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REBECA PRISCILA FLORA CATARINO

CELULOSE BACTERIANA: EXPLORANDO INFLUÊNCIAS NA PRODUÇÃO POR BACTÉRIAS DO ÁCIDO ACÉTICO

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Tese de doutorado apresentada à Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutora em Ciência de Alimentos.

Orientador: Profa. Dra. Wilma Aparecida Spinosa

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À Deus, em Quem depositei minha fé e caminhei na certeza de que Ele abre caminhos. És o começo, meio e fim!

You may write me down in history
With your bitter, twisted lies,
You may trod me in the very dirt
But still, like dust, I'll rise […].

Maya Angelou (Poema: Still I Rise)

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RESUMO

Bioprocessos permitem a obtenção de novos produtos de importância econômica nos diversos setores industriais. Na categoria dos biopolímeros a celulose bacteriana (CB) se destaca como um exopolissacarídeo obtido por fermentação acética com estrutura semelhante a celulose vegetal e características que permitem sua aplicação na indústria alimentícia como espessante, estabilizante, filmes e matriz para imobilização de compostos bioativos. Entretanto, a viabilidade econômica deste produto está fortemente atrelada ao rendimento e ao elevado custo de produção. Dentre os fatores que limitam a produção de CB em larga escala destacam-se o cultivo e manutenção de células viáveis, a formulação de meio de cultivo de baixo custo, e o controle do método de cultivo empregado. O objetivo deste trabalho foi investigar a influência da cepa, meio de cultivo e método no bioprocesso para a produção de celulose bacteriana por bactérias do ácido acético e otimizar a produção de CB partir de um meio de baixo custo formulado a base de sacarose e soro de leite. A otimização da produção de CB pela cepa *Komagaeibacter intermedius V-05* a partir do meio alternativo foi conduzida utilizando Metodologia de Superfície de Resposta (MSR) por meio de delineamento central composto (CCD), considerando as variáveis concentração de soro de leite, concentração de sacarose e pH. A fermentação foi conduzida em cultivo agitado (150 RPM) à 30 ºC por 7 dias. O material obtido foi caracterizado por espectroscopia de infravermelho (FTIR), difração de raios-X (DRX), termogravimetria (TG/DTG) e capacidade de retenção de água (CRA). Neste estudo, utilização de fontes de C e N alternativas influenciou na forma da CB obtida, sendo observada a formação de suspenção fibrosa e massa irregular dispersa no meio de cultivo. O rendimento (1,05 g L^{-1}) a partir do meio otimizado (22.52 g L^{-1} sacarose, 43.80 g L^{-1}, soro de leite e pH 5.39) foi equivalente ao meio padrão Hestrin-Schramm (0.96 g L^{-1}), demonstrando que o meio formulado pode substituir o meio convencional nas condições de cultivo utilizadas para a cepa *K.intermedius V-05*. A caracterização da CB comprovou sua elevada cristalinidade (82.40% 89.12%), estabilidade térmica (235 ºC - 310 ºC), capacidade de retenção de água (223.41 - 227.11g) e pureza. Apesar da versatilidade do biopolímero, o rendimento e o custo da produção são fatores limitante para a consolidação da CB em algumas áreas de aplicação. Entretanto, a utilização de cepas eficientes, matéria-prima de baixo custo, adequação e controle do método de fermentação, bem como o conhecimento sobre a interação destes fatores são estratégias que contribuem para a viabilidade econômica do processo em escala industrial.

Palavras-chave: fermentação acética; *Komagataeibacter*; otimização; resíduos agroindustriais; biopolímeros.

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ABSTRACT

Bioprocesses can develop new products for industrial applications. In this context, cacterial cellulose (BC) is a biopolymer resulting from acetic fermentation, and its structure is similar to plant cellulose. The exopolysaccharide characteristics allow its application in the food industry as a thickener, stabilizer, edible film, and matrix for bioactive compound immobilization. However, the economic viability of this product depends on the yield and production cost. Scale-up BC production is affected by cell cultivation and viability, the cost of the culture medium, and the process control. This study investigated the influence of strain, culture media, and cultivation methods on bacterial cellulose production by acetic acid bacteria and aimed to optimize biopolymer production using a low-cost medium formulated with sucrose and whey. Response Surface Methodology (RSM) and central composite design (CCD) were applied for BC production the optimization using *Komagaeibacter intermedius* V-05 strain and considering the variables pH and sucrose and milk whey concentration. The production was carried out under agitated culture (150 RPM) at 30°C for seven days. The material was characterized by infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetry (TG/DTG), and water-holding capacity (WHC). The use of alternative C and N sources influenced the shape of the BC obtained, resulting in a fibrous suspension and an irregular mass dispersed in the culture medium. The optimized medium (22.52 g L-1 sucrose, 43.80 g L-1 whey, and pH 5.39) provides a yield (1.05 g L-1) similar to the standard Hestrin-Schramm medium (0.96 g L-1), indicating that the formulated medium can replace the conventional under the cultivation conditions used in this work for the *K.intermedius* V-05 strain. BC characterization confirmed its high crystallinity (82.40% - 89.12%), thermal stability (235 ºC - 310 ºC), water holding capacity (223.41 - 227.11 g), and purity. Despite its versatility, the biopolymer yield and the cost of production are limiting factors for the application in some areas. However, using efficient strains and low-cost raw materials in addition to the control of the fermentation system and understanding the interaction of these factors can improve the economic viability of the process on an industrial scale.

Key-words: acetic acid fermentation; *Komagataeibacter*; production optimization; agro-industrial waste; biopolymers.

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1 INTRODUÇÃO

Os bioprocessos permitem a obtenção de produtos de interesse econômico e industrial através da transformação de fontes de carbono e nitrogênio, bem como outros nutrientes presentes no meio de cultura (Mandenius; Brundin, 2008). Dentre os produtos obtidos por processos biotecnológicos destacam-se os biopolímeros oriundos do metabolismo de diversos microrganismos, por exemplo, goma xantana, goma dextrana, pululana e celulose bacteriana (Pinto *et al.*, 2021).

A celulose bacteriana (CB) é um exopolissacarídeo obtido por diversos microrganismos dos quais as espécies de bactérias do ácido acético (BAA) se destacam devido à alta produtividade. É um homopolímero com estrutura similar a celulose vegetal e com propriedades que permitem sua utilização em alimentos, na indústria farmacêutica, biomédica e eletrônica, e tratamento de efluentes. Sua estrutura tridimensional origina-se da associação entre cadeias de glicose conectadas por ligações de hidrogênio entre grupamentos hidroxil da molécula de glicose (Phruksaphithak; Kaewnun;O-Thong, 2019; Salari *et al*., 2019). Dentre as características físico-químicas e estruturais destacam-se a alta cristalinidade, força mecânica, estabilidade térmica e capacidade de retenção de água (Foresti; Vázquez; Boury, 2017).

O primeiro relato deste biopolímero data de 1886 por Adrian Brown que observou a presença de uma película branca e gelatinosa originada na superfície do meio durante a fermentação acética para produção de vinagre. O material era denominado "mãe-do-vinagre" e apresentava a mesma estrutura, composição e reatividade que a celulose vegetal (Cacicedo *et al.*, 2016). Durante a produção do vinagre, o acúmulo de grandes volumes de celulose nos fermentadores é fator indesejável, uma vez que resulta em limpeza constante pelo operador. Do ponto de vista comercial, a presença de películas de celulose em vinagres orgânicos ou convencionais afeta a aceitação por parte do consumidor (Gomes *et al.*, 2018). Por outro lado, quando purificado, este biopolímero é uma fonte de celulose pura, além de ser biodegradável e biocompatível, o que permite sua reinserção na cadeia produtiva de diversas maneiras.

As bactérias do ácido acético (BAA), principal grupo de microrganismo associados à produção de celulose bacteriana, são reconhecidas por sua habilidade em oxidar etanol a ácido acético. São bactérias gram-negativas ou gram-variável, não formam esporos, apresentam forma de bastonete, com ocorrência em pares, cadeias ou isoladas. São catalase positiva e oxidase negativa, aeróbias estritas, mesófilas (28-30ºC) e capazes de sobreviver em ambientes extremante ácidos, entretanto, a faixa de pH ótima para seu crescimento é de 5,3 a 6,3 (Andrés-Barrao *et al.*, 2013; Hutkins, 2019; Mamlouk; Gullo, 2013). Pertencem à família

Acetobacteraceae que compreende os gêneros *Acetobacter*, *Gluconobacter, Gluconacetobacter, Komagataeibacter, Asaia, Neokomagataea, Granulibacter, Kozakia, Neoasaia, Swaminathania, Saccharibacter, Acidomonas, Tanticharoenia, Ameyamaea, Nguyenibacter, Swingsia, Commensalibacter, Endobacter* e *Bombella*, totalizando 19 gêneros (Kim *et al*., 2019; Malimas *et al*. 2017).

As espécies do gênero *Gluconacetobacter* foram anteriormente divididas em dois grupos de acordo com suas características filogenéticas e fenotípicas: *Gluconacetobacter xylinus* e *Gluconacetobacter liquefaciens*. O gênero *Komagataeibacter* surgiu a partir da reclassificação de espécies do grupo *Gluconacetobacter xylinus* (Yamada *et al*., 2012). As espécies deste gênero se destacam pela eficiência na conversão de diferentes fontes de nitrogênio na produção de celulose bacteriana (Malimas *et al*. 2017). Esse grupo de microrganismos é frequentemente encontrado em ambientes ácidos e ricos em açúcares, condições típicas de frutas, alimentos e bebidas fermentadas como *kombucha*, *kefir*, nata de coco e vinagre, principal produto obtido por BAA (Lynch *et al*., 2019).

Nos últimos anos, a produção de celulose a partir de microrganismos é alvo de pesquisas em diversos países uma vez que essa via de produção origina um material com propriedades superiores a celulose vegetal e de alta pureza, fator que permite sua aplicação em produtos de diversas áreas, por exemplo, produtos alimentícios, biomédicos e farmacêuticos (Lin *et al*., 2013; Vasconcelos *et al*., 2017). O interesse na utilização de microrganismos para obtenção de biopolímeros se deve a possibilidade de controle da produção e obtenção de materiais de alta pureza e com características desejadas, além de ser uma via de produção não-sazonal.

Na indústria de alimentos, a celulose é considerada uma fibra alimentar por ser uma substância não digerível por enzimas digestivas humanas. Neste sentido, a celulose obtida por via microbiana (celulose bacteriana) pode ser utilizada na elaboração de produtos de baixo valor calórico e ricos em fibra alimentar. Além dos benefícios nutricionais, este polissacarídeo pode exercer funções tecnológicas em alimentos, por exemplo, estabilizante, espessante, formação de gel, imobilização de compostos bioativos e desenvolvimento de filmes comestíveis (Fennema; Damodaran; Parkin, 2017; Oliveira; Roman, Seibel, 2018; Azeredo *et al.*, 2019).

Os fatores que influenciam no rendimento e característica da CB produzida podem estar relacionados ao microrganismo utilizado, a composição do meio de cultura, o método de produção (agitado, estático ou em biorreator) e parâmetros como pH, temperatura, velocidade de agitação e tempo de fermentação. A possibilidade de variar os parâmetros do processo permite a otimização da produção a fim de maximizar o rendimento em CB ou ainda obter

materiais com características e propriedades diferentes. Nos últimos anos, pesquisas realizadas em escala laboratorial visam otimizar a produção de celulose bacteriana bem como a sua aplicação. O Brasil está entre os dez países com maior número de publicações sobre o tema, acompanhado de China, Estados Unidos, Japão, Índia, Coréia do Sul, Alemanha, Espanha, Irã e Canada. A obtenção de celulose por via microbiana, é conduzida em condições controladas favorecendo as rotas metabólicas que levam a formação do biopolímero, possibilitando ainda obter características necessárias às aplicações desejadas (Marestoni *et al.*, 2021; Sperotto *et al.*, 2021).

O aumento da produção é o ponto de partida para explorar as diversas aplicações de celulose bacteriana. Nesse sentido, a seleção de microrganismos com elevado potencial de produção e o uso de meio de cultivo de baixo custo é primordial para a viabilidade econômica desse biopolímero, sendo essencial o conhecimento dos diversos fatores que envolvem a obtenção de CB, bem como a compreensão dos desafios e estratégias para a otimização e controle do bioprocesso. Neste contexto, metodologias estatísticas têm sido utilizadas para avaliar o efeito das diversas variáveis no rendimento, permitindo a otimização dos parâmetros de processo e redução de custos atrelados a componentes como fonte de carbono e nitrogênio (Bagewadi et al., 2020; Bilgi et al., 2016; Singh et al., 2016).

A Metodologia de Superfície de Resposta (RSM) é uma alternativa para a otimização de processos biotecnológicos. Esta técnica se destaca pela possibilidade de avaliar uma ampla faixa de parâmetros em tempo reduzido e sem utilização excessiva de recursos, uma vez que requer um número reduzido de experimentos (Aytekin; Demirbag; Bayrakdar, 2016; Yolmeh; Jafari, 2017). A definição das condições ótimas de cultivo passa pela seleção das variáveis que influenciam no processo, otimização, onde são construídos os modelos preditivos considerando a região ótima, e por fim, validação das condições otimizadas (Rodrigues; Lemma, 2009; Calado; Montgomery, 2003).

Portanto, este trabalho explora os principais fatores que influenciam na produção de celulose bacteriana por bactérias do ácido acético, em especial as espécies do gênero *Komagataeibacter* visando a produção em larga escala e disseminação desde biopolímeros nas diversas áreas de aplicação.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar a influência da cepa, meio de cultivo e método no bioprocesso para a produção de celulose bacteriana por bactérias do ácido acético e otimizar a produção de CB em cultivo agitado partir de um meio de baixo custo formulado a base de sacarose e soro de leite por *Komagataeibacter intermedius* V-05 (CMRP 4501), previamente isolada de caldos de fermentação de vinagre.

2.1.1 Objetivos Específicos

- Revisar os principais efeitos do método de cultivo, cepa e concentração e tipo de fonte de nutrientes na produção de celulose bacteriana;
- Otimizar o meio de cultura formulado com sacarose e soro de leite variando o pH inicial e a concentração de substrato utilizando Metodologia de Superfície de Resposta;
- Avaliar o efeito da alteração da fonte de carbono e nitrogênio no rendimento e características da celulose bacteriana em relação ao meio de cultivo convencional Hestrin-Schramm (HS).

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3 CAPÍTULOS DA TESE

Esta tese será apresentada na forma de dois capítulos descritos abaixo, seguidos de uma conclusão geral.

Capítulo I – Artigo de Revisão: Bioprocess for bacterial cellulose production by acetic acid bacteria: the influence of strain, culture media and cultivation

Capítulo II – Artigo científico: Low-cost medium for bacterial cellulose production by *Komagataeibacter intermedius* V05 under shaken cultivation

CAPÍTULO I

BIOPROCESS FOR BACTERIAL CELLULOSE PRODUCTION BY ACETIC ACID BACTERIA: THE INFLUENCE OF STRAIN, CULTURE MEDIA AND CULTIVATION

Review Article

Bioprocess for bacterial cellulose production by acetic acid bacteria: the influence of strain, culture media and cultivation

Abstract

Biodegradable biopolymers obtained by fermentation techniques have been widely required in industrial applications. Due to its characteristics bacterial cellulose (BC) is a biopolymer that fits in medical, bioengineering, electronics, and food products. The functional properties provided by BC are associated with the three-dimensional structure, the high purity, high crystallinity degree, mechanical strength, and porosity. Among BC producers, the acetic acid bacteria (AAB) group are the most used due to the high production rate, mainly the strains from *Komagataeibacter* genus. This biodegradable material is a metabolite excreted by AAB during oxidative fermentation, an alternative metabolic pathway for energy supply to cells. The demand for BC is driving alternatives to improve the cost-effectiveness of production. Currently, the more challenging aspects are improving the yield, reducing the production cost, and scaling-up production. This review emphasizes the main features of bacterial cellulose production, highlighting the challenges and strategies of this bioprocess concerning the three fundamental factors: microorganism, substrate, and cultivation methods. The review aims to provide insights into the upstream steps (microorganism selection and conservation, inoculum preparation, and culture medium selection) and the fermentation methods for BC production.

Keywords: upstream; oxidative fermentation; microbial biopolymer; agro-industrial waste; food application.

1 Introduction

26 The biotechnology industry obtains innumerous products from microorganism
27 metabolism to benefit humans, animals, and the environment. Among this type of products,
28 bacterial cellulose (BC) is emerging in industrial scope and has shown characteristics useful for
29 different fields. BC is a biodegradable biopolymer, biocompatible, with high purity, and
30 approved as generally recognized as safe (GRAS) by the Food and Drug Administration – FDA
31 (Azeredo *et al*., 2019; Mandenius; Brundin, 2008; Shi *et al*., 2014).

32 Since it was discovered in 1886 in the vinegar production, BC still attracting
33 researchers around the world aiming optimize the production and polymer functionality to
34 develop high value-added products (Cacicedo *et al*., 2016; Marestoni *et al*., 2021). Some of the
35 possible BC application are food additive and dietary fiber-enriched products (Li; Nie, 2016;
36 Xavier; Ramana, 2022), food packaging (Cazón; Velázquez; Vázquez, 2019), bioengineering,
37 aiming cell and bioactive compound immobilization and biosensor production (Cai *et al*., 2018;
38 Jayani *et al*., 2020; Moradi *et al*., 2019), cosmetics (Bilgi *et al*., 2021), wound dressings
39 (Pasaribu *et al*., 2023), and electronic field (Legnani *et al*., 2019). Besides the expressive efforts
40 on research and academic areas to attest potential applications, the commercial use of BC is
41 still limited due the high cost of the production. Improving BC commercialization includes
42 explore alternatives to increase the yield and reduces the cost of the production, consequently
43 making this biopolymer profitable to application in other areas. In this context, optimize the
44 microorganism activity, and adequate the nutrient source and the fermentation technique
45 (method) is essential to overcome these limitations.

46 In terms of metabolism of acetic acid bacteria, bacterial cellulose is a product of the
47 oxidative fermentation. These microorganisms can metabolize carbon sources such as sugar,
48 ethanol, and alcoholic sugars to produce energy by following a sequence of enzymatic reactions
49 (He *et al*., 2022). As a product of the metabolism, it is essential to understand the mechanisms
50 and agents involved on BC formation as well as how to control the fermentation parameters to

51 optimize the metabolite production. Briefly, a fermentative process includes three sections:
52 upstream steps (microorganism selection and conservation, inoculum preparation, and culture
53 media formulation), fermentation, and downstream steps (product purification and residues
54 treatment). Each step will influence direct or indirectly on the bioproduct yield and
55 characteristic, consequently in the bioprocess efficiency and cost-effective. Nowadays,
56 researchers have been focused on the isolation of news strains with high BC productivity,
57 optimization of culture media and improving fermentation methods (Chen *et al*., 2019; Gomes;
58 Ida; Spinosa, 2022; Lotfy *et al*., 2021). This review provides insights into the upstream steps
59 and the fermentation method for bacterial cellulose production using acetic acid bacteria. Also,
60 emphasizes the main features of BC production, highlighting the challenges and strategies of
61 this bioprocess concerning the three fundamental factors: microorganism, substrate, and
62 cultivation methods.
63
64 **2 Bacterial cellulose**
65
66 Bacterial cellulose is an extracellular polysaccharide produced by microorganisms
67 with structure similar to plant cellulose. The material was first reported in 1886 when Adrian
68 Brown observed the presence of a white and gelatinous film at the surface of the medium during
69 acetic fermentation for vinegar production (Fig. 1). At the time, BC was known as vinegar-plant
70 or vinegar-mother. Later, the chemical and structural evaluation confirms the similarity with
71 cellulose (Cacicedo *et al*., 2016; Lin *et al*., 2020; Salari *et al*., 2019). Nowadays, BC is also
72 named as biocellulose, microbial cellulose, or bacterial nanocellulose.
73 The main BC-producing strains belong to acetic acid bacteria (AAB), and mostly from
74 the *Komagataeibacter* genus due to the higher yields compared to another AAB genus (Foresti,
75 Vázquez; Boury, 2017; Lin *et al*., 2013). For the microorganisms, BC is a metabolite produced

76 during acetic acid fermentation (oxidative fermentation) and assists in flotation acting as a cell-
77 support in the air-liquid interface, considering the aerobic metabolism this mechanism ensures
78 the cell survival under stress condition. In addition, BC helps in cell protection against
79 dehydration, ultraviolet radiation, and acetic acid diffusion to the cytoplasmic membrane
80 (Armitano *et al*., 2014; Aydın; Aksoy, 2014; Knchanarach *et al*., 2010; Valera *et al*., 2015; Wu;
81 Liu, 2013). In vinegar production, large volumes of BC might become an issue once requires
82 extra cleaning steps in the fermenters. Also, the presence of AAB in organic vinegar or
83 remaining cells in conventional vinegar (not completely removed by filtration) can affect the
84 visual aspect of the final product (Gomes, Borges, Maria de Fátima, *et al*., 2018).

85 However, investigation on chemical composition and structural proprieties suggested
86 that after purification steps the biopolymer has the potential to produce biotechnological
87 products. Since then, researchers have been focusing on understanding the mechanism of BC
88 synthesis, isolation of BC-producing microorganisms, and production optimization aiming a
89 controlled production in laboratorial and industrial scale.

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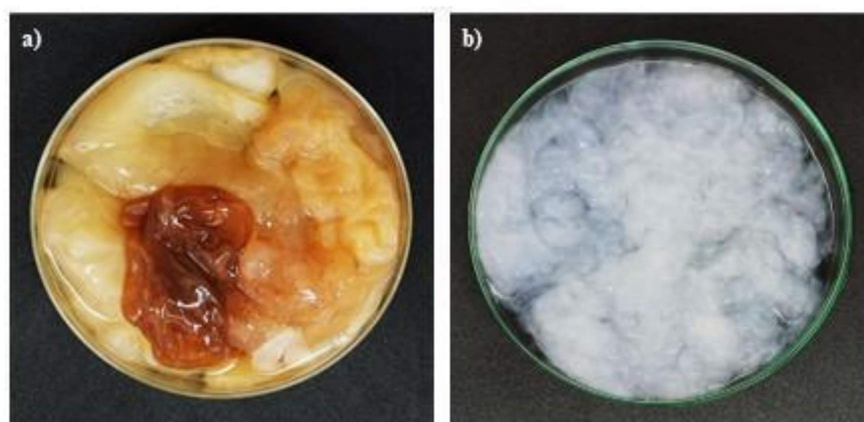


91

92 **Fig.1.** Bacterial cellulose from vinegar production before (a) and after (b) purification with
93 NaOH 1M.

94

95 2.1 Bacterial cellulose-producing microorganisms

96 Several microorganisms have been identified as producers of BC, such as *Aerobacter*,
97 *Acetobacter*, *Komagataeibacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*,
98 *Pseudomonas*, *Rhizobium* and *Sarcina* (Foresti, Vázquez and Boury, 2017; Lin *et al*., 2013).
99 Among the AAB species, *Komagataeibacter xylinus* is considered a model for obtaining this
100 biopolymer from different carbon and nitrogen sources due to the high BC yield. However, new
101 *Komagataeibacer* species have been continuously isolated for this purpose, for example,
102 *Komagataeibacter medellinensis*, *Komagataeibacter intermedius*, *Komagataeibacter hansenii*,
103 *Komagataeibacter europaeus* and *Komagataeibacter rhaeticus* (Lima *et al*., 2017; Lin *et al*.,
104 2016; Machado *et al*., 2018; Molina-Ramírez *et al*., 2018).These microorganisms are usually
105 isolated from Kombucha, fruits and vegetables, and vinegar (Du *et al*., 2018; Fernández *et al*.,
106 2019; Li *et al*., 2019; Rangaswamy, Vanitha and Hungund, 2015; Semjonovs *et al*., 2017).
107 Apart from AAB, new genres and species of microorganisms have been reported as BC
108 producers, for example *Bacillus licheniformis* (Bagewadi *et al*., 2020), *Enterobacter* sp. FY-07
109 (Liu *et al*., 2019), and *Lactobacillus hilgardii* IITRKH159 (Khan; Kadam; Dutt, 2020).
110
111 2.2 Acetic fermentation and bacterial cellulose synthesis
112
113 Bacterial cellulose is a product of the oxidative metabolism. Acetic acid bacteria obtain
114 energy alternatively through the oxidative fermentation (acetic fermentation) pathway to supply
115 the low energy yield from aerobic respiration and improve biomass formation by incomplete
116 substrate oxidation (Lynch *et al*., 2019; Saichana *et al*., 2015). During acetic fermentation, the
117 organic substrates, such as ethanol, glucose, organic acids, and polyols, are incompletely
118 oxidized to CO_2 and H_2O. The residual products from this metabolism find a place in the
119 biotechnology industry to obtain high-added value products (e. g. ketones, organic acids, and
120 exopolysaccharides, such as bacterial cellulose (China, La *et al*., 2018).

121 BC biosynthesis is a process of high precision and specificity controlled by catalytic
122 and regulatory enzymatic complexes using uridine diphosphate glucose (UDP-Glucose) as a
123 precursor. (Jacek *et al*., 2019). In this mechanism, the initial stage is the phosphorylation of
124 glucose to glucose-6-phosphate by the enzyme glucokinase. Then, the phosphoglucomutase
125 promotes the isomerization of glucose-6-phosphate to glucose-1-phosphate, which will be
126 converted to UDP-glucose by UDPG-pyrophosphorylase. The polymerization of glucose into
127 β-glucan chains occurs from UDP-glucose by cellulase synthase, a complex of four subunits,
128 namely *BcsA*, *BcsB*, *BcsC*, and *BcsD*, coded by three (*bcsAB, bcsC*, and *bcsD*) or four (*bcsA*,
129 *bcsB*, *bcsC*, and *bcsD*) genes. Finally, β-glucan chains are crystalized into cellulose (Lin *et al*.,
130 2013; Ross; Mayer; Benziman, 1991; Ullah; Santos; Khan, 2016).

131 The synthesized chains are excreted to the medium through pores in the microorganism
132 cell allowing the elongation and association of the chains in the extracellular medium resulting
133 in BC sub-fibrils (1.5 nm wide). These sub-fibrils give rise to nanofibrils (3–4 nm thick), which
134 ultimately form cellulose ribbons (40–60 nm wide and 3–8 nm thick). The random arrangement
135 between the BC ribbons originates the three-dimensional, porous, and highly crystalline
136 network. The length of cellulose fibers can vary from 1 to 9 meters (Cacicedo *et al*., 2016; Costa
137 *et al*., 2017; Foresti *et al*., 2017; Shah *et al*., 2013; Shi *et al*., 2014). The material observed in
138 the culture medium could have different shapes depending on the cultivation method, the strain,
139 and nutrient sources.

140

141 2.3 Structure, function, and application

142

143 As previously mentioned, BC has a structure similar to that obtained from plants.
144 Cellulose is formed by β-glucopyranosyl units linked by glycosidic bonds (1→ 4) resulting in
145 a long-chain polymer with a degree of polymerization greater than 20.000 (Habibi *et al*., 2010).

146 The association between β-glucopyranosyl units originates a planar structure forming a ribbon.
147 This planar and linear structure allows the formation of fibrous and polycrystalline bundles
148 along extensive zones due to the association between cellulose molecules by hydrogen bonds.
149 These structures present amorphous and crystalline zones (Gibson, 2012; Habibi; Lucia; Rojas,
150 2010; Nascimento *et al*., 2018).

151 In recent years, the production of cellulose from microorganisms has been the subject
152 of research in several countries since the fermentation process originates a material with
153 superior properties and high purity, allowing its application in products such as food,
154 biomedicine, and pharmaceuticals (Blanco Parte *et al*., 2020; Chen *et al*., 2019; Ul-Islam *et al*.,
155 2019). The BC structure formed by the three-dimensional network ensures remarkable
156 properties, namely high mechanical strength, and crystallinity, stability to chemical agents and
157 high temperature, high water retention capacity, and resistance to degradability. BC is lignin
158 and hemicellulose-free, nor requiring intense purification, and is also a biocompatible and
159 biodegradable biopolymer (Foresti; Vázquez; Boury, 2017; Kwak *et al*., 2015; Rani; Appaiah,
160 2013).

161 High crystallinity is one of the main characteristics of BC, and the degree of
162 crystallinity can range from approximately 60 to 90% depending on the cultivation conditions
163 and strain ability to convert the substrate and adaptation to the fermentation system. The
164 crystallinity influences other characteristics of the biopolymer such as mechanical properties
165 and thermal stability (Li *et al*., 2019; Machado *et al*., 2016; Vasconcelos *et al*., 2017).
166 Concerning the crystalline structure, cellulose I (Iα and Iβ) and cellulose II forms are frequently
167 obtained under fermentation culture. The Iα (triclinic) and Iβ (monoclinic) forms correspond to
168 crystalline structures and differ by the distribution of intra and interunit hydrogen bonds. In
169 cellulose II, the random arrangement of chains results in highly amorphous regions, which also
170 differs by its high thermodynamic stability. In most cases, higher crystallinity is observed in

171 static culture, while amorphous content is more evident in agitated cultivation (Bi *et al*., 2014;
172 Brandes *et al*., 2017; Foresti *et al*., 2017).

173 The large surface area, the high number of hydroxyl groups, and its porosity enable
174 BC to interact with water and polymers allowing the application as a support material for
175 enzymes, cell, and nanoparticle immobilization. BC has a high-water holding capacity (WHC)
176 retaining about 90% of its weight. This property is due to the strength of the hydrogen bonds
177 involved in the adsorption of water molecules on the surface of the fibers and the density of the
178 bond between the crosslinked fibers. The presence of thin and long ribbons in the BC structure
179 also explains the greater water retention capacity, the moldability and the high tensile strength
180 (de Oliveira Barud *et al*., 2016; Feng *et al*., 2015; Fijałkowski *et al*., 2016; Machado *et al*.,
181 2018).

182 Regarding these characteristics, bacterial cellulose showed potential for food,
183 bioengineering, cosmetics, biomedical, and electronic fields. The material can be used in
184 different shapes, for example, nanofibers, nanocrystals, dried or wet pellicle and sphere. In food
185 applications (Table 1) BC act as a multifunctional ingredient and its addition in food products
186 do not affect sensorial characteristics since its can be colored and flavored (Lima *et al*., 2018;
187 Marchetti *et al*., 2020). From the nutritional aspect, cellulose has a health-promotion function
188 and can be used as a dietary fiber source, and low calorie or gluten-free products. As a food
189 additive these biopolymers have been suggested as a stabilizer, thickener agent and texture
190 modifier (Azeredo *et al*., 2019; Shi *et al*., 2014).

191 The three-dimensional, porous, and crystalline structure added to the presence of
192 hydroxyl groups in the surface area allows BC association to other polysaccharides and proteins
193 by hydrogen, *Van der Waals*, and hydrophilic bonds improving their functionalities. Otherwise,
194 the crystalline structure allows hydrophobic interactions ensuring BC amphiphilic features
195 (Guo *et al*., 2018; Yan *et al*., 2017). The formation of nanocomposites is useful to biodegradable

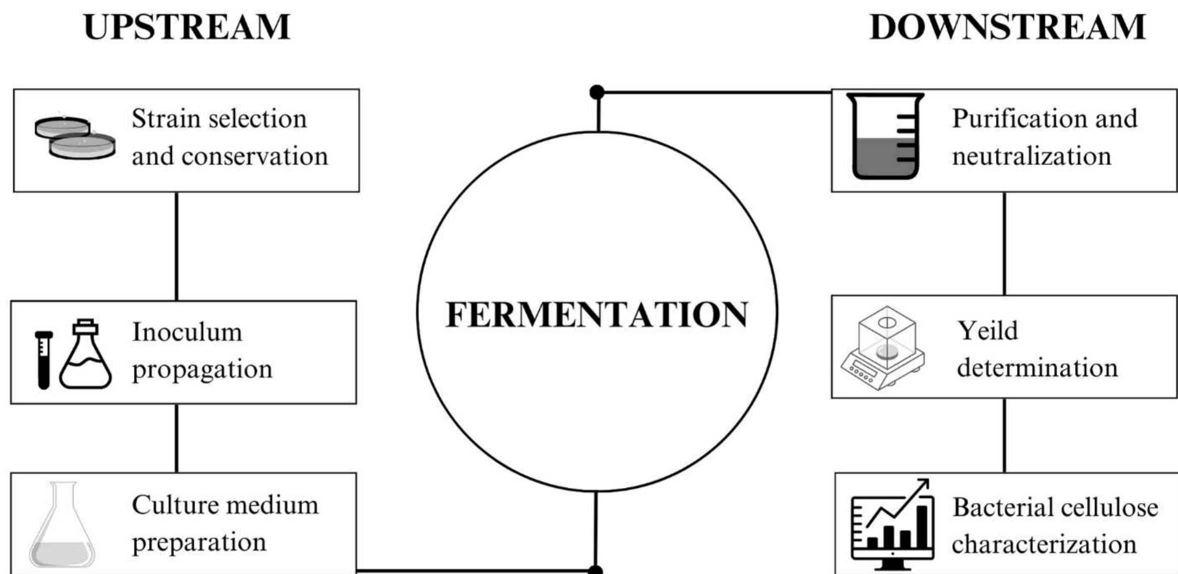
food packaging and edible film development, in this case BC could be associated to other polymers, proteins, bioactive composites, and inorganic nanoparticles to enhance mechanical and thermal properties, barrier performance, and antimicrobial properties (Jang *et al*., 2023; Ju *et al*., 2020; Salari *et al*., 2018). Some research reports the use of chemical, mechanical, and enzymatic modification to improve or development specific properties allowing more applications (Blanco Parte *et al*., 2020). Besides the several applications field, the low yield and high production costs are a limiting factor. In this context, it is crucial to understand and optimize the production parameters to improve the bioprocess cost-effective.

204 **Table 1** – Bacterial Cellulose properties and their potential application in food

Function	Structure-properties	Food application	Reference
Fat replacer	Water holding capacity; emulsion stabilization; amphiphilic nature.	Meat products; ice cream.	Guo *et al*., 2018; Marchetti *et al*., 2017; Oliveira *et al*., 2022, 2023
Functional food ingredients	Insoluble dietary fiber; high water holding; ion exchange capacities.	Dietary fiber source; low calorie products; low cholesterol diet.	Lin *et al*., 2020; Ullah *et al*., 2016; Xavier & Ramana, 2022
Food packaging and support for bioactive compounds	High surface area; porosity; high pore volume; gelling behavior; high crystallinity; hydrophilicity; rehydration property; chemical, thermal, and mechanical stability; barrier properties.	Food packaging, edible films and coatings, active and intelligent packaging film; immobilization of cell, enzyme, antimicrobial agents	Farooq *et al*., 2020; Lin *et al*., 2020; Liu *et al*., 2021; Yordshahi *et al*., 2020
Thickener and stabilizing agent in emulsion, suspensions, and foam stabilizer	Amphiphilic nature; high surface area; crystallinity; three-dimensional structure.	Pickering emulsion; edible foam; beverages; bakery products; dairy products.	Gao *et al*., 2020; Marchetti *et al*., 2020; Martins *et al*., 2020; Paximada *et al*., 2016; Yan *et al*., 2017; Zhai *et al*., 2018; Zhang *et al*., 2020

205

206 Briefly, BC production comprises three stages, (1) upstream, (2) fermentation and (3)
207 downstream. Upstream includes strain selection and conservation, inoculum and medium
208 preparation, and definition of cultivation condition. During the fermentation several parameters
209 can affect the yield and product characteristics, therefore they should be controlled, for
210 example, pH, oxygen, temperature, agitation. Otherwise, downstream step requires the product
211 purification, neutralization, characterization, and effluent treatment. A summary scheme of BC
212 production is shown in the Fig. 2
213



214
215
216 **Fig. 2.** Scheme of bacterial cellulose production
217
218 Different strategies to overcome or minimize limiting factors can be used for each
219 process step, consequently, optimizing BC production. The following sections discuss the
220 features of the central factors of BC production (microorganism, substrate, and cultivation
221 methods), including some strategies to improve this bioprocess.
222

223 **3 Acetic acid bacteria for bacterial cellulose production**

224

225 The cultivation of BC producers is a crucial stage considering the nutritional
226 requirements for cell metabolism and microorganism adaptation to the fermentation condition.
227 In this context, the researchers are constantly trying to find new strains with high capacity to
228 produce BC. AAB strains are fastidious microorganisms to cultivate and isolate and have been
229 challenged researchers to find out alternatives to optimize the cell growth and BC production
230 (Gullo et al., 2006; Kim et al., 2019; Vegas et al., 2010). *Komagataeibacter* species has been
231 previously described as the most efficient on BC production due their ability to growth and
232 produce BC from different carbon and nitrogen sources (Nascimento et al., 2021; Ryngajłło et
233 al., 2020). *K. intermediuns, K.rhaeticus, K. hansenii and K. medellinensis* are frequently
234 referred as highly BC producers, similar to the *K. xylinus* strain, the role-model on BC
235 production. The main sources of BC producers are vinegar (Fernández et al., 2019; Gomes et
236 al., 2021), kombucha (Jacek et al., 2021) fermented juice (Lin et al., 2016), and fruits and
237 vegetables (He et al., 2020).

238 BC producers has developed mechanisms to survive in stressful conditions, usually
239 similar to the isolation environment, for example, resistance to high acid and ethanol
240 concentration. All these mechanisms make AAB attractive microorganisms to industrial
241 process and are useful to improve the biopolymer formation extending the rate for bioprocess
242 design (Nascimento et al., 2021; Zhang et al., 2017). Also, some *Komagataeibacter* strains
243 presented a remarkably ability to produce BC under alkaline condition, which could be
244 associated with the protective nature of the material (Lin et al., 2016; Thongwai et al., 2022).
245 Additionally, resistance mechanisms have been investigated at different cultivation, such as
246 static and agitated, and the adaptation to highest rotational speed is referred as a strain-
247 dependent characteristic (Ryngajłło et al., 2020). The cultivation under laboratory conditions

248 and synthetic media can reduce the microorganism resistance suggesting that these mechanisms
249 are inducible or transient (Barja et al., 2016; Gullo; Giudici, 2008). However, the product
250 formation must be evaluated considering the interaction between strain, culture medium and
251 cultivation method once the performance of the microorganisms is affected by the cell
252 adaptation to the cultivation conditions. In this case, the same strain cultured in different
253 bioprocess not always reproduce the same yield and productivity (Table 2).

254 Differences observed in bacterial cellulose production are frequently associated to the
255 influence of stress conditions on cell metabolism. Atypical cell resulting from spontaneous
256 mutation influence the yield and material characteristic due the inactivation of essential
257 enzymes for the polymer synthesis (AydIn; Aksoy, 2014; Dubey; Singh; Singh, 2018). Besides
258 reducing BC production, the effect of cellulose nonproducing cells can be investigated by the
259 polymer structure, and the crystallinity is the most affected (Singhsa; Narain; Manuspiya,
260 2018). Additionally, the presence of mutant cell influence fiber assembly process due the
261 formation of soluble polysaccharides such as acetan since both use the same starter molecule,
262 the UDP glucose (Chen et al., 2017). Regarding microorganism activity, it is also important to
263 consider the effects of the cultivation parameters on the emergence of mutant cells. Changes in
264 process parameters, pH, temperature, culture medium volume and oxygen availability affect the
265 cell metabolism and product formation (Araújo et al., 2020).

266 Similarly, stress condition such as high sugar concentration, anaerobic conditions, and
267 high temperature can induce the viable but non culturable (VBNC) state which will influence
268 cell monitoring and product formation. Briefly, in VBNC state even if the cells are alive, they
269 do not grow on conventional media (nonselective) used to form colonies. The mainly affected
270 metabolic and morphologic characteristics are the modification of wall cell components,
271 reduction in respiration rate, nutrient transport, macromolecular synthesis. Nevertheless, cells
272 in VBNC state are more resistant to chemical, physical and antibiotics. Modification in the

273 environmental conditions and the media composition could favor the cell growth and reverse
274 this cell state (Dong et al., 2020; Lynch et al., 2019; Shafiei et al., 2017).

275 Aiming to improve the material formation, BC producing strain can also be obtained
276 using genetic engineering techniques. This tool has been used to enhance the BC production or
277 properties and ensure the control of the process that is essential to industrial applications. Yang
278 et al. (2023) developed a recombinant strain for BC production in mannose-rich media by
279 introducing genes from the *Escherichia coli* K-12 strain, which increased BC production on
280 84% compared with the wild-type strain. Jacek et al. (2019) modified *K. hansenii* motility and
281 cell size, which are suggested to influence the yield and network organization in BC structure.
282 The use of genetic engineering resulted in thicker ribbons of cellulose arranged in looser
283 network, and the biopolymer are suggested to scaffolds production.

284 **Table 2** - Acetic acid bacteria performance on bacterial cellulose production under different conditions

Strain	Source	Nutrient sources	Method	Parameters	BC (g L)	BC (g L h)	Reference
K. rhaeticus AF-1	Kombucha tea	Hestrin-Schramm with ethanol	Static	28 °C/96 h	6.70	0.0698	Machado *et al*., 2016
		Cashew tree exudate	Static	28 °C/168 h	2.80	0.0167	Pacheco *et al*., 2017
		Cashew gum	Static	28 °C/168 h	2.30	0.0137	
		Hestrin-Schramm	Static	28 °C/168 h	~ 6.0	0.0357	
		HSCTE	Static	28 °C/168 h	~ 6.0	0.0357	
		HSCG	Static	28 °C/168 h	~ 6.0	0.0357	
		Sugarcane molasses-supplemented	Static	30 °C/120 h	3.46 - 4.01	0.0288 - 0.0334	Machado *et al*., 2018
		Sugarcane molasses	Static	30 °C /120 h	1.90	0.0158	
		Hestrin Schramm	Static	30 °C/120 h	3.00	0.0250	
K. intermedius BCRC 910677	Fermented fruit juice	Hestrin Schramm	Static	28 ºC/120 h	1.20	0.0100	Lin *et al*., 2016
		Synthetic optimized medium	Static	28 ºC/144 h	3.91	0.0271	Santoso *et al*., 2020

285 (continue)

286

287 **Table 2** - Acetic acid bacteria performance on bacterial cellulose production under different conditions (continued)

Strain	Source	Nutrient sources	Method	Parameters	BC (g L)	BC (g L h)	Reference
K. europaeus SGP37	Rotten grapes	Hestrin Schramm (glucose)	Static	30 ºC/384 h	5.61	0.0146	Dubey *et al*., 2017
		Hestrin Schramm (fructose and ethanol)	Static	30 ºC/384 h	9.98	0.0260	
		Sweet lime pulp waste	Static	30 ºC/384 h	6.30	0.0164	Dubey *et al*., 2018
		Sweet lime pulp waste supplemented with HS	Static batch	30 ºC/384 h	26.20	0.0682	
		Sweet lime pulp waste supplemented with HS	Static intermittent fed-batch	30 ºC/384 h	38.00	0.0990	
K. intermedius V-05	Vinegar	Soy molasses with ethanol	Static	30 °C/336 h	10	0.0297	Gomes *et al*., 2021
		Hestrin Schramm	Static	30 °C/336 h	3.7	0.0110	
		Synthetic with amino acids (optimized)	Static	30 °C/240 h	3.02	0.0125	Gomes *et al*., 2022
		Hestrin Schramm	Static	30 °C/240 h	3.31	0.0138	Catarino, 2019
		Hestrin Schramm	Agitated	30 °C/240 h	0.82	0.0034	
		Soy molasses with ethanol	Static	30 °C/240 h	3.39	0.0140	
		Soy molasses with ethanol	Agitated	30 °C/240 h	0.44	0.0018	

288

289 3.1 Monitoring and control of cell growth

290

291 The isolation, cultivation, and cell preservation are crucial steps in the bioprocess due
292 the influence in the biopolymer formation. The success of AAB cultivation for bacterial
293 cellulose production have been associated with the nutritional requirements of the
294 microorganism and the cell growth control (Gomes *et al*., 2018; Zou *et al*., 2020).

295 The culture media for microorganisms are classified depending on their composition,
296 namely chemically defined (synthetic), complex, selective, differential, and enrichment
297 medium (Tortora; Funke; Case; 2017). Using complex medium (composition not exactly
298 known) is a strategy to evaluate the growth characteristics of unknown strains or to create an
299 environment able to supply the complex nutritional requirements some microorganisms
300 (Sperotto *et al*., 2021).

301 Culture media for AAB isolation, pre-activation and inoculum propagation are
302 formulated to satisfy the nutritional demand, providing components that simulate the
303 characteristics of the isolation environment, such as rich-sugar, acetic acid and ethanol that are
304 observed in fermentation bioreactors, fruits, vinegar, or fermented beverage, the most common
305 sources for AAB isolation (De Vero; Gullo; Giudici, 2017)). The main elements to cell
306 formation are Carbon (C), Nitrogen (N), Hydrogen (H), Oxygen (O), Sulfur (S), And
307 Phosphorus (P) since these components are used in proteins, nucleic acids, carbohydrates, and
308 lipids synthesis. (Santos Júnior *et al*., 2022; Sperotto *et al*., 2021).

309 Carbon and nitrogen are the most significant considering the structural function of
310 several cell components (Gomes; Ida; Spinosa, 2022). Carbon is an essential element to the
311 synthesis of organic components in cell metabolism and to energy production (Tortora; Funke;
312 Case, 2017). In the culture medium this macronutrient is provided through sugar which
313 represents a large percentage in the formulation. Although AAB can metabolize several carbon

314 sources, glucose, mannitol, glycerol, and ethanol are the most common substrates to these
315 metabolic pathways since they are oxidized by the membrane-bound (periplasmic)
316 dehydrogenases, not requiring previous hydrolysis reaction which would mean an extra cell
317 work (Lynch *et al*., 2019; Saichana *et al*., 2015). Nitrogen is another essential element in the
318 culture medium and are required to produce proteins, enzymes, nucleic acid, and biomass
319 formation (Tortora; Funke; Case, 2017). To AAB cultivation and BC production this nutrient
320 may be supplied by organic (yeast extract, peptone, malt extract and amino acids) and inorganic
321 sources, for example, ammonium sulfate ($(NH_4)_2SO_4$) and ammonium nitrate (NH_4NO_3)
322 (Gomes; Ida; Spinosa, 2022; Gopu; Govindan, 2018; Santoso *et al*., 2020)

323 Also, minerals and vitamin play essential roles in cell growth. Mineral components
324 influence enzyme activity, nitrogen fixation, and electron transference from substrate to
325 oxygen. Some essential minerals for AAB metabolism are molybdenum (Mo), boron (B) and
326 manganese (Mn). In addition, vitamins such as p-aminobenzoic acid, pyridoxine (B_6),
327 cyanocobalamin (B_{12}), nicotinamide (B_3), and ascorbic acid have been shown significant effect
328 on cell growth and BC production (Leonarski *et al*., 2021; Santos Júnior *et al*., 2022; Souza *et*
329 *al*., 2020).

330 Table 3 shows the culture medium most used to AAB and its composition. These
331 culture media are mainly formulated by sugar (carbon source) and yeast extract or peptone
332 (nitrogen source). Frequently, some additives have been incorporated into culture media to
333 supply the microorganism requirement for nutrients. For example, ethanol has been suggested
334 as an alternative energy source to microorganism growth, also supporting cell recovery from
335 viable but non culturable (VBNC) state and inhibit the non-producing cells (Li *et al*., 2019;
336 Shafiei *et al*., 2017) Similarly, organic acids such as acetic, citric, malic, lactic, pyruvic, and
337 succinic acid could be metabolized by AAB and used as intermediate metabolites to energy
338 production (Mamlouk; Gullo, 2013b; Revin *et al*., 2018).

339 Besides the media composition it is also useful apply alternatives methods in isolation
340 or enrichment stages to solid media preparation, for example, double-layer agar. For AAB
341 strains from industrial vinegar production this method simulates the growth conditions in
342 fermentation tanks (Mamlouk; Gullo, 2013) This technique was described by Entani *et al*.
343 (1985) and consists in creating an inferior layer with broth with 0.5% agar. Further, the surface
344 will be coated by the broth with 1.0% agar. The use of double-layer agar plate supplies a high
345 humidity environment, for the cells favoring the growing of high-acidity colonies (Entani *et al*.,
346 1985; Mamlouk; Gullo, 2013).

347 **Table 3** – Main culture media for acetic acid bacteria isolation, cultivation, and preservation

Culture media	Composition	Function	Reference
AE	1.5 % glucose; 0.2 % yeast extract; 0.3 % peptone; 2.0 % ethanol; 6.5 % acetic acid.	Isolation and enrichment	Entani *et al*., 1985
Glucose-Yeast extract-Carbonate (GYC)	10 % glucose; 1.0% yeast extract; 2.0 % $CaCO_3$.	Isolation and enrichment	Gullo; Giudici, 2008
Hestrin-Schramm (HS)	2.0 % glucose; 0.5 % yeast extract; 0.5 % peptone; 0.27 % Na_2HPO_4; 0.115 % citric acid.	Isolation, cultivation, and bacterial cellulose production	Fernández *et al*., 2019 Hestrin; Schramm, 1954 Lotfy *et al*., 2021
Mannitol, Yeast extract, Peptone (MYP)	2.5 % mannitol; 0.5 % yeast extract; 0.3 % peptone.	Isolation and enrichment Preservation	Mamlouk; Gullo, 2013
Carr	2.5 % yeast extract; 2.0 % ethanol; 0.02 % peptone.	Preservation	Vashisht *et al*., 2019
Glycerol	15% glycerol	Preservation	Gupte *et al*., 2021
Malt extract	20 % malt extract	Preservation	Sokollek; Hertel; Hammes, 1998

348

349 3.1.2 Alternatives to cell enumeration

350

351 Estimate the cell population is essential to understand the microorganism growth
352 profile, the metabolic aspects concerning BC production, also for fermentation control. Briefly,
353 the cell growth profile has four stages: lag, exponential (log), stationary and death. In the first
354 stage the cell concentration increases slowly as a result of the microorganism adaptation to the
355 cultivation conditions. The exponential growth phase reflets the most intense cell activity and
356 substrate consumption. During the stationary stage the nutrients availability is reduced, and the
357 growth rate is equal to the death rate, however the cell still active and producing metabolites,
358 such as bacterial cellulose. Finally, the nutrient limitation results in cell death (Reiniati;
359 Hrymak; Margaritis, 2017; Vazquez *et al*., 2013). Traditionally, the AAB population can be
360 determined by cell enumeration in plating or microscopy, and turbidimetric and gravimetric
361 methods (Table 4). Since these techniques are well established to vinegar and fermented
362 beverages production, they could be applied in BC production to improve the cell growth
363 control and product formation. However, alternative techniques are useful to cell monitoring
364 and to analyze the microorganism behavior. These methodologies should be applicable to
365 fermentation routine to monitoring cell growth and ensure the bioprocess control.

366 Plating methods to cultivate and enumerate AAB in synthetic culture media could be
367 affected by the presence of cells in VBNC which leads to an underestimate cell count and limits
368 the cultivate, isolation and cell maintenance (Bartowsky; Henschke, 2008; De Roos; De Vuyst,
369 2018; Mamlouk; Gullo, 2013). VBNC state was associated to discrepancies between AAB
370 target inoculation rate and plate counting results in fermentation systems, and can interrupt the
371 direct correlation between biomass formation, substrate consumption, and product since the
372 VBNC cells cannot be enumerated (Hutchinson *et al*., 2019; Lynch *et al*., 2019; De Roos; De
373 Vuyst, 2018; Tran *et al*., 2020).

374 Another limiting factor cell determination is the cell attachment at the BC during the
375 inoculum propagation and fermentation. During the inoculum preparation on a liquid medium
376 the simultaneous cell growth and BC formation result in the cell holding inside of the
377 biopolymer structure. An alternative to overcome this limitation is the use of cellulase to release
378 cells attached in BC fibers, increasing free cell in liquid media also improving cell enumeration
379 (Wang *et al*., 2016; Zou *et al*., 2020).

380 Besides growth monitoring, it is also crucial to use rapid methods to quantify and
381 identify the cell state (i.e., live, dead, or VBNC). Some techniques have been used to quantify
382 both live and dead cells in acetic fermentation. The fluorescence technology might be useful to
383 this purpose and showed good results for AAB compared to the plating method (Zou *et al*.,
384 2020). In the same way, flow cytometer was used to assist researchers in cell enumeration in
385 acetic fermentation (Baena-Ruano *et al*., 2006), and to analyze the cell viability after exposure
386 to stress factors (Shafiei *et al*., 2013). Moreover, real-time polymerase chain reaction (RT-PCR)
387 is another alternative to the traditional method of AAB enumeration (González *et al*., 2006;
388 Torija *et al*., 2010).

389 **Table 4 –** Methods of cell estimation for acetic acid bacteria

Method	Principle	Material	Main characteristics	Reference
Biomass (dry weight)	Gravimetric method. Determinate the cell dry weight.	Centrifuge; drying oven;a analytical balance.	Low cost and quick method. Overestimate the cell population (total cell concentration).	Reiniati; Hrymak; Margaritis, 2017
Plating	Direct cell counting. Viable colonies quantification by plating in solid culture media.	Agar plates.	Efficient indicator of inoculum viability since the cells which grow in plate have greater metabolic activity. Large standard deviations; aggregates formation; inability to detect VBNC; underestimate the cell population; time-consuming (72-96h).	Baena-Ruano *et al*., 2006; De Roos; De Vuyst, 2018; Gomes *et al*., 2018; Santos Júnior *et al*., 2022; Torija *et al*., 2010
Microscopy	Direct cell counting.	Neubauer chamber; trypan blue vital dye; optical microscope	Monitoring the multiplication and differentiation of cell viability;	Santos Júnior *et al*., 2022
Turbidimetric	Spectrophotometric-turbidimetric method that estimates cell population by optical density (OD_{600}).	Spectrophotometer	Quick method; useful to cell growth control; overestimate the cell population.	Kuo; Teng; Lee, 2015; Mat Isham *et al*., 2019; Santos Júnior *et al*., 2022

390

(continue)

391 **Table 4 –** Methods of cell estimation for acetic acid bacteria (continued)

Method	Principle	Material	Main characteristics	Reference
Epifluorescence microscopy	Live and dead cell are by differed by staining.	Epifluorescence microscope	Rapid method; estimates total and viable cell; precise control of the living cell concentration at the beginning of fermentation.	Baena-Ruano *et al*., 2006; Zhou *et al*., 2019
Flow cytometry	Molecular approach. Cell population is determined by fluorescence using an optical system.	Flow cytometer	Appropriate for the determination of cell viability.	Baena-Ruano *et al*., 2006; Shafiei *et al*., 2013)

392

4 Designing culture media to bacterial cellulose production

Culture media impacts significatively the total cost in bacterial cellulose production and requires strategies to overcome this limitation and increase the bioprocess economic feasibility (Reiniati; Hrymak; Margaritis, 2017; Revin *et al*., 2018). The conventional medium used for BC production was proposed by Hestrin and Schramm (1954). Hestrin-Schramm (HS) composition consists in (%, w/v): glucose (2%), peptone (0.5%), yeast extract (0.5%), anhydrous sodium phosphate (0.27%), and acid citrus (0.115%). Culture media composition must supply sufficient macro and micronutrients for cell growth and biopolymer synthesis. Considering the HS composition, each component has an essential role on microorganism metabolism and BC formation. Carbon is provided by glucose which is the ideal precursor for the formation of BC chains (Bilgi *et al*., 2016; Cacicedo *et al*., 2016; Jozala *et al*., 2015). Peptone and yeast extract provides amino acids for protein synthesis and essential compounds for the microorganism growth, such as vitamins and minerals (Campano *et al*., 2016; Gopu; Govindan, 2018; Lee *et al*., 2014). Finally, anhydrous sodium phosphate (Na_2HPO_4) and acid citric have buffering effect during the cell cultivation (Molina-Ramírez *et al*., 2017).

Increase BC yield and reduces the media cost is essential to bioprocess viability. Nowadays, different approaches have been used to design economically feasible nutrient sources to BC production. These strategies consist in change individual components on the standard media, culture media supplementation, synthetic media formulation, and use of low-cost materials. All approaches must consider an ideal carbon and nitrogen ratio for BC production. In microbial biopolymer production the excess of N increases biomass formation and limits biopolymer production while the excess of carbon over nitrogen leads to a decrease in protein synthesis, reducing the growth of the microorganism. Thus, the energy from excess carbon is used to produce the polysaccharide (Lima *et al*., 2017; Miqueleto *et al*., 2010; Rastogi; Banerjee, 2020).

419 Many BC studies reported higher production when HS carbon and nitrogen sources
420 were modified by changing the concentration or the type of the sources. Basu, Vadanan and
421 Lim (2019) used response surface methodology to find the optimal HS composition for *G.*
422 *hansenii* strain. In this case, glucose and sucrose were evaluated on different concentration and
423 the author found higher BC yields with sucrose, a cheaper carbon source compared to glucose.
424 Similarly, Jacek *et al*. (2021) reported the increase on BC production when replaced glucose by
425 eucalyptus biomass hydrolysate in HS supplemented with ethanol. Considering N influence on
426 cell growth and biopolymer formation, Santoso *et al*., (2020) used different nitrogen source,
427 namely, yeast extract, peptone, malt extract, and ammonium sulfate as substitute for the
428 nitrogen source in HS composition. The results suggested peptone was the more suitable source
429 for *K. intermedius* (BCRC 910677) while no BC was produced using ammonium sulfate as N
430 substitute.

431 Synthetic media formulation is another alternative to improve BC yield from different
432 strains. Gomes, Ida and Spinosa (2022) evaluated the effect of amino acid supplementation on
433 *K.intermedius* V-05 metabolism for BC production. The authors reported aspartic acid (1.5 g
434 L^{-1}), phenylalanine (1.5 g L^{-1}), and serine (3.0 g L^{-1}) as essential elements on the media
435 formulated (50 g L^{-1}sucrose, 10 g L^{-1} $(NH_4)_2SO_4$), 2 g L^{-1} NaH2PO4, 1 g L MgSO4.7H2O,
436 and 10 mL L^{-1} ethanol) achieving 3.02 g L^{-1} from the optimized media.

437 In addition, low-cost materials have been successfully applied on BC biosynthesis
438 using agro-industrial waste, for example, cashew apple juice and soybean molasse (Souza *et*
439 *al*., 2020), potato peel wastes (Abdelraof; Hasanin; El-Saied, 2019), sugar beet molasses and
440 cheese whey media (Salari *et al*., 2019), tabacco waste (Ye *et al*., 2019), oat hulls (Skiba *et al*.,
441 2020a), and brewing by-products (Tsouko *et al*., 2023). Despite the cost reduction the use of
442 agro-industrial waste in fermentation still has limitations. These materials have complex
443 component, consequently, its undefined composition reduces the bioprocess reproducibility and

444 could affect BC quality. On the other hand, the use of synthetic media (defined media) can
445 improve the control and monitoring aspects, the process scale-up, and the product recovery and
446 purification (Skiba *et al*., 2020a; Sperotto *et al*., 2021). Also, some agro-industrial substrates
447 require pre-treatment such as acid or enzymatic hydrolysis to increase the fermentable sugar
448 concentration and extensive purification which means an extra cost in the process. However,
449 more studies can explore this application considering the low-cost, and positive effect on
450 environmental, and the possibility to obtain high added value (Qi *et al*., 2017; Salari *et al*.,
451 2019a; Saleh *et al*., 2021).
452
453 **5 Cultivation method**
454
455 Bacterial cellulose fermentation could be carried out under static and agitated
456 cultivation and the method used influence in the yield and material properties. However, the
457 success of each process depends on strain adaptation and how these variables interact with the
458 culture media. Under static condition, AAB are inoculated into the fermentation flacks or
459 bioreactor filled with the sterile culture medium and incubated statically at the predefined
460 condition of temperature and time. In this method, BC formation occurs into the air-liquid
461 interface as a gelatinous pellicle in the shape of the flask used to cultivation (Khattak *et al*.,
462 2015).
463 Although static cultivation is the most used technique to BC production, the agitated
464 (stirred or shaking) culture have been suggested as an alternative due the possibility to
465 overcome some limitations inherent to static method. In agitated cultivation the culture medium
466 inoculated with the AAB is incubated under a wide rate of agitation speed and the biopolymer
467 will be synthetized as ellipsoidal, stellate, or fibrous component dispersed in the culture medium
468 (Azeredo *et al*., 2019) Compared to static cultivation the crystallinity is the most significant

469 characteristic affected by agitation, meanly in high-speed rotation. This parameter reflects the
470 structure organization that would be affected by shear force resulting in a less organized
471 network. Under agitation system the spherical BC formation consist in cell aggregation around
472 the air bobble following ribbon arrangement. However, the mechanism is also influenced by
473 inoculum, carbon sources, media volume temperature (Brandes *et al.*, 2018; Mohite; Patil,
474 2014).

475 The evaluation of the strain performance under both systems should consider the effect
476 of the culture medium on cell viability and cell ability to adapt the metabolic mechanics under
477 each cultivation method used. Therefore, it is useful to consider the media composition and the
478 strain when comparing the static and agitated methods. The main differences observed in
479 studies comparing both methods considering the same strain and nutrient sources in both
480 cultivations are the yield, the crystallinity index, water retention, porosity, and BC form (Table
481 5). These aspects are essential to define the final biopolymer application (Chen *et al.*, 2019;
482 Gao *et al.*, 2020b).

483 BC production is influenced by surface area, volume of culture medium, and nutrient
484 availability. Under static cultivation, the surface area-volume ratio has a significant influence
485 on the oxygen availability thereby, the greater area favors the oxygen consumption. Since AAB
486 are aerobic microorganism, the oxygen supply is essential for cell growth. In this case, cells are
487 suggested to use the BC synthesized, as well as the remaining from inoculum, as a support to
488 access the air–liquid interface improving their oxygen access. In contrast, the aeration promoted
489 by the agitated method ensure a greater oxygen supply and improve the nutrient availability,
490 consequently improving the cell growth (Gullo *et al.*, 2017; Li *et al.*, 2019; Rodrigues *et al.*,
491 2019; Singhsa; Narain; Manuspiya, 2018).

492 Despite the higher oxygen diffusion under agitation, BC production may be negatively
493 affected by emerging mutant cell (non-producing cells), by-product formation, and

494 simultaneous production of water-soluble polysaccharides (WSPS), resulting in lower
495 production compared to static method (Chen *et al*., 2019; Lu *et al*., 2020; Singhsa; Narain;
496 Manuspiya, 2018). The by-product formation is a consequence of the nutrient consumption,
497 carbon source metabolization and aeration. For example, using glucose as a carbon source Chen
498 *et al*. (2019) reported higher glucose consumption and gluconic acid production in shaking
499 system. Krusong *et al*. (2021) observed gluconic acid formation increased with the aeration rate
500 while BC production and cell content were reduced. Besides these limiting-factors, recent
501 research confirms the ability of some AAB strains to grow and produce BC under agitation, and
502 the yield could be similar or higher than static method (AydIn; Aksoy, 2014; Gao *et al*., 2020;
503 Singhsa; Narain; Manuspiya, 2018).

504 **Table 5 –** Bacterial cellulose production by acetic acid strains under static and agitated cultivation

Strain	Static g L^{-1}	Agitated g L^{-1}	BC properties Static	BC properties Agitated	Reference
A. xylinum BCA263	3.97	1.70	Higher crystallinity; stronger tensile strength; denser network structure; higher temperature resistance.	Larger porous; lower crystallinity; higher water retention.	Gao *et al*., 2020
K. xylinus BCC529	2.48	1.66			
G. xylinus P1	1.40	1.72			
K. xylinus (KX)	1.14–1.84	0.60 - 1.20 (~)	Higher crystallinity and smaller crystallite sizes;	Disorderly reticulated structures of microfibrils; higher cellulose Iα content in the flocky asterisk-like BC than in the solid sphere-like.	Singhsa; Narain; Manuspiya, 2018
K. xylinus (TISTR 086)	0.14–0.39	0.00 - 0.10 (~)			
K. xylinus (428)	0.09–0.22	0.20 - 0.40 (~)			
K. xylinus (975)	1.11–1.55	(~) 2.40 - 3.54			
K. xylinus (1011)	0.57–1.46	(~) 3.20 - 4.69			
Komagataeibacter sp. nov. CGMCC 17276	8.85	3.22	Higher crystallinity; high water-holding capacity; denser network.	Network structure looser and more porous; higher porosity.	Lu *et al*., 2020
K. hansenii JR-02	4.62	3.14	Thicker fibers; higher thermal degradation temperature and lower moisture content; higher crystallinity.	Higher weight loss; higher moisture content and amorphous proportion.	Li *et al*., 2019

505 **Table 5 –** Bacterial cellulose production by acetic acid strains under static and agitated cultivation (continued)

Strain	**Static g L^{-1}**	**Agitated g L^{-1}**	**BC properties Static**	**BC properties Agitated**	**Reference**
G. hansenii P2A	1.89	3.25	Ordered and dense network of fibrils with (8–10 nm diameter); the network was composed of interconnected layers.	Slight decrease in the crystallinity index; looser clump of disordered short and thin fibrils; lower molecular weights; increased thermal due the gradual increase in Iβ phase content.	Aydin; Aksoy, 2014

506

507 **6 Bioprocess control, optimization and scale-up**

508

509 As mentioned, bacterial cellulose has unique properties to industrial applications.
510 However, the implementation of highly productivity bioprocess is required to scale-up the
511 biopolymer production. Each fermentation process can be optimized by using statistics tools to
512 evaluate the effect of critical parameters on BC production or properties. Besides the one-factor-
513 at-a-time approach, the statistical optimization is frequently used to define the ideal condition
514 for BC production by analyzing a widely rate of a parameter, including the interaction of the
515 process variables. The statistical models are less time-consuming and profitable alternative to
516 explore the fermentation conditions aiming large scale production. Considering their influence
517 on BC yield, the mean parameters used on bioprocess optimization are the type of carbon and
518 nitrogen sources and concentration, ethanol, pH, temperature, method of cultivation, rotation
519 speed, inoculum concentration, and volume of the culture media (Bilgi *et al*., 2016; Du *et al*.,
520 2020; Hu; Catchmark; Demirci, 2021; Rastogi; Banerjee, 2020).
521 Nowadays, the limitation faced on large-scale implementation are meanly linked to
522 raw materials cost, energy and water consumption, by-products formation, carbon source
523 metabolization, and reproduces the yield obtained in the initial stages (Araújo *et al*., 2020; Skiba
524 *et al*., 2020; Zhong, 2020). Scale-up studies and alternatives operation mode have been
525 successfully carried using different nutrient sources and acetic acid bacteria, confirming that
526 this bioprocess can be more explored to improve BC application (Dubey; Singh; Singh, 2018;
527 Jahan; Kumar; Saxena, 2018).

528

529 **Conclusion**

530

531 Bacterial cellulose is a high-value product obtained from acetic acid fermentation

532 which could be widely used in industrial applications due to its characteristics. For this purpose,
533 the main question is how to optimize the yield to ensure a production system able to supply the
534 industrial demand. Designing a profitable bioprocess should consider the interaction between
535 the three main elements of the fermentation, the strain, the culture media, and the cultivation
536 method. The purpose of this review was to present the general aspects of BC production and
537 summarize the main challenges and strategies to increase production and reduce the bioprocess
538 cost. The findings presented in this review address insights into the alternatives to improve
539 bacterial cellulose production. Design a bioprocess for bacterial cellulose production requires
540 (1) a high-productivity strain either wild-type or using genetic engineering; (2) a low-cost
541 nutrient source, which could be achieved by using agro-industrial waste or replacing the carbon
542 sources in a synthetic medium; and (3) optimized cultivation method. An effective combination
543 of these strategies must be explored to ensure bacterial cellulose production and application on
544 industrial scale.
545
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547

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CAPÍTULO II

LOW-COST MEDIUM FOR BACTERIAL CELLULOSE PRODUCTION BY *Komagataeibacter intermedius* V-05 UNDER SHAKEN CULTIVATION

Original Research Article to be submitted to **Carbohydrate Polymers**

Low-cost medium for bacterial cellulose production by *Komagataeibacter intermedius* V-05 under shaken cultivation

Abstract

Bacterial cellulose (BC) is a natural and renewable polymer produced by microorganisms especially acetic acid bacteria. BC can be used in food, cosmetics, and biomedical fields however, the high cost of production mainly due the cost of culture medium components limits the application. Thus, the study aimed to formulate a low-cost medium with saccharose, and whey powered applying response surface methodology (RSM) using central composite design (CCD). *Komagaeibacter intermedius* V-05 was used to BC production under agitation (150 rpm/7days and 30 ºC). The optimized medium provides the yield (1.05 g L^{-1}) equivalent to Hestrin–Schramm (HS) medium (0.96 g L^{-1}). BC presented fibrous suspensions and irregular masses shape; and the physicochemical properties were similar to standard medium. CCD allowed the obtention of a low-cost culture medium that supply the nutrient requirements to BC production by the strain *Komagaeibacter intermedius* V-05.

Keywords: milk waste; exopolysaccharide; agitated fermentation; biopolymer; statistical model.

1 Introduction

Bacterial cellulose (BC) is a biopolymer with a structure similar to vegetal cellulose. This exopolysaccharide is produced by different species of microorganism, especially by acetic acid bacteria from the *Komagataeibacter* genus (Salari, Sowti Khiabani, Rezaei Mokarram, Ghanbarzadeh, & Samadi Kafil, 2019; Wang, Tavakoli, & Tang, 2019). Structurally, BC shows a three-dimensional network due to the association of the glucan chain by hydrogen bonds between hydroxyl groups of glucose units (Phruksaphithak, N.; Kaewnun, C.; O-Thong, 2019). The structure affords high crystallinity, porosity, high mechanical strength, thermal and chemical degradation stability, and high water holding capacity. Furthermore, BC is polymer

29 biodegradable and biocompatible and does not have lignin and hemicellulose, representing
30 high-purely cellulose (Foresti, Vázquez, & Boury, 2017).

31 The selection of microorganism strain, the media composition, and the cultivation
32 method, i.e., static or shaken, influence on yield and properties of the biomaterial and allows
33 the BC application in different areas, for example, the food industry, cosmetics, and
34 biomedicine (Gromovykh et al., 2020; Wang et al., 2019). Although the high potential of
35 application, the high cost of the culture medium, mostly carbon and nitrogen, is a limitation on
36 the fermentation process. The use of low-cost raw materials is a strategy to make BC production
37 economically feasible (Gupte et al., 2021; Marestoni et al., 2021).

38 Alternative culture medium must supply the essential nutrients as carbon and nitrogen
39 to bacterial growing and BC production (Reiniati, Hrymak, & Margaritis, 2017). The
40 *Komagataeibacter* strains are known for their ability to metabolize diverse carbohydrates. This
41 feature allows glucose replacement by carbon and nitrogen sources, such as saccharose and
42 vital components from low-cost raw materials, for example, fruits residues (Pacheco et al.,
43 2017), sugar industry waste (Qi et al., 2017), and coproducts of milk processing (Revin,
44 Liyaskina, Nazarkina, Bogatyreva, & Shchankin, 2018). Whey presents sugar, proteins,
45 vitamins, minerals, and organic acids whose components should support cell growth and BC
46 production (Kolesovs & Semjonovs, 2020), thus, is an attractive nutrient source to use in for
47 the composition of culture medium

48 Enhancing the yield of the biopolymer is as important as cost reduction. In this context,
49 using statistical tools could support the evaluation of the process variables and allow production
50 optimization (Khan, Saroha, Raghuvanshi, Bharti, & Dutt, 2021). Response surface
51 methodology (RSM) is an alternative to enhance BC production. RSM corresponds to a group
52 of mathematical and statistical techniques applied to create a functional relationship between a
53 response and independent variables (Khuri & Mukhopadhyay, 2010). RSM tool allows the

54 analyses of parameters that influence BC production, such as pH, temperature, and substrate
55 concentration. In addition, it is possible to define the great combination of each variable to
56 enhance BC yield (de Andrade Arruda Fernandes et al., 2020).

57 The reports on optimization of BC production from alternative mediums in agitated
58 cultivation are still limited. Therefore, this work aimed to formulate a low-cost medium using
59 saccharose and milk whey to produce BC by *Komagataeibacter intermedius* V-05 and
60 investigate the effects of the alternative medium on the structural and physicochemical
61 properties of the biopolymer.

62

63 **2 Material and methods**

64

65 2.1 Material

66 Glucose, saccharose and mannitol (Synth, Diadema, Brazil). Yeast extract (HiMedia,
67 Indaiatuba, Brazil). Bacteriological peptone (HiMedia, West Chester, PA, USA).
68 Bacteriological agar (Vetec, Rio de Janeiro, Brazil). Citric acid (Fmaia, Belo Horizonte, Brazil).
69 Disodium phosphate (Na_2HPO_4) (Cinética, Itapevi, Brazil). Sodium hydroxide (NaOH) (Biotec,
70 São José dos Pinhais, Brazil). Whey powder (2.30 g 100g moisture, 5.08 g 100g ashes, 8.11 g
71 100g protein, 0.35 g 100g lipids, and 84.16 g 100g carbohydrates) was supplied by a local
72 cooperative (Londrina, Brazil).

73

74 2.2 Microorganisms

75 *Komagataeibacter intermedius* V-05 was isolated by Gomes et al. (2021) from fruit and
76 cereal vinegar fermentation broth at a local food industry (Tecnologia em Saúde Indústria de
77 Alimentos, Assis, São Paulo State, Brazil). The industry produces vinegar by the fast method
78 in vinegar generators. The strain was deposited (CMRP4501) in the microbial collection of the

79 Laboratory of Microbial Genetics (LabGeM) of the Federal University of Paraná (UFPR),
80 Brazil. Bacterial cultures were stored in 20% (w/v) malt extract in a freezer at −18 °C and
81 maintained on MYP agar (25 g L^{-1} mannitol, 5 g L^{-1} yeast extract, and 3 g L^{-1} peptone). Plates
82 were prepared by the double-layer technique using 1% agar in the top layer and 0.5% agar in
83 the bottom layer (Entani et al., 2008), stored at 4 °C, and periodically subcultured.
84

85 2.3 Inoculum preparation
86
87 Cells were picked from the MYP agar plates, inoculated into 100 mL of Hestrin–
88 Schramm (HS) broth (20 g L^{-1} glucose, 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 2.7 g L^{-1}
89 Na_2HPO_4, 1.15 g L^{-1} citric acid, and distilled water) with 1 mL of cellulase at 0.2 g mL^{-1}
90 (Prozyn, São Paulo, Brazil), incubated or under agitation at 120 rpm on an orbital shaker
91 (Tecnal TE-4200, Brazil) for 48 hours at 30 °C. The inoculum was centrifuged (5000 rpm, 4
92 ºC, 15 minutes), washed with distilled water (5000 rpm, 4 ºC, 15 minutes), and resuspended
93 with distilled water (OD_{600}= 0.740; 1.28×10^8 UFC mL^{-1}).
94

95 2.4 Optimization study
96
97 The response surface methodology (RSM) using the Central Composite Rotatable
98 Design (CCRD) was performed in the optimization of three variables: saccharose concentration
99 (X_1), whey powder concentration (X_2), and pH (X_3). The ranges of the independent variables
100 were chosen based on preliminary experiments (data not shown). The effects of each variable
101 and the interactions were evaluated using a 2^3 factorial design with six axial points and three
102 replicates at the central point, resulting in a total of 17 experiments. BC production (g L^{-1}) was
103 used as response in CCD results. The actual and coded levels of the independent variable and
104 the CCD design are shown in Table 1 and 2.
105

106 Table 1. Range values of independent variables

Variable	Levels				
	-α	-1	0	1	+α
Saccharose concentration g.L^{-1} (X_1)	11.59	15.00	20.00	25.00	28.41
Whey powder concentration g.L^{-1} (X_2)	27.55	33.00	41.00	49.00	54.45
pH (X_3)	4.66	5.00	5.50	6.00	6.34

107

108 Table 2. Central Composite Rotatable Design and response variables expressed in bacterial

109 cellulose production (g.L^{-1})

No.	Independent variables coded and uncoded[a]			BC[b]
	X_1 (x_1)	X_2 (x_2)	X_3 (x_3)	
1	15.00 (-1)	33.00 (-1)	5.00 (-1)	0.55
2	15.00 (-1)	33.00 (-1)	6.00 (+1)	0.67
3	15.00 (-1)	49.00 (+1)	5.00 (-1)	1.14
4	15.00 (-1)	49.00 (+1)	6.00 (+1)	0.20
5	25.00 (+1)	33.00 (-1)	5.00 (-1)	0.96
6	25.00 (+1)	33.00 (-1)	6.00 (+1)	0.74
7	25.00 (+1)	49.00 (+1)	5.00 (-1)	0.89
8	25.00 (+1)	49.00 (+1)	6.00 (+1)	0.91
9	11.59 (-α)	41.00 (0)	5.50 (0)	0.85
10	28.41 (+α)	41.00 (0)	5.50 (0)	1.26
11	20.00 (0)	27.55 (-α)	5.50 (0)	1.01
12	20.00 (0)	54.45 (+α)	5.50 (0)	1.24
13	20.00 (0)	41.00 (0)	4.66 (-α)	0.60
14	20.00 (0)	41.00 (0)	6.34 (+α)	0.42
15	20.00 (0)	41.00 (0)	5.50 (0)	1.25
16	20.00 (0)	41.00 (0)	5.50 (0)	1.30
17	20.00 (0)	41.00 (0)	5.50 (0)	1.41

110 [a] X_1 (Saccharose g L^{-1}), X_2 (Whey g L^{-1}), X_3 (pH). [b] bacterial cellulose production (g.L^{-1})

111

112

113 The optimum point was predicted by second-order model represented by the quadratic

114 equation (1):

115

116 $$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum\sum_{i<j} \beta_{ij} x_i x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 \quad (1)$$

117

118

119 where **Y** is the predicted response; $\boldsymbol{\beta_0}$, $\boldsymbol{\beta_j}$, $\boldsymbol{\beta_{ij}}$ and $\boldsymbol{\beta_{jj}}$ refer to the coefficients of constant, linear,

120 interaction effects and quadratic, respectively; $\boldsymbol{x_i}$ and $\boldsymbol{x_j}$ are the coded value of the independent

121 variables. The model was evaluated by analysis of variance (ANOVA) as the Fisher value (F

122 value). The significance level was considered as $\alpha = 0.10$. The software Statistic 10.0 (Statsoft,

123 USA) was used to experimental design, statistical analysis and to generate the three-

124 dimensional curves of the response surface and contour plots.

125

126 2.5 Preparation of the culture media with whey powder

127

128 The culture media was formulated following the composition of HS. Glucose as

129 replaced by saccharose as a carbon source. Yeast extract and peptone were replaced by whey.

130 After dissolution and pH adjustment, each media was added to sterile *Erlenmeyer* flasks and

131 pasteurized (65 ºC/ 30 min) in a heating bath (Quimis Q215M, Brazil).

132

133 2.6 BC production and quantification

134 10 mL of inoculum was transferred to a Falcon tube of 15 mL and centrifuged (5000

135 rpm, 4 ºC, 5 minutes). Then, 10 mL from respective medium was used to dissolve the cells

136 precipitated. After homogenization, the inoculum was transferred to 250 mL *Erlenmeyer* flasks

137 (final volume: 100 mL). Fermentation was carried under agitation at 150 rpm on an orbital

138 shaker (Tecnal TE-4200, Brazil) for 7 days at 30 °C. The produced BC was separated from the

139 medium and purified by immersion in 1 M NaOH solution at 80 °C for 40 min and successively
140 washed until neutralization. Purified BC was oven-dried (Nova Ética, Brazil) at 105 °C to
141 constant weight, which was determined using an analytical balance (FA2104N, Bioprecisa,
142 Brazil). BC production is expressed as the dry weight of BC produced per liter.

143

144 2.8 Physicochemical and structural evaluations
145
146 2.8.1 Fourier-transform infrared spectroscopy (FTIR)
147
148 Fourier-transform infrared spectroscopy (FTIR) was performed using an IR Prestige-21
149 spectrophotometer (Shimadzu, Japan) using the KBr (≥ 99%, Sigma-Aldrich) pellet technique.
150 Spectra were obtained over the wavenumber range of 4000–400 cm^{-1} at 1 cm^{-1} resolution.

151

152 2.8.2 X-ray diffraction (XRD)
153
154 X-ray diffraction (XRD) spectra of BC was recorded using a X´Pert PRO MPD
155 diffractometer (PANalytical, Reino Unido), using Cu-Kα at 40 kV and 30 mA. The data were
156 collected at a scan of 2θ from 5˚ to 40˚ in steps of 0.05º. The crystallinity indices (CrI) were
157 calculated using Segal equation (Segal et al., 1959):

158

159 $$\text{CrI (\%)} = \frac{(I200 - Iam)}{I200} x100 \quad (2)$$

160

161 2.8.3 Thermogravimetry
162
163 Thermogravimetric/derivative thermogravimetric analysis (TGA/DTG) was performed
164 on a TGA 50 (Shimadzu, Japan) under nitrogen atmosphere (50 mL min^{-1}). Samples were
165 heated from 30 to 600 °C at a heating rate of 10 °C min^{-1}. DTG curves are expressed as mass
166 variation as a function of temperature.

167

168 2.8.4. Determination of water-holding capacity
169
170 The water-holding capacity (WHC) of BC samples was determined according to the
171 method described by Lin, Lopez-Sanchez, Li, & Li (2014). WHC was calculated using Eq. (3)
172 and expressed in grams of water held per gram of sample.

173

174 $$WHC\ (g) = \frac{water\ weight\ loss}{BC\ dry\ weight} \quad (3)$$

175

176 **3 Results and discussion**
177

178 3.1 Central Composite Rotatable Design

179 A Central Composite Rotatable Design (CCRD) was used to investigate the optimum
180 concentration (g L^{-1}) of saccharose (X_1) and whey powder (X_2), and the optimum initial pH
181 (X_3) on the production of BC by *K. intermedius* V-05 under agitated condition. The yield of BC
182 was obtained within a range from 0.42 to 1.41 g L^{-1} (Table 2). The ANOVA results (Table 3)
183 showed that the second-order model was significant at a 10 % level ($F_{cal} > F_{tab}$; p-value =
184 0.0086). Also, exhibited a non-significant lack of fit and R^2 was 0.8206 indicating that 82,06%
185 of the data variability can be explained by the model. This R^2 value was enough to BC
186 production since is a bioprocess with high variability.

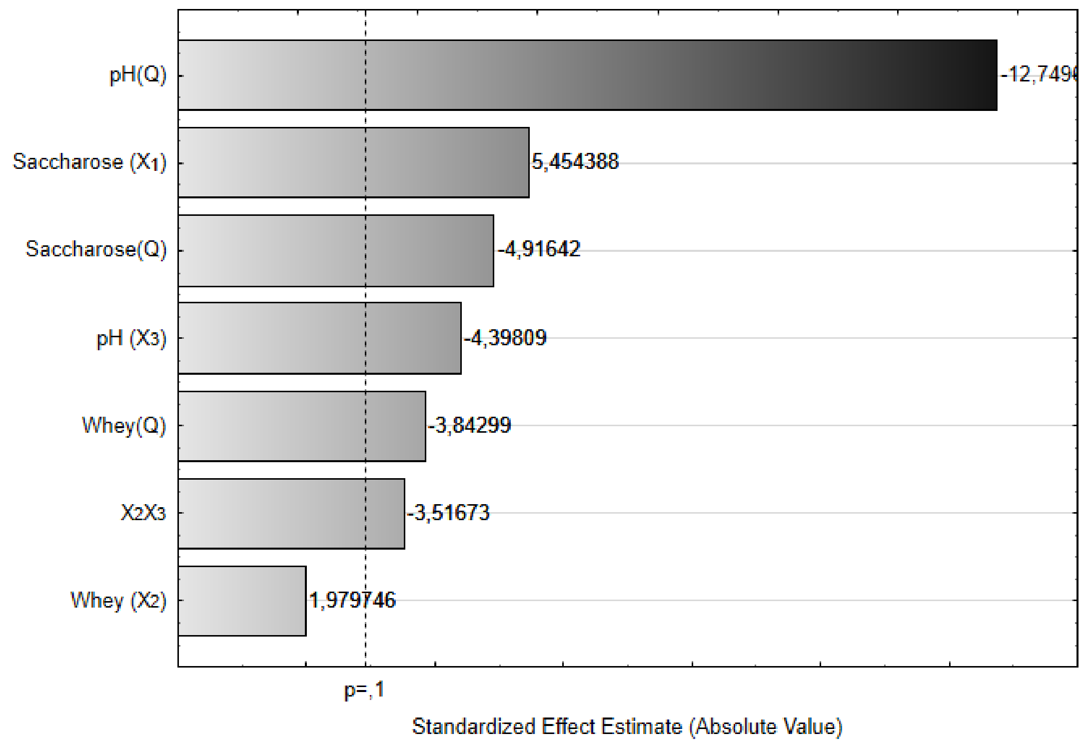
187

188 Table 3 - ANOVA analysis of the model

Source of variation	SS	Df	MS	Fvalue	*p*-value
x_1	0.199238	1	0.199238	29.7504	0.032008*
x_1^2	0.161875	1	0.161875	24.1712	0.038969*
x_2	0.026248	1	0.026248	3.9194	0.186288**

x_2^2	0.098905	1	0.098905	14.7686	0.061528*
x_3	0.129542	1	0.129542	19.3432	0.048006*
x_3^2	1.088608	1	1.088608	162.5516	0.006096*
$x_2.x_3$	0.082825	1	0.082825	12.3674	0.072209*
Residual	0.335885	9	0.037321		
Lack of Fit	0.322491	7	0.046070	6.8792	0,132750
Pure error	0.013394	2	0.006697		
Total SS	1.872317	16			
R^2	0.8206				
Adj R^2	0.6811				

189 X_1 (Saccharose g L^{-1}), X_2 (Whey g L^{-1}), X_3 (pH). (*) Significant at 0.10. (**) Maintained for
190 being present in a significant interaction. Df: degrees of freedom; SS: Sum of squares; MS:
191 Mean square.
192
193 Fig. 1 shows the Pareto charts of effects (considering statistical significance p-level of
194 0.10) that impact BC production. The pH quadratic term (Q) had the most pronounced effect
195 on the response. Both linear and quadratic terms of pH negatively affect BC production,
196 indicating that lower initial pH values provide a higher yield. The interaction between pH and
197 whey also had a negative effect. For saccharose (X_1) and whey powder (X_2), the linear effects
198 were positive, while quadratic terms were negative. The non-significant interactions (X_1X_2 and
199 X_1X_3) were removed.
200



201

202 **Fig. 1.** Pareto charts for the bacterial cellulose production as a function saccharose (X_1), whey

203 powder (X_2), and the pH (X_3).

204

205 The model equation for BC production after elimination of non-significant terms ($p >$

206 0.10) was provided in the coded form (Eq. 2). The linear term X_2 was maintained for being

207 present in a significant interaction.

208

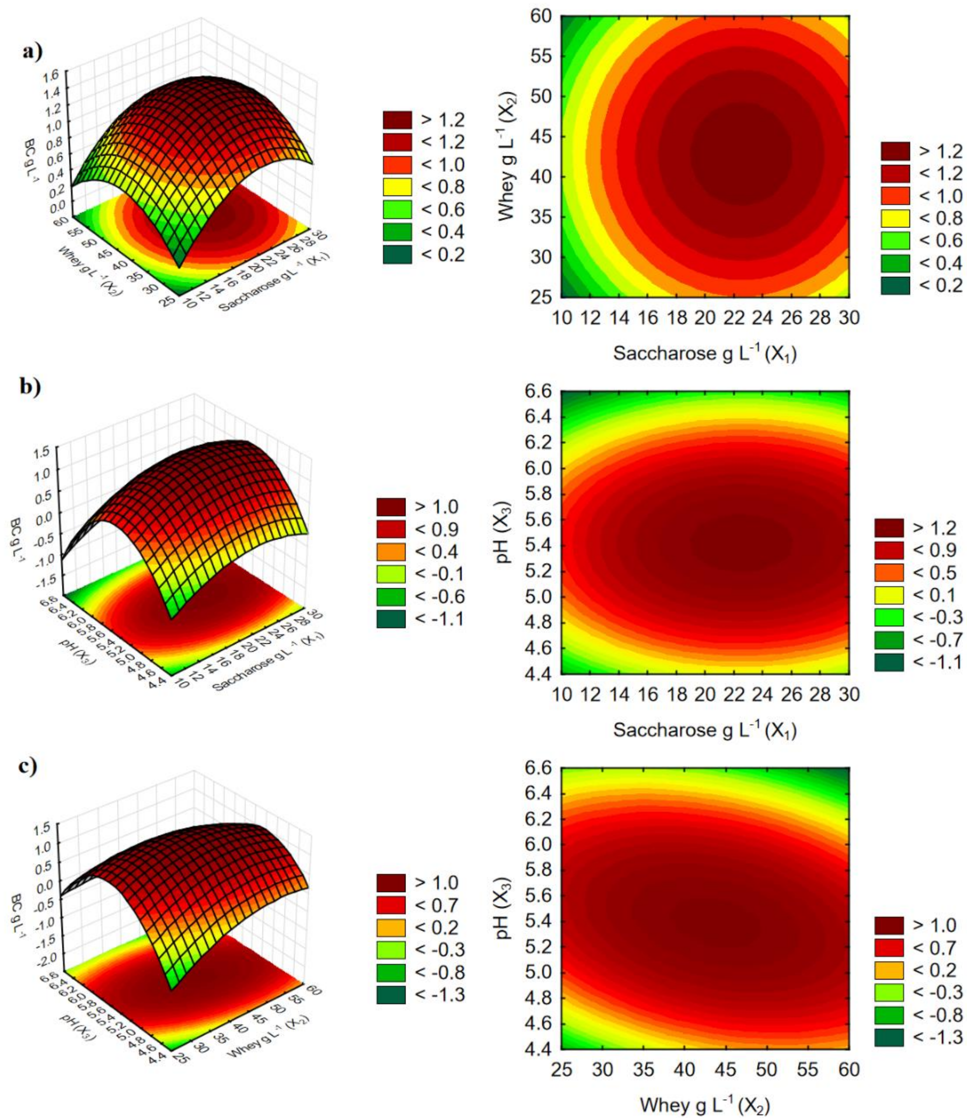
209 $$\text{BC (g L}^{-1}) = 1.33 + 0.12X_1 - 0.12X_1^2 + 0.04X_2 - 0.09X_2^2 - 0.10X_3 - 0.31X_3^2 - 0.10X_2X_3 \quad (4)$$

210

211 3.1.1 Response surfaces and contour curves

212 The three-dimensional graphs and contour surfaces based on equation 2 were plotted

213 to show the effect of each two variables on BC production (Fig. 2).



214

215 **Fig. 2.** Response surfaces and contour plots of the bacterial cellulose production as a function
216 saccharose (X_1) and whey powder (X_2), and the optimum pH (X_3)

217

218 Fig. 2a shows the effects of saccharose and whey on BC production. These data
219 indicate that the higher BC yields were found in the central point region for saccharose (20 –
220 26 g L^{-1}) and whey powder (36 – 48 g L^{-1}). The graph shows that the BC production increase
221 with the concentration of both variables until the mentioned range and then decreases,
222 indicating the excess of the carbon and nitrogen sources in the medium does not favor the yield.
223 As illustrated in Fig. 2b, BC production was higher in pH 5.2 to 5.6. According to the model,

224 at extreme values of pH (> 6.0 and < 4.8) the production was not possible in any saccharose
225 concentrations, indicating this is outside the suitable pH range to V-05 strain. Finally, Fig. 2c
226 shows the significant interaction of the effect of pH and whey powder concentration (Table 3)
227 in which the higher whey concentration, lower pH was required to increase BC production.

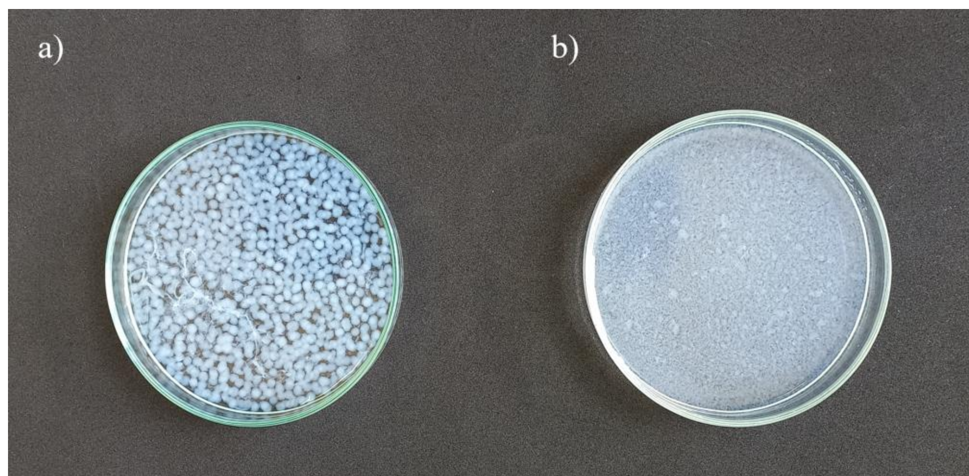
228 Concerning saccharose concentration, while low concentrations are not enough for the
229 cell growth and BC syntheses, the excess of carbon source could reduce the oxygen supply and
230 decrease bacterial activity, consequently limiting substrate consumption (Lotfiman, Awang
231 Biak, Ti, Kamarudin, & Nikbin, 2018). The carbon source used in this study is a disaccharide
232 and needs to be hydrolyzed into glucose and fructose to start BC (Lee, Buldum, Mantalaris, &
233 Bismarck, 2014). The hydrolyses occur enzymatically, wherefore, the excess of saccharose
234 could result in substrate saturation and invertase inhibition. Considering the metabolism of
235 BAA, one of the features of this carbon source is that saccharose may address the pH decrease
236 by the accumulation of acid residues such as gluconic acid since glucose is not the primary
237 carbon source (Santoso et al., 2020). V-05 has already showed potential of grow and produce
238 BC in a alternative culture medium were saccharose was the main sugar (Gomes et al., 2021).

239 Besides nitrogen and amino acids support, whey also contributes with another essential
240 component for cell growth and BC production, such as, vitamins, minerals, organic acids, and
241 additional sugar (e.g. glucose) that could be used by the microorganism as a carbon source
242 (Kolesovs & Semjonovs, 2020). In this context, the higher BC production with an increase of
243 the whey concentration (Fig 2) was possibly due to the nutrient apport from this by-product.
244 Revin et al. (2018) reported that the BC production from a whey medium was influenced by the
245 consumption of lactose, hexoses (including glucose), and monocarboxylic acids.

246 The optimum initial pH to V-05 strain was found to be 5.39, which is close to optimum
247 range suggested for *K. intermediuns* strains by some authors (Fernández et al., 2019; Santoso
248 et al., 2020). However, *K. intermediuns* were also able to produce BC at alkaline pH values

249 (Fernández et al., 2019; Lin et al., 2016). The initial pH to BC production has been found at 4-
250 7, depending on the strain and media composition (Reiniati et al., 2017; Santoso et al., 2020).
251 Besides the effects on cell activity, pH plays an essential function in nutrient solubility
252 and uptake. In this study, the optimum initial pH (5.39) is higher than the isoelectric pH of milk
253 proteins and may influence protein accessibility by furthering the solubility. In contrast, the low
254 BC productions close to the isoelectric pH (~4.5) may be associated with the minimum
255 solubility that occurs at this point (Pelegrine & Gasparetto, 2005; Damodaran, 2017). Also, the
256 negative interaction between pH and whey concentration suggests a buffering effect of this
257 nutrient source. In other words, the lower pH values require higher concentrations of whey to
258 keep the pH at the optimum range during BC production. This buffering effect was observed in
259 other studies using alternative nutrient sources (Abdelraof, Hasanin, & El-Saied, 2019; Santoso
260 et al., 2020).
261
262 3.1.1 Validation of the statistical model
263 The optimized values for each variable predicted by the model were saccharose (X_1)
264 22.52 g L^{-1}, whey powder (X_2) 43.80 g L^{-1}, and initial pH (X_3) 5.39. Under these optimal
265 conditions, the BC production (Y) predicted was 1.37 g L^{-1} and experimental value obtained
266 was 1.05 ± 0.05 g L^{-1}. This value was considered acceptable considering the R^2 (0.8206)
267 obtained from the adjusted model. Given the BC production in HS medium (0.96 ± 0.01 g L^{-1}
268), the optimized medium could be used as a low-cost alternative to supply nutrients to cell
269 growth and biopolymer production.
270 Aiming to prospect the use of this strain in a cost-effective process for BC production,
271 *K. intermedius* V-05, isolated from a vinegar industry, was previously cultivated in soybean
272 molasses medium (Gomes et al., 2021) and shows high-capacity production in static cultivation
273 (10 g L^{-1}) compared to HS medium (3.70 g L^{-1}). Under shaking, this alternative medium
274 provided a yield similar to HS (data not published yet). In this work, *K. intermedius* V-05 was

275 not able to produce BC under static conditions using saccharose and whey as carbon and
276 nitrogen sources. Other low-cost materials have been studied to replace the nutrient sources in
277 BC production such as bagasse hydrolysates (1.09 g L^{-1} – 0.0022 g L^{-1} h^{-1}) (Qi et al., 2017);
278 sugar beet molasses (4.56 g L^{-1} – 0.0135 g L^{-1} h^{-1}) and cheese whey enzymatically treated (3.55
279 g L^{-1} – 0.0106 g L^{-1} h^{-1}) (Salari et al., 2019); aqueous extract of fruit peel wastes (11.44 g L^{-1} –
280 0.0298 g L^{-1} h^{-1}) (Khan et al., 2021). Under agitation, Revin et al. (2018) also used wheat thin
281 stillage (6.19 g L^{-1} – 0.0859 g L^{-1} h^{-1}) and whey (5.45 g L^{-1} – 0.0757 g L^{-1} h^{-1}) for BC production.
282
283 3.2 BC characterization
284
285 3.2.1 Morphology
286
287 Firstly, the shape of BC obtained from the optimized medium (Fig. 3b) was fibrous
288 suspensions and irregular masses. In contrast, BC produced in HS medium (Fig. 3a) was in a
289 small sphere shape. In the agitated method, the bacteria can produce BC in different particles
290 sizes and shapes, such as spherical, stellate, fibrous suspensions, pellets, or irregular masses.
291 However, the medium composition, the strain, and the rotation speed can influence the shape
292 of BC, resulting in a biomaterial with different characteristics and useful to different application
293 (Gromovykh et al., 2020; Singhsa, Narain, & Manuspiya, 2018; Wang et al., 2019). After the
294 purification, BC produced in this study is a raw-material that could later be processed, for
295 example, disassembled by physical or chemical methods to obtain a suspension or powder to
296 use in different applications, such as food industry (Azeredo, Barud, Farinas, Vasconcellos, &
297 Claro, 2019).

298 

299 **Fig. 3.** Bacterial cellulose from the HS (a) and optimized medium (b)

300

301 3.2.2 WHC
302

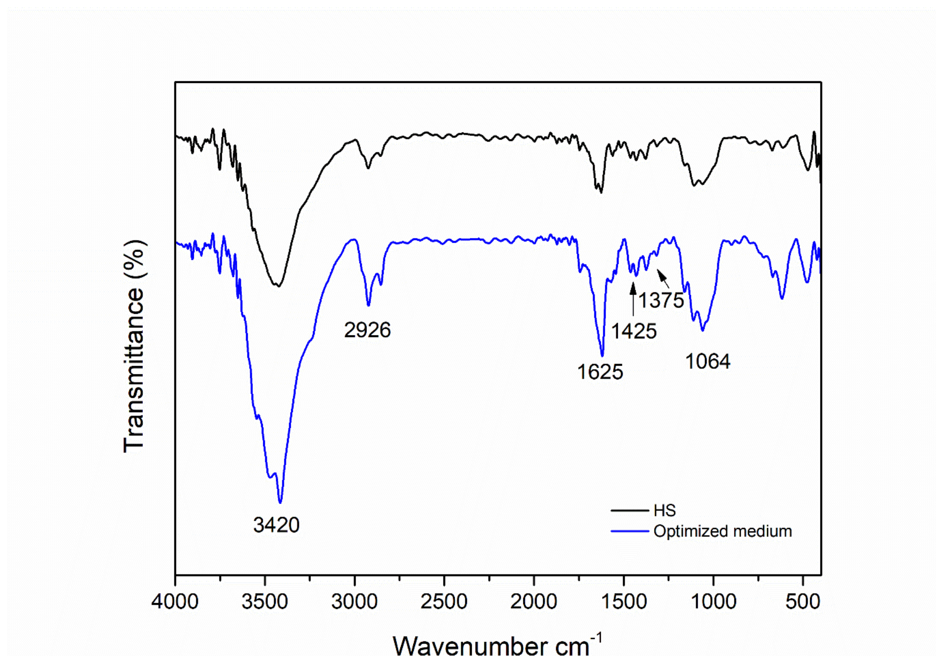
303 BC showed a great capacity to entrapment of water molecules. WHC of BC from
304 optimized medium (223.41 ± 1,19 g) was similar to that cultivated in HS (227.11 ± 4.46 g).
305 This event may be supported by the less organized structure of BC from the alternative medium,
306 contributing to the formation of hydrogen bonds between β-D-glucopyranose monomers and
307 water molecules (Campano, Balea, Blanco, & Negro, 2016; Lotfiman et al., 2018). The WHC
308 is a useful property to many applications such as support for cell immobilization (Żywicka,
309 Banach, Junka, Drozd, & Fijałkowski, 2019), low-lipid low-sodium food application
310 (Marchetti, Muzzio, Cerrutti, Andrés, & Califano, 2017), and drugs and bioactive molecules
311 delivery (Portela, Leal, Almeida, & Sobral, 2019).

312

313 3.2.3 Fourier-transform infrared spectroscopy

314 The FTIR spectrum of BC produced in the optimized medium showed similar peaks
315 to standard HS medium (Fig.4), indicating the typical bands of bacterial cellulose: stretching
316 vibration absorption peak of -OH (3420 cm^{-1}); stretching vibration absorption peaks of C-H
317 (2926 cm^{-1}); absorption peak near 1625 cm^{-1} represented the expansion of C=O; symmetric

318 angular deformation vibration peak of methylene (-CH_2) (1425 cm^{-1}); C–H angular deformation
319 of methyl groups (1375 cm^{-1}); C–O symmetric stretching and C–H angular stretching (1064
320 cm^{-1}). The alternative culture medium did not change the structure of the BC and the spectrums
321 were consistent with previous reports, confirming the material purity (Gao et al., 2020; Khan et
322 al., 2021; Lu et al., 2020).



323

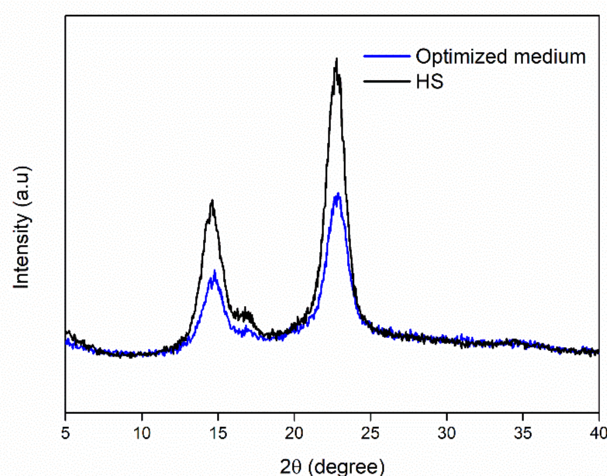
324 **Fig. 4.** Fourier-transform infrared spectra of bacterial cellulose produced by *Komagataeibacter*
325 *intermedius* V-05 from Hestrin–Schramm medium (HS) and optimized medium.

326

327 3.2.4 X-ray diffraction (XRD)

328 X-ray diffraction pattern (Fig. 5) of BC from optimized medium and HS revealed three
329 diffraction peaks located at 14,73º, 16,97º, and 22,77º, usually attributed to crystallographic
330 planes of 101 (amorphous region), 10 (amorphous region), and 200 (crystalline region),
331 respectively. The presence of these diffraction peaks characterizes cellulose type I (triclinic)
332 (Sederavičiūtė, Bekampienė, & Domskienė, 2019; Vasconcelos et al., 2017). The crystallinity
333 index was 82.40% for BC obtained from the optimized medium and 89.12% for HS. In this
334 study, the replacement of carbon and nitrogen sources kept the material high crystallinity.

335 Despite increasing the BC production, changing nutrients sources can influence BC
336 organization, consequently, affecting its properties (Gromovykh et al., 2020). This was reported
337 when the HS medium replacement by whey resulted in a crystallinity index reduction from 79.7
338 to 50.2 % was observed (Revin et al., 2018). The same trend was observed by Salari et al.
339 (2019), where the crystallinity decrease (from 79.07 to 61.86 %) was a refer to the presence of
340 galactose in a whey enzymatically tread. The crystalline index could influence polymers
341 properties, for example, thermal stability and mechanical resistance, and is required for some
342 applications, such as food packaging and support for bioactive compounds (Mohite & Patil,
343 2014; Lin et al., 2020; Souza et al., 2020).



344

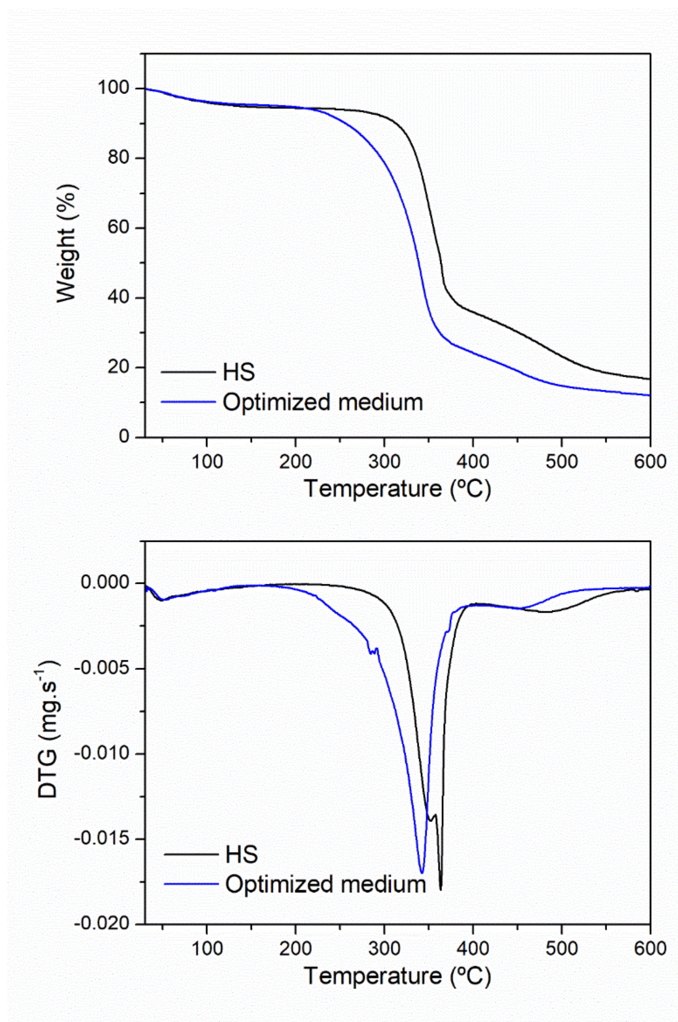
345 Fig. 5 X-ray diffractogram of bacterial cellulose produced by *Komagataeibacter intermedius*
346 V-05 from Hestrin–Schramm medium (HS) and optimized medium.

347

348 3.2.4 TGA

349 TGA and DTG curves of BC (Fig. 6) showed a similar thermal degradation pattern.
350 As usual, the mass loss occurred in three main events in both samples: evaporation of water
351 molecules physically adsorbed onto the cellulose structure (30 to 100 ºC); degradation of
352 cellulose and depolymerization into glucose units (~200 to 400 ºC); oxidation degradation and

353 breakdown of carbonaceous compounds (~400 to 600 ºC). The main weight loss started at about
354 235 ºC and 310 ºC to BC from alternative medium and HS, respectively. The maximum rate of
355 weight loss was around 340 ºC to BC produces using the optimized medium, and around 360
356 ºC to BC from HS. During this degradation stage (second event), the mass reduction of BC was
357 higher (70 %) than BC cultured in the standard medium (59%). The final residue value of BC
358 from optimized medium and HS was about 12% and 16.73%, respectively. Thermal degradation
359 is influenced by molecular weight, crystallinity, and cellulose chain orientation (Mohite & Patil,
360 2014). In this case, the lower crystallinity index provide by the optimized medium could explain
361 the thermal stability reduction; however, BC produced still a highly resistant material. The
362 results were according to previous reports (He et al., 2020; Li et al., 2019; Ye et al., 2019).
363 These thermal stability profile suggest the material is applicable in biomedical field, film, and
364 biopolymer composites (Campano et al., 2016).



365
366 **Fig. 6.** TGA/DTG curves of bacterial cellulose produced by *Komagataeibacter intermedius* V-
367 05 from Hestrin–Schramm medium (HS) and optimized medium.

368

369 **4. Conclusion**

370 The alternative medium provided nutrients for cell growing and BC production under
371 agitation, resulting in a similar yield to the standard HS medium. The optimized medium
372 resulted in a highly pure material, which was validated by the FTIR spectrum, the thermal
373 stability, and the crystallinity. The Response Surface Methodology allowed to find the optimal
374 combination of saccharose (22.52 g L^{-1}), whey (43.80 g L^{-1}) and pH (5.39) to BC production.
375 The use of low-cost nutrient source contributes to increasing the use of this renewable polymer
376 in food, cosmetic and biomedical fields.

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378

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4 CONCLUSÃO GERAL

O presente trabalho abordou os principais aspectos atrelados a produção de celulose bacteriana por bactérias do ácido acético. Os dados apresentados reforçam que a otimização da produção de celulose bacteriana deve abranger os três pilares do bioprocesso: cepa, meio de cultivo e método. Tal objetivo pode ser atingido por meio da implementação de métodos de monitoramento do crescimento e metabolismo microbiano, utilização de cepas de alta produtividade, resistentes ou geneticamente modificadas, adequação da fonte de nutrientes e adaptação da cepa às condições de cultivo.

Um dos desafios da produção de celulose bacteriana em larga escala é a formulação de meio de cultivo de baixo custo e que se adequem às necessidades nutricionais do microrganismo utilizado. Neste trabalho, um meio de cultura formulado com sacarose e soro de leite foi otimizado utilizando Metodologia de Superfície de Resposta para produção de celulose bacteriana pela cepa *Komagataeibacter intermedius* V-05 em cultivo agitado. A condição ótima estabelecida para os parâmetros avaliados foi 22,52 g L^{-1} de sacarose, 43.80 g L^{-1} soro de leite e pH inicial 5,39. O rendimento obtido a partir do meio otimizado (1,05 g L^{-1}) foi similar ao meio padrão Hestrin- Schramm cultivo (0.96 g L^{-1}). Além disso, o material obtido na condição otimizada destacou-se pela alta pureza, alta cristalinidade e elevada estabilidade térmica.

Embora as bactérias do ácido acético, em especial as espécies do gênero *Komagataeibacter* sejam capazes de metabolizar inúmeras matérias-primas ricas em açúcares, o cultivo dessas bactérias é um fator desafiador uma vez que requer a avaliação do desempenho do microrganismo nos diferentes métodos de cultivo. O emprego de agitação na fermentação, ainda que eficiente na produção de CB, pode induzir o surgimento de células mutantes e afetar diretamente no rendimento uma vez que essas células têm sua capacidade de síntese de celulose inibida. A cepa *Komagataeibacter intermedius* V-05 isolada de uma unidade produtora de vinagres mostrou-se adequada para cultivo em método agitado utilizando o meio formulado. O aprimoramento dos parâmetros da fermentação, do preparo de inóculo à obtenção do produto, é essencial para que essa cepa se consolide como alta produtora de CB e por fim, dissemine seu emprego em processos fermentativos em escala industrial.

Considerando a importância econômica deste biopolímero, explorar técnicas e parâmetros de cultivo do microrganismo que maximizem o rendimento bem como o desenvolvimento de meios de cultivo de baixo custo são estratégias primordiais para a produção e utilização da celulose bacteriana em larga escala.