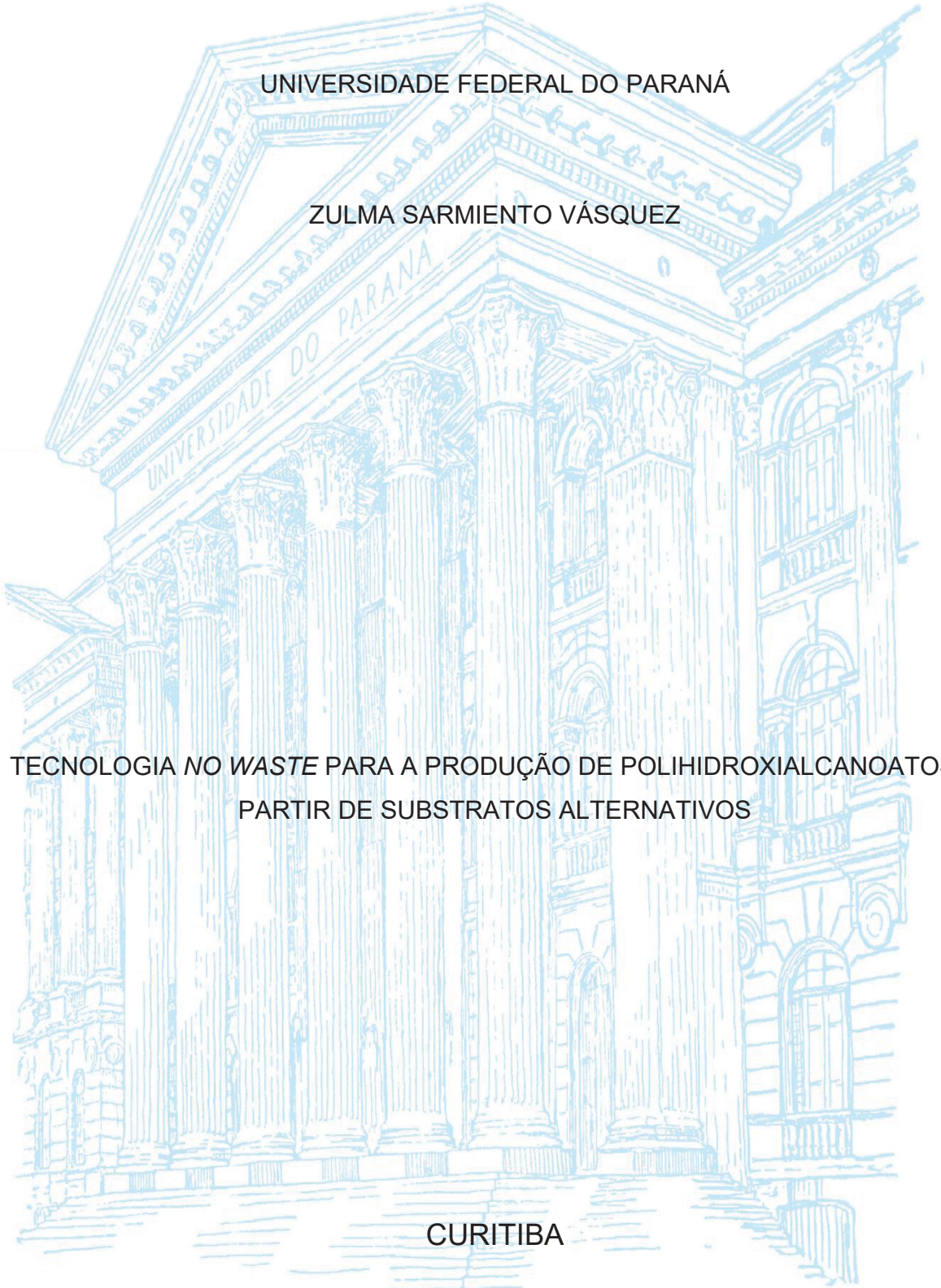
UNIVERSIDADE FEDERAL DO PARANÁ

ZULMA SARMIENTO VÁSQUEZ



TECNOLOGIA *NO WASTE* PARA A PRODUÇÃO DE POLIHIDROXIALCANOATOS A PARTIR DE SUBSTRATOS ALTERNATIVOS

CURITIBA

2022

ZULMA SARMIENTO VÁSQUEZ

TECNOLOGIA *NO WASTE* PARA A PRODUÇÃO DE POLIHIDROXIALCANOATOS A PARTIR DE SUBSTRATOS ALTERNATIVOS

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós- Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientadora:
Profª. Drª. Luciana P. S. Vandenberghe,

Co-orientador:
Prof. Dr. Carlos Ricardo Soccol

Curitiba

2022

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA CIÊNCIA E TECNOLOGIA

Sarmiento Vásquez, Zulma.
Tecnolgia *no waste* para a produção de polihidroxialcanoatos a partir de substratos alternativos. / Zulma Sarmiento Vásquez. – Curitiba, 2022.
1 recurso on-line : PDF.

Tese (Doutorado) – Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia.
Orientadora: Profa. Dra. Luciana P. S. Vandenberghe.
Coorientador: Prof. Dr. Carlos Ricardo Soccol.

1. Biotecnologia. 2. Biopolímeros. 3. Soja. 4. Glicerol. I. Vandenberghe, Luciana P. S. II. Soccol, Carlos Ricardo. III. Universidade Federal do Paraná. Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia. IV. Título.

Bibliotecário: Nilson Carlos Vieira Junior CRB-9/1797

ATA Nº163

ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTORA EM ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

No dia vinte e oito de abril de dois mil e vinte e dois às 16:00 horas, na sala virtual (fechada), remota via Microsoft Teams , foram instaladas as atividades pertinentes ao rito de defesa de tese da doutoranda **ZULMA SARMIENTO VÁSQUEZ**, intitulada**: Tecnologia *no waste* para a produção de polihidroxialcanoatos a partir de substratos alternativos**, sob orientação da Profa. Dra. LUCIANA PORTO DE SOUZA VANDENBERGHE. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: LUCIANA PORTO DE SOUZA VANDENBERGHE (UNIVERSIDADE FEDERAL DO PARANÁ), RAFAELA DE OLIVEIRA PENHA Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ), DÃO PEDRO DE CARVALHO NETO (INSTITUTO FEDERAL DO PARANÁ), CARLOS RICARDO SOCCOL (UNIVERSIDADE FEDERAL DO PARANÁ), SUSAN GRACE KARP (UNIVERSIDADE FEDERAL DO PARANÁ), WALTER JOSE MARTINEZ BURGOS Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutora está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, LUCIANA PORTO DE SOUZA VANDENBERGHE, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.
Observações: Aprovada com mérito científico.

CURITIBA, 28 de Abril de 2022.

Assinatura Eletrônica
29/04/2022 12:35:15.0
LUCIANA PORTO DE SOUZA VANDENBERGHE
Presidente da Banca Examinadora

Assinatura Eletrônica
29/04/2022 12:34:04.0
RAFAELA DE OLIVEIRA PENHA
Avaliador Interno Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
02/05/2022 14:07:05.0
DÃO PEDRO DE CARVALHO NETO
Avaliador Externo (INSTITUTO FEDERAL DO PARANÁ)

Assinatura Eletrônica
29/04/2022 11:39:17.0
CARLOS RICARDO SOCCOL
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
02/05/2022 10:24:33.0
SUSAN GRACE KARP
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
29/04/2022 12:16:28.0
WALTER JOSE MARTINEZ BURGOS
Avaliador Interno Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ)

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ZULMA SARMIENTO VÁSQUEZ** intitulada: **Tecnologia *no waste* para a produção de polihidroxialcanoatos a partir de substratos alternativos**, sob orientação da Profa. Dra. LUCIANA PORTO DE SOUZA VANDENBERGHE, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Abril de 2022.

Assinatura Eletrônica
29/04/2022 12:35:15.0
LUCIANA PORTO DE SOUZA VANDENBERGHE
Presidente da Banca Examinadora

Assinatura Eletrônica
29/04/2022 12:34:04.0
RAFAELA DE OLIVEIRA PENHA
Avaliador Interno Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
02/05/2022 14:07:05.0
DÃO PEDRO DE CARVALHO NETO
Avaliador Externo (INSTITUTO FEDERAL DO PARANÁ)

Assinatura Eletrônica
29/04/2022 11:39:17.0
CARLOS RICARDO SOCCOL
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
02/05/2022 10:24:33.0
SUSAN GRACE KARP
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
29/04/2022 12:16:28.0
WALTER JOSE MARTINEZ BURGOS
Avaliador Interno Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ)

A meus pais, Ricardo e Marina
Dedico.

AGRADECIMENTOS

Aos Diretores, Coordenadores e Professores do Programa de Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná, pela oportunidade de fazer parte do curso e por contribuir com a minha formação acadêmica e profissional.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudo.

À Professora Luciana Vandenberghe, por sua orientação, exemplo, ensinos, confiança e estímulos para o desenvolvimento deste estudo. E por ser um suporte importante durante esta etapa do meu desenvolvimento profissional.

A minha família, em especial aos meus pais Ricardo e Marina, e as minhas irmãs Lucia e Tatiana, e ao meu sobrinho Sebastian pelo amor, confiança e apoio. A Luiz Eduardo, pelo amor, compreensão e apoio incondicional.

Aos meus amigos e colegas pelo apoio no dia-dia e pelo intercâmbio de ideias.

A todas as pessoas, que contribuíram para que este estudo se desenvolvesse adequadamente, o meu agradecimento.

RESUMO

As atuais problemáticas ambientais requerem urgentemente a aplicação de tecnologias e conhecimento para encontrar soluções que viabilizem a continuidade dos processos produtivos de forma sustentável. Biorrefinarias baseadas na cadeia produtiva da soja (*Glycine max*) têm sido estudadas e apresentam boas perspectivas para a geração de biomoléculas de médio e alto valor agregado. Neste contexto, o presente projeto teve como objetivo o aproveitamento de resíduos agroindustriais gerados em grandes quantidades no território brasileiro, neste caso a casca de soja e o glicerol residual, para a produção biotecnológica de biopolímeros, especificamente os polihidroxialcanoatos (PHA). Este trabalho faz parte do projeto BRICS-BEST CNPq/ MCTIC/BRICS-STI N º 18/2016 proposta 271. A primeira parte deste trabalho tem como foco a revisão e análise das tecnologias, avanços e desafios relacionados com a produção de polihidroxialcanoatos microbianos a partir de hidrolisados de biomassa lignocelulósica. Por sua vez, a segunda parte deste estudo descreve a implementação da casca de soja e do glicerol residual como fontes de carbono para a produção de PHA. Assim, com o intuito de explorar a alternativa de valorizar resíduos agroindustriais do território brasileiro presentes em grandes quantidades, para a produção de PHA, desenvolveram-se as etapas de caracterização estrutural da casca de soja para depois definir, de a cordo com a composição encontrada (celulose 31,0 ± 1,6%; hemicelulose 11,8 ± 1,3%; lignina 6,18 ± 0,8%; proteínas 10,5%; extrativos 9,3 ± 1,0%; cinzas 3,6 ± 0,03%; umidade 6,0 ± 0,10%; outros 21,62%), a escolha do melhor tratamento para a recuperação dos açúcares presentes no material lignocelulósico. A bioconversão da biomassa lignocelulósica em biopolímeros envolve três grandes operações unitárias de *up-stream*: pré-tratamento do material, sacarificação e fermentação. Desta forma, para a primeira etapa foi definido um pré-tratamento alcalino [NaOH 2% (m/v)] do substrato [10% (m/v)] em condições brandas (60 min - 121 °C - 1 atm.). Para o processo de sacarificação, implementou-se um coquetel enzimático não comercial, composto por complexos de celulase (B1), xilanase (B1-XylA), e por β-glucosidase (F10). Desenvolveu-se a otimização das condições do processo implementando a Metodologia de Superfície de Resposta baseada na ferramenta de Desenho Composto Central, onde foram consideradas as variáveis de concentração enzimática, concentração de substrato e tempo de processamento. Como resultado, na melhor condição da otimização foi obtido um hidrolisado com 115,9 $g \cdot L^{-1}$ de açúcares redutores, adicionando 62% (m/v) de casca de soja pré-tratada, com tempo de processamento de 42h e com a carga enzimática da enzima B1 correspondente a 11,52 mg proteína$\cdot g^{-1}$ biomassa seca, da enzima B1-XylA de 2,88 mg proteína$\cdot g^{-1}$ biomassa seca e da enzima F10 de 57,59 U/g biomassa seca. Posteriormente, foi realizada a etapa de sacarificação aumentando a escala de processamento, utilizando um tanque de 1 L com agitação e temperatura controladas. Implementando as mesmas concentrações das enzimas em pH 4,5, temperatura de 45 °C, volume de trabalho de 260 mL e tempo de incubação de 42 h, sob operação em batelada alimentada com alimentação de substrato após 14 h e 22 h, foi obtido um hidrolisado com concentração de 185,7 $g \cdot L^{-1}$ de açúcares redutores, representando um incremento de 37,6% quando comparado com os resultados obtidos na etapa previa de otimização. De igual forma, o rendimento de glucose foi de 0,38 g/g_{SBH}, o que correspondeu a 60% de conversão de celulose. Com o hidrolisado produzido e com o glicerol residual, o microrganismo *Cupriavidus necator* DSMz 545, efetuou a fermentação em cultivo tipo batch em escala de bancada (inóculo 10% (v/v), 30 °C, 150 rpm, pH 6-7, 96 h) obtendo um acúmulo máximo de 39% de PHB. Assim, comprovou-se pela primeira vez, que a casca de soja e o glicerol residual, são matérias primas plausíveis e de baixo custo para a produção de moléculas de médio a alto valor agregado.

Palavras-chave: casca de soja; glicerol residual; pré-tratamento alcalino; sacarificação enzimática; polihidroxialcanoatos; biorrefinaria.

ABSTRACT

Current environmental problems urgently require the application of technologies and knowledge to find solutions that enable the continuity of production processes in a sustainable way. Biorefineries based on the soybean (*Glycine max*) production chain have been studied and show good perspectives for the generation of middle to high-value-added biomolecules. In this context, the present project aimed to use agro-industrial residues present in large quantities in Brazilian territory, in this case, soybean hulls and residual glycerol, for the biotechnological production of biopolymers, specifically polyhydroxyalkanoates (PHA). This work is part of the BRICS-BEST CNPq/MCTIC/BRICS-STI project No. 18/2016 proposal 271.The first part of this work focuses on the review and analysis of technologies, advances, and challenges related to the production of microbial PHA from lignocellulosic biomass hydrolysates. The second part of this study describes the implementation of soybean hulls and residual glycerol as carbon sources for PHA production. Thus, to explore the alternative of valuing agro-industrial residues from Brazilian territory present in large quantities, to produce PHA, the stages of structural characterization of the soybean hull were developed to then define, according to the composition found (cellulose 31.0 ± 1.6%; hemicellulose 11.8 ± 1.3%; lignin 6.18 ± 0.8%; proteins 10.5%, extractives 9.3 ± 1.0%; ash 3.6 ± 0. 03%; humidity 6.0 ± 0.10%; other 21.62%), the best treatment to follow for the recovery of sugars present in the lignocellulosic material. The bioconversion of lignocellulosic biomass into biopolymers involves three major upstream unit operations: material pretreatment, saccharification, and fermentation. Thus, for the first step, an alkaline pre-treatment [NaOH 2% (w/v)] of the substrate [10% (w/v)] was defined under mild conditions (60 min - 121 °C - 1 atm.). For the saccharification process, a non-commercial enzymatic cocktail was implemented, composed of cellulase (B1), xylanase (B1-XylA), and β-glucosidase (F10) complexes. For the enzymatic saccharification optimization was implemented the Response Surface Methodology based on the Central Composite Design tool, where the variables of enzyme concentration, substrate loading, and processing time were analyzed. As a result, in the best optimization condition, a hydrolysate with 115.9 $g \cdot L^{-1}$ of reducing sugars was obtained, adding 62% (w/v) of pre-treated soybean hulls, with a processing time of 42 h, and with the enzymatic load of 11.5 mg protein/g dry substrate for enzyme preparation B1, 2.88 mg protein/g dry substrate for B1-XylA, and 57.6 U/g dry substrate for F10, after 42 h at 45°C and pH 4.5. Subsequently, the saccharification step increased the processing scale, using a 1L tank with controlled temperature and agitation. Implementing the same enzyme concentrations at pH 4.5, 45 °C, working volume of 260 mL, and incubation time of 42 h, under fed-batch operation with substrate feed after 14h and 22h, was reached a hydrolysate with a concentration of 185.7 $g \cdot L^{-1}$ reducing sugars, representing an increase of 37.6% when compared to the results obtained in the previous optimization step. As well, glucose yield was 0.38 g/gSBH, which corresponded to 60% cellulose conversion. Using the soybean hydrolysate and waste glycerol, the microorganism *Cupriavidus necator* DSMz 545 carried out the fermentation in batch culture (inoculum 10% (v/v), 30 °C, 150 rpm, pH 6-7, 96 h) obtaining a maximum accumulation of 39% of PHA. Thus, it was proved for the first time that soybean hulls and residual glycerol are plausible and low-cost raw materials to produce molecules of medium to high-added value.

Keywords: soybean hull; waste glycerol; alkaline pretreatment; enzymatic saccharification; polyhydroxyalkanoates; biorefinery.

LISTA DE FIGURAS

SUMÁRIO

1. INTRODUÇÃO

1.1. A casca de soja e o glicerol residual como fontes de carbono para processos biotecnológicos

As atividades agroindustriais desenvolvidas ao redor do mundo geram grandes quantidades de material residual. Porém, considerando-se as tendências e necessidades ambientais, de produtividade e sustentabilidade, estes materiais agora são vistos por uma nova perspectiva. Atualmente, devido à sua interessante composição físico-química, podem ser aproveitados e reintegrados em processos produtivos que garantem sua valorização. Mundialmente o Brasil destaca-se na agroindústria, a qual representa um dos principais pilares da economia nacional. Cabe mencionar à produção de biodiesel e soja, como importantes exemplos desta área. Em 2021 o Brasil produziu 6,76 milhões de m^3 de biodiesel (ANP, 2022) e 135,409 milhões de toneladas de soja, sendo o principal produtor mundial deste grão (EMBRAPA, 2021). Nestes casos em particular, são gerados, como consequência do processo produtivo, materiais residuais que precisam ser explorados adequadamente, aproveitando o potencial como matérias primas para novos processos.

Em sua maioria, os resíduos do setor agrícola são de natureza lignocelulósica (palha, espiga, talos, caules, bagaço, casca) e possuem como principais compostos estruturais a celulose (40-60%), a hemicelulose (20-40%) e a lignina (10-25%) (SU et al., 2020). Estes polissacarídeos possuem um alto potencial de transformação e valorização, pois são usados como matéria prima para a geração de biocombustíveis, ácidos orgânicos e diversas moléculas de alto custo (NIJU et al., 2020). Dado que anualmente são geradas milhões de toneladas deste tipo resíduos, é notório o incremento de pesquisas sobre as possíveis aplicações que permitam o reaproveitamento de ditos compostos estruturais. Calcula-se que para 2020, foram geradas em torno de 7 milhões de toneladas de casca de soja no Brasil. Tradicionalmente esta casca tem sido utilizada como base para ração animal devido as propriedades nutricionais que apresenta e que tem sido amplamente estudada (STEIN et al., 2008). Este material possui baixa recalcitrância devido ao reduzido conteúdo de lignina (2-13%), o que facilita o

processamento para a exploração da hemicelulose (19-34%) e da celulose (29-52%) presentes na estrutura. Diversos pré-tratamentos físicos, químicos e enzimáticos têm sido implementados para a sacarificação destes compostos, e assim, obter os açúcares fermentescíveis que permitem a produção, através de processos biotecnológicos, de moléculas de médio a alto valor agregado como enzimas, ácidos orgânicos e biocombustíveis (BITTENCOURT et al., 2021).

Por outro lado, para cada 10 kg de biodiesel produzido é gerado como subproduto 1 kg de glicerol (RAMAN et al., 2019). Em 2020, Brasil gerou aproximadamente 600 mil m^3 de glicerol como resultado da produção de biodiesel, cujo aumento tem sido de 140% durante a última década (ANP, 2021). A grande disponibilidade de glicerol perfila suas aplicações em escala industrial. Assim, tem sido utilizado amplamente nas indústrias cosmética, alimentar, farmacêutica e de energias renováveis. As perspectivas de futuras aplicações ambientalmente amigáveis são de grande interesse nas áreas de P&D da indústria. Assim, moléculas de alto valor agregado (i.e., 1,3-propanediol, ácido málico, lipídeos, biopolímeros, dentre outras), têm sido produzidas por microrganismos (GOYAL et al., 2021; JU et al., 2020).

1.2. Polihidroxialcanoatos como alternativa aos plásticos tradicionais

Os polihidroxialcanoatos (PHAs) são poliésteres que se acumulam dentro de uma ampla variedade de microrganismos, quando expostos a condições de estresse nutricional ou ambiental específicos. Os polímeros biodegradáveis são a solução mais promissora para uma das principais preocupações ambientais deste século: a poluição gerada por plásticos descartáveis. Assim, biodegradabilidade dos PHA abre novos horizontes para os problemas ambientais enfrentados atualmente. É por isso que desde a descoberta destes compostos microbianos, o objetivo de cientistas e indústrias tem sido induzir a produção de biopolímeros com as mesmas propriedades dos plásticos tradicionais, agregando a vantagem da biodegradabilidade.

Dentro do citoplasma celular de diversos microrganismos, os PHAs são sintetizados em decorrência de condições de estresse (fatores ambientais ou limitação de nutrientes) e são considerados materiais de armazenamento de carbono e energia. Esses compostos são estruturalmente formados por monômeros de ácidos graxos 3-hidroxi, que

formam poliéster linear da cabeça à cauda e acumulados como inclusões insolúveis de 0,2 a 0,5 μm de diâmetro. As unidades de monômero (R)-3HA são polimerizadas em moléculas de alta massa molecular em uma escala de 2×10^5 a 3×10^5 Da, sendo que o núcleo de poliéster é cercado por fosfolipídios ou proteínas (RAZA et al., 2018, KESHAVARZ e ROY, 2010; SURIYAMONGKOL et al., 2007; SUDESH et al., 2000).

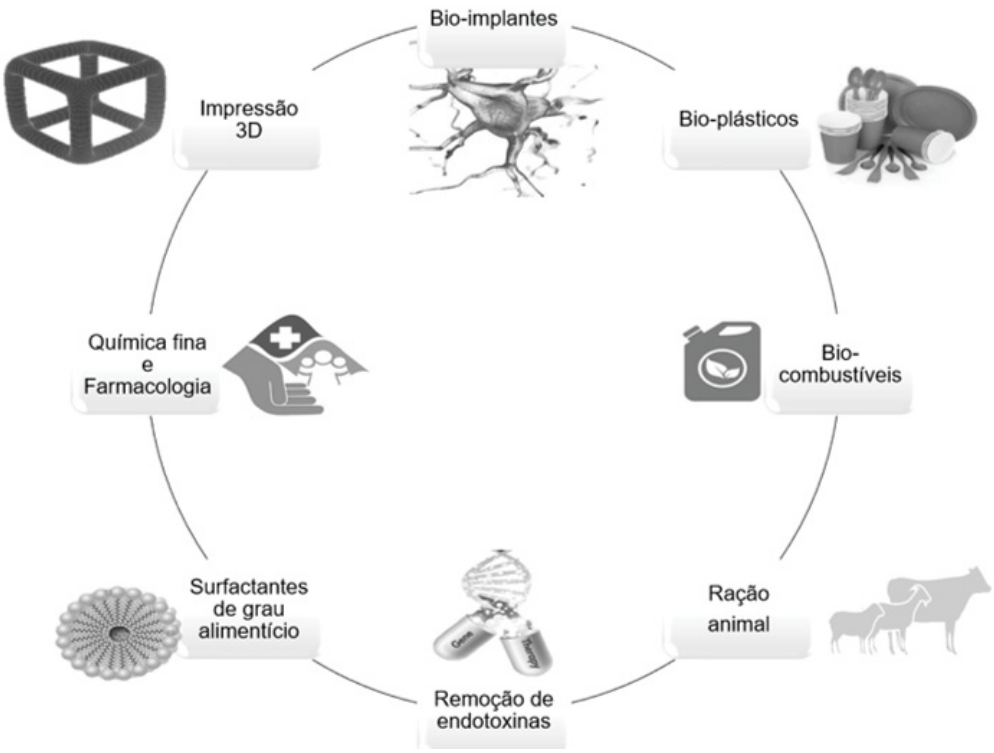
De acordo com o número de unidades monoméricas de átomos de carbono nas cadeias laterais, os PHAs são geralmente divididos em dois grupos: cadeia longa curta (scl) e cadeia longa média (mcl). O número de átomos de carbono varia de 3-5 para Scl-PHA e 6-14 para mcl-PHA (ANJUM et al., 2016; ALBUQUERQUE & MALAFAIA, 2018). Incorporando diferentes unidades de monômero, diferentes tipos de PHA podem ser produzidos. Essa alteração está diretamente ligada ao substrato, em conjunto com a atividade de polimerização da PHA sintase (HUONG et al., 2017). Assim, além do número de átomos de carbono, de acordo com sua composição química, os PHAs podem ser identificados como homopolímeros [poli(3-hidroxihexanoato) P(3HHx), poli(3-hidroxioctanoato) P(3HO)] ou copolímeros [P(3HHx-co-3HO), P(3HB-co-3HV)]. A natureza das fontes de carbono utilizadas durante a biossíntese de PHA influência nesta composição. Diferente dos homopolímeros, os copolímeros de PHA apresentam características físicas e térmicas, devidas ao arranjo de estruturas moleculares, ou pela presença de monômeros aromáticos na cadeia de PHA o que representa vantagens na hora da comercialização devido a que existem faixas de preços mais competitivas (ALBUQUERQUE e MALAFAIA, 2018, SUDESH et al., 2000, FUKUI e DOI, 1998).

Para se tornarem interessantes para a fabricação industrial, os PHAs devem possuir algumas propriedades físico-químicas. Por exemplo, o homopolímero polihidroxibutirato (PHB) que é o tipo de PHA mais comumente produzido na indústria, apresenta como principais características a rigidez e fragilidade e ao mesmo tempo, exibe boa resistência aos raios ultravioleta e impermeabilidade ao oxigênio, alto grau de cristalinidade (60-80%), temperatura de fusão em torno de 180°C, pouca elasticidade e pouca resistência a ácidos e bases (POIRIER et al., 1995). Uma das propriedades mais notáveis dos PHAs é a sua biodegradabilidade. Quando exposto ao solo, lodo, composto ou sedimento marinho, os PHAs se degradam. A taxa deste processo depende de diferentes fatores ambientais (e.g., temperatura, umidade, pH, atividade microbiana) e características

estruturais do material (por exemplo, natureza da unidade monomérica, cristalinidade, composição do polímero de peso molecular, aditivos e área de superfície exposta) (BUGNICOURT et al., 2014). O baixo acúmulo no meio ambiente, a não toxicidade, a redução do custo do gerenciamento de resíduos e a potencialidade de permitir a preservação de recursos limitados são algumas vantagens que os plásticos biodegradáveis oferecem (TOKIWA et al., 2009).

Devido à sua origem renovável e de baixo custo como matéria-prima, biodegradabilidade, biocompatibilidade, não toxicidade, propriedades de hidrofobicidade e diversidade de monômeros (o que confere amplos atributos térmicos e mecânicos), os PHAs são materiais de base interessantes para uma ampla gama de aplicações (MOHANDAS et al. al., 2018). Até agora, os PHAs têm sido aplicados em diversas áreas como medicina, farmacologia, biotecnologia, nanotecnologia, biomateriais, agricultura, embalagens, fibras têxteis, entre outras (FIGURA 1).

FIGURA 1 – Aplicações industriais dos polihidroxialcanoatos



FONTE: Adaptada de Tan, Yin & Yen (2017).

A aplicação mais promissora dos PHAs e suas misturas está sendo construída, através da interação dos campos da medicina, nanotecnologia, biomateriais e engenharia de tecidos, para o desenvolvimento da saúde. A biocompatibilidade e a baixa resposta inflamatória são características valiosas e úteis em aplicações médicas, como a geração de ossos, válvulas cardíacas, condutas nervosas, cartilagens, tendões e vasos sanguíneos artificiais, bio-implantes, detecção de câncer, curativos e andaimes (EL-MALEK et al., 2020, RAZA et al., 2018).

1.3. Pesquisa e tecnologia aplicadas à produção de polihidroxialcanoatos

Nos últimos anos, muitos artigos de revisão analisaram extensivamente as estratégias implementadas em todo o processo de produção de PHA para sua otimização. Como em qualquer bioprocesso, deve-se levar em consideração a seleção do microrganismo e das fontes de carbono, as etapas *upstream* e *downstream*. Começando com as etapas de biossíntese, Sagong et al. (2018) expuseram os *insights* estruturais relacionados à produção microbiana do bio-polímero. Em termos da importância que os substratos baratos exercem em todo o processo de produção de PHA dado que representam aproximadamente 50% do custo total de processamento, Jiang et al. (2016) e Sirohi et al. (2020) fizeram uma revisão crítica das fontes de carbono e matérias-primas de biomassa usadas como substrato sustentável, sugerindo os subprodutos e resíduos agroindustriais como os mais promissores e relevantes. Os autores mencionam o material lignocelulósico, os hidrolisados de hemiceluloses, o glicerol cru, os substratos ricos em amido, os óleos residuais, os resíduos da indústria láctea, dentre outros, e apontam que, embora as fontes de carbono puras (glucose e glicerol puros) tenham dado melhores resultados nas etapas de *downstream*, os custos relacionados não são competitivos no final do processo produtivo.

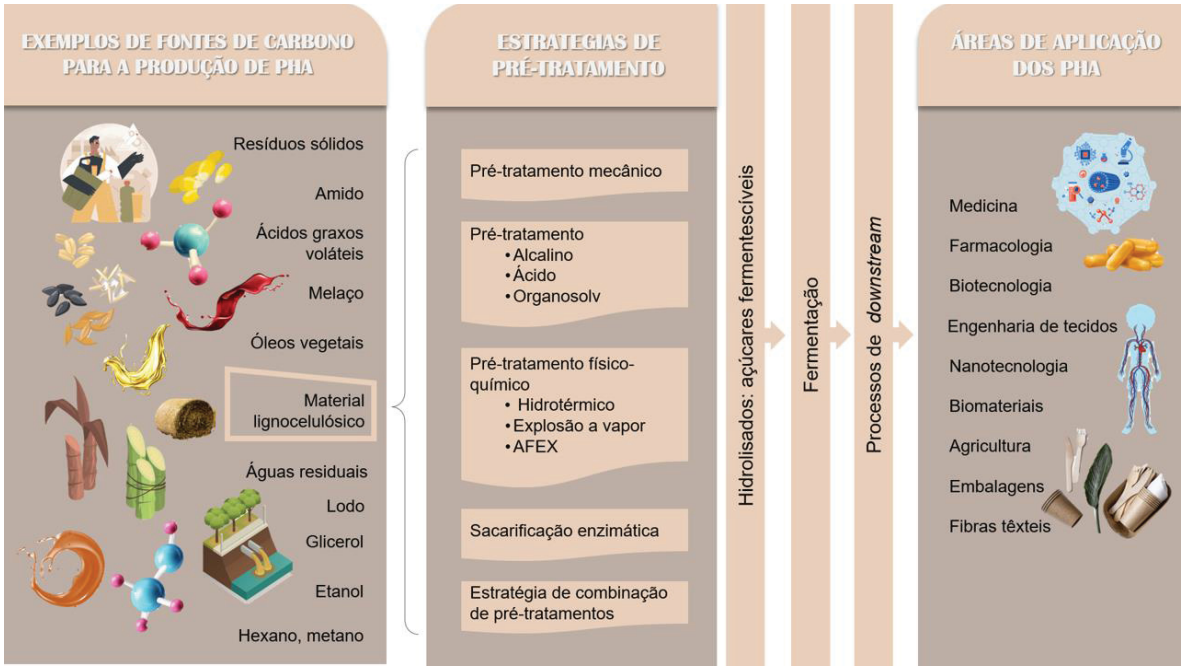
Por outro lado, um resumo substancial das estratégias de operação de biorreatores para melhorar a produtividade do PHA foi apresentado por Blunt et al. (2018). Por fim, Koller et al. (2013) examinaram profundamente as estratégias de recuperação e purificação de PHA e, da mesma forma, Kosseva e Rusbandi (2018) analisaram as tendências na produção de PHA com foco no processamento *downstream*. Porém, na revisão bibliográfica realizada, nenhum artigo tem focado exclusivamente na produção

de hidrolisados a partir de material lignocelulósico, para a posterior produção de biopolímeros. É por isso, que o primeiro capítulo deste trabalho é dedicado ao tema. Na FIGURA 2 apresentam-se alguns exemplos das fontes de carbono que têm sido utilizadas para a produção microbiana de PHAs, dentre os que se encontram os resíduos sólidos, amido, ácidos graxos voláteis, melaço de cana de açúcar, óleos, óleos e gorduras residuais, material lignocelulósico, lodos, lixiviados, águas residuais, glicerol, etanol, metano e hexano. Nesta mesma Figura, mencionam-se os possíveis tratamentos para a transformação da matéria prima quando é de natureza lignocelulósica para a obtenção de hidrolisados. Uma vez produzidos, os hidrolisados servem como fonte de carbono para os microrganismos que irão utilizar os açúcares e nutrientes no metabolismo para a produção de compostos de interesse para diversas áreas da indústria.

Embora o processo de produção de PHA tenha sido revisto com bastante ênfase, do ponto de vista da pesquisa acadêmica, pouca atenção tem sido dada ao conteúdo das patentes que exploram a implementação de ferramentas inovadoras na perspectiva das indústrias. A continuação, apresentam-se brevemente os dados relevantes de patentes publicadas que exploram as inovações na área de produção microbiana de PHA e as tecnologias relevantes e estabelecem perspectivas em torno dos avanços neste campo.

A busca de patentes foi feita na plataforma *Derwent Innovations Index* com as palavras-chave em inglês: *polyhydroxyalkanoate*, *3-hydroxyalkanoic acid*, *polyhydroxybutyrate*, *polyhydroxyvalerate*, no título (TI), tema (TS) e, C12P-007/62 para o campo *International Patent Classification code* (PI). O exame foi realizado entre janeiro de 1990 e março de 2020, sendo esta última data, na qual realizou-se a busca. Aproximadamente 500 documentos de patentes foram recuperados da busca. Após verificar manualmente e filtrar o material relevante, reduziu-se o grupo em 334 patentes, diretamente relacionadas à produção de PHA e que foram utilizadas para a análise que será apresentada a seguir. Em relação as publicações acadêmicas, para ter acesso ao banco de dados de documentos relacionados, foi implementado o site *Web of Science*, utilizando as palavras-chave: produção e polihidroxialcanoatos. Neste caso, foi utilizado o lapso de tempo entre os anos 2000 e 2022. Ao todo, foram encontrados mais de 3 mil documentos.

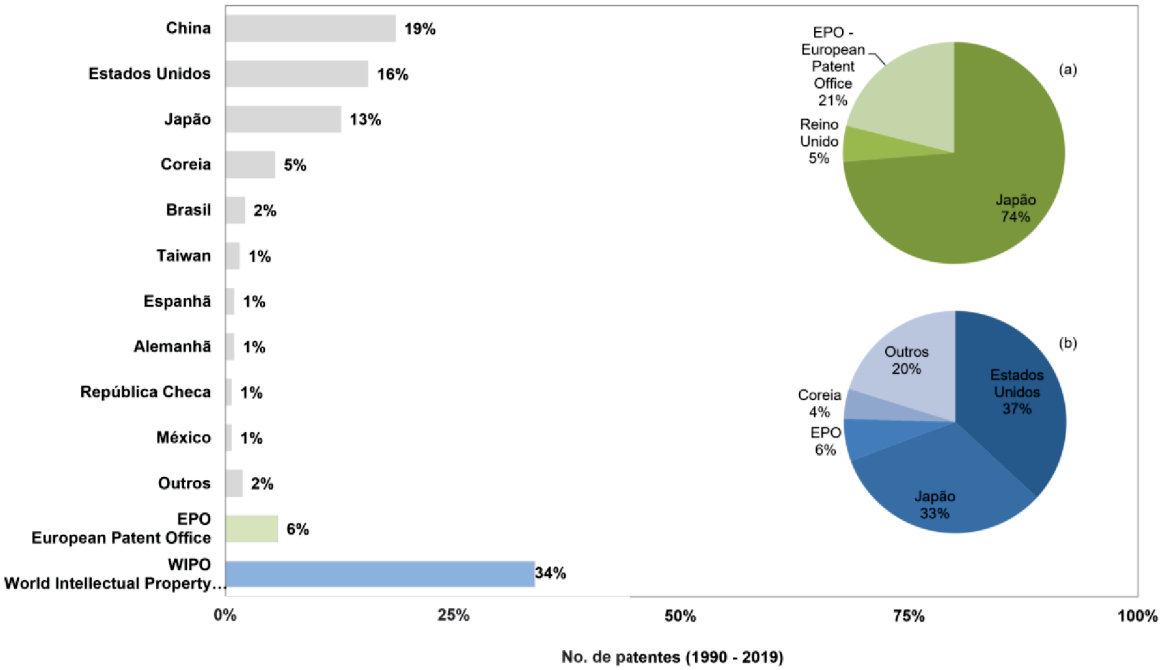
FIGURA 2 - Produção de polihidroxialcanoatos a partir de biomassa lignocelulósica.



FONTE: A Autora (2022).

Os principais países detentores da tecnologia PHA em todo o mundo são a China, os Estados Unidos e o Japão (FIGURA 3). As empresas que registram mais patentes na área de PHA são Kaneka Corp. (13%), Canon Inc. (9%) e Yield10 Bioscience (antes Metabolix Inc.) (4%). As duas primeiras empresas, sediadas no Japão, e a última, localizada nos Estados Unidos. A empresa francesa Veolia Water Solutions & Technologies também está entre as primeiras detentoras de patentes, dado seu foco no uso de águas residuais e lodos para a produção do bio-polímero. Por outro lado, as universidades com maior participação na produção de patentes são a Universidade de Jiangnan (China), a Universidade de Hohai (China) e a Universidade de Stanford (EUA).

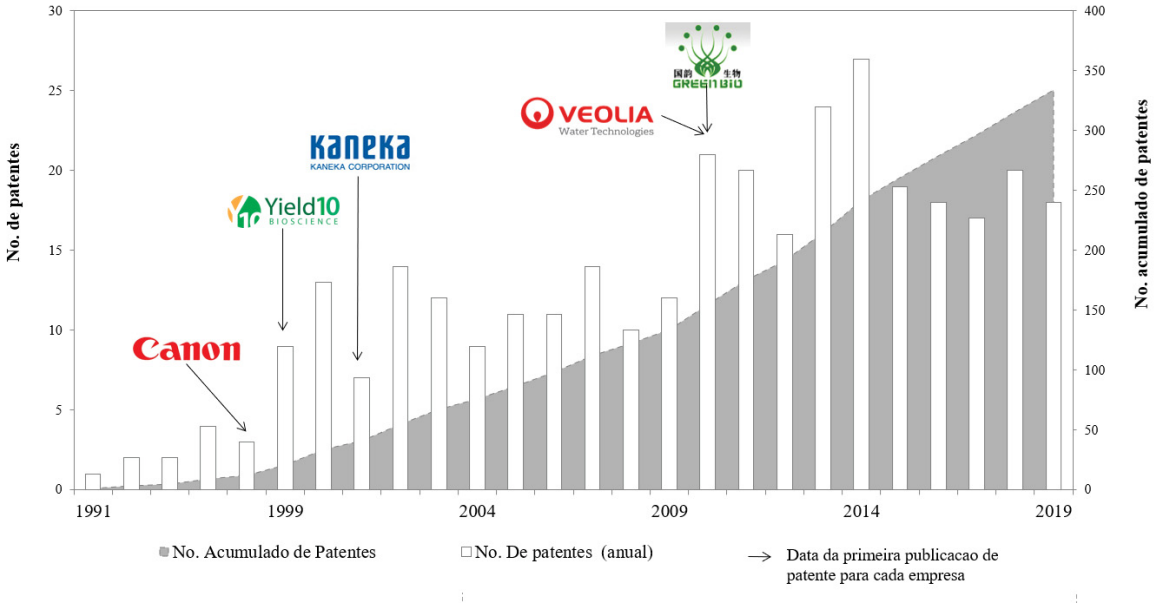
FIGURA 3 - Principais países detentores da tecnologia de produção de PHA. a) Origem de patentes depositadas na EPO; b) Origem de patentes depositadas na WIPO



FONTE: A Autora (2022).

O crescente interesse pela produção de PHA é evidente tanto na área de pesquisa acadêmica quanto na indústria. Durante as últimas duas décadas, foram publicados mais de 3 mil documentos relacionados à produção deste tipo de biopolímero. A FIGURA 4 mostra a evolução das patentes na área de produção de PHA e mostra as principais empresas que deram contribuições importantes para o desenvolvimento de tecnologias de produção de PHA. A concessão de patentes teve uma taxa de crescimento superior a 70% durante os últimos 20 anos, passando de cerca de 113 patentes entre 2000 e 2009 para mais de 200 entre 2010 e 2019.

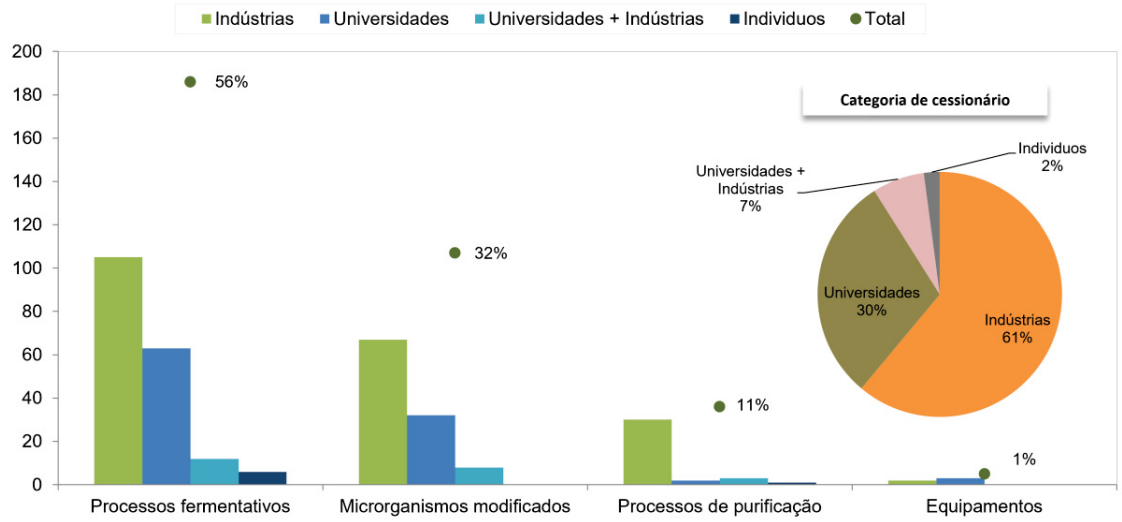
FIGURA 4 - Cronologia de publicações de patente relacionadas com a produção de PHA (1990-2019).



FONTE: A Autora (2022).

Simultaneamente, foi identificado que, de acordo com a categoria de cessionários, as indústrias depositam mais do que o dobro de patentes (61%) do que as universidades (30%). Sabe-se comumente que a junção de forças proporciona resultados mais relevantes do que quando o trabalho é individual. Assim, os avanços e resultados do trabalho de pesquisa científica são uma das bases para o desenvolvimento tecnológico. Desta forma, verificou-se que a associação entre duas ou mais empresas é mais usual do que entre várias universidades. Ao longo das últimas décadas, as inovações desenvolvidas em parceria entre os setores acadêmico e industrial representaram 7% dos documentos de patentes analisados (FIGURA 5).

FIGURA 5 - Classificação do processo de produção de polihidroxialcanoatos por categoria de cessionário (Industria, universidades, indivíduos ou parcerias).



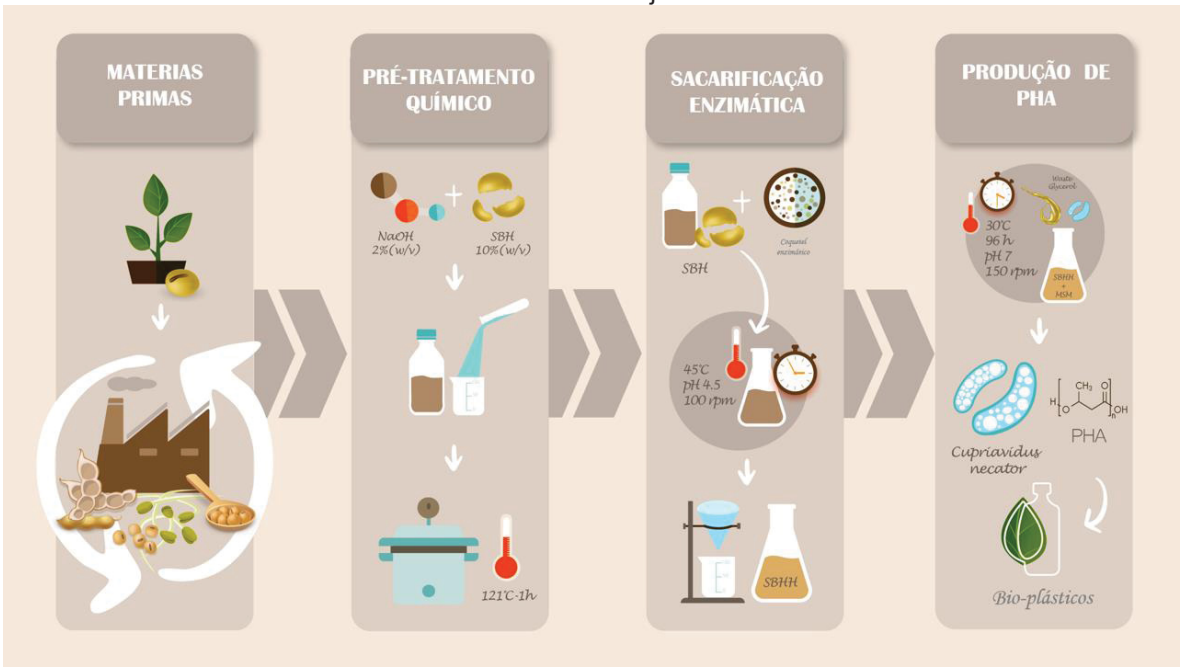
FONTE: A Autora (2022).

Como parte da associação e interação entre a pesquisa acadêmica e a atividade industrial, é fundamental ter uma formação solidária entre as partes. A identificação, reconhecimento e aplicação de trabalhos anteriores em ambos os setores, é a chave para acelerar o real desenvolvimento deste campo, que está em contínua evolução. Assim, para sustentar esta ideia verificou-se o suporte bibliográfico (patentes relacionadas ou artigos científicos), reportado pelos autores para cada patente de documento. Observou-se que mais de 85% das patentes não adicionam nenhuma citação bibliográfica, apenas 12% incluem entre uma e cinquenta referências, e os 3% restantes incorporam mais de cinquenta referências. Isso revela uma aparente falta de ligação entre as atividades de pesquisa de P&D industrial e instituições acadêmicas.

Os resultados obtidos nesta busca de tecnologias demonstram que os documentos de patentes são classificados em três áreas temáticas: Química, Ciência dos Polímeros e Biotecnologia. De acordo com o tipo de inovação proposta, foi constatado que 56% das patentes estavam diretamente relacionadas ao processo de fermentação, 33% estavam associadas a novos microrganismos geneticamente modificados, 15% descreveram etapas de recuperação de polímeros e 1 % trabalhou em equipamentos para a produção de PHA (FIGURA 5). Os microrganismos mais implementados para a produção

biotecnológica do PHA são as bactérias com 58%, sendo que as culturas mistas representam 5,1% dos documentos e outros não definidos nas patentes completam o registro com 36,9%. Foram também verificadas as fontes de carbono utilizadas nas patentes analisadas, encontrando mais de cinquenta opções. Alguns exemplos são glucose, glicerol, óleos, óleos e gorduras residuais, melaço de cana de açúcar, hidrolisados lignocelulósicos, xilose, amidos, ácidos graxos voláteis, lodos e águas residuais, metanol, hexano, succinatos, ácidos orgânicos e lixiviados. Tanto nas patentes consultadas como nos artigos científicos verificou-se que a exploração da casca de soja para a produção de polihidroxialcanoatos não havia sido realizada previamente, de modo tal que no segundo capítulo deste trabalho apresentam-se os resultados obtidos ao respeito e encontram-se resumidos na FIGURA 6.

FIGURA 6 – Etapas de produção de polihidroxialcanoatos a partir de glicerol residual e hidrolisados de casca de soja



FONTE: A Autora (2022).

2. CAPÍTULO I

Manuscrito não submetido

MICROBIAL POLYHYDROXYALKANOATES PRODUCTION FROM BIOMASS HYDROLYSATES: TECHNOLOGIES, PROGRESS AND CHALLENGES

2.1. MICROBIAL POLYHYDROXYALKANOATES PRODUCTION FROM BIOMASS HYDROLYSATES: TECHNOLOGIES, PROGRESS AND CHALLENGES

Zulma Sarmiento-Vásquez[a], Luciana Porto de Souza Vandenberghe[a*], Ariane Fátima Murawski de Mello[a], Carlos Ricardo Soccol[a]

[a]Federal University of Paraná, Department of Bioprocess Engineering and Biotechnology, Centro Politécnico, 81531-980, Curitiba, Paraná, Brazil

2.2. Abstract

Since 1920, when the microbial polyhydroxyalkanoates (PHA) were identified as an eco-friendly material, a wide spectrum of processes, products, and technologies have been designed, studied, and eventually implemented for its application in some industries for the society benefit. To achieve the sustainable production of this bioplastic, the application of the concept of biorefineries could be considered as an alternative, since the use of biomass for the generation of this biopolymer would allow cost reduction, large-scale production and make it accessible to all industries. The current production process must consider reducing the cost of producing PHA and increasing yields using low-cost carbon sources. In addition, green and sustainable product recovery methods should be considered to decrease pollution and promote rational energy usage throughout the whole process. In this way, this study highlights the recently published articles and patents on PHA production using biomass hydrolysates. This review is not limited to condensing innovations in this area, but rather presents and examines data on the relevant technologies, which will benefit audiences belonging to academy and research areas, even industry professionals. The analyzed information allowed establishing the recent progress in PHA production and outlined new perspectives to overcome the challenges to scale up the developed processes by offering low production costs, for a product that aims to provide a solution to one of the major environmental problems afflicting humanity today.

Keywords: polyhydroxyalkanoates; biomass hydrolysates; biorefinery; bioplastics; patents.

*Corresponding author: lvandenberghe@ufpr.br

2.3. Introduction

Extensive use that has been given to the oil-based polymers, with their great versatility, makes their production infeasible to stop immediately. Rubber, celluloid, and Bakelite are the predecessors of plastics, as we know them today. The history of the plastics industry began more than a hundred years ago and will likely persist for a long time. Efficient large-scale production, low costs, versatility of use, physical characteristics, strength, and durability have made plastic one of the preferred materials for the manufacture of a large number of products that currently make part of the daily life of society. An estimated 9.2 billion tons of plastics have been manufactured between 1950 and 2017, 91% of which have not been recycled. Currently, 32% of plastics end up directly in environmental ecosystems, 40% is dumped in landfills, 14% is burned and only 14% is recycled (WILLIAMS AND RANGEL-BUITRAGO, 2022). The development of the human population meant that plastics were used in various productive sectors, including building and construction, appliances, mechanical engineering, furniture, medical and pharmaceutical, automotive industry, electrical and electronic, household, transportation, industrial machinery, leisure sports, and packaging (PLASCTICSEUROPE, 2021). The problem emerged when this last use, packaging, fill up the world of many plastic-made products for single-use or immediately disposable. Because of the extensive use of this kind of product, the number of residues generated during the last decades now is unmanageable and the move towards sustainable packaging in the food and beverage sector is slow and inconsistent (PHELAN et al., 2022). The excessive molecular size of plastics turns difficult its final disposal, and its harmful and toxic effects on ecosystems became a strong environmental and political concern around the world (DENG et al., 2021; KASAVAN et al., 2021; OKEKE et al., 2022; SIMUL BHUYAN et al., 2021; VERED and SHENKAR, 2021).

Currently, the proposed solution consists of the rational and responsible use of plastic that is part of the daily life of the population (WILLIS et al., 2021). However, it is necessary to strengthen and encourage new incentives laws, political regulations, and the large-scale production of alternative materials such as biopolymers (PELLIS et al., 2021). The biodegradability of these compounds opens new horizons for the reduction of environmental problems that are currently being faced. Nevertheless, as many authors

have pointed out, to ensure that real and positive environmental impacts are achieved in the transition from petrochemicals to bioplastics, more comprehensive and increasingly accurate life cycle assessments (LCAs) of the sustainability of bioplastics are needed to avoid trading one problem for another (BISHOP et al., 2021, IOANNIDOU et al., 2020). Despite the advantages that the use of bioplastics may represent, due to the incipient and immature development of the production processes of PHAs, their environmental impact could be greater than that caused by petroleum-derived plastics. This is expected to change as processes were optimized, scaling up the production efficiently and thus, becoming economically viable (ATIWESH et al., 2021; VEA et al., 2021). Nitkiewicz et al. (2020) pointed out that the results obtained in the development of LCAs, by evaluating and comparing different scenarios for the production of biopolymers, allow for a more complete analysis and contribute to better decision making. Those decisions should be aimed at reducing the environmental impact of both the processes and the final product, which is achieved through the management of operations and the implementation of environmentally friendly innovations. They suggest that the production of microbial polymers should be integrated into biorefineries and conclude that, work must be done on issues of economic viability, and this requires optimizing the production processes. Along the same lines, Haque et al. (2022) conclude that emphasis should be placed on research to make the downstream process simpler, more efficient, and more economical in order to make it environmentally sustainable.

Thus, since the discovery of microbial PHAs, the purpose of scientists and industries was to induce the production of biopolymers with the same properties as traditional plastics, adding the advantage of biodegradability (MEDEIROS-GARCIA-ALCÂNTARA et al., 2020; MOSHOOD et al., 2022a). The first industrial microbial production of PHA was led by ICI Ltda., in 1970, using a mutant of *Cupriviadus necator* where polyhydroxybutyrate (PHB) and polyhydroxybutyrate-valerate (PHBV) was produced on an industrial scale (200.000 L) (BYROM, 1992). Since then, to make the process viable, several companies around the world have taken on the task of producing biopolymers at large scale, trying to overcome the principal hindrances, reducing production costs, increasing productivities, and correcting deficiencies in thermal properties (MOSHOOD et al., 2022b). Renewable resources, such as residual biomass, have been studied as

alternatives to produce hydrolysates that can serve as a substrate for microbial PHA production. Biomass is a raw material available around the world in large quantities and at low prices, due to the high production of commodities and the lack of large-scale reuse. When the biomass has lignocellulosic characteristics, it is necessary to break down the structure to gain access to compounds that are rich in fermentable sugars. This is achieved through mechanical, chemical, enzymatic pretreatments, or a combination of these (ZHOU and TIAN, 2022).

In 2020, global plastics production reached 367 million tons (PLASCTICSEUROPE, 2021), of which less than 1% corresponds to bioplastics. According to the European Bioplastics, the forecast for the bioplastics market is continuously growing and diversifying. In 2021, the production capacity reached 2.42 million tons, where almost 50 percent of bioplastics are currently being produced in Asia. It is expected that global bioplastics production capacity will increase to approximately 7.6 million tons in 2026 (EUROPEAN BIOPLASTICS AND NOVA-INSTITUTE, 2021). Additional and joint efforts must be made by academic research, industry, and policymakers, to accelerate, strengthen, and make economically successful the large-scale biopolymer industry around the world, and to overcome the present obstacles in the PHA production process (CUCINA et al., 2021; ESCOBAR AND BRITZ, 2021; TAN et al., 2021). This review aims to highlight the recently published articles and patents to update the state of the art about PHA production from biomass hydrolysates, analyze the relevant technologies, main challenges and establish perspectives around the advancements in this field.

2.4. Microbial PHA: biosynthesis, general properties, and applications

Inside the cell cytoplasm of various organisms, PHAs are considered as carbon and energy storage materials and are synthesized because of stress conditions such as environmental factors or limitation of nutrients (ADELEYE et al., 2020; OBRUCA et al., 2010). There is not a single metabolic pathway for microbial PHA synthesis; in fact, fourteen metabolic pathways have been analyzed and explored (SAGONG et al., 2018). About twenty different enzymes involved in PHA metabolism have been identified for these pathways and among the most important ones, PhaA (acetyl-CoA acetyltransferase), PhaB (acetoacetyl-CoA reductase) and PhaC (PHA synthase) are

worth mentioning (SAGONG et al., 2018; SINDHU et al., 2021; STEINBÜCHEL and LÜTKE-EVERSLOH, 2003). At least, four pathways were well defined, and they are directly associated with the nature of the carbon source (SUDESH et al., 2000). However, the characteristics and structure of the final product (PHAs homopolymer and/or copolymer and number of monomer units of carbon atoms in the side chains) will be defined by the intracellular substrate availability and PHA synthase substrate specificity (THOMAS et al., 2022). When the carbon sources are sugars, fatty acids, or proteins, the primary metabolic precursor is the acetyl-CoA, and the possible biosynthesis pathways are Glycolysis, Pentose-Phosphate pathway, β-oxidation, or Amino acid catabolism. On the other hand, when the carbon sources are fatty acids, the primary metabolic precursors could be Enoyl-CoA, 3-Ketoacyl-CoA, and the Fatty Acid β-oxidation is the biosynthesis pathway. Additionally, Acyl-ACP becomes the primary metabolic precursor when the fatty acid biosynthesis is activated in presence of sugars, fatty acids, or proteins as possible carbon sources (THOMAS et al., 2022).

The most used organisms for PHA production or extraction are bacteria (usually strains of the genus *Pseudomonas* and *Cupriavidus*), mixed cultures (bacteria + bacteria, bacteria + fungus communities), or plants (Nawrath & Poirier, 2008; Pinto-Ibieta et al., 2020; Thomas et al., 2022). Other organisms, such as yeast, archaea, and algae, are also used. In some cases, those microorganisms are recombinant or genetically modified to improve PHA production throughout the upstream steps (KUTRALAM-MUNIASAMY and PERÉZ-GUEVARA, 2018). As an example, it is well known that *C. necator* DSM 545, is not able to grow on starch. However, Brojanigo et al. (2022) developed a recombinant amylolytic strain in which glucodextranase G1d from *Arthrobacter globiformis* I42 and the α-amylase amyZ from *Zunongwangia profunda* SM-A87 were co-expressed into C. necator DSM 545 to develop one-step PHAs production from rice (5.78 g/L 3HB - 43.32 % CDM), corn (5.92 g/L 3HB - 48.22 % CDM) and potato (3.65 g/L 3HB - 35.95 % CDM) as starchy residues. On the other hand, Murugan et al. (2017) implemented the genetically modified *C. necator* strain Re2058/pCB113, which unlike the wild-type microorganism, can accumulate P(3HB-co-3HHx) containing a relatively high proportion of 3HHx (>20 mol%) monomer when fed with palm olein as the sole carbon source. In this study, the authors co-feeding palm olein and fructose as the carbon sources and obtained as a result

the production of P(3HB-co-3HHx) copolymer (CDW of 7.41 g/L and 80% PHA/CDW) and reached a high molecular weight of 17 mol% 3HHx monomer fraction.

PHAs are structurally formed of 3-hydroxy fatty acid monomers, which form linear, head-to-tail polyester and they accumulate as insoluble inclusions of 0.2–0.5 μm in diameter. The (R)-3HA monomer units are polymerized into high molecular weight polymers in a range of 2×10^5 to 3×10^5 Da depending on the microorganism and growth circumstances, and the core of polyester is surrounded by either phospholipids or proteins (KESHAVARZ and ROY, 2010; RAZA et al., 2018; SUDESH et al., 2000; SURIYAMONGKOL et al., 2007). According to the number of monomer units of carbon atoms in the side chains, PHAs are usually divided into two groups: short long-chain (scl) and medium long-chain (mcl). The number of carbon atoms varies from 3-5 for scl-PHA and 6-14 for mcl-PHA (ANJUM et al., 2016). This feature is directly linked with the added substrate in the fermentation medium, in conjunction with the PHA synthase polymerizing activity (HUONG et al., 2017). Thus, besides the number of carbon atoms, according to their chemical composition, the PHAs can be identified as homopolymers [poly (3-hydroxyhexanoate) P (3HHx), poly (3-hydroxyoctanoate) P (3HO)] or copolymers [P (3HHx-co-3HO), P (3HB-co-3HV)] (MUTHURAJ et al., 2021).

The wide range of properties of PHA copolymers, such as their physical and thermal characteristics, depends on the arrangement of molecular structures or the presence of aromatic monomers in the PHA chain (FUKUI and DOI, 1998; SUDESH et al., 2000; ALBUQUERQUE AND MALAFAIA, 2018). Besides, the polymerization degree and, as consequence, the general characteristics of the biopolymer can be defined from the nature of the carbon sources used during PHA biosynthesis. To become interesting for industrial manufacture, PHAs must have some physicochemical properties (Table 1). For example, the polyhydroxybutyrate (PHB) homopolymer, which is the most common type of PHA, presents as its main characteristics stiffness and brittleness and also shows good ultra-violet resistance and oxygen impermeability, a high degree of crystallinity (60-80%), melting temperature around 180 °C, poor elasticity, and poor resistance to acids and bases (POIRIER et al., 1995; SAMUI and KANAI, 2019). Depending on the final purpose, the biopolymer and its blends with other materials are selected considering typical characteristics such as Young's modulus (E), tensile strength (σ), stiffness, toughness,

density, light transmittance, glass transition temperature (T_g), melting temperature (T_m), crystallinity degree (X_{cr}), flexural strength, dielectric strength, water vapor transmission rate (WVTR) or oxygen transmission rate (O_{TR}). The suitable conjugation of those properties will concede specific performance and functionality to the material, which will reflect in attributes such as flexibility, durability, printability, transparency, permeability, and chemical, and heat resistance to the final product (ANJUM et al., 2016; NTAIKOU et al., 2018; WRÓBLEWSKA-KREPSZTUL et al., 2018; YEO et al., 2018).

TABLE 1 PROPERTIES OF DIFFERENT PHAs

Polymer	**P3HB**	**P4HB**	**(3HB-co-4HB)**	**PHBV**	**PBHH**
Material class					
Semicrystalline thermoplastic	X		X	X	X
Thermoplastic elastomer		X	X		
Properties					
Brittle	X			X	
Ductile		X	X		X
Easy to process					X
Flexible		X			X
High melt viscosity		X			
High softening temperature	X			X	
Large thermal processing window			X	X	
Small thermal processing window	X				
Low softening and melting temperature					X
Strong	X	X	X	X	
Tough			X		

Poly (3hydroxybutyrate-co-4hydroxybutyrate) = P(3HB-co-4HB); Poly(3-hydroxyalkanoate-3-hydroxyvalerate) = PHBV; Poly(3-hydroxybutyratehexanoate) = PBHH; Poly(3-hydroxybutyrate) = P3HB; Poly(4-hydroxybutyrate) = P4HB.

Source: Adapted from Ilyas et al., (2020).

One of the most remarkable properties of PHAs is their biodegradability when they are exposed to soil, sludge, compost, or marine sediment. The rate of this process depends on different environmental factors (e.g., temperature, moisture, pH, microbial activity) and structural characteristics of the material (e.g., nature of monomer unit, crystallinity, molecular weight polymer composition, additives, and exposed surface area) (BUGNICOURT et al., 2014). Low accumulation in the environment, non-toxicity,

reduction in the cost of waste management, and the potentiality to allow the preservation of limited resources, are some advantages that biodegradable plastics offer (TOKIWA et al., 2009; VANDENBERGHE et al., 2021).

Due to their renewable and low-cost feedstocks origin, biodegradability, biocompatibility, non-toxicity, hydrophobicity properties, and diversity of monomers (which confers broad thermal and mechanical attributes), PHAs are interesting base materials for a wide range of applications (MOHANDAS et al., 2018). Until now, PHAs have been applied in diverse areas such as medicine, pharmacology, biotechnology, nanotechnology, biomaterials, agriculture, packaging, and textile fibers, among others (KALIA et al., 2021). The most promising application of PHAs and their blending are being built up, through the interaction of medicine, nanotechnology, biomaterials, and tissue engineering fields, for healthcare development. Biocompatibility and low inflammatory response are valuable characteristics that are helpful in medical applications such as the generation of bones, heart valves, nerve conduct, cartilage, tendon, and artificial blood vessels, bio-implants, cancer detection, wound dressings, and scaffolds (EL-MALEK et al., 2020; RAZA et al., 2018).

2.5. Biomass for polyhydroxyalkanoates (PHAs) production

Carbohydrates are the main component of biomass - it is estimated that these molecules are responsible for up to 75% of biomass' weight (CHATTERJEE et al., 2015) - and are constituted by monomers linked by specific bonds that grant characteristics to each material. The main carbohydrates are lignocellulose, starch, and pectin. Lignocellulose is the major constituent of the plant cell wall, and it is composed of a network of cellulose (monomers of glucose linked with β-(1,4) glycosidic bonds having amorphous and crystalline regions in its structure), hemicellulose (a complex polysaccharide composed by a variety of C5 and C6 sugars, like xylose, arabinose, galactose, and mannose) and lignin, which is constituted by a high molecular weight aromatic polymer and contains numerous biologically stable ether or ester linkages (KUMAR and CHANDRA, 2020; ABDEL-HAMID et al., 2013). On the other hand, starch can act as an energy reserve for plants, and it is constituted by amylose and amylopectin, both having glucose monomers linked with α-(1,4) bonds and being connected with α-

(1,6) bonds creating branches in its structure (BERTOFT, 2017). Finally, in higher plants, pectin is located between the cell wall and the middle lamella and is a complex structural polysaccharide composed of a backbone of alpha-(1-4)-D-galacturonic acid (Yang et al., 2021).

Studies carried out for the validation of raw materials in the production of bioplastics have focused on the exploration of biomass of lignocellulosic origin (BRODIN et al., 2017). This resource has advantages such as large quantities and the possibility of being employed in integrated processes for the utilization of all its fractions. In addition, lignocellulosic biomasses do not compete with food availability, and above all, it is of low cost (GOVIL et al., 2020; USMANI et al., 2021). TABLE 2 shows some examples of implementation of lignocellulosic material to produce PHA, where the applied treatments, process conditions, and the results obtained in each case are described.

The composition of PHA produced by bacteria is dependent on the substrate and specificities of enzymes that act in the PHA's biosynthetic pathway. A huge variety of carbon sources have been stated as suitable raw materials to produce this kind of biopolymers. Some of them are molasses, starch, volatile fatty acid, vegetable oils, lignocellulosic material, solid waste, ethanol, acetic acid, glycerol, biomass-derived synthesis gas, hexane, methane originating from landfill gas, wastewater, and sludge, among others (ADELEYE et al., 2020; ESTÉVEZ-ALONSO et al., 2021; JIANG et al., 2016; KAWAGUCHI et al., 2022; KUMAR et al., 2019; LI and WILKINS, 2020; LOW et al., 2021; SEN and BAIDURAH, 2021; SILVA et al., 2022). PHA-producing microorganisms are not able to metabolize complex networks of carbohydrates, which implies the need of breaking down the polysaccharides into their forming monomeric units, usually fermentable sugars. To produce them, it is necessary to implement pre-treatment strategies for biomass conversion, and the most relevant technologies will be described below.

TABLE 2 BIOMASS SUBSTRATES FOR PHA PRODUCTION

Biomass	Pretreatment conditions	Reducing sugars (RS)	Microorganism	Production conditions	PHA type	PHA/DCW content (%)	References
Rice Straw	H_2SO_4 6%, 121°C, 1h	11,3 g·L^{-1} of glucose	*Cupriavidus necator* ATCC 17697	Batch, Flasks, 12h	P(3HB)	21 ± 3.1	Ahn et al., 2015
Sawdust	HCl 1M, 110°C, 40 min	8.9 g·L^{-1} glucose	*Halomonas halophila* CCM 3662	Batch, Flasks, 72h	PHB	46.85 ± 4.29	Kucera et al., 2018
Spent Coffee Grounds	HCl 1M, 110°C, 40 min	19.2 g·L^{-1} glucose	*H. halophila* CCM 3662	Batch, Flasks, 72h	PHB	61.95 ± 1.34	Kucera et al., 2018
Softwood	Dilute H_2SO_4, 160°C, 15 min	19.8 ± 0.4 g·L^{-1} glucose	*Paraburkholderia sacchari* DSM 17165	Batch, Flasks, 51h	PHB	71	Dietrich et al., 2018
Rice Straw	H_2SO_4 0.5%, 121°C, 40 min	16.6 g·L^{-1} of RS	*Bacillus megaterium* B-10	Batch, Flasks, 48h	P(3HB)	32.56	Li et al., 2021
Sunflower Stalk	Hydrothermal, 190°C, 5 min	78.8 g·L^{-1} of glucose	Recombinant *R. eutrhopha*	Batch, reactor, 108h	P(3HB)	79.02	Kim et al., 2016
Sugar Maple Wood	H_2SO_4 2%, 95°C, 120 min	71.9 g·L^{-1} of xylose	*Burkholderia cepacia* ATCC 17759	Fed-batch, reactor, 96h	PHB	51.4	Pan et al., 2012
Wheat Bran	NaOH 1%, 121°C, 30 min	48.4 g·L^{-1} glucose	*R. eutropha NCIMB 11599*	Batch, Flasks, 48h	PHB	62.55 ± 0.85	Annamalai and Sivakumar, 2016
Sunflower husk	NaOH 2%, 121°C, 30 min	545 ±11.45 mg/g of the substrate	*R. eutropha* ATCC 17699	Batch, Flasks, 48h	PHB	67.18 ± 1.12	Saratale and Oh, 2015
Soybean straw	NaOH 2%, 121°C, 30 min	425 ±8.12 mg/g of the substrate	*R. eutropha* ATCC 17699	Batch, Flasks, 48h	PHB	62.26 ± 1.22	Saratale and Oh, 2015
Wood Straw	NaOH 2%, 121°C, 30 min	325 ±10.25 mg/g of the substrate	*R. eutropha* ATCC 17699	Batch, Flasks, 48h	PHB	59.54 ± 1.25	Saratale and Oh, 2015
Rice Paddy Straw	NaOH 2%, 121°C, 30 min	703 ±18.12 mg/g of the substrate	*R. eutropha* ATCC 17699	Batch, Flasks, 48h	PHB	70.15 ± 1.34	Saratale and Oh, 2015
Hardwood chips	Thermomechanical pulping	100 ± 5 g/L of glucose	*Paraburkholderia sacchari* IPT 101	Fed-batch, reactor, 52h	PHB	58	Dietrich et al., 2020
Wheat Straw	AFEX	49.8 g L-1 of RS	*Burkholderia sacchari* DSM 17165	Fed-batch, reactor, 40h	P(3HB)	72	Cesário et al., 2014

Source: Authors (2022).

2.6. Pre-treatment strategies for biomass conversion to hydrolysates

Lignocellulosic biomass usually passes through different stages for hydrolysate production: mechanical milling and pretreatment (acid, alkaline, hydrothermal) for disruption of the complex polysaccharides' matrix for further enzymatic hydrolysis with specific enzymes for the release of fermentable sugars and other components (AGBOR

et al., 2011; ARORA et al., 2020; LORENCI WOICIECHOWSKI et al., 2020; RABEMANOLONTSOA and SAKA, 2016; TU and HALLETT, 2019). Each biomass has a different structure and composition of carbohydrates and, therefore, will require a particular method of pretreatment and enzymatic hydrolysis.

2.7. Chemical pretreatments.

Chemical pretreatments are largely applied in laboratorial and industrial scales for biomass hydrolysis since the reagents used in these processes are usually cheap and the techniques are well established (BEHERA et al., 2014; BENSAH and MENSAH, 2013). Among the diverse methods of chemical pretreatments, the most applied is the acid treatment that involves the solubilization of hemicellulose through the hydrolysis of its amorphous bonds mediated by the ion H^+ as catalyzer (KAPU et al., 2016). Acidic pretreatments can be conducted either with the use of concentrated acid and low temperatures – has a lower energetic cost, however, equipment corrosion can occur at a higher rate – and with dilute acids and high temperatures (HAGHIGHI MOOD et al., 2013; KUMAR and SHARMA, 2017). Inorganic acids, such as sulphuric, phosphoric, and hydrochloric, are largely employed in this type of pretreatment. However, organic acids, such as formic, acetic, and maleic, can also be applied. According to the type of biomass, organic acids may promote a lower rate of sugar conversion, but also a lower formation of fermentation inhibitors, which can be an advantage for further processes . Inhibitors are considered as compounds or by-products of pretreatment process that negatively affect the performance of enzymes and microorganisms during fermentation or saccharification processes and its generation must be avoided (JUNG and KIM, 2015). Besides, acids can also be applied for starch biomass hydrolysis (WOICIECHOWSKI et al., 2002). Kucera et al. (2018) applied acidic pretreatment to various lignocellulosic biomasses for further production of PHA. Sawdust and spent coffee grounds were treated with HCl 1M for 40 minutes at 110 °C. After cooling down, the pH was adjusted for enzymatic hydrolysis with Viscozyme L at 45 °C for 18 hours. The hydrolysates were recovered with filtration and applied to the cultivation of *Halomonas halophila* CCM 3662. Using the spent coffee grounds' hydrolysate, an accumulation of 61.95% of PHA was reached, while with the sawdust hydrolysate, 46.85%.According to the authors, the PHA yields obtained on

sawdust hydrolysate are lower than those obtained in spent coffee grounds' hydrolysate maybe due to the fact that the last one is a hexose-rich substance, which was identified as one of the best convertible sugars for *H. halophila*.

On the other hand, in alkaline pretreatments, the reagents can act on side chains and glycosidic bonds (KUMAR and SHARMA, 2017), leading to a general disruption of the lignocellulosic matrix with significant modification of its structure and cellulose de-crystallization (KUMAR and SHARMA, 2017; CHEN et al., 2017; BEHERA et al., 2014). The range of conditions as temperature, pressure, and time of alkaline pretreatments is high (the process can last minutes or hours, in temperatures that can vary from ambient temperature to 121 °C in autoclaves) (SINDHU et al., 2015), but generally, the residence time is higher when compared to acid pretreatments (HAGHIGHI MOOD et al., 2013), which can be a disadvantage due to the influence that this could have on the final costs of the process. The most applied reagents are sodium, potassium, calcium, and ammonia hydroxides (CHEN et al., 2017). Saratale and Oh (2015) carried out an alkaline pretreatment using different lignocellulosic biomasses to obtain enzymatic hydrolysates for further PHA production. The pretreatment conditions were: 2% (w/v) NaOH, 121 °C and 30 minutes with a solid-liquid ratio of 1:10, while further enzymatic hydrolysis was performed in two steps using Celluclast 1.5 L. PHA production reached 8.82 $g \cdot L^{-1}$ (67.18% accumulation) with sunflower husk hydrolysate, 7.54 $g \cdot L^{-1}$ (62.26% accumulation) with soybean straw hydrolysate, 6.79 $g \cdot L^{-1}$ (59.54% accumulation) with wood straw hydrolysate and 10.87 $g \cdot L^{-1}$ (70.15% accumulation) with rice paddy straw hydrolysate.

Another type of chemical pretreatment is the application of organic solvents (Organosolv), in which the lignin is removed during pretreatment, leaving cellulose and hemicellulose exposed for enzymatic hydrolysis. Besides, it can be combined with acid and alkali catalyzers to be more efficient in disrupting the polysaccharides matrix. A large range of temperatures can be applied, but extreme conditions of catalyzer concentration, high temperature, and reaction time can lead to inhibitors' formation (KUMAR and SHARMA, 2017). The most applied organic solvents are methanol, ethanol, acetone, and ethylene glycol, and systems of evaporation for solvent recovery can be applied (HAGHIGHI MOOD et al., 2013; CHEN et al., 2017). The synthesis and application of deep eutectic solvents (DES) have been recently used as an option for biomass

pretreatment, taking advantage of their main characteristics: biocompatible, non-toxic, non-corrosive, and recyclable (TOMÉ et al., 2018). However, there are no references mentioning the use of DES as a pretreatment of biomass for the subsequent production of PHA, which is an opportunity to explore the junction of these technologies in the process.

The main disadvantages of chemical pretreatments, when compared to other methods, are residue management (the applied reagents can be toxic, therefore, the residues obtained in the process need to be properly discarded), need to neutralize the hydrolysate (which can lead to unwanted salt formation) (HAGHIGHI MOOD et al., 2013) and fermentation inhibitors generation. The most common inhibitors found in lignocellulosic hydrolysates are 5-(Hydroxymethyl) furfural (5-HMF) that can also be degraded into levulinic, formic acid, and acetic acid derived from the dehydration of acetyl groups of hemicellulose, phenols, and vanillin (KUMAR et al., 2020). Some downstream methods can be applied for the removal of these inhibitors from the hydrolysate, which involve mainly adsorption methods such as the application of ion exchange resins or activated charcoal, but also the application of separation membranes, liquid-liquid extraction, and salting-out methods. However, the addition of a step to obtain the hydrolysate can lead to a more expensive process and, therefore, less competitive. Another alternative is the use of recombinant microorganisms that can be resistant to inhibitors of the media (KUMAR et al., 2020). Besides, alternative pretreatments that are carried out under mild conditions, can lead to great yields of sugars after enzymatic hydrolysis with lower inhibitors generation.

2.8. Physicochemical pretreatments

Hydrothermal or autohydrolysis pretreatment resembles acid treatments in its mechanism of action, however, the main catalyzer is water or steam. In these cases, with high temperatures (that can reach up to 230 °C) and pressure (up to 20 bars), water itself ionizes into H_3O^+ and OH^- ions, which are responsible for breaking down glycosidic bonds of hemicellulose, leading to its solubilization and depolymerization (RUIZ et al., 2019). Generally, cellulose is not hydrolyzed, but this treatment can enhance the enzymatic hydrolysis of this polysaccharide with cellulolytic enzymes. Liquid hot water (LHW) and

steam explosion can be considered variations of hydrothermal pretreatment when there is no application of different catalyzers (RUIZ et al., 2019; KUMARI et al., 2018). Kim et al. (2016) applied the hydrothermal pretreatment to sunflower husks with water at 190 °C for 5 minutes under agitation. The solid fraction was recovered through centrifugation and submitted to enzymatic hydrolysis with Cellic® CTec3 at 50 °C during 72 hours under 200 rpm agitation. The obtained hydrolysate was used to cultivate a recombinant *Ralstonia eutropha* (engineered to metabolize xylose) and obtain, after 108 hours of fed-batch process, 33.70 $g \cdot L^{-1}$ of P(3HB), representing a DCW accumulation of 79.02%.

Ammonia fiber explosion (AFEX) pretreatment is the combination of alkaline pretreatment using liquid ammonia as a catalyst and steam explosion. In this system, solid biomass gets in touch with liquid ammonia in a pressurized reactor with a temperature that can reach up to 100 °C (CHEN et al., 2017). After a settled residence time (HAGHIGHI MOOD et al., 2013), pressure is released leading to reagent evaporation and sudden temperature loss. The effect on biomass is similar to that of alkaline pretreatment: cellulose de-crystallization and change in lignin structure (KUMAR and SHARMA, 2017). This pretreatment is indicated for biomasses with high cellulose content (CHEN et al., 2017), but not for biomasses with high lignin content (BEHERA et al., 2014). Cesário et al. (2014) applied AFEX pretreatment to ground wheat straw to obtain a hydrolysate for PHA production with *Burkholderia sacchari* DSM 17165, a bacterial strain able to metabolize C5 and C6 sugars. A fed-batch operation was applied and, after 61 hours of process, PHA accumulation reached 72% of DCW, representing productivity of 1.6 g L^{-1} h^{-1}.

2.9. Enzymatic hydrolysis

Enzymatic hydrolysis is a crucial step, after pretreatment, for obtaining hydrolysates since the enzymes are responsible for breaking down polysaccharides' molecules into fermentable sugars that will be consumed by microorganisms. Lignocellulosic biomasses require not only a severe pretreatment for disruption of its matrix but a set of enzymes for the total hydrolysis of carbohydrates. Therefore, to properly hydrolyze lignocellulosic biomass, its composition needs to be deeply studied for the best combination of pretreatment and set of enzymes to be chosen. Usually, commercial

formulations with various enzymes are applied to facilitate hydrolysis. Cellic® CTec and Htec, from Novozymes, are examples of commercial products that are largely used for research purposes and in industrial processes (LOPES et al., 2018).

In the case of cellulose, usually, 3 enzymes are applied synergistically: endoglucanases (which act internally on cellulose, exposing non-reductive terminals), cellobiohydrolases or exoglucanases (release cellobiose and other oligosaccharides), and β-galactosidases (generate glucose monomers) (PINO et al., 2018; NEGI, 2019). While cellulose is a complex but well-defined molecule, hemicellulose can present a challenge since its structure can vary for each biomass. The most common type of hemicellulose is xylan, a polysaccharide composed of xylose monomers and hydrolyzed by xylanases, enzymes responsible for breaking down β-1,4 glycosidic bonds of xylan (GODOY et al., 2018). However, xylan may have branches on its main chain with glucuronic acid, arabinofuranose, acetyl groups, and even ferulic acid esters from lignin (MADEIRA et al., 2017; BAJPAI, 2014). In these cases, a larger set of enzymes needs to be applied for total hydrolysis of the molecule, releasing other C5-sugars, such as arabinose (ZABEL and MORREL, 2020). Hemicellulose can also be constituted by mannans, a polysaccharide composed mainly of mannose monomers linked by β-1,4 bonds that can have branches with glucose (glucomannan), galactose (galactomannan), or a combination of both residues (galactoglucomannan) (OJIMA, 2013). For mannan hydrolysis, mannanase (random cleavage of β-1,4 bonds, releasing oligomers) and mannosidase (process the oligosaccharides, releasing monomers) act together (OJIMA, 2013). Lastly, lignin usually is not hydrolyzed, but some enzymes – such as laccases, manganeses, and peroxidases – can help to reduce recalcitrance and detoxify the biomass (KUMAR and CHANDRA, 2020).

On the other hand, starch biomasses do not require pretreatment and the obtained hydrolysate contains mainly C6-sugars (glucose). Enzymatic hydrolysis of starch occurs in two steps: solubilization and saccharification. The first one happens due to the action of α-amylase enzymes, responsible for breaking down α-1,4 glycosidic bonds of starch, releasing oligomers of glucose, mainly maltose, in the media. Saccharification occurs due to the action of glucoamylase or amyloglucosidase enzymes that cleave α-1,4 and α-1,6

bonds of non-reductive terminals of starch and release glucose in the media (BIJTTEBIER et al., 2009).

Some processes, such as bioethanol production, apply the saccharification steps simultaneously to fermentation (SSF), reducing operation costs (KÁDÁR et al., 2004; KROUMOV et al., 2006). This kind of process can also be applied to PHA production since the accumulation of this biopolymer happens with nutrient imbalance (usually excess of carbon source and limitation of nitrogen and phosphorus sources). With the gradual release of sugars in SSF, the nutrient imbalance occurs naturally and can, therefore, enhance PHA accumulation by microorganisms. Dahman and Ugwu (2014) applied SSF to produce PHA. Wheat straw was pretreated with dilute sulphuric acid (1% v/v) during 30 minutes, 120°C at 1 atm and the obtained hydrolysate was submitted to enzymatic hydrolysis with cellulase and β-glucosidase and simultaneous fermentation with *Cupriavidus necator* ATCC 17699. After 72 hours of process, 15.3 $g \cdot L^{-1}$ of biomass was obtained and 10 $g \cdot L^{-1}$ of PHA, representing an accumulation of 65%. García-Torreiro et al. (2016) applied starch biomass (corn kernel mash) to the production of PHA under SSF conditions. The biomass had already passed through the liquefaction step with α-amylase and the liquid fraction was submitted to simultaneous hydrolysis with glucoamylase and fermentation with *Halomonas boliviensis*. After 72 hours of process, 26 $g \cdot L^{-1}$ of PHA was obtained, representing a yield of 23.5%.

2.10. Recently developed technologies and innovation landscape

Over the years, the concern to make the bioplastics production process more efficient has led to the search for raw materials that allow the reduction of the process' cost. Contributing to that purpose, recent research papers and patents refer to the use of hydrolysates obtained from different feedstocks for PHA production, and some developed technologies and methodologies will be described below. The most employed feedstocks are of lignocellulosic composition (sugar cane bagasse, wood, wheat straw, banana fronds, rice bran, hemp hurd, waste office paper, hardwood, corn stover). In addition, municipal solid waste, starch, fruit residues, algae biomass, spent coffee grounds, and others, also appear among the possible raw materials to produce hydrolysates that can be used as a carbon source in biopolymers' production.

Some deposited patents report the use of alternative sources of feedstock that have the potential to be converted into PHA after their hydrolysis. For example, the utilization of a plant, the *Jerusalem artichoke*, is described in patent FR2951460-A1 (DEVER and BRIAND, 2009). This patent specified the transformation of raw material to obtain a hydrolysate rich in fermentable sugars, by its extrusion at 120 °C, in the presence of water and strong acid or base (0.5-4 vol.%). The obtained hydrolysate was filtered, sterilized at 120°C, and neutralized. The hydrolysate was then employed in PHA production, which was extracted by extrusion at a temperature of 120-200°C. According to the inventors, the process is simple, inexpensive, and eco-friendly.

In another example, the invention CN111676253-A (JIA and LI, 2020) described a mixed microbial fermentation method using four recombinant microorganisms for the synthesis of mcl-PHA. First, starch was used as an initial substrate and was hydrolyzed by *Aspergillus niger* to obtain glucose. Then, the glucose hydrolysate was employed as a substrate by a mixed culture of *Saccharomyces cerevisiae* L2612 and *Aspergillus orientalis*, performing mixed fermentation to generate an acetic acid-containing solution. Finally, the acetic acid fermentation broth was used as a substrate by *Pseudomonas putida-acs* KT2440, which efficiently accumulates mcl-PHA intracellularly. This system, created by four bacteria, changed the biological reaction into a simple modularization work. As a result, the multistage flow of materials and energy consumption was reduced.

Patent KR2019128395-A (CHUNG et al., 2018) explores the use of the residual starch-rich sediments (lees), from the production process of Korean rice wine, the makgeolli. A hydrolysate (MLEH) was obtained from this residue by using α-amylase (20 U/g - at 50 °C for 2 h) and glucoamylase (10 U/g - at 60 °C for 24 h). After centrifugation at 1000 rpm for 10 min, and washing, the supernatant MLEH was obtained. According to the authors, the lees hydrolysate presented a protein content of 35-43 $g \cdot L^{-1}$ and a glucose content of approximately 77-85 $g \cdot L^{-1}$. Fermentation of Mineral Salt Medium with this carbon source was carried out for PHA production by *C. necator* at 30°C during 96 h, maintaining a pH of 7, using NaOH and HCl. The oxygen supply was set at 1.5 vvm. Initial carbon source (30 $g \cdot L^{-1}$) was used to develop a fermentation in fed-batch, with the addition of glucose (2%) after 24 h and 48 h. Thus, the authors claimed that approximately 30 $g \cdot L^{-}$

^{1} of the biopolymer were obtained. Hence, an improvement in the PHA production yield was reached and the process can be applied on an industrial scale.

Patent JP2020031631-A (MIWA et al., 2019) discloses a method for culturing bacteria of the genus *Cobetia* (which was isolated from seawater samples) that can assimilate alginic acid and synthesize PHA. The microorganism was isolated and then identified by staining and GC-MS techniques, and PHA synthesis was confirmed by ^{1}H-NMR analysis. The extraction method includes the obtaining of an alginic acid hydrolysate from seaweed through enzymatic (alginate lyase) or an acid (hydrochloric acid, phosphoric acid, sulfuric acid, acetic or formic acid can be used) hydrolysis. As a result of the biopolymer production step, the accumulation rate of P (3HB) using 3% alginic acid and 2% NaCl in an acidic culture medium reached 46.6% after 36 hours. Finally, polymer purification was performed by extraction with chloroform/hexane.

In 2019, around 931 million tons of food waste were generated, which is equivalent to 17% of total world food production including fruit waste (UNITED NATIONS ENVIRONMENT PROGRAMME, 2021). This type of waste is available worldwide, making it a suitable candidate for circular reuse approaches. Its use for PHA microbial production was protected in patent WO2021113995-A1 (ANDLER, 2019). The heat-dried, crushed, sifted, and selected fruit residues were subjected to mild acidic hydrolysis with H_2SO_4 at high pressure and temperature, obtaining a hydrolysate rich in reducing sugars and levulinic acid. A medium composed of the hydrolysate was fermented by *R. eutropha*, at 30 °C, pH 6.5-7.5, 400-700 rpm, and aeration of 1 vvm and oxygen pressure (pO_2) greater than 20%. As a result, after freeze-drying of biomass, a copolymer of PHBV was extracted using a solution of sodium hypochlorite, methanol, and ethanol.

Patent WO2012168410-A2 (ZHANG et al., 2012) describes the pretreatment of lignocellulosic material, but this time using ionic liquids. As it was described above, this technology has good prospects for the efficient treatment of feedstock of this nature. In this case, a two-step pretreatment method of cane bagasse was carried out at 130°C, stirred at 500 rpm for 30 minutes, using an ionic liquid [imidazolium cation: l-n-butyl-3-memylimidazolium chloride (BMIMCl)] at 79% by weight and 20% of water. The acid catalyst was conducted with HCl (1.2% by weight of the pretreatment solution). As an advantage, this pretreatment step reduces the production of 5- hydroxymethylfurfural,

furfural, and/or acetic acid. After separation of the pretreatment solution, the lignocellulosic material was washed with a basic solution (pH >11) and enzymatic hydrolysis with the enzyme Accellerase® (50% w/v) was conducted at 50°C for 72 hours, obtaining a yield of 91% of glucose.

Other patents describe the process to obtain hydrolysates and only mention the production of PHA as a possible application. That is the case of patent WO2012031270-A1 (JANSEN and EYAL, 2011), in which lignocellulosic material was hydrolyzed to obtain sugar mixtures to optionally produce PHA-based products. Patent WO2013170017-A2 (JU et al., 2013) describes an enzymatic hydrolysis method for soybean meal, where three streams of useful materials were obtained as a result and, one of them, the soluble one, contains saccharides and hydrolyzed carbohydrates (releasing sugars) that can be converted by fermentation into various valuable bioproducts such as PHA.

2.11. Current challenges and outlook

Different aspects of the PHA production chain must be considered to improve the scenario of commercial bioplastics based on this molecule. It is crucial to include in the agenda the study and application of emerging technologies to trigger innovation and evolution in the biotechnological field. It is not valid from any point of view, strive to produce a biodegradable molecule that reduces world plastic pollution, while in its production process, solvents are used or highly toxic or polluting chemical residues are generated, or if energy needs are high, due to processes carried out at high temperatures or pressures. Moreover, the main future challenge in bioplastics' research is to improve biodegradability characteristics and processes. The production of this type of molecule must be consistent from start to finish, with the ultimate environmental purpose.

Although it was identified an increment over the last years in research using hydrolysates for PHA production, the number of patents in this area is still incipient. The importance of encouraging joint work between companies, research centers, and universities is highlighted since this type of collaboration will accelerate the evolution of this industry. Moreover, a reinforcement of the incentives to encourage current plastics producers to invest in research and include biopolymers into their portfolio of products is an alternative. International guidelines to reduce the consumption of traditional plastics

and to incentive, the massive use of biopolymers are extremely relevant to accelerate the transition from traditional plastics to the biopolymer's era.

2.12. Conclusion

To obtain relevant, scalable, and sustainable results in the production of bioplastics from alternative medium, it is necessary to evaluate some aspects related to the entire production process, from the hydrolysates' production to the purification stages of the final material. In the first place, it is necessary to explore and implement at large scale, techniques that apply the principles of green chemistry to obtain hydrolysates, efficiently using renewable raw materials, avoiding the use of toxic and hazardous reagents, and solvents, thus, reducing the environmental impact and increasing sustainable development. On the other hand, the new generation of PHA production processes must focus on strategies that adapt the bioreactor design to a more productive controlled process, as this factor can also significantly reduce production costs. Besides, other aspects such as the increment of PHA production yield throughout the implementation of Genetic engineering, Metabolic engineering, or Synthetic biology, must be fundamentally studied.

Now, concerning downstream processes, for PHA extraction, the cell disruption is usually carried out using several consecutive processes: implementation of the alkaline solution at high temperatures, subsequent application of heating, and the addition of solvents. Moreover, the most frequently reported technologies during the purification process include the use of chemical compounds (acids, bases, organic solvents, and oxidizing agents), implementation of high temperatures, and mechanical disintegration, among others. Although obtaining bioplastics is a relatively recent industrial activity, it is necessary to adequate the process to environmental and production urgent requirements.

Regarding the industrial application of biopolymers, when the purity or the physicochemical characteristics must be highly conserved, due to the type of application (in the biomedical area, for example), the PHA molecule has a high commercial value. Thus, the costs of the coupled production processes end up being covered and highly justified. Nevertheless, one of the main challenges in PHA manufacture in the mid-term is to reduce the production costs to achieve its massive use in daily and single-use products.

Fulfilling the requirements of price and performance is the most important issue for the future of bioplastics. As aforementioned, one strategy to reach this objective is reducing the associated cost of raw materials for microbial PHA production. For this reason, this work examined the use of hydrolysates, considering that this type of raw material allows the use of biomass with different physical characteristics (lignocellulosic material, residual biomass, fruit waste, starch, lees), facilitating its use by microorganisms and its implementation at an industrial scale.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Luciana Porto de Souza Vandenberghe: Conceptualization, writing - review & editing; Zulma Sarmiento-Vásquez: Conceptualization, writing - review & editing; Ariane Fátima Murawski de Mello: writing - review & editing; Carlos Ricardo Soccol: Funding acquisition.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research scholarship.

2.13. References

Abdel-Hamid, A.M., Solbiati, J.O., Cann, I.K.O. (2013). Insights into Lignin Degradation and its Potential Industrial Applications, Advances in Applied Microbiology. Elsevier. https://doi.org/10.1016/B978-0-12-407679-2.00001-6

Adeleye, A.T., Odoh, C.K., Enudi, O.C., Banjoko, O.O., Osiboye, O.O., Toluwalope Odediran, E., Louis, H. (2020). Sustainable synthesis and applications of polyhydroxyalkanoates (PHAs) from biomass. Process Biochemistry. https://doi.org/10.1016/j.procbio.2020.05.032

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. (2011). Biomass pretreatment: Fundamentals toward application. Biotechnology Advances 29, 675–685. https://doi.org/10.1016/j.biotechadv.2011.05.005

Ahn, J., Jho, E.H., Nam, K. (2015). Effect of C/N ratio on polyhydroxyalkanoates (PHA) accumulation by Cupriavidus necator and its implication on the use of rice straw hydrolysates. Environ Eng Res 20, 246–253. https://doi.org/10.4491/eer.2015.055

Albuquerque, P.B.S., Malafaia, C.B. (2018). Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2017.09.026

Andler, R.J. (2019). Hydrolysate for microbial culture, comprising high levels of levulinic acid and reducing sugars. WO2021113995-A1.

Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S. (2016). Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2016.04.069

Annamalai, N., Sivakumar, N. (2016). Production of polyhydroxybutyrate from wheat bran hydrolysate using Ralstonia eutropha through microbial fermentation. J Biotechnol 237, 13–17. https://doi.org/10.1016/j.jbiotec.2016.09.001

Arora, A., Nandal, P., Singh, J., Verma, M.L. (2020). Nanobiotechnological advancements in lignocellulosic biomass pretreatment. Materials Science for Energy Technologies 3, 308–318. https://doi.org/10.1016/j.mset.2019.12.003

Atiwesh, G., Mikhael, A., Parrish, C.C., Banoub, J., Le, T.-A.T. (2021). Environmental impact of bioplastic use: A review. Heliyon 7, e07918. https://doi.org/10.1016/j.heliyon.2021.e07918

Bajpai, P. (2014). Xylan. Xylanolytic Enzym 9–18. https://doi.org/10.1016/b978-0-12-801020-4.00002-0

Behera, S., Arora, R., Nandhagopal, N., Kumar, S. (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew Sustain Energy Rev 36, 91–106. https://doi.org/10.1016/j.rser.2014.04.047

Bensah, E.C., Mensah, M. (2013). Chemical pretreatment methods for the production of cellulosic ethanol: Technologies and innovations. Int J Chem Eng 2013. https://doi.org/10.1155/2013/719607

Bertoft, E. (2017). Understanding starch structure: Recent progress. Agronomy 7. https://doi.org/10.3390/agronomy7030056

Bijttebier, A., Goesaert, H., Delcour, J.A. (2008). Amylase action pattern on starch polymers. Biologia (Bratisl) 63, 989–999. https://doi.org/10.2478/s11756-008-0169-x

Bishop, G., Styles, D., Lens, P.N.L. (2021). Environmental performance comparison of bioplastics and petrochemical plastics: A review of life cycle assessment (LCA) methodological decisions. Resources, Conservation and Recycling. https://doi.org/10.1016/j.resconrec.2021.105451

Brodin, M., Vallejos, M., Opedal, M.T., Area, M.C., Chinga-Carrasco, G. (2017). Lignocellulosics as sustainable resources for production of bioplastics – A review. Journal of Cleaner Production. https://doi.org/10.1016/j.jclepro.2017.05.209

Brojanigo, S., Gronchi, N., Cazzorla, T., Wong, T.S., Basaglia, M., Favaro, L., Casella, S. (2022). Engineering Cupriavidus necator DSM 545 for the one-step conversion of starchy waste into polyhydroxyalkanoates. Bioresource Technology 347, 126383. https://doi.org/10.1016/J.BIORTECH.2021.126383

Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V. (2014). Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Express Polymer Letters 8, 791–808. https://doi.org/10.3144/expresspolymlett.2014.82

Byrom, D. (1992). Production of poly-/3-hydroxybutyrate • poly-/3-hydroxyvalerate copolymers, FEMS Microbiology Reviews.

Cesário, M.T., Raposo, R.S., de Almeida, M.C.M.D., van Keulen, F., Ferreira, B.S., da Fonseca, M.M.R. (2014). Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. N Biotechnol 31, 104–113. https://doi.org/10.1016/j.nbt.2013.10.004

Chatterjee, C., Pong, F., Sen, A. (2015). Chemical conversion pathways for carbohydrates. Green Chem 17, 40–71. https://doi.org/10.1039/c4gc01062k

Chen, H., Liu, J., Chang, X., Chen, D., Xue, Y., Liu, P., Lin, H., Han, S. (2017). A review on the pretreatment of lignocellulose for high-value chemicals. Fuel Process Technol 160, 196–206. https://doi.org/10.1016/j.fuproc.2016.12.007

Chung, C.W., Kim, J., Ho, K., Lee, W.S., Kim, T. (2018). Medium composition useful for microbial fermentation to produce polyhydroxyalkanoates, comprises enzyme hydrolyzate of lees. KR2019128395-A.

Cucina, M., de Nisi, P., Tambone, F., Adani, F. (2021). The role of waste management in reducing bioplastics' leakage into the environment: A review. Bioresource Technology. https://doi.org/10.1016/j.biortech.2021.125459

Dahman, Y., Ugwu, C.U. (2014). Production of green biodegradable plastics of poly(3-hydroxybutyrate) from renewable resources of agricultural residues. Bioprocess Biosyst Eng 37, 1561–1568. https://doi.org/10.1007/s00449-014-1128-2

Deng, H., He, J., Feng, D., Zhao, Y., Sun, W., Yu, H., Ge, C. (2021). Microplastics pollution in mangrove ecosystems: A critical review of current knowledge and future directions. Science of the Total Environment. https://doi.org/10.1016/j.scitotenv.2020.142041

Dever, C., Briand, A. (2009). Preparing polyhydroxyalkanoate comprises transformation of raw vegetable material of carbon source by extrusion in water to obtain sweet juice, bacterial fermentation of juice, centrifugation to recover bacterial biomass and extraction. FR2951460-A1.

Dietrich, K., Dumont, M.J., Schwinghamer, T., Orsat, V., Del Rio, L.F. (2018). Model Study to Assess Softwood Hemicellulose Hydrolysates as the Carbon Source for PHB Production in Paraburkholderia sacchari IPT 101. Biomacromolecules 19, 188–200. https://doi.org/10.1021/acs.biomac.7b01446

Dietrich, K., Oliveira-Filho, E.R., Dumont, M.J., Gomez, J.G.C., Taciro, M.K., Silva, L.F. da Orsat, V., Rio, L.F.D. (2020). Increasing PHB production with an industrially scalable hardwood hydrolysate as a carbon source. Ind Crops Prod 154, 112703. https://doi.org/10.1016/j.indcrop.2020.112703

El-malek, F.A., Khairy, H., Farag, A., Omar, S. (2020). The sustainability of microbial bioplastics, production and applications. International Journal of Biological Macromolecules 157, 319–328. https://doi.org/10.1016/j.ijbiomac.2020.04.076

Escobar, N., Britz, W. (2021). Metrics on the sustainability of region-specific bioplastics production, considering global land use change effects. Resources, Conservation and Recycling 167. https://doi.org/10.1016/j.resconrec.2020.105345

Estévez-Alonso, Á., Pei, R., van Loosdrecht, M.C.M., Kleerebezem, R., Werker, A. (2021). Scaling-up microbial community-based polyhydroxyalkanoate production: status and challenges. Bioresource Technology. https://doi.org/10.1016/j.biortech.2021.124790

European Bioplastics, Nova-Institute. (2021). Bioplastics market development - Update 2021.

Fukui, T., Doi, Y. (1998). Efficient production of polyhydroxyalkanoates from plant oils by Alcaligenes eutrophus and its recombinant strain. Appl Microbiol Biotechnol 49, 333–336. https://doi.org/10.1007/s002530051178

García-Torreiro, M., López-Abelairas, M., Lu-Chau, T.A., Lema, J.M. (2016). Production of poly(3-hydroxybutyrate) by simultaneous saccharification and fermentation of cereal mash using Halomonas boliviensis. Biochem Eng J 114, 140–146. https://doi.org/10.1016/j.bej.2016.07.002

Godoy, M.G., Amorim, G.M., Barreto, M.S., Freire, D.M.G. (2018). Agricultural Residues as Animal Feed, Current Developments in Biotechnology and Bioengineering. Elsevier B.V. https://doi.org/10.1016/b978-0-444-63990-5.00012-8

Govil, T., Wang, J., Samanta, D., David, A., Tripathi, A., Rauniyar, S., Salem, D.R., Sani, R.K. (2020). Lignocellulosic feedstock: A review of a sustainable platform for cleaner production of nature's plastics. Journal of Cleaner Production. https://doi.org/10.1016/j.jclepro.2020.122521

Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H., Gholami, M., Ardjmand, M. (2013). Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renew Sustain Energy Rev 27, 77–93. https://doi.org/10.1016/j.rser.2013.06.033

Haque, M.A., Priya, A., Hathi, Z.J., Qin, Z.H., Mettu, S., Lin, C.S.K. (2022). Advancements and current challenges in the sustainable downstream processing of bacterial polyhydroxyalkanoates. Current Opinion in Green and Sustainable Chemistry 36, 100631. https://doi.org/10.1016/J.COGSC.2022.100631

Huong, K.H., Azuraini, M.J., Aziz, N.A., Amirul, A.A.A. (2017). Pilot scale production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) biopolymers with high molecular weight and elastomeric properties. Journal of Bioscience and Bioengineering 124, 76–83. https://doi.org/10.1016/j.jbiosc.2017.02.003

Ilyas, R.A., Sapuan, S.M., Kadier, A., Kalil, M.S., Ibrahim, R., Atikah, M.S.N., Nurazzi, N.M., Nazrin, A., Lee, C.H., Faiz Norrrahim, M.N., Sari, N.H., Syafri, E., Abral, H., Jasmani, L., Ibrahim, M.I.J. (2020). Properties and Characterization of PLA, PHA, and Other Types of Biopolymer Composites. Advanced Processing, Properties, and Applications of Starch and Other Bio-Based Polymers 111–138. https://doi.org/10.1016/B978-0-12-819661-8.00008-1

Ioannidou, S.M., Pateraki, C., Ladakis, D., Papapostolou, H., Tsakona, M., Vlysidis, A., Kookos, I.K., Koutinas, A. (2020). Sustainable production of bio-based chemicals and polymers via integrated biomass refining and bioprocessing in a circular bioeconomy context. Bioresource Technology 307. https://doi.org/10.1016/j.biortech.2020.123093

Jansen, R., Eyal, A. (2011). Methods and systems for processing sugar mixtures and resultant compositions. WO2012031270A1.

Jia, X., Li, S. (2020). Mixed fermentation method of four microorganisms for synthesizing mcl-PHA. CN111676253-A.

Jiang, G., Hill, D.J., Kowalczuk, M., Johnston, B., Adamus, G., Irorere, V., Radecka, I. (2016). Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. International Journal of Molecular Sciences. https://doi.org/10.3390/ijms17071157

Ju, L.-K., Loman, A., Coffman, A., Li, Q., Koganti, S. (2013). Enzyme-based protein separation and enrichment from soy meal, wheat meal, and other protein-rich materials derived from plant seeds, fruits and other biomass. WO2013170017-A2.

Jung, Y.H., Kim, K.H. (2015). Acidic Pretreatment, Pretreatment of Biomass: Processes and Technologies. Elsevier B.V. https://doi.org/10.1016/B978-0-12-800080-9.00003-7

Kádár, Z., Szengyel, Z., Réczey, K. (2004). Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Ind Crops Prod 20, 103–110. https://doi.org/10.1016/j.indcrop.2003.12.015

Kalia, V.C., Singh Patel, S.K., Shanmugam, R., Lee, J.K. (2021). Polyhydroxyalkanoates: Trends and advances toward biotechnological applications. Bioresource Technology. https://doi.org/10.1016/j.biortech.2021.124737

Kapu, N.S., Yuan, Z., Chang, X.F., Beatson, R., Martinez, D.M., Trajano, H.L. (2016). Insight into the evolution of the proton concentration during autohydrolysis and dilute-acid hydrolysis of hemicellulose. Biotechnol Biofuels 9, 1–10. https://doi.org/10.1186/s13068-016-0619-6

Kasavan, S., Yusoff, S., Rahmat Fakri, M.F., Siron, R. (2021). Plastic pollution in water ecosystems: A bibliometric analysis from 2000 to 2020. Journal of Cleaner Production 313. https://doi.org/10.1016/j.jclepro.2021.127946

Kawaguchi, H., Takada, K., Elkasaby, T., Pangestu, R., Toyoshima, M., Kahar, P., Ogino, C., Kaneko, T., Kondo, A. (2022). Recent advances in lignocellulosic biomass white biotechnology for bioplastics. Bioresource Technology. https://doi.org/10.1016/j.biortech.2021.126165

Keshavarz, T., Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. Current Opinion in Microbiology. https://doi.org/10.1016/j.mib.2010.02.006

Kim, H.S., Oh, Y.H., Jang, Y.A., Kang, K.H., David, Y., Yu, J.H., Song, B.K., Choi, J. il, Chang, Y.K., Joo, J.C., Park, S.J. (2016). Recombinant Ralstonia eutropha engineered to utilize xylose and its use for the production of poly(3-hydroxybutyrate) from sunflower stalk hydrolysate solution. Microb Cell Fact 15, 1–13. https://doi.org/10.1186/s12934-016-0495-6

Kroumov, A.D., Módenes, A.N., Tait, M.C.D.A. (2006). Development of new unstructured model for simultaneous saccharification and fermentation of starch to ethanol by recombinant strain. Biochem Eng J 28, 243–255. https://doi.org/10.1016/j.bej.2005.11.008

Kucera, D., Pernicová, I., Kovalcik, A., Koller, M., Mullerova, L., Sedlacek, P., Mravec, F., Nebesarova, J., Kalina, M., Marova, I., Krzyzanek, V., Obruca, S. (2018). Characterization of the promising poly(3-hydroxybutyrate) producing halophilic

bacterium Halomonas halophila. Bioresour Technol 256, 552–556. https://doi.org/10.1016/j.biortech.2018.02.062

Kumar, A., Chandra, R. (2020). Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. Heliyon 6, e03170. https://doi.org/10.1016/j.heliyon.2020.e03170

Kumar, A.K., Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. Bioresour Bioprocess 4. https://doi.org/10.1186/s40643-017-0137-9

Kumar, G., Ponnusamy, V.K., Bhosale, R.R., Shobana, S., Yoon, J.J., Bhatia, S.K., Rajesh Banu, J., Kim, S.H. (2019). A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. Bioresource Technology. https://doi.org/10.1016/j.biortech.2019.121427

Kumar, V., Yadav, S.K., Kumar, J., Ahluwalia, V. (2020). A critical review on current strategies and trends employed for removal of inhibitors and toxic materials generated during biomass pretreatment. Bioresour Technol 299, 122633. https://doi.org/10.1016/j.biortech.2019.122633

Kumari, D., Singh, R. (2018). Pretreatment of lignocellulosic wastes for biofuel production: A critical review. Renew Sustain Energy Rev 90, 877–891. https://doi.org/10.1016/j.rser.2018.03.111

Kutralam-Muniasamy, G., Peréz-Guevara, F. (2018). Genome characteristics dictate poly-R-(3)-hydroxyalkanoate production in Cupriavidus necator H16. World Journal of Microbiology and Biotechnology. https://doi.org/10.1007/s11274-018-2460-5

Li, J., Yang, Z., Zhang, K., Liu, M., Liu, D., Yan, X., Si, M., Shi, Y. (2021). Valorizing waste liquor from dilute acid pretreatment of lignocellulosic biomass by Bacillus megaterium B-10. Ind Crops Prod 161, 113160. https://doi.org/10.1016/j.indcrop.2020.113160

Li, M., Wilkins, M.R. (2020). Recent advances in polyhydroxyalkanoate production: Feedstocks, strains and process developments. International Journal of Biological Macromolecules 156, 691–703. https://doi.org/10.1016/j.ijbiomac.2020.04.082

Lopes, A.M., Ferreira Filho, E.X., Moreira, L.R.S. (2018). An update on enzymatic cocktails for lignocellulose breakdown. J Appl Microbiol 125, 632–645. https://doi.org/10.1111/jam.13923

Lorenci Woiciechowski, A., Dalmas Neto, C.J., Porto de Souza Vandenberghe, L., de Carvalho Neto, D.P., Novak Sydney, A.C., Letti, L.A.J., Karp, S.G., Zevallos Torres, L.A., Soccol, C.R. (2020). Lignocellulosic biomass: Acid and alkaline pretreatments and their effects on biomass recalcitrance – Conventional processing and recent advances. Bioresource Technology 304, 122848. https://doi.org/10.1016/j.biortech.2020.122848

Low, T.J., Mohammad, S., Sudesh, K., Baidurah, S. (2021). Utilization of banana (Musa sp.) fronds extract as an alternative carbon source for poly(3-hydroxybutyrate)

production by Cupriavidus necator H16. Biocatalysis and Agricultural Biotechnology 34. https://doi.org/10.1016/j.bcab.2021.102048

Madeira, J.V., Contesini, F.J., Calzado, F., Rubio, M.V., Zubieta, M.P., Lopes, D.B., de Melo, R.R. (2017). Agro-Industrial Residues and Microbial Enzymes: An Overview on the Eco-Friendly Bioconversion into High Value-Added Products, Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. Elsevier Inc. https://doi.org/10.1016/B978-0-12-803725-6.00018-2

Medeiros Garcia Alcântara, J., Distante, F., Storti, G., Moscatelli, D., Morbidelli, M., Sponchioni, M. (2020). Current trends in the production of biodegradable bioplastics: The case of polyhydroxyalkanoates. Biotechnology Advances. https://doi.org/10.1016/j.biotechadv.2020.107582

Miwa, Y., Hiroki, M., Hitoshi, S. (2019). Methods for producing polyhydroxyalkanoic acid using alginic acid as a carbon source. JP2020031631-A.

Mohandas, S.P., Balan, L., Jayanath, G., Anoop, B.S., Philip, R., Cubelio, S.S., Bright Singh, I.S. (2018). Biosynthesis and characterization of polyhydroxyalkanoate from marine Bacillus cereus MCCB 281 utilizing glycerol as carbon source. International Journal of Biological Macromolecules 119, 380–392. https://doi.org/10.1016/j.ijbiomac.2018.07.044

Moshood, T.D., Nawanir, G., Mahmud, F., Mohamad, F., Ahmad, M.H., AbdulGhani, A. (2022a). Biodegradable plastic applications towards sustainability: A recent innovations in the green product. Cleaner Engineering and Technology 6, 100404. https://doi.org/10.1016/j.clet.2022.100404

Moshood, T.D., Nawanir, G., Mahmud, F., Mohamad, F., Ahmad, M.H., AbdulGhani, A. (2022b). Sustainability of biodegradable plastics: New problem or solution to solve the global plastic pollution? Current Research in Green and Sustainable Chemistry 100273. https://doi.org/10.1016/j.crgsc.2022.100273

Murugan, P., Gan, C.Y., Sudesh, K. (2017). Biosynthesis of P(3HB-co-3HHx) with improved molecular weights from a mixture of palm olein and fructose by Cupriavidus necator Re2058/pCB113. International Journal of Biological Macromolecules 102, 1112–1119. https://doi.org/10.1016/J.IJBIOMAC.2017.05.006

Muthuraj, R., Valerio, O., Mekonnen, T.H. (2021). Recent developments in short- and medium-chain- length Polyhydroxyalkanoates: Production, properties, and applications. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2021.07.143

Nawrath, C., Poirier, Y. (2008). Pathways for the Synthesis of Polyesters in Plants: Cutin, Suberin, and Polyhydroxyalkanoates. Advances in Plant Biochemistry and Molecular Biology 1, 201–239. https://doi.org/10.1016/S1755-0408(07)01008-9

Negi, S. (2019). Green Bio-processes. Springer Singapore. https://doi.org/10.1007/978-981-13-3263-0

Nitkiewicz, T., Wojnarowska, M., Sołtysik, M., Kaczmarski, A., Witko, T., Ingrao, C., Guzik, M. (2020). How sustainable are biopolymers? Findings from a life cycle assessment of polyhydroxyalkanoate production from rapeseed-oil derivatives. Science of The Total Environment 749, 141279. https://doi.org/10.1016/J.SCITOTENV.2020.141279

Ntaikou, I., Koumelis, I., Tsitsilianis, C., Parthenios, J., Lyberatos, G. (2018). Comparison of yields and properties of microbial polyhydroxyalkanoates generated from waste glycerol-based substrates. International Journal of Biological Macromolecules 112, 273–283. https://doi.org/10.1016/j.ijbiomac.2018.01.175

Obruca, S., Marova, I., Svoboda, Z., Mikulikova, R. (2010). Use of Controlled Exogenous Stress for Improvement of Poly(3-hydroxybutyrate) Production in Cupriavidus necator, Folia Microbiol.

Ojima, T. (2013). Polysaccharide-degrading enzymes from herbivorous marine invertebrates, Marine Enzymes for Biocatalysis: Sources, Biocatalytic Characteristics and Bioprocesses of Marine Enzymes. Woodhead Publishing Limited. https://doi.org/10.1533/9781908818355.3.333

Okeke, E.S., Okoye, C.O., Atakpa, E.O., Ita, R.E., Nyaruaba, R., Mgbechidinma, C.L., Akan, O.D. (2022). Microplastics in agroecosystems-impacts on ecosystem functions and food chain. Resources, Conservation and Recycling. https://doi.org/10.1016/j.resconrec.2021.105961

Pan, W., Perrotta, J.A., Stipanovic, A.J., Nomura, C.T., Nakas, J.P. (2012). Production of polyhydroxyalkanoates by Burkholderia cepacia ATCC 17759 using a detoxified sugar maple hemicellulosic hydrolysate. J Ind Microbiol Biotechnol 39, 459–469. https://doi.org/10.1007/s10295-011-1040-6

Pellis, A., Malinconico, M., Guarneri, A., Gardossi, L. (2021). Renewable polymers and plastics: Performance beyond the green. New Biotechnology. https://doi.org/10.1016/j.nbt.2020.10.003

Phelan, A. (Anya), Meissner, K., Humphrey, J., Ross, H. (2022). Plastic pollution and packaging: Corporate commitments and actions from the food and beverage sector. Journal of Cleaner Production 331, 129827. https://doi.org/10.1016/j.jclepro.2021.129827

Pino, M.S., Rodríguez-Jasso, R.M., Michelin, M., Flores-Gallegos, A.C., Morales-Rodriguez, R., Teixeira, J.A., Ruiz, H.A. (2018). Bioreactor design for enzymatic hydrolysis of biomass under the biorefinery concept. Chem Eng J 347, 119–136. https://doi.org/10.1016/j.cej.2018.04.057

Pinto-Ibieta, F., Cea, M., Cabrera, F., Abanto, M., Felissia, F.E., Area, M.C., Ciudad, G., (2020). Strategy for biological co-production of levulinic acid and polyhydroxyalkanoates by using mixed microbial cultures fed with synthetic hemicellulose hydrolysate. Bioresource Technology 309. https://doi.org/10.1016/j.biortech.2020.123323

PlascticsEurope (2021). Plastics: the Facts 2021. An analysis of European plastics production, demand and waste data.

Poirier, Y., Nawrath, C. & Somerville, C. (1995). Production of Polyhydroxyalkanoates, a Family of Biodegradable Plastics and Elastomers, in Bacteria and Plants. Nat Biotechnol 13, 142–150. https://doi.org/10.1038/nbt0295-142

Rabemanolontsoa, H., Saka, S. (2016). Various pretreatments of lignocellulosics. Bioresource Technology 199, 83–91. https://doi.org/10.1016/j.biortech.2015.08.029

Raza, Z.A., Abid, S., Banat, I.M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. International Biodeterioration and Biodegradation. https://doi.org/10.1016/j.ibiod.2017.10.001

Ruiz, H.A., Conrad, M., Sun, S.N., Sanchez, A., Rocha, G.J.M., Romaní, A., Castro, E., Torres, A., Rodríguez-Jasso, R.M., Andrade, L.P., Smirnova, I., Sun, R.C., Meyer, A.S. (2020). Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour Technol 299, 122685. https://doi.org/10.1016/j.biortech.2019.122685

Samui, A.B., Kanai, T. (2019). Polyhydroxyalkanoates based copolymers. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2019.08.147

Sagong, H.-Y., Son, H.F., Choi, S.Y., Lee, S.Y., Kim, K.-J. (2018). Structural Insights into Polyhydroxyalkanoates Biosynthesis. Trends Biochem. Sci. 43, 790–805. https://doi.org/10.1016/j.tibs.2018.08.005

Saratale, G.D., Oh, M.K. (2015). Characterization of poly-3-hydroxybutyrate (PHB) produced from Ralstonia eutropha using an alkali-pretreated biomass feedstock. Int J Biol Macromol 80, 627–635. https://doi.org/10.1016/j.ijbiomac.2015.07.034

Sen, K.Y., Baidurah, S. (2021). Renewable biomass feedstocks for production of sustainable biodegradable polymer. Current Opinion in Green and Sustainable Chemistry. https://doi.org/10.1016/j.cogsc.2020.100412

Silva, F., Matos, M., Pereira, B., Ralo, C., Pequito, D., Marques, N., Carvalho, G., Reis, M.A.M. (2022). An integrated process for mixed culture production of 3-hydroxyhexanoate-rich polyhydroxyalkanoates from fruit waste. Chemical Engineering Journal 427. https://doi.org/10.1016/j.cej.2021.131908

Simul Bhuyan, M., Venkatramanan, S., Selvam, S., Szabo, S., Maruf Hossain, M., Rashed-Un-Nabi, M., Paramasivam, C.R., Jonathan, M.P., Shafiqul Islam, M. (2021). Plastics in marine ecosystem: A review of their sources and pollution conduits. Regional Studies in Marine Science. https://doi.org/10.1016/j.rsma.2020.101539

Sindhu, R., Pandey, A., Binod, P. (2015). Alkaline Treatment. Pretreat Biomass Process Technol 51–60. https://doi.org/10.1016/B978-0-12-800080-9.00004-9

Sindhu, R., Madhavan, A., Arun, K.B., Pugazhendhi, A., Reshmy, R., Awasthi, M.K., Sirohi, R., Tarafdar, A., Pandey, A., Binod, P. (2021). Metabolic circuits and gene

regulators in polyhydroxyalkanoate producing organisms: Intervention strategies for enhanced production. Bioresource Technology 327, 124791. https://doi.org/10.1016/J.BIORTECH.2021.124791

Steinbüchel, A., Lütke-Eversloh, T. (2003). Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. Biochemical Engineering Journal 16, 81–96. https://doi.org/10.1016/S1369-703X(03)00036-6

Sudesh, K., Abe, H., Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters.

Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M., Shah, S. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants - A review. Biotechnology Advances. https://doi.org/10.1016/j.biotechadv.2006.11.007

Tan, D., Wang, Y., Tong, Y., Chen, G.Q. (2021). Grand Challenges for Industrializing Polyhydroxyalkanoates (PHAs). Trends in Biotechnology. https://doi.org/10.1016/j.tibtech.2020.11.010

Thomas, C.M., Kumar, D., Scheel, R.A., Ramarao, B., Nomura, C.T. (2022). Production of Medium Chain Length polyhydroxyalkanoate copolymers from agro-industrial waste streams. Biocatalysis and Agricultural Biotechnology 102385. https://doi.org/10.1016/J.BCAB.2022.102385

Tokiwa, Y., Calabia, B.P., Ugwu, C.U., Aiba, S. (2009). Biodegradability of plastics. International Journal of Molecular Sciences. https://doi.org/10.3390/ijms10093722

Tomé, L.I.N., Baião, V., da Silva, W., Brett, C.M.A. (2018). Deep eutectic solvents for the production and application of new materials. Applied Materials Today. https://doi.org/10.1016/j.apmt.2017.11.005

Tu, W.C., Hallett, J.P. (2019). Recent advances in the pretreatment of lignocellulosic biomass. Current Opinion in Green and Sustainable Chemistry 20, 11–17. https://doi.org/10.1016/j.cogsc.2019.07.004

United Nations Environment Programme. (2021). UNEP Food waste Index, Report 2021.

Usmani, Z., Sharma, M., Awasthi, A.K., Lukk, T., Tuohy, M.G., Gong, L., Nguyen-Tri, P., Goddard, A.D., Bill, R.M., Nayak, S.C., Gupta, V.K. (2021). Lignocellulosic biorefineries: The current state of challenges and strategies for efficient commercialization. Renewable and Sustainable Energy Reviews 148. https://doi.org/10.1016/j.rser.2021.111258

Vandenberghe, L.P. de S., Oliveira, P.Z. de, Bittencourt, G.A., Mello, A.F.M. de, Vásquez, Z.S., Karp, S.G., Soccol, C.R. (2021). The 2G and 3G bioplastics: an overview. Biotechnology Research and Innovation 5, e2021004. https://doi.org/10.4322/biori.202104

Vea, E.B., Fabbri, S., Spierling, S., Owsianiak, M. (2021). Inclusion of multiple climate tipping as a new impact category in life cycle assessment of polyhydroxyalkanoate (PHA)-based plastics. Science of The Total Environment 788, 147544. https://doi.org/10.1016/J.SCITOTENV.2021.147544

Vered, G., Shenkar, N. (2021). Monitoring plastic pollution in the oceans. Current Opinion in Toxicology 27, 60–68. https://doi.org/10.1016/j.cotox.2021.08.005

Williams, A.T., Rangel-Buitrago, N. (2022). The past, present, and future of plastic pollution. Marine Pollution Bulletin 176, 113429. https://doi.org/10.1016/J.MARPOLBUL.2022.113429

Willis, K.A., Hardesty, B.D., Wilcox, C. (2021). State and local pressures drive plastic pollution compliance strategies. Journal of Environmental Management 287. https://doi.org/10.1016/j.jenvman.2021.112281

Woiciechowski, A.L., Nitsche, S., Pandey, A., Soccol, C.R. (2002). Acid and enzymatic hydrolysis to recover reducing sugars from cassava bagasse: An economic study. Brazilian Arch Biol Technol 45, 393–400. https://doi.org/10.1590/S1516-89132002000300018

Wróblewska-Krepsztul, J., Rydzkowski, T., Borowski, G., Szczypiński, M., Klepka, T., Thakur, V.K. (2018). Recent progress in biodegradable polymers and nanocomposite-based packaging materials for sustainable environment. International Journal of Polymer Analysis and Characterization 23, 383–395. https://doi.org/10.1080/1023666X.2018.1455382

Yang, N., Li, Y., Xing, F., Wang, X., Li, X., Li, L., Yang, J., Wang, Y., Zhang, M. (2021). Composition and structural characterization of pectin in micropropagated and conventional plants of Premma puberula Pamp. Carbohydrate Polymers 260. https://doi.org/10.1016/j.carbpol.2021.117711

Yeo, J.C.C., Muiruri, J.K., Thitsartarn, W., Li, Z., He, C. (2018). Recent advances in the development of biodegradable PHB-based toughening materials: Approaches, advantages and applications. Materials Science and Engineering C. https://doi.org/10.1016/j.msec.2017.11.006

Zabel, R.A., Morrell, J.J. (2020). Chemical changes in wood caused by decay fungi. Wood Microbiol 215–244. https://doi.org/10.1016/b978-0-12-819465-2.00008-5

Zhang, Z., O`hara, I.M., Doherty, W.O.S. (2012). Methods for treating lignocellulosic material. WO2012168410-A2.

Zhou, M., Tian, X. (2022). Development of different pretreatments and related technologies for efficient biomass conversion of lignocellulose. International Journal of Biological Macromolecules 202, 256–268. https://doi.org/10.1016/j.ijbiomac.2022.01.036

3. CAPÍTULO II

Manuscrito não submetido

PRODUCTION OF POLYHYDROXYALKANOATES THROUGH SOYBEAN HULL VALORIZATION: SUBSEQUENT ALKALINE PRETREATMENT AND ENZYMATIC HYDROLYSIS

3.1. PRODUCTION OF POLYHYDROXYALKANOATES THROUGH SOYBEAN HULL AND WASTE GLYCEROL VALORIZATION: SUBSEQUENT ALKALINE PRETREATMENT AND ENZYMATIC HYDROLYSIS

Zulma Sarmiento-Vásquez[a], Luciana Porto de Souza Vandenberghe[a*], Susan Grace Karp[a], Carlos Ricardo Soccol[a]

[a]Federal University of Paraná, Department of Bioprocess Engineering and Biotechnology, Centro Politécnico, 81531-980, Curitiba, Paraná, Brazil

3.2. Abstract

Alkaline pretreatment and sequential enzymatic hydrolysis of soybean hull were investigated to obtain fermentable sugars for polyhydroxyalkanoates production along with residual glycerol as low-cost carbon sources. Soybean hull is composed of approximately 32% cellulose, 12% hemicellulose, 6% lignin, and 11% protein. Alkaline pretreatment was carried out with 2% NaOH con-centration, 10% (w/v) biomass loading, and 60 min incubation time in an autoclave at 120 °C. The response surface methodology (RSM) based on the central composite design (CCD) tool was employed to optimize the enzymatic hydrolysis process, where the variables of biomass loading, enzymes' concentration, and time were considered. The maximum total reducing sugars concentration obtained was 115.9 $g{\cdot}L^{-1}$ with an enzyme concentration of 11.5 mg protein/g dry substrate for enzyme preparation B1, 2.88 mg protein/g dry substrate for XylA, and 57.6 U/g dry substrate for β-glucosidase, after 42 h at 45°C, and pH was 4.5. Subsequently, the saccharification step was conducted by increasing the processing scale, using a 1 L tank with stirring with a controlled temperature. Implementing the same enzyme concentrations at pH 4.5, temperature of 45 °C, 260 mL working volume, and incubation time of 42 h, under fed-batch operation with substrate feeding after 14 h and 22 h, a hydrolysate with a concentration of 185.7 $g{\cdot}L^{-1}$ was obtained. Initially, to verify the influence of different carbon sources on *Cupriavidus necator* DSMz 545 in biomass pro-duction, batch fermentations were developed, testing laboratory-grade glucose, soybean hull hydrolysate, and waste glycerol (a by-product of biodiesel processing available in large quantities) as carbon sources in one-factor-at-a-time assays, and the mixture of soybean hull hydrolysate and waste glycerol. Then, the hydrolysate and waste glycerol were consumed by *C. necator*, producing 12.1 $g{\cdot}L^{-1}$ of biomass and achieving 39% of polyhydroxyalkanoate (PHB) accumulation. To the best of our knowledge, this is the first time that soybean hull hydrolysate has been used as a carbon source to produce polyhydroxyalkanoates, and the results suggest that this agro-industrial by-product is a viable alternative feedstock to produce value-added components.

Keywords: soybean hull; alkaline pretreatment, saccharification; biomass hydrolysate; fermentable sugars; polyhydroxyalkanoates; biorefinery.

*Corresponding author: lvandenberghe@ufpr.br

3.3. Introduction

Agro-industrial activities around the world generate enormous quantities of waste material. However, considering environmental, productivity, and sustainability trends these materials are now seen from a new perspective. Currently, due to their interesting physical-chemical composition, they can be used and reintegrated in productive processes that guarantee their valorization. Agricultural sectors produce lignocellulosic waste materials (straw, cob, stalks, bagasse, hulls), which have as main structural compounds cellulose (40-60%), hemicellulose (20-40%), and lignin (10-25%) (SU et al., 2020). Given that around 200 billion metric tons of these wastes are generated annually (NAHAK et al., 2022), it is remarkable the increase in research on applications, that allow the reuse of these structural compounds. These polysaccharides have a high potential for transformation and valorization, as they are used as feedstock for the generation of biofuels, organic acids, and various high-cost molecules (NIJU et al., 2020).

Worldwide, Brazil stands out in agribusiness, which represents one of the main pillars of the national economy. It is worth mentioning important examples in this area, such as soybean production. In 2020/21, Brazil produced 135.409 million tons of soybean (*Glycine max*) which corresponds to 37.3% of world production, being the world's leading producer of this grain (Embrapa, 2022). It is estimated that around seven million tons of soybean hulls were generated in Brazil by 2020. This material has low recalcitrance due to its low lignin content (2-13%), which facilitates processing for the utilization of hemicellulose (19-34%) and cellulose (29-52%). Various physical, chemical, and enzymatic pretreatments have been implemented for the saccharification of these compounds to obtain fermentable sugars that allow the production, through biotechnological processes, of medium to high value-added molecules such as enzymes, organic acids, and biofuels (AMARO BITTENCOURT et al., 2021).

On the other hand, in biodiesel production industry, for every 10 kg of biodiesel 1 kg of glycerol is generated as a by-product (Raman et al., 2019). In 2020, approximately 600,000 m^3 of glycerol were generated in Brazil, representing an increase of 140% over the last decade (ANP, 2021). The wide availability of glycerol outlines its application on an industrial scale. Thus, it has been widely used in the cosmetics, food, pharmaceutical, and renewable energy industries. The prospects for future environmentally friendly

applications are of great interest in the R&D areas of the industry. Thus, high value-added molecules (i.e., 1,3-propanediol, malic acid, lipids, biopolymers, among others) have been produced through microorganisms (GOYAL et al., 2021; JU et al., 2020).

In addition to the problem of the excessive generation of waste materials because of the production processes that currently support human needs (food, fuel, clothing, construction), there is also the excessive accumulation of millions of tons of plastic. It is estimated that more than 8.3 billion tons of plastic have been produced since 1950, about 60% ends up in landfills or in the natural environment and, approximately eleven million tons, are dumped into the sea each year (UNEP, 2018). In 2020, global plastics production reached 367 million tons (PLASTICSEUROPE, 2021), of which less than 1% corresponds to bioplastics. In the United States, for example, only 9% of this material was recycled (Environmental Protection Agency, 2020). A plausible alternative to this situation focuses on the production of biodegradable polymers, which are generated from renewable raw materials and can be degraded by biological processes. Polyhydroxyalkanoates (PHA) belong to this group of biopolymers and could be produced intracellularly by microorganisms. They have characteristics of conventional plastics and, most importantly, are biodegradable (GAHLAWAT, 2019). However, the marketing value is affected by the high cost of raw materials within the production process, which has been a major obstacle to large-scale production (LI and WILKINS, 2020). Thus, as an alternative for the use of available waste material in the region and to mitigate the environmental problem generated by the use of polymers produced from petroleum, this work explores the use of agro-industrial by-products, such as soybean hulls and wasted glycerol, as raw materials to produce PHAs by *Cupriavidus necator*. To the best of our knowledge, this is the first time that soybean hull hydrolysate is used as a carbon source to produce polyhydroxyalkanoates, and the results suggest that both this agro-industrial by-product and glycerol are viable alternative feedstocks to produce value-added components.

3.4. Material and methods

3.4.1. Characterization of soybean hull

The soybean hulls (SBH) used in this study were supplied by the company Imcopa S.A (Araucária, Paraná, Brazil). Following the NREL/TP-510-42620 standard on sample preparation for compositional analysis (HAMES et al., 2008), the SBH were subjected to drying processes at 45±3°C for 24 h (Thot drying oven - Mod. 510.480, Brazil), milling (Marconi-MA 580/E electric knife mill) and sieving (USS/ASTM No. 20 mesh). In the analyses described below, particle size between 0.84 - 2.00 mm was employed. The total solids and moisture of the SBH were analyzed using a balance for moisture determination by Infrared (Top Ray - BEL). Ash content was determined by applying the methodology established in NREL/TP-510-42622 (SLUITER et al., 2008a) using a muffle furnace at 575 ± 25 °C to calcine the material for 6 h. For the determination of extractables in water and ethanol, the methodology established in the standard NREL/TP-510-42619 (SLUITER et al., 2008b) was adopted. The extraction was carried out during 8 h with the addition of 190 ± 5 mL of each solvent (water and ethanol consecutively) in the Soxhlet equipment. The determination of structural carbohydrates and lignin was performed following the methodology established in NREL/TP-510-42618 (SLUITER et al., 2011).

For the determination of reducing sugars (RS) and ions analyses, it was necessary to prepare the aqueous extract of the SBH. Thus, in an incubator water bath with circular orbital stirring (Ethiktechnology-Labstore, Brazil), the mixture of 1 g of SBH and 50 mL of deionized water was heated at 100 °C for 10 minutes. This content was carefully transferred to a 100 mL volumetric flask and the volume was completed with deionized water. The sample was filtered using qualitative filter paper and stored at 4 °C until analysis. The followed stages in this work are indicated in Fig 7.

3.4.2. Pretreatment conditions of soybean hulls

Considering the physicochemical characteristics of SBH it was determined that the implementation of chemical pretreatment followed by enzymatic hydrolysis, to obtain a sugars-rich hydrolysate to be employed in further fermentation processes (QING et al.,

2017). The chemical pretreatment was developed using 2% (w/v) of NaOH, SBH in a ratio of 10% (w/v) and at 121 °C for 1 hour. After pretreatment, once the material reached room temperature, it was neutralized with HCl, filtered, washed with distilled water to remove soluble components, and filtered again (KARP et al., 2021). The resulting wet solid material was reserved for the following steps once the total solids and moisture were analyzed using an infrared moisture determination balance (Top Ray - BEL).

3.4.3. Optimization of enzymatic hydrolysis

3.4.3.1. First step of SBH enzymatic hydrolysis optimization

For the saccharification step of alkaline pretreated SBH, was used an enzyme cocktail (produced at the Federal Research Centre "Fundamentals of Biotechnology" at Lomonosov Moscow State University) composed of: cellulase and xylanase complexes of *Penicillium verruculosum* (B1 host preparation), xylanase B1-XylA preparation, which was obtained through the recombinant expression of *Penicillium canescens* xylanase A in the *P. verruculosum* B1 host strain, and β-glucosidase F10 preparation, which was obtained by the recombinant expression of *Aspergillus niger* β-glucosidase in the B1 host strain (MOROZOVA et al., 2010; OSIPOV et al., 2011; DOTSENKO et al., 2015; KARP et al., 2020). SBH saccharification conditions through enzymatic hydrolysis were optimized using the Response Surface Methodology (RSM). Following a Central Composite Design (CCD) approach, eighteen essays were performed (with 6 axial points and 2 center point runs), using the software *Statistica ® 5.0* for data analysis. The substrate loading [%(w/v)], saccharification time (h), and enzyme cocktail concentration (w/w) with B1, β-glucosidase F10 and XylA, were studied, keeping pH 4.5 and temperature constant (45 °C). The independent factors in the design were studied at five different levels (-α, -1, 0, +1 and + α) as shown in TABLE 3. The analysis of variance was evaluated at a significance level of 95% ($p < 0.05$) for all compounds. In an orbital agitated water bath (Ethiktechnology-Labstore, Brazil) samples were incubated at 100 rpm, from 12 h to 72 h, using 20 mL as working volume in 125 mL Erlenmeyer flasks. Then, the heat shock was performed to disactivate the enzyme activity. Samples of enzymatically treated SBH were filtered through qualitative filter paper and stored under refrigeration until further use.

TABLE 3 - THE INDEPENDENT AND CODING LEVELS EMPLOYED IN THE CCD.

Independent variable	-1.682	-1	0	+1	+1.682
Substrate loading [%(w/v)]	15.0	24.5	38.5	52.5	62.0
Saccharification time (h)	12.0	24.2	42.0	59.8	72.0
Enzyme concentrations (w/w)					
B1	3.28	6.62	11.52	16.42	19.76
β-glucosidase F10	16.40	33.10	57.59	82.08	98.78
XylA	0.82	1.66	2.88	4.10	4.49

Source: Authors (2022).

3.4.3.2. Second step of SBH fed-batch enzymatic hydrolysis optimization

Additional optimization of SBH enzymatic hydrolysis essays were performed in a 1L bioreactor (Mod. TE-054-MAG, Tecnal, Brazil) adapted to a mechanical stirrer with a spiral propeller blade (Mod. TE-139, Tecnal, Brazil). The SBH enzymatic hydrolysis was conducted with the use of a reaction volume of 260 mL of a sodium acetate buffer (1M, pH 4.5), incubated at 45°C with constant stirring for 42 h, was developed. The previously optimized enzyme cocktail was employed, which was composed of: 11.52 $mg \cdot g^{-1}$ dry substrate of B1, 2.88 $mg \cdot g^{-1}$ dry substrate of B1-XylA, and 56.7 U /g of dry substrate of F10 β-glucosidase. In this case, the final substrate concentration (in dry basis) was set at 38.5% (w/v) by applying a fed-batch strategy, with the addition of alkaline-pretreated SBH for the saccharification reaction. The saccharification process began with 12.9 % (w/v) solids content and 12.8 % and 12.8 % of substrate were fed at 14 h and 22 h to achieve 38.5 % (w/v) of total solids content. Then, the heat shock was performed to disactivate the enzymatic reaction, the hydrolyzed SBH material was filtered through qualitative filter paper and the hydrolysate was stored under refrigeration until further use (directly for PHB production without detoxification) and/or analysis. The degree of conversion from cellulose to glucose (C_{CG}%) was determined as the ratio of the glucose obtained to the theoretical yield based on the amount of cellulose in soybean hulls, where Y_G is the glucose yield (g/g) and C is the amount of cellulose in 1 g SBH (YOO et al., 2011).

$$C_{CG} = \frac{Y_G}{C \times 1.1} \times 100 \qquad (1)$$

3.4.4. PHA production

3.4.4.1. Culture condition and PHB synthesis

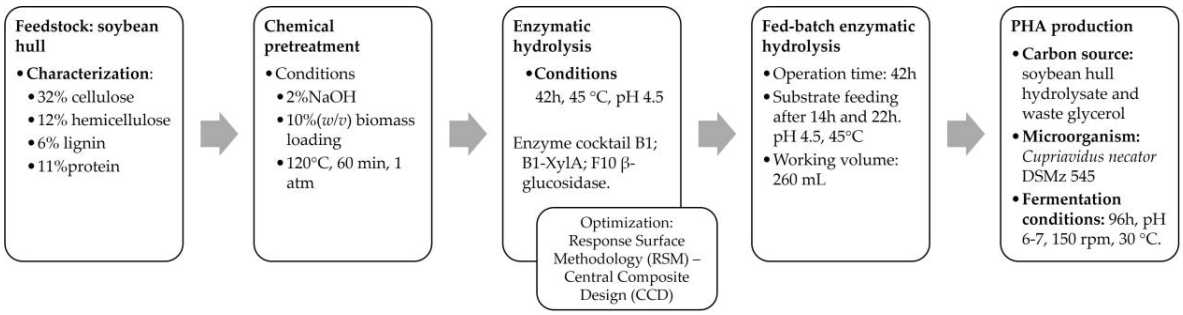
The *C. necator* strain DSMz 545 was purchased from DSMz (Braunschweig, Germany) and used throughout this study. This bacterial strain was selected for its capacity for PHA production, which has been widely studied (KUMAR et al., 2020; MOZUMDER et al., 2014). Culture stocks were generated from the lyophilized cells and stored at -80 °C in glycerol [20% (v/v)]. Initially, to improve the rate of glycerol consumption by *C. necator*, successive cultures were carried out in Petri dishes at gradually increasing glycerol concentrations (5–25 $g \cdot L^{-1}$). Besides glycerol, the medium was composed of ($g \cdot L^{-1}$): yeast extract 10, peptone 5, and agar 15. Inoculum for shake flask was prepared by transferring bacterial cells to a liquid medium containing ($g \cdot L^{-1}$): carbon source 20 (according to the experiments described as follows), yeast extract 10, and peptone 5. The maintenance medium was prepared on slant agar containing the described medium and 15 $g \cdot L^{-1}$ agar (GARCÍA et al., 2013).

The culture medium implemented to produce the biopolymer - Salt Mineral Medium (MSM) (CAVALHEIRO et al., 2009) was composed of the following elements ($g \cdot L^{-1}$): $(NH_4)_2SO_4$, 4; KH_2PO_4, 13.3; $MgSO_4$-$7H_2O$, 1.2; carbon source 20; yeast extract 1; citric acid 1.7. 10 $mL \cdot L^{-1}$ of trace element solution, consisting of ($g \cdot L^{-1}$): $FeSO_4$, 10; $ZnSO_4$, 2.25; $CuSO_4$, 1; $CaCl_2$, 2; $Na_2B_4O_7$, 0.23; $MnSO_4$, 0.5; $(NH_4)_6Mo_7O_{24}$, 0.1 and 10 $mL \cdot L^{-1}$ of HCl (35%). For PHA production, different carbon sources were tested: laboratory-grade glucose, laboratory-grade glycerol, waste glycerol (WG), and reducing sugars from the obtained hydrolysate of SBH. For each case, the final concentration was calculated based on 8 g/mol of C (considering that 20 g L^{-1} of glucose are equivalent to 8 g/mol of C). When used simultaneously, 50% of SBH hydrolysate (SBHH) and 50% of WG were added to the medium. Carbon sources and components susceptible to precipitation under sterilization conditions were sterilized separately (20 min - 120 °C – 1 atm). Prior to

inoculation of the medium, the pH between 6.0 – 7.0 was checked and, when necessary, adjusted with KOH solution. In order to avoid results' variability, the experiments were performed in triplicate. Standard deviation was calculated from the mean of the three values.

For the inoculum, the effect of different C:N ratios (1:1, 5:1 and 11:1), nitrogen source (yeast extract, ammonium sulfate, and urea), and laboratory-grade glucose on biomass production was studied. In these cases, the inoculated flask (10% v/v), with a total volume of 50 mL of the growth media, was added to 250 mL flasks and incubated in an orbital shaker (Excella E24 Incubator Shaker Series - New Brunswick Scientific) for 24 h at 30 °C with 100 rpm agitation. Batch fermentation for biopolymer synthesis was performed in 250 mL Erlenmeyer flasks: 5 mL of bacterial suspension, obtained in the exponential phase of inoculum culture (10% v/v) was added to 45 mL of MSM, with an initial pH between 6.0 and 7.0, and incubated on an orbital shaker at 30 °C and 150 rpm for 96 h. The kinetics of PHA and biomass production and substrate consumption were monitored periodically.

FIGURE 7 - Stages followed for the biotechnological production of PHB through the valorization of soybean hulls and residual glycerol



SOURCE: Authors (2022)

3.4.5. Analytical procedures

The elemental composition parameters of SBHH and fermented medium were determined prior to the use of HPLC and ion chromatograph, with the following conditions. The Metrohm CH-9101 ion analyzer was used to determine the SBH composition of ions. A Metrosep C3 250/4.0 column (250 x 4.0 mm ID; No. 5607002) was employed to quantify

cations, under the following conditions: eluent 5.0 mM HNO_3; flow rate: 1.0 $mL \cdot min^{-1}$; detector: CD; temperature 40 °C; injection volume: 20 µL. For anion analysis, a Metrosep-A Supp 5 250/4.0 column (250 x 4.0 mm ID; No. 7610789) was used, with the following analysis conditions: eluent 3.2 mM Na_2CO_3 + 1.0 mM $NaHCO_3$; flow rate: 0.7 $mL \cdot min^{-1}$; detector: suppressed CD; room temperature (25 °C); injection volume: 20 µL. Quantification of sugars, organic acids, glycerol, and determination of WG purity were performed in an Agilent 1260 Infinity HPLC equipment of the Analytical Chemistry Laboratory of Bioprocess and Engineering Department of Federal University of Paraná-UFPR. Analytical conditions were: Hi-Plex H column (300 x 7.7 mm) was used and operated at 60 °C, 0.005 MH_2SO_4 as the mobile phase at 0.6 $mL \cdot min^{-1}$ flow rate. Ajinomoto Brazil Ltda. Supplied WG, which was filtered through qualitative filter paper and, using a densimeter, the density was determined. The purity and elemental composition parameters of WG were established before use in the processes, using HPLC and ion chromatograph, under the conditions described above.

The 3,5-dinitrosalicylic acid (DNS) methodology was implemented for the determination of reducing sugars (MILLER, 1959) for both SBH aqueous extract and hydrolyzed SBH. Cells' dry weight was determined by adding 2 mL of culture medium in a previously weighed Eppendorf tube and after centrifugation at 6000 rpm, the pellet was washed with deionized water and taken to the drying oven (80 °C) for 12 h. After 1 hour in the desiccator, the material was weighed on an analytical balance and calculated. All analyses were done in triplicate.

The accumulated PHB content was estimated through gas chromatography (GC) analysis (EVANGELINE & SRIDHARAN, 2019), in which approximately 10 mg of lyophilized cells were subjected to methanolysis in the presence of methanol, 3% (v/v) sulfuric acid, and chloroform. The mixture was incubated at 100°C for 140 minutes (Thermoreaktor TR300 - Merck). After cooling, 1 mL of Na_2CO_3 solution was added. The organic layer was separated, and 1 mL of a solution was obtained. The resulting hydroxyacyl methyl esters were analyzed by GC (GC2010 Plus Capillary GC, Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector (FID), an autoinjector (AOC-20i), and SH-RTX ™-Wax capillary column (Shimadzu, 30 m, 0.32 mm ID, 0.25 µm). The injection volume was 1 µL and the split ratio 1:75. The carrier gas

(helium) was controlled at a linear speed of 45 cm/s. The injector and FID detector temperature was maintained at 240 °C and 250 °C, respectively. The column oven temperature was set to start at 100°C for 2 min, then increased to 280 °C at a rate of 40°C/min and maintained for 4 min. The chromatogram data were analyzed using GCsolution Workstation Version 2.32 software (Shimadzu, LabSolutions). The internal standard was benzoic acid, and the external standard was poly[(R)-3-hydroxybutyric acid (Sigma-Aldrich). For identification and quantification, the standards were also subjected to methanolysis as described above, and the methyl esters were analyzed.

The extraction and purification of the biopolymer were performed using solvents as described below. Six percent sodium hypochlorite and chloroform (1:1) were added to the freeze-dried biomass, maintaining a temperature of 37 °C at 1500 rpm for 2 h. Subsequently, the material was centrifuged (3000 rpm, 10 min) and the top layer was removed. The solution obtained was filtered to remove cellular debris and poured into a tube of known mass. By adding methanol (1:9) the biopolymer was precipitated and separated by centrifugation (3000 rpm, 10 min), and the methanol-chloroform mixture was decanted. The precipitated polymer was again dissolved in chloroform and precipitated with methanol and then dried to obtain a highly purified polymer (ANNAMALAI and SIVAKUMAR, 2016).

3.4.6. Results and discussion

3.4.6.1. Characterization of soybean hull and waste glycerol

The SBH was characterized to identify the structure and composition of the material to determine the best pretreatment strategy to be implemented. The selection of the most suitable pretreatment is of most importance because it allows proper exposition of cellulose structure before enzymatic action, thus obtaining the highest concentration of fermentable sugars.

The composition of the analyzed SBH was determined as follows. The amount of cellulose 31.0 ± 1.6%, hemicellulose 11.8 ± 1.3%, and lignin 6.18 ± 0.8% are close to values reported by other authors related to SBH analysis (QING et al., 2017; KARP et al., 2020). The low lignin content showed that mild pretreatment conditions could be employed

without reducing the cellulose-rich fraction loss and favoring higher enzymatic hydrolysis conversion to fermentable sugars (KIM et al., 2016; XU et al., 2016). Other components such as protein (10.5%), extractives (9.3 ± 1.0%), ash (3.6 ± 0.03%), moisture (6 ± 0.10%), and others (≈21.62%) mostly represented by pectin, lipids, organic acids, were determined.

The determination of soluble sugars showed a concentration of 6.02 $mg \cdot g^{-1}$ SBH $_{(bs)}$ in the aqueous extract, while for the hydrolysate, the concentration reached 115.9 $g \cdot L^{-1}$. Thus, the results show an increase of more than 100% in the availability of fermentable sugars for application in subsequent processes. The composition of ions present in SBHH was compared with the composition of the aqueous extract obtained directly from the non-treated SBH (*in natura)*, (TABLE 4).

TABLE 4 - QUANTIFICATION OF IONS IN THE AQUEOUS EXTRACT, SBHH, AND IN THE WG

Ions	Concentration ($mg \cdot L^{-1}$)		
	Aqueous extract SBH	**SBH hydrolysate**	**Waste glycerol**
Soluble anions			
Cl^-	34	135	12,150
F^-	2	278	nd
SO_4^{-2}	9	84	170
PO_4^{-3}	6	nd	nd
Br	nd	nd	nd
NO_x^-	nd	nd	nd
Soluble Cations			
Na^+	2	630	10,350
Ca^{+2}	6	110	nd
Mg^{+2}	6	60	nd
K^+	58	nd	nd
NH_4^+	nd	nd	nd

Source: Authors (2022) *nd= not determined.

For both, anions and cations, in all cases (except for phosphate and potassium, respectively) a significant increase in the concentration of each analyzed component was observed. In addition, WG density was 1.12 $g \cdot mL^{-1}$, the purity corresponds to 39±1%, ions content of WG was also determined where the high amount of the anion Cl^- (≈12 $g \cdot L^{-1}$)

and the cation Na^+ (≈10 $g \cdot L^{-1}$) stands out. These elements may be present in high concentrations given that they are used as catalysts in production and downstream processes where glycerol is generated as waste (GANESAN et al., 2021). Taking these parameters into account the formulation of the culture medium was possible for the subsequent fermentative processes.

3.4.6.2. Alkaline pretreatment of soybean hull

Alkaline pretreatment has been considered a viable method for the bioconversion of lignocellulosic biomass (KIM et al., 2016) and is recommended when the substrate has a low lignin content (SINGH et al., 2015). Moreover, it reduces the formation of inhibitory compounds, and it is useful even when lower temperatures are implemented (JÖNSSON and MARTÍN, 2016). Thus, considering that the structural compounds of the SBH used in this research correspond to 6.18% of lignin, a mild alkaline pretreatment was selected. Qing et al., (2017) analyzed acid and alkaline solutions to compare their performance during pretreatment and subsequent enzymatic hydrolysis. They found that alkaline pretreatment, at 100 °C, 120 min, with a solid to liquid ratio set at 1:20 and NaOH 1% (w/w) was more effective in delignification and improved the enzymatic digestibility of pretreated substrate, when compared with acid pretreatment.

After the alkaline pretreatment of SBH, a mass loss of 48% (w/w) was quantified, which reflects the structural effect caused by sodium hydroxide. NaOH promotes the opening of hydrogen bonds between cellulose and hemicellulose, and between the latter and lignin stimulates the cleavage of ester bonds (WU et al., 2022). In consequence, lignin, small portions of hemicellulose, and other components such as proteins, extractives, and ash were removed. Thus, the hydrolysis of other SBH constituents could also occur. In addition, during the procedures, was observed a loss of the pretreated biomass when the material was transferred between the flasks or when it was filtered since it remained stuck, and this material was not directly quantified. Saha and Cotta, (2007) quantified after an alkaline pretreatment for the conversion of rice hull cellulose and hemicellulose to simple sugars, a mass loss of 53% (w/w). Karp et al, (2020) described similar results in SHB, where after an alkaline pretreatment with a substrate

concentration of 10% (w/v) of solids, NaOH 2%, at 121 °C for 1 h, a mass loss of 51.3% was quantified. Comparatively, in the present work, the NaOH concentration was lower (1%) becoming the main difference that could have influenced the slightly lower mass loss, 48% compared to 51% when NaOH was used at a concentration of 2%.

Biomass swelling of biomass and surface alteration was observed after the alkaline pretreatment (data not shown), which was also previously reported by Camiscia et al., (2018). The swelling of lignocellulosic biomass represents the degradation of the ester and glycosidic chain and suggests an increase in the internal surface area, and solvation effect on the structural composition of the material (BASTOS et al., 2021; JIN et al., 2013). The swelling effect promotes a larger contact surface for higher efficiency of the enzymatic action. Therefore, with the subsequent enzymatic treatment of the resultant solid material, which is the cellulose-rich fraction, monomeric fractions are obtained for further utilization in fermentation processes (BEHERA et al., 2014; QING et al., 2017).

3.4.6.3. Optimization of enzymatic hydrolysis of soybean hull

3.4.6.3.1. Batch enzymatic hydrolysis process

CCD tool of RSM methodology was used to analyze the effects and the interactions between the selected factors that influence the enzymatic hydrolysis of SBH as is shown in Table 5 and represented in Fig 8. R^2 was 0.7941, indicating that the model could explain 79.4% of the response variability. This model is presented below: $z = -16.539586971434 + 6.9365911630221 \times x - 0.065286327941407 \times x^2 + 0.45024633546848 \times y - 0.0041949471036503 \times y^2 - 0.025583940331375 \times x \times y - 0.086229983660131 \times 42 \times x + 0.02828167597832 \times 42 \times y + 21.6274308$. According to the results, linear substrate loading and linear interaction between enzyme cocktail loading and time were significantly positive factors in the release of RS from SBH as showed in the Pareto chart (Fig 8a), while the other variables and their possible interactions do not represent statistical significance. Important concentrations of RS were observed when the interaction between processing time and enzymatic cocktail loading are considered as showed in the plotted response surface (Fig 8b), showing an optimum region near the established range. During the experimental processes it was observed that once in the reaction medium, the substrate (SBH) presented a swollen

appearance, and with the passage of time and thanks to the enzymatic action, there was a reduction in the size of the material until a relatively homogeneous mixture was obtained in cases where the substrate concentration was around 38.5%. When the substrate concentration was higher, this effect was not achieved, and the reaction medium was not completely homogenized, due to the low agitation rate during this stage (100 rpm). These observations may be related to the results obtained in the RSM for the interaction between the concentration of the substrate and the enzymatic cocktail (Fig 8c), and between substrate concentration and processing time (Fig 8d), in which a possible inhibition of the substrate on the enzymatic action can be suggested, which would require additional studies to be confirmed.

The highest concentration of reducing sugars (115,9 $g \cdot L^{-1}$), was reached by adding 62% (w/v) of pretreated SBH (d.m.) after 42h of process and using the enzyme cocktail composed of B1 enzyme with 11.52 mg protein$\cdot g^{-1}$ dry substrate, B1-XylA enzyme, with 2.88 mg protein$\cdot g^{-1}$ dry substrate, and F10 β-glucosidase enzyme, with 57.59 U/g of dry substrate (standard run 12). It should be noted that since it is a non-commercial enzymatic cocktail resulting from the drying of a raw fermentation broth, residual sugars (not quantified) remain and that they could possibly interfere with the value mentioned above. In this case the glucose yield was 0.12 g/g_{SBH}, which corresponded to 18% cellulose conversion. Meanwhile, in the central point (where the substrate loading was 38.5%), using the same concentration of the enzyme cocktail and in the same period, an average glucose yield was 0.13 g/g_{SBH} and cellulose conversion corresponded to 21%, obtaining an average concentration of reducing sugars of 81 $g \cdot L^{-1}$. Considering these results, the next stage of scaling, was developed under these processing conditions.

Chemical pretreatment followed by enzymatic hydrolysis of SBH has been previously tested by different authors to recover the greatest amount of reducing sugars from lignocellulosic biomass. Karp et al, (2020) analyzed the application of the enzyme combination of B1 host and B1-XylA to hydrolyze SBH, the same enzymes used in the present work. They described that after an alkaline pretreatment with NaOH followed by enzymatic hydrolysis with this preparation, the SBH was efficiently saccharified, yielding a concentration of reducing sugars between 92–96 $g \cdot L^{-1}$; the glucose recovery

corresponded to 81 g·L^{-1}, 74 g/100 g; glucose and reducing sugar yields from initial substrate mass were 36% and 42–47%, respectively. On the other hand, Dall Cortivo et al. (2020) carried out the pretreatment of SBH by dilute acid hydrolysis using 1 % (mass concentration) of sulfuric acid and heat treatment for 40 min in an autoclave (121 °C), using a solid-liquid ratio of 1:10. The solid fraction was subsequentially enzymatically hydrolyzed by the commercial enzyme Celluclast® 1.5 L (Novozymes, Brazil) and the resulted hydrolysate had a final concentration of 25.7 ± 0.7 g·L^{-1} of glucose.

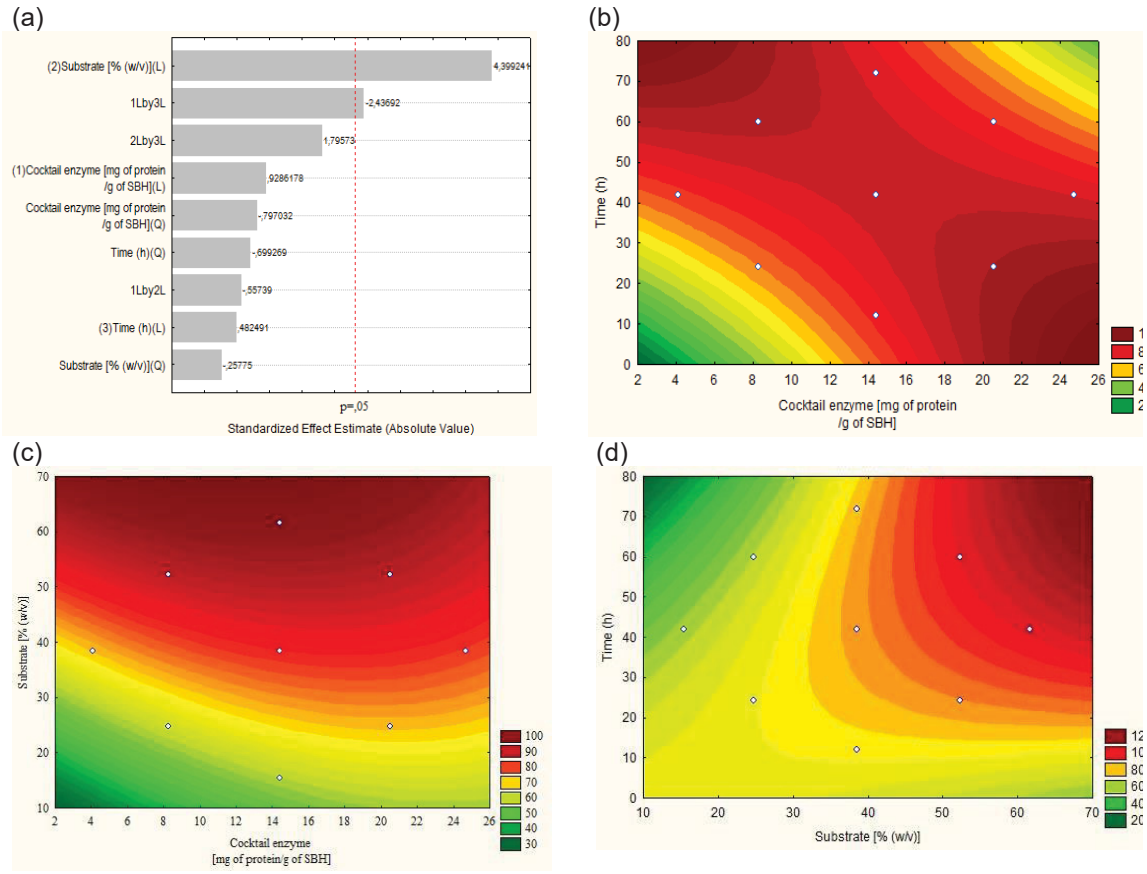
TABLE 5 - CENTRAL COMPOSITE DESIGN MATRIX FOR THE SBH ENZYMATIC HYDROLYSIS OPTIMIZATION

Standard Run	Enzyme cocktail			Substrate (d.m.) [% (w/v)]	Time (h)	Reducing sugars (g·L^{-1})	Yield (g glucose /g SBH)
	B1 (mg of protein/g of substrate)	β-glucosidase (U/g of substrate)	Xylanase A (mg of protein/ g of substrate)				
1	6.62	33.10	1.66	24.5	24.2	60.7	0.15
2	6.62	33.10	1.66	24.5	59.8	64.9	0.16
3	6.62	33.10	1.66	52.5	24.2	61.9	0.07
4	6.62	33.10	1.66	52.5	59.8	99.3	0.12
5	16.42	82.08	4.10	24.5	24.2	91.9	0.23
6	16.42	82.08	4.10	24.5	59.8	63.9	0.16
7	16.42	82.08	4.10	52.5	24.2	89.9	0.11
8	16.42	82.08	4.10	52.5	59.8	84.2	0.10
9	3.28	16.40	0.82	38.5	42.0	76.0	0.12
10	19.76	98.78	4.94	38.5	42.0	72.7	0.12
11	11.52	57.59	2.88	15.0	42.0	42.3	0.17
12	11.52	57.59	2.88	62.0	42.0	115.9	0.12
13	11.52	57.59	2.88	38.5	12.0	71.8	0.12
14	11.52	57.59	2.88	38.5	72.0	78.6	0.13
15 (C)	11.52	57.59	2.88	38.5	42.0	86.6	0.14
16 (C)	11.52	57.59	2.88	38.5	42.0	82.7	0.13
17 (C)	11.52	57.59	2.88	38.5	42.0	79.4	0.13
18 (C)	11.52	57.59	2.88	38.5	42.0	79.2	0.13

Source: Authors (2022).

In other experiments, YOO et al. (2011) performed an alkaline pretreatment of SBH (10%, w/w) in which 1% (w/w) sodium hydroxide solution was added and the mixture was processed at 121 °C for 30 min and 1 atm. For the subsequent enzymatic saccharification, a combination of commercial enzymes (cellulase, Celluclast 1.5L; β-glucosidase, Novozyme 188, and Viscozyme® L, a cell wall degrading enzyme complex) was implemented and incubated at 50 °C for 72 h in 0.05 M sodium acetate buffer at pH 5. As a result, after enzymatic hydrolysis of alkaline pretreated SBH, glucose yield was 0.36 g/g, which corresponded to 93.3% cellulose conversion. In the present work, where a non-commercial enzyme cocktail was implemented, at the same time of processing (72h) glucose yield was ≈0.13 g/g, which corresponded to ≈20% cellulose conversion.

FIGURE 8 - Influence of enzyme cocktail loading, substrate loading, and process time on saccharification of SBH. Response surface plot for optimal concentration for reducing sugars ($g \cdot L^{-1}$) showing interactive effects of: a) Pareto chart of standardized effects on reducing sugars production; (p-value = 5%); b) cocktail enzyme loading [mg of protein /g of substrate] *vs.* time (h); c) cocktail enzyme loading [mg of protein /g of substrate] *vs.* substrate [%(w/v)]; d) substrate [%(w/v)] *vs.* time (h).



SOURCE: Authors (2022)

3.4.6.3.2. Fed-batch enzymatic hydrolysis process

After validation of the results of the optimization of enzymatic hydrolysis in a batch process, a fed-batch strategy was implemented, as described in the previous section, in a 1L stirred enzymatic bioreactor. Hence, a considerable increment in reducing sugars concentration (37.6%) was obtained going from 115.9 $g \cdot L^{-1}$ to 185.7 $g \cdot L^{-1}$. As well, glucose yield was 0.38 g/g_{SBH}, which corresponded to 60% cellulose conversion. In addition to the fed-batch approach, one of the main factors, which improved the efficiency of SBH enzymatic hydrolysis was the stirring with the help of a propeller that contributed to a better homogenization of the material and better contact of the enzymes with the substrate. Hernández-Beltrán and Hernández-Escoto, (2018) developed the enzymatic hydrolysis of sorghum straw biomass (pretreated with an alkaline-oxidative medium) at high-solids loading (20% $w \cdot v^{-1}$) through fed-batch operation, using a stirred tank bioreactor (150 rpm), and applying a commercial enzyme complex (Cellic® CTec2, Novozymes®). The produced hydrolysate after 10 h, reached a reducing sugars concentration of 126 $g \cdot L^{-1}$.

The higher loading of the SBH substrate used in this study, 38.5%, with lower enzymes' concentrations, in the same system and volume of bioreactor would promote lower process's costs, which is certainly the main bottleneck of enzymatic processes. Mukasekuru et al., (2020) implemented a high-solids fed-batch enzymatic saccharification method, in alkaline-catalyzed atmospheric glycerol organosolv pretreated sugarcane bagasse. They began with 10% (w/v) of solids content and 6%, 6%, and 8% of substrates were fed at 6, 12, and 24h to achieve 30% (w/v) of the total solids content. The saccharification process mediated by the cellulase enzyme was carried out at 50 °C and 180 rpm. In this study, they used additives (Tween 80, tea saponin, BSA) and accessory enzymes (xylanase and AA9 enzyme) to facilitate cellulase activity. As a result, the saccharification process released 180 $g \cdot L^{-1}$ of fermentable sugars with 70 % glucose yield after 72 h. On the other hand, Gonzalez-Rios et al., (2021) studied the quick fed-batch saccharification strategy of pretreated wheat straw (by autohydrolysis followed by a steam explosion stage) at high solid loadings, developed to achieve high sugar concentrations. After optimization of variables (pretreatment time, number of feedings, and the time

between them), total sugar concentrations after 60 h with 30% (w/w) of solids were 163.5 $g \cdot L^{-1}$, 42% higher than its batch saccharification counterpart.

Other authors have previously tested the performance of high-solids fed-batch enzymatic saccharification, in different lignocellulosic matrices but we are not aware of references that applied this method in SBH. In the previous examples, the authors used various factors independently, such as optimization tools, additives to enhance the enzymatic activity, and stirred tank bioreactors applying commercial enzyme complexes during the process, achieving similar results for RS to those obtained in the present work. Thus, the optimization of the enzymatic hydrolysis of SBH was relevant to achieving a high release of sugars to be further used as substrate in PHB production. This operational alternative allowed the saccharification process to be made viable, reducing operating costs, with possible process scale-up.

3.4.6.4. PHA production from alternative substrates

3.4.6.4.1. Inoculum development and preparation

C. necator strain DSMz 545 was first cultivated using the medium described for inoculum development using laboratory-grade glucose, where the C:N ratio was tested (1:1, 5:1, and 11:1). In this case, the concentration of yeast extract and peptone was fixed in the inoculum medium, varying glucose concentrations between 5 and 50 $g \cdot L^{-1}$. The highest biomass concentration, 5.6±0.3 $g \cdot L^{-1}$ was achieved at a C:N ratio of 5:1, corresponding to 20 $g \cdot L^{-1}$ of glucose, with a consumption of 42% of the carbon source. At a C:N ratio of 1:1, corresponding to 5 $g \cdot L^{-1,}$ and a C:N ratio of 11:1, corresponding to 50 $g \cdot L^{-1}$ were obtained 4.9±0.4 $g \cdot L^{-1}$ and 4.5±0.2 $g \cdot L^{-1}$ of biomass respectively. Thus, in the following step the 5:1 ratio was maintained. Annamalai and Sivakumar. (2016) studied the effect of different C:N ratios (20:1–20:5) on cell growth of mutant strain *R. eutropha* NCIMB 11599, founding that at C:N ratio of 20:5 the cell growth was the maximum obtained reaching 6.23 $g \cdot L^{-1}$, a value close to that of the cell growth obtained in this work.

Then, the effect of three different nitrogen sources: yeast extract, ammonium sulfate, and urea in the biomass development was studied, using 20 $g \cdot L^{-1}$ of glucose. The best results were obtained with yeast extract that was added to the culture medium

allowing the production of 7.6 ± 0.1 $g \cdot L^{-1}$ of biomass, with 100% consumption of the carbon source. Using ammonium sulfate 5.4±0.3 $g \cdot L^{-1}$ of biomass were reached, while 4.6±0.4 $g \cdot L^{-1}$ were produced using urea. In both cases, lower consumption of the carbon source was observed with 63% and 47%, respectively. In the same study, Annamalai and Sivakumar (2016) analyzed the effect of both inorganic and organic nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, peptone, and tryptone) on cell growth. They found that the ammonium sulfate permits a higher amount of biomass corresponding to 6.14 $g \cdot L^{-1}$, and using yeast extract, biomass production reached 5.52 $g \cdot L^{-1}$, contrarily to what was found in this study, where yeast extract was shown to be the best source of nitrogen for the microorganism.

On the other hand, Cavalheiro et al. (2012) evaluated the mechanisms of cell membrane adaptation during the various stages involved in the production of PHA when different carbon sources, glucose (30 $g \cdot L^{-1}$) or waste glycerol (30 $g \cdot L^{-1}$), were used in the seeding medium and in the basal mineral medium. They used a stock culture of *C. necator* to inoculate the pre-culture, used as inoculum for the fed-batch experiments. They found that the degree of saturation of the fatty acids of the phospholipids in the cell membranes of *C. necator* varied during the time course of the fermentation, and cell membranes adapted to the environment by increasing their fluidity, which facilitates substrate uptake and cell division.

As a tentative to better adapt *C. necator* strain DSMz 545 cells during inoculum development, for subsequent PHB production, the influence in biomass generation of different carbon sources was studied. Laboratory-grade glucose and laboratory-grade glycerol were used as the control/reference carbon sources. SBHH, WG, and their combinations were evaluated as presented in TABLE 5. Laboratory grade glucose provided the best biomass production (10.3±0.2 $g \cdot L^{-1}$) followed by SBHH (9.0±0.4 $g \cdot L^{-1}$), showing consumptions of 81% and 91%, respectively. The combination of SBHH and WG was promising with a biomass production of 6.7 $g \cdot L^{-1}$ and total consumption of 48%. In this case, the microorganism showed a preference for SBHH sugars consumption (80%) and a lower consumption rate of WG, for which, it was found consumption of only 21% after 24 h of culture (data not shown). Although the microorganism metabolized glycerol (both commercial and waste), biomass production was only 4.5 $g \cdot L^{-1}$ and the consumption

of this carbon source reached 23%. SBHH showed to be a very good alternative for inoculum preparation, which may reduce the lag phase of the microorganism in the following step of biopolymer production.

TABLE 5 - *C. necator* STRAIN DSMz 545 WITH DIFFERENT CARBON SOURCES (INOCULUM AND PRODUCTION MEDIA)

Carbon source	Inoculum		PHB production		
	Biomass ($g \cdot L^{-1}$)	Carbon source consumption (%)	Biomass ($g \cdot L^{-1}$)	Carbon source consumption (%)	PHB (%)
SBHH + WG	6.7 ± 0.4	48	8.7 ± 0.1	50	39.0 ± 0.8
SBHH	9.0 ± 0.4	91	7.8 ± 0.2	77	31.8 ± 0.2
WG	4.5 ± 0.2	17	6.9 ± 0.2	44	25.4 ± 1.6
LGG	10.3 ± 0.2	81	9.2 ± 0.2	90	39.4 ± 1.1

SBHH: soybean hull hydrolysate; WG: waste glycerol; LGG: Laboratory grade glucose

SOURCE: Authors (2022).

3.4.6.4.2. PHB production using alternative substrate

It has been reported in the literature the use of different carbon sources (e.g.) for polyhydroxyalkanoates production with good perspectives for implementation and improvement of yields (SIROHI et al., 2020). Specifically, the use of hydrolysates from different lignocellulosic feedstocks (e.g., rice bran and straw, wheat bran, sunflower hull, sugar cane, among others), have been implemented, obtaining results of accumulation between 20 and 80% of PHA using non-genetically modified strains (AHN et al., 2015; ANNAMALAI AND SIVAKUMAR, 2016; OH et al., 2015; SARATALE and OH, 2015; SILVA et al., 2004). To the best of our knowledge, this is the first time that SBHH is used as a carbon source to produce polyhydroxyalkanoates. Initially, to verify the influence of different carbon sources on *C. necator* DSMz 545 in biomass production, batch fermentations in MSM medium were developed during 96h, testing laboratory-grade glucose, SBHH, and WG as carbon sources in one-factor-at-a-time essays, and the mixture of SBHH and WG (TABLE 5). Then, all samples were analyzed, after performing the acid methanolysis, by gas chromatography. It was observed that the retention time of the obtained methyl esters, both from the commercial PHB standard and the samples

obtained after fermentation, coincided, allowing the conclusion that the generated biopolymer belongs to the polyhydroxybutyrate (PHB) type.

Biomass production reached 9.2±0.1 $g \cdot L^{-1}$ with the use of laboratory-grade glucose, as well as the best accumulation percentage of 39.4, which was then used as a reference value. When Sen et al. (2019) implemented the same strain, in 400 mL MSM culture medium with glucose as carbon source (10 $g \cdot L^{-1}$) in batch fermentation at 30±1 °C, 72h, 250±10 rpm, achieved 2.81 $g \cdot L^{-1}$ of biomass and PHB accumulation of 27%. For SBHH used individually, although a high consumption was observed (77%), biomass concentration reached 7.8±0.2 $g \cdot L^{-1}$ and was found to lead to an accumulation of 31.8%. Silva et al. (2004) tested sugarcane bagasse hydrolysate (after adsorption treatment with 20% active charcoal for the elimination of toxic compounds) as a carbon source for *Burkholderia sacchari* for PHB production. The microorganism was cultured in batch fermentation using MSM medium (50 mL, 150 rpm, 30 °C), reaching a final cell dry weight of 6.13 $g \cdot L^{-1}$ and 23.33% PHB accumulation. Superior results were attained by Annamalai and Sivakumar, (2016), using the mutant strain *C. necator* NCIMB 11599 which reached 25 $g \cdot L^{-1}$ and 63% of PHB accumulation using an alkaline pretreated wheat bran hydrolysate (incubated at 150 rpm for 72 h at 30 °C). In this case, although the implemented strain is genetically modified, it allows verifying that the hydrolysate is feasible to be used by microorganisms for later use in the production of biopolymers.

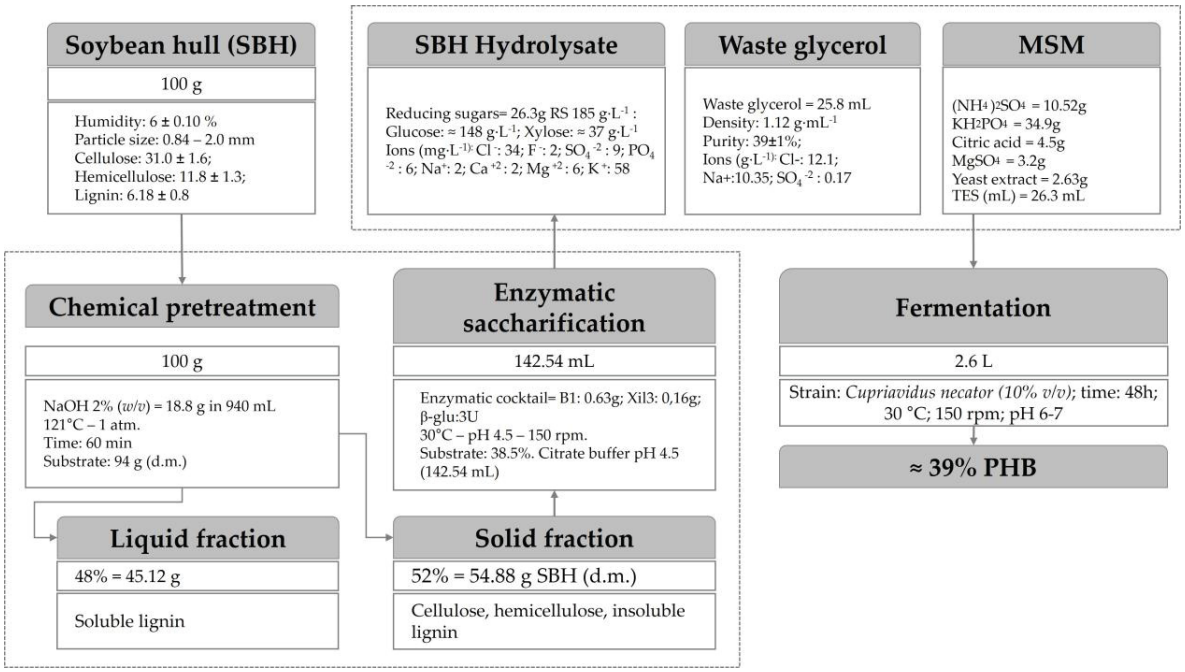
The low consumption of WG (44%), when used independently, was observed, which directly influenced a lower biomass growth (6.9±0.2 $g \cdot L^{-1}$) and lower accumulation of the biopolymer (25.4%) when compared with the other results. Similarly, Gahlawat and Soni, (2017) have also reported the use of WG as the sole carbon source (20 $g \cdot L^{-1}$) for PHB production by *C. necator* DSM 545 in batch fermentation, reaching 5.7 $g \cdot L^{-1}$ of biomass and 3.42 $g \cdot L^{-1}$ with. As pointed out by these authors, the content of contaminants mainly methanol and sodium ions, affects the growth rate of the microorganism during the fermentation. In this study, the WG used has a considerable content of Na^+ and Cl^- ions which could have an adverse impact on the growth rate of the strain and PHB production (MOTHES et al., 2007).

Finally, when SBHH and WG were used simultaneously (4 g/mol C each one), the consumption of the total carbon source was 50% with a biomass production of 8.7±0.1

$g \cdot L^{-1}$, which was higher than the value achieved when SBHH or WG were used independently. In this case, when this mixture of carbon sources was used for inoculum development, biomass generation (6.7±0.4) was lower than that observed during the biopolymer production stage (8.7±0.1). According to Wang et al. (2019) glucose is a highly preferred carbon source for some microorganisms, and that also allows it to support relevant growth rates. For the PHB production process, this variety of carbon sources can allow the microorganism to initially consume the most accessible carbon source during the lag and exponential phase and once this is exhausted, the cell will adapt to the consumption of the other available sources to continue with its development (RODRÍGUEZ-CONTRERAS et al., 2015).

Finally, and meeting expectations, this combination of carbon sources, enabled the generation of PHAs content of 39%. Fig 9 shows an example of mass balance that exemplifies the process presented in this work. This is a very good result when a large-scale PHB production is projected, bringing important alternatives that allow overcoming the problem of high process costs of biopolymers' production (BHATIA et al., 2021). García et al. (2013) developed a study exploring the production of PHAs by *C. necator* DSM 545 in rapeseed meal hydrolysates with MSM medium using a free amino nitrogen solution (400 $mg \cdot L^{-1}$) and crude glycerol (25 $g \cdot L^{-1}$). Bach fermentation was carried out at 180 rpm, 30 °C, pH 6.7 – 6.9, for 57h reaching a total cell dry weight of 13.7 $g \cdot L^{-1}$ with total consumption of glycerol and 46.2% of PHB accumulation. The authors concluded that the hydrolysate, rich in amino acids acted as a nutrient supplement to achieve desirable microbial growth and PHA production.

FIGURE 9 - Mass balance of the production process of PHB by *C. necator* DSMz 545 in MSM medium containing SBHH and WG as carbon sources.

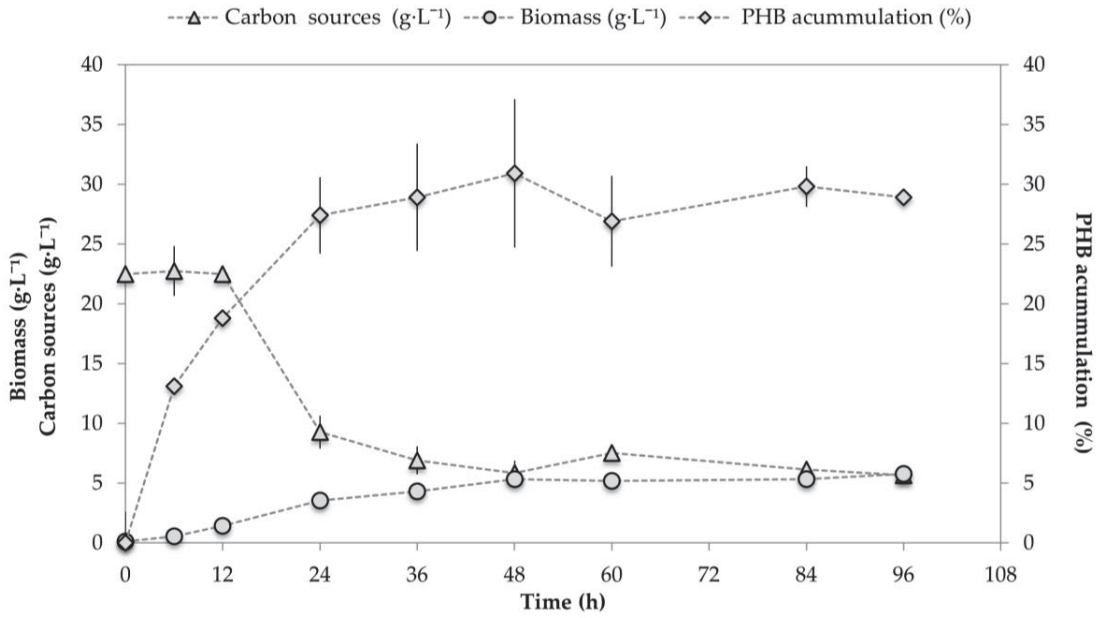


SOURCE: Authors (2022)

3.4.6.4.3. PHB production kinetics

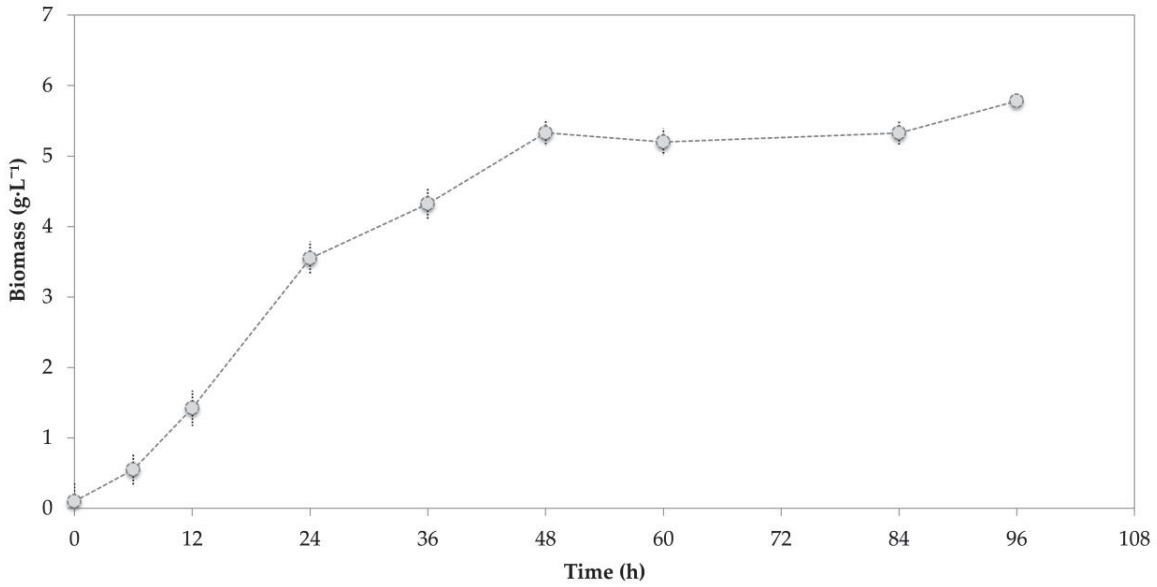
Considering the results obtained in the previous phase, the kinetics of PHB production using simultaneously SBHH and WG as carbon sources were conducted as shown in Fig 10. It is observed that the cells of *C. necator* were in the lag phase during the initial 6h of fermentation since, although they showed slow cell growth and the consumption of carbon sources was null, their metabolism is adapting to the new conditions and is active (Fig 11). The consumption of available carbon sources only started after 12 hours of the process. Then, it is observed that up to 48h of fermentation the microorganism showed exponential growth until it reached 5.3 ± 0.23 $g \cdot L^{-1}$ of biomass, with productivity of 0.110 $g \cdot L^{-1} \cdot h^{-1}$ to then enter the stationary phase.

FIGURE 10 - Kinetics of PHB production by *C. necator* DSMz 545 in MSM medium containing SBHH and WG as carbon sources, performed in 250 mL flask.



SOURCE: Authors (2022)

FIGURE 11 - Kinetics of biomass production by *C. necator* DSMz 545 in MSM medium containing SBHH and WG as carbon sources, performed in 250 mL flask.

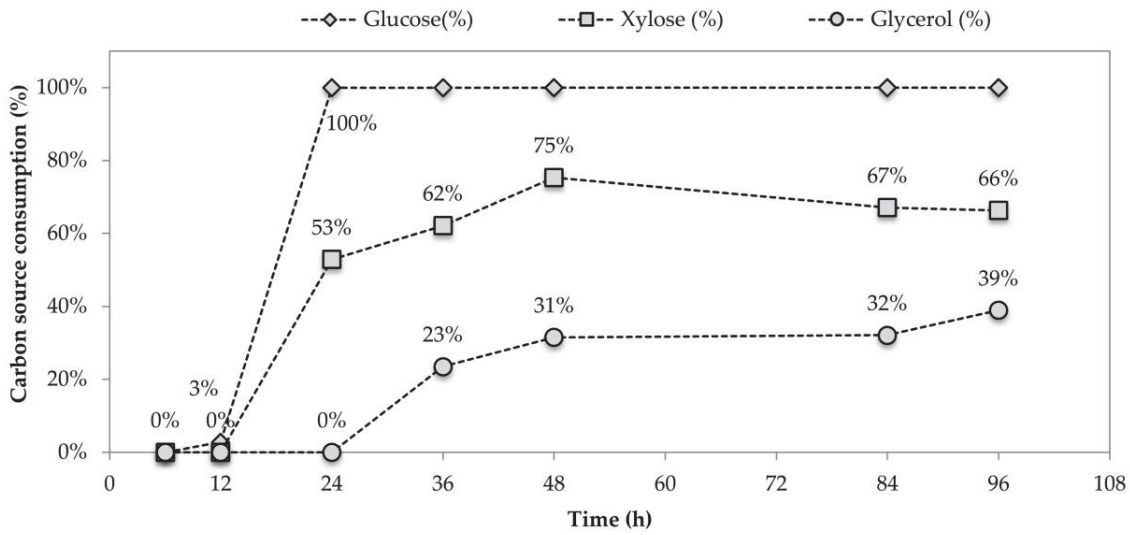


SOURCE: Authors (2022)

The consumption profile for each SBHH and WG were followed (Fig 12). For SBHH, glucose and xylose were the main sugars, which were metabolized by the microorganism. Glucose was completely consumed during the first 24h of the process, during which time

the microorganism simultaneously used 53% of the xylose, of which it had a total consumption of 66% at the end of the process. Since the alkaline pretreatment of SBH preserves the hemicelluloses and promotes the generation of pentoses, the fact that the microorganism implemented in the process has the capacity to metabolize these sugars represents a great advantage, because in this case a greater use of this low-cost raw material is obtained. Previously, Poomipuk et al 2014 also described the consumption of this pentose by another strain of Cupriavidus sp. KKU38. In this case, the hydrolysate was produced from cassava starch reaching a PHA concentration of 46%.

FIGURE 12 - Kinetics of SBHH and WG consumption by *C. necator* DSMz 545 in MSM medium, performed in 250 mL flask.



SOURCE: Authors (2022)

In Fig 12 is showed that *C. necator* started the consumption of WG after 24h (coinciding with the total depletion of the glucose source), showing a total consumption of 39% after 96 h. Maximum carbon sources consumption reached after 36 h (72%) that remained almost stabilized until the end of the fermentation. This behavior indicates the preference of *C. necator* toward glucose compared to xylose and WG. This fact shows the need to implement optimization methods or fed-batch culture strategies, where a great imbalance of C:N would be created to favor PHB production as previously demonstrated by Kim et al. (2005). In their study, *C. necator* ATCC 17699 was grown in flask culture to produce PHA using fructose and γ-butyrolactone as carbon sources. Initially, they

established that in batch fermentation, when fructose (20 $g \cdot L^{-1}$) was used as the sole carbon source, it was reached 4.95 $g \cdot L^{-1}$ and 53.7% of biomass and PHA accumulation respectively. Then, to improve those results, they implemented a fed-batch culture for 44 h, where cells were grown by feeding fructose using the DO-stat method. During the polymer accumulation phase, the feeding solution was changed to a mixture of fructose and γ-butyrolactone and nitrogen limitation was applied. Under these culture conditions, the maximum biomass and PHA accumulation were 48.5 $g \cdot L^{-1}$ and 50.2% respectively, reaching maximum productivity of 0.55 $g \cdot L^{-1} \cdot h^{-1}$.

Regarding the PHB accumulation (Fig 10), it was found that the production of the compound started in the exponential phase and continue until the beginning of the stationary phase. In the first 6 h of the process, 13% was accumulated with a maximum accumulation of 31% after 48 h and was constant until the end of the process. Sen et al. (2019) observed similar results using molasses pretreated with hydrothermal acid as a carbon source for *C. necator* (Batch culture, work volume 400 mL, 30 °C, 250 rpm), where during the first 24 hours of fermentation a generation of approximately 10% of PHB was quantified, to later complete after 60h a maximum production of 27.3% of PHB. Although the data obtained are slightly low, they are still within the ranges reported in the literature for native strains on a laboratory scale in batch culture, for which fermentation optimization processes have not been applied (SARMIENTO-VÁSQUEZ et al., 2022 *unpublished article*). On the other hand, the low agitation during the fermentation process (150 rpm) reduces the interactions between the cell and the substrate, in addition to affecting the ability of cell membranes to adapt to the conditions of the medium, reducing the absorption capacity of nutrients from the medium and consequent cell division, as it was observed (CAVALHEIRO et al., 2009; GARCÍA et al., 2013). Furthermore, some factors such as the presence of possible compounds detrimental to the growth of microorganisms both in the hydrolysate and in the glycerol (which were not quantified) to a certain extent, affect the performance of the microorganism during the PHB accumulation stage.

3.4.7. Conclusions

The present study demonstrated for the first time that the agro-industrial residues soybean hull hydrolysate and waste glycerol are alternative carbon sources for the biotechnological production of polyhydroxyalkanoates, molecules of medium-high added value. The efficacy of alkaline pretreatment for the hydrolysis of soybean hull biomass allowed the removal of lignin and increases the subsequent enzyme accessibility. In order to reduce the future cost associated with the enzymatic saccharification of the SBH biomass, a crude enzyme cocktail was effectively used instead of a purified commercial preparation, thus improving the economic feasibility of the process. For the saccharification stage, the operational conditions were optimized by implementing the response surface methodology (RSM), through the central composite design (CCD) tool. Then, the *C. necator* DSMz 545 strain was able to metabolize the sugars (hexoses and pentoses) present in the obtained hydrolysate and waste glycerol, accumulating 39% of polyhydroxybutyrate within 96h at batch culture.

Highlights

- Fermentable sugars can be generated by chemical-enzymatic pretreatment of lignocellulose residues as renewable carbon resources.
- The production of PHB from soybean hulls and glycerol residues as co-substrates is a sustainable and economical approach.
- The biopolymer was identified as poly(3-hydroxybutyrate).
- Production by *C. necator* yielded 39% PHB accumulation from residual glycerol and chemical-enzymatically pretreated soybean hull hydrolysate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Zulma Sarmiento-Vásquez: Conceptualization, writing - review & editing; Luciana Porto de Souza Vandenberghe: Conceptualization, writing - review & editing; Susan Grace Karp: enzymatic pretreatment of SBH methodology; Carlos Ricardo Soccol: Funding acquisition.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research scholarship. The authors thank professors Arkady Sinitsyn, Alexandra Rozhkova, Ivan Zorov and Olga Sinitsyna for donating the cocktail enzyme samples.

3.5. References

Ahn, J., Jho, E. H., & Nam, K. (2015). Effect of C/N ratio on polyhydroxyalkanoates (PHA) accumulation by Cupriavidus necator and its implication on the use of rice straw hydrolysates. *Environmental Engineering Research*, *20*(3), 246–253. https://doi.org/10.4491/eer.2015.055

Amaro Bittencourt, G., Porto de Souza Vandenberghe, L., Valladares-Diestra, K., Wedderhoff Herrmann, L., Fátima Murawski de Mello, A., Sarmiento Vásquez, Z., Grace Karp, S., & Ricardo Soccol, C. (2021). Soybean hulls as carbohydrate feedstock for medium to high-value biomolecule production in biorefineries: A review. In *Bioresource Technology* (Vol. 339). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2021.125594

Annamalai, N., & Sivakumar, N. (2016). Production of polyhydroxybutyrate from wheat bran hydrolysate using Ralstonia eutropha through microbial fermentation. *Journal of Biotechnology*, *237*, 13–17. https://doi.org/10.1016/j.jbiotec.2016.09.001

ANP. (2021). *Anuário Estatístico 2021 - Agência Nacional do Petróleo, Gás Natural e Biocombustíveis*.

Bastos, J. A., Remor, P. V., Alino, J. H. L., Frare, L. M., Lofhagen, J. C. P., & Edwiges, T. (2021). Hydrolysate recycling improves economic feasibility of alkaline pretreatment for bioenergy production. *Journal of Environmental Chemical Engineering*, *9*(5). https://doi.org/10.1016/j.jece.2021.105935

Behera, S., Arora, R., Nandhagopal, N., & Kumar, S. (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. In *Renewable and Sustainable Energy Reviews* (Vol. 36, pp. 91–106). Elsevier Ltd. https://doi.org/10.1016/j.rser.2014.04.047

Bhatia, S. K., Otari, S. v., Jeon, J. M., Gurav, R., Choi, Y. K., Bhatia, R. K., Pugazhendhi, A., Kumar, V., Rajesh Banu, J., Yoon, J. J., Choi, K. Y., & Yang, Y. H. (2021). Biowaste-to-bioplastic (polyhydroxyalkanoates): Conversion technologies, strategies, challenges, and perspective. In *Bioresource Technology* (Vol. 326). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2021.124733

Camiscia, P., Giordano, E. D. V., Brassesco, M. E., Fuciños, P., Pastrana, L., Cerqueira, M. F., Picó, G. A., & Woitovich Valetti, N. (2018). Comparison of soybean hull pre-treatments to obtain cellulose and chemical derivatives: Physical chemistry characterization. *Carbohydrate Polymers*, *198*, 601–610. https://doi.org/10.1016/j.carbpol.2018.06.125

Cavalheiro, J. M. B. T., de Almeida, M. C. M. D., da Fonseca, M. M. R., & de Carvalho, C. C. C. R. (2012). Adaptation of Cupriavidus necator to conditions favoring polyhydroxyalkanoate production. *Journal of Biotechnology*, *164*(2), 309–317. https://doi.org/10.1016/j.jbiotec.2013.01.009

Cavalheiro, J. M. B. T., de Almeida, M. C. M. D., Grandfils, C., & da Fonseca, M. M. R. (2009). Poly(3-hydroxybutyrate) production by Cupriavidus necator using waste glycerol. *Process Biochemistry*, *44*(5), 509–515. https://doi.org/10.1016/j.procbio.2009.01.008

Dall Cortivo, P. R., Hickert, L. R., Rosa, C. A., & Záchia Ayub, M. A. (2020). Conversion of fermentable sugars from hydrolysates of soybean and oat hulls into ethanol and xylitol by

Spathaspora hagerdaliae UFMG-CM-Y303. *Industrial Crops and Products*, *146*. https://doi.org/10.1016/j.indcrop.2020.112218

Embrapa. (2022). *Soja em números (safra 2020/21); Empresa Brasileira de Pesquisa Agropecuária Embrapa Soja Ministério da Agricultura, Pecuária e Abastecimento*. https://www.embrapa.br/soja/cultivos/soja1/dados-economicos

Environmental Protection Agency. (2020). *Facts and Figures about Materials, Waste and Recycling. Plastics: Material-Specific Data*.

Evangeline, S., & Sridharan, T. B. (2019). Biosynthesis and statistical optimization of polyhydroxyalkanoate (PHA) produced by Bacillus cereus VIT-SSR1 and fabrication of biopolymer films for sustained drug release. *International Journal of Biological Macromolecules*, *135*, 945–958. https://doi.org/10.1016/j.ijbiomac.2019.05.163

Gahlawat, G. (2019). *Polyhydroxyalkanoates Biopolymers - Production Strategies* (Navard, Patrick). Spinger. www.epnoe.eu

Gahlawat, G., & Soni, S. K. (2017). Valorization of waste glycerol for the production of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer by Cupriavidus necator and extraction in a sustainable manner. *Bioresource Technology*, *243*, 492–501. https://doi.org/10.1016/j.biortech.2017.06.139

Ganesan, R., Manigandan, S., Shanmugam, S., Chandramohan, V. P., Sindhu, R., Kim, S. H., Brindhadevi, K., & Pugazhendhi, A. (2021). A detailed scrutinize on panorama of catalysts in biodiesel synthesis. In *Science of the Total Environment* (Vol. 777). Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2021.145683

García, I. L., López, J. A., Dorado, M. P., Kopsahelis, N., Alexandri, M., Papanikolaou, S., Villar, M. A., & Koutinas, A. A. (2013). Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production by Cupriavidus necator. *Bioresource Technology*, *130*, 16–22. https://doi.org/10.1016/j.biortech.2012.11.088

Gonzalez-Rios, J. A., Valle-Pérez, A. U., Amaya-Delgado, L., & Sanchez, A. (2021). A quick fed-batch saccharification strategy of wheat straw at high solid loadings improving lignocellulosic ethanol productivity. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-021-01580-0

Goyal, S., Hernández, N. B., & Cochran, E. W. (2021). An update on the future prospects of glycerol polymers. In *Polymer International* (Vol. 70, Issue 7, pp. 911–917). John Wiley and Sons Ltd. https://doi.org/10.1002/pi.6209

Hames, B., Ruiz, R., Scarlata, C., Sluiter, A., Sluiter, J., & Templeton, D. (2008). *Preparation of Samples for Compositional Analysis: Laboratory Analytical Procedure (LAP); Issue Date 08/08/2008*. www.nrel.gov

Hernández-Beltrán, J. U., & Hernández-Escoto, H. (2018). Enzymatic hydrolysis of biomass at high-solids loadings through fed-batch operation. *Biomass and Bioenergy*, *119*, 191–197. https://doi.org/10.1016/j.biombioe.2018.09.020

Jin, Y., Huang, T., Geng, W., & Yang, L. (2013). Comparison of sodium carbonate pretreatment for enzymatic hydrolysis of wheat straw stem and leaf to produce fermentable sugars. *Bioresource Technology*, *137*, 294–301. https://doi.org/10.1016/j.biortech.2013.03.140

Jönsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. In *Bioresource Technology* (Vol. 199, pp. 103–112). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2015.10.009

Ju, J. H., Wang, D., Heo, S. Y., Kim, M. S., Seo, J. W., Kim, Y. M., Kim, D. H., Kang, S. A., Kim, C. H., & Oh, B. R. (2020). Enhancement of 1,3-propanediol production from industrial by-product by Lactobacillus reuteri CH53. *Microbial Cell Factories*, *19*(1). https://doi.org/10.1186/s12934-019-1275-x

Karp, S. G., Osipov, D. O., Semenova, M. v, Rozhkova, A. M., Zorov, I. N., Sinitsyna, O. A., Soccol, C. R., & Sinitsyn, A. P. (2020). Effect of Novel Penicillium verruculosum Enzyme Preparations on the Saccharification of Acid-and Alkali-Pretreated Agro-Industrial Residues. *Agronomy*, *10*. https://doi.org/10.3390/agronomy10091348

Karp, S. G., Rozhkova, A. M., Semenova, M. v., Osipov, D. O., de Pauli, S. T. Z., Sinitsyna, O. A., Zorov, I. N., de Souza Vandenberghe, L. P., Soccol, C. R., & Sinitsyn, A. P. (2021). Designing enzyme cocktails from Penicillium and Aspergillus species for the enhanced saccharification of agro-industrial wastes. *Bioresource Technology*, *330*. https://doi.org/10.1016/j.biortech.2021.124888

Keshavarz, T., & Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. In *Current Opinion in Microbiology* (Vol. 13, Issue 3, pp. 321–326). https://doi.org/10.1016/j.mib.2010.02.006

Kim, J. S., Lee, B. H., & Kim, B. S. (2005). Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by Ralstonia eutropha. *Biochemical Engineering Journal*, *23*(2), 169–174. https://doi.org/10.1016/j.bej.2005.01.016

Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. In *Bioresource Technology* (Vol. 199, pp. 42–48). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2015.08.085

Kosseva, M. R., & Rusbandi, E. (2018). Trends in the biomanufacture of polyhydroxyalkanoates with focus on downstream processing. In *International Journal of Biological Macromolecules* (Vol. 107, Issue PartA, pp. 762–778). Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2017.09.054

Kumar, M., Rathour, R., Singh, R., Sun, Y., Pandey, A., Gnansounou, E., Andrew Lin, K.Y., Tsang, D.C.W., Thakur, I.S. (2020). Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects. Journal of Cleaner Production 263, 121500. https://doi.org/10.1016/J.JCLEPRO.2020.121500

Li, M., & Wilkins, M. (2020). Fed-batch cultivation and adding supplements to increase yields of polyhydroxybutyrate production by Cupriavidus necator from corn stover alkaline pretreatment liquor. *Bioresource Technology*, *299*. https://doi.org/10.1016/j.biortech.2019.122676

Medeiros-Garcia-Alcântara, J., Distante, F., Storti, G., Moscatelli, D., Morbidelli, M., & Sponchioni, M. (2020). Current trends in the production of biodegradable bioplastics: The case of

polyhydroxyalkanoates. In *Biotechnology Advances* (Vol. 42). Elsevier Inc. https://doi.org/10.1016/j.biotechadv.2020.107582

Miller, G. L. (1959). Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Anal. Chem*, *31*, 426–428.

Moshood, T. D., Nawanir, G., Mahmud, F., Mohamad, F., Ahmad, M. H., & AbdulGhani, A. (2022). Biodegradable plastic applications towards sustainability: A recent innovations in the green product. *Cleaner Engineering and Technology*, *6*, 100404. https://doi.org/10.1016/j.clet.2022.100404

Mothes, G., Schnorpfeil, C., & Ackermann, J. U. (2007). Production of PHB from crude glycerol. *Engineering in Life Sciences*, *7*(5), 475–479. https://doi.org/10.1002/elsc.200620210

Mozumder, M.S.I., de Wever, H., Volcke, E.I.P., Garcia-Gonzalez, L. (2014). A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production. Process Biochemistry 49, 365–373. https://doi.org/10.1016/J.PROCBIO.2013.12.004

Mukasekuru, M. R., Kaneza, P., Sun, H., Sun, F. F., He, J., & Zheng, P. (2020). Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. *Industrial Crops and Products*, *146*. https://doi.org/10.1016/j.indcrop.2020.112156

Nahak, B. K., Preetam, S., Sharma, D., Shukla, S. K., Syväjärvi, M., Toncu, D.-C., & Tiwari, A. (2022). Advancements in net-zero pertinency of lignocellulosic biomass for climate neutral energy production. *Renewable and Sustainable Energy Reviews*, *161*, 112393. https://doi.org/10.1016/J.RSER.2022.112393

Nawrath, C., & Poirier, Y. (2008). Pathways for the Synthesis of Polyesters in Plants: Cutin, Suberin, and Polyhydroxyalkanoates. *Advances in Plant Biochemistry and Molecular Biology*, *1*(C), 201–239. https://doi.org/10.1016/S1755-0408(07)01008-9

Niju, S., Swathika, M., & Balajii, M. (2020). Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production. *Lignocellulosic Biomass to Liquid Biofuels*, 301–324. https://doi.org/10.1016/B978-0-12-815936-1.00010-1

Oh, Y. H., Lee, S. H., Jang, Y. A., Choi, J. W., Hong, K. S., Yu, J. H., Shin, J., Song, B. K., Mastan, S. G., David, Y., Baylon, M. G., Lee, S. Y., & Park, S. J. (2015). Development of rice bran treatment process and its use for the synthesis of polyhydroxyalkanoates from rice bran hydrolysate solution. *Bioresource Technology*, *181*, 283–290. https://doi.org/10.1016/j.biortech.2015.01.075

Pinto-Ibieta, F., Cea, M., Cabrera, F., Abanto, M., Felissia, F. E., Area, M. C., & Ciudad, G. (2020). Strategy for biological co-production of levulinic acid and polyhydroxyalkanoates by using mixed microbial cultures fed with synthetic hemicellulose hydrolysate. *Bioresource Technology*, *309*. https://doi.org/10.1016/j.biortech.2020.123323

PlasticsEurope. (2021). *An analysis of European plastics production, demand and waste data*. PlasticsEurope: Bruxelles, Belgium, 2021.

Qing, Q., Guo, Q., Zhou, L., Gao, X., Lu, X., & Zhang, Y. (2017). Comparison of alkaline and acid pretreatments for enzymatic hydrolysis of soybean hull and soybean straw to produce

fermentable sugars. *Industrial Crops and Products*, *109*, 391–397. https://doi.org/10.1016/j.indcrop.2017.08.051

Poomipuk, N., Reungsang, A., Plangklang, P. (2014). Poly-β-hydroxyalkanoates production from cassava starch hydrolysate by Cupriavidus sp. KKU38. International Journal of Biological Macromolecules 65, 51–64. https://doi.org/10.1016/J.IJBIOMAC.2014.01.002

Raman, A. A. A., Tan, H. W., & Buthiyappan, A. (2019). Two-Step Purification of Glycerol as a Value Added by Product From the Biodiesel Production Process. *Frontiers in Chemistry*, *7*(November 2019), 1–9. https://doi.org/10.3389/fchem.2019.00774

Rodríguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell-Monfort, M., Braunegg, G., & Marqués-Calvo, M. S. (2015). Influence of glycerol on poly(3-hydroxybutyrate) production by Cupriavidus necator and Burkholderia sacchari. *Biochemical Engineering Journal*, *94*, 50–57. https://doi.org/10.1016/j.bej.2014.11.007

Saha, B. C., & Cotta, M. A. (2007). Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