medium.

Sample	2 θ	FWMH ($^{\circ}$)	d-spacing (nm)	Crystallite size (nm)	Crystallin- ity (%)
HS	14.62	3.02	0.60	2.65	85.9
	22.37	1.77	0.40	4.58	
AC	15.05	2.80	0.59	2.86	90.8
	22.88	2.07	0.39	3.91	

Regarding crystallite size, both samples indicated higher crystallite for the peak 22.5 $^{\circ}$, being this value higher for the HS medium (4.58 nm). For peak 14.5 $^{\circ}$, the samples revealed closer values. However, AC presented a higher value (2.86 nm). The average crystallite size was 3.61 nm for HS and 3.38 nm for AC, indicating a similar crystalline structure. Compared to the literature, higher values were reported for bacterial cellulose produced by *K. rhaeticus* equal to 4.5 nm and by *K. xylinus* equal to 4.8 nm (He et al., 2020). Commonly, BC presents small crystallite sizes and high crystallinity (Singhsa et al., 2018). Usually, the widening of the peaks is a significant contributor to the increase in the amorphous phase of cellulose. However, the crystallite size and non-uniform strain within the crystal are two intrinsic factors that influence peak broadening (Park et al., 2010).

The crystallinity of both samples was high, 85.9% and 90.8% for HS and AC medium, respectively. He et al. (2020) found 85% of crystallinity using *K. rhaeticus* strain for cellulose production, whereas Güzel and Akpınar (2019) found 87.5% using the *K. hansenii* strain for cellulose production, both in HS medium. The authors also verified the cellulose crystallinity using lemon peel, mandarin peel, orange peel, and grapefruit peel as a medium source, obtaining values between 79-92%. In previous work, (Leonarski et al., 2021), crystallinity between 81.4-96.7% was found for BC using acerola by-product as a substrate for kombucha-like beverage production.

The alkaline treatment applied to remove bacterial, proteins, and other fermentation residues is used to achieve cellulose purification (Dima et al., 2017). However, if this treatment is intense, it can cause cellulose to mercerize, changing it from type I to type II (Moharram and Mahmoud, 2008; Vazquez et al., 2013). According to Bandyopadhyay et al.

(2018), type I cellulose has better mechanical properties than type II cellulose. In this study, the alkaline treatment did not change the cellulose structure to transform it into type II.

4.4 CONCLUSION

The bacterium isolated from the kombucha was identified as an *Acetobacter* genus by biochemical analysis. Considerable cellulose production after 12 days of fermentation was obtained, both in HS and AC medium. In addition, the medium containing AC did not change the properties of the polymer and revealed greater crystallinity compared to that produced in the HS medium. Although the production was considerable, the parameters for the production were not optimized. Therefore, this would be the next step to use the by-product of acerola more appropriately.

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CAPÍTULO 5

5 CONCLUSÕES

Neste trabalho foi avaliado a utilização de subproduto de acerola como substituto do chá para produção de bebida tipo kombucha. A substituição apresentou resultados interessantes, houve a produção de ácido acético, etanol e celulose bacteriana comum na kombucha tradicional. Entretanto, quanto maior foi a quantidade de extrato adicionada (m/v), maior foi a produção de ácido acético e de celulose bacteriana, enquanto o etanol apresentou valores similares independentes da concentração do extrato.

Além disso, durante a fermentação da kombucha houve o aumento de compostos bioativos, verificado pelo aumento de compostos fenólicos e atividade antioxidante. Com relação a vitamina C, ocorreu a degradação durante a fermentação. Entretanto, esta foi inferior nas amostras que continham maior quantidade de extrato (maior quantidade de vitamina C inicial), sugerindo que a maior quantidade de ácido ascórbico auxilia na proteção deste composto.

A celulose bacteriana produzida apresentou morfologia e características similares para as três amostras. Entretanto, foi observado maiores espaços fibrilares para a mostra com 1% do substrato de acerola. Além disso, esta amostra foi a que apresentou menor cristalinidade se comparada com as demais amostras de 3 e 5% de subproduto de acerola.

A segunda etapa do trabalho iniciou-se pelo isolamento da bactéria produtora de celulose, identificada na primeira etapa do trabalho como *Komagataeibacter rhaeticus* presente no consórcio da kombucha utilizada. Através de análises bioquímicas foi verificado que a cepa isolada é do gênero *Acetobacter*. A identificação da bactéria isolada precisa ainda ser confirmada.

A produção de celulose em meio contendo 5% de subproduto de acerola (anteriormente verificado como a melhor entre as testadas (1, 3 e 5% m/v) para a produção de celulose na kombucha), foi igual a 2,26 g/L após 12 dias de fermentação. Em meio HS, a produção foi igual a 2,91 g/L, superior ao meio AC. Entretanto, considerando que o meio AC possui custo inferior ao meio tradicional, pode-se considerar este vantajoso quanto a sua utilização para produção da celulose.

A caracterização da celulose não demonstrou diferença na produção em diferentes meios. Entretanto, a celulose produzida pelo meio AC apresentou maior cristalinidade

comparada ao meio tradicional. Embora os resultados utilizando o meio AC tenham sido promissores, deve-se procurar otimizar as condições do processo para um possível aumento na produção de celulose.

Os resultados obtidos neste trabalho demonstraram o subproduto da acerola como um material potencial para aplicação no desenvolvimento de produtos e também para a produção da celulose bacteriana. Ambos apresentaram resultados interessantes e provaram de fato ser uma alternativa viável para a utilização deste subproduto.

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ANEXO A

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Production of kombucha-like beverage and bacterial cellulose by acerola byproduct as raw material

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ABSTRACT

In this study, acerola byproduct was used as a new raw material for the production of kombucha-like beverage and bacterial cellulose. The amount of acerola byproduct (1, 3 and 5% w/v) in the preparation of the beverage affected the product formation. The final concentration of ethanol was similar in all samples, approximately 9.5 g/L, but it was achieved at 12, 9 and 6 days, respectively, suggesting that yeast metabolism was accelerated in the presence of higher amounts of acerola byproduct. In addition, the highest productions of acetic acid (16.3 g/L) and bacterial cellulose (4.0 g/L) were achieved with 5% byproduct. Less porous and denser membranes were obtained with a highly crystalline structure (96.4%), also in higher amounts of acerola byproduct. These results suggest that the increase in the content of polyphenols and vitamin C, naturally present in the acerola byproducts, increased the concentration of acetic acid, and improved the environment for bacterial cellulose production.

1. Introduction

Kombucha is a fermented tea drink of Asian origin, commonly prepared with black or green tea (Jayabalan, Marimuthu, & Swaminathan, 2007). A symbiotic culture of bacteria and yeasts (SCOBY) is responsible for the fermentation process, resulting in a refreshing, bittersweet, lightly carbonated drink. The characteristics of kombucha can vary greatly with several factors, such as the type of tea or the raw material, the microorganisms present in SCOBY, and the fermentation time and temperature (Chakravorty et al., 2016; Jayabalan, Malbaia, Lončar, Vitas, & Sathishkumar, 2014). Typically, the fermentation time is 7–15 days. Extended periods can yield a drink with an intense acid flavor (similar to vinegar). In addition to acetic acid, different components during the fermentation of kombucha are produced, due to the presence of various strains of bacteria and yeast. Ethanol, acetic, lactic, and glucuronic acids are the most common products found in kombucha fermentation. The final drink also has a large quantity of tea-derived polyphenols (Chakravorty et al., 2016; Jayabalan et al., 2007). Another important product of kombucha fermentation is the biofilm produced by acetic acid bacteria. It consists of pure cellulose fibrils, has

great water retention capacity, high crystallinity, and thermostability (Emiljanowicz & Malinowska-Pańczyk, 2019). The cellulose yield varies according to the fermentation conditions and the species of producing bacteria present in SCOBY (Goh et al., 2012).

Currently, some studies have presented alternative raw materials for the fermentation of kombucha-like beverages, such as milk (Hrnjez et al., 2014); coffee (Watawana, Jayawardena, and Waisundara, 2015); cactus pear juice (Ayed & Hamdi, 2015); grape juice (Ayed, Ben Abid, & Hamdi, 2017); snake fruit (Zubaidah et al., 2018); rooibos tea (Gaggla et al., 2019); black and red goji berry (Abuduabifia & Tamer, 2019); African mustard leaves (Rahmani et al., 2019); and black carrot juice, cherry laurel, blackthorn, and red raspberry (Ulusoy and Tamer, 2019). The use of alternative raw materials can promote the development of new beverages with novel functional properties due to their diverse composition, mainly focused on polyphenols (Emiljanowicz & Malinowska-Pańczyk, 2019).

An alternative to be considered is the use of industrial byproducts from fruit processing, such as acerola. Acerola is considered a "superfruit" due to the content of vitamin C and polyphenols (Prakash and Baskaran, 2018). The compounds present in acerola have been

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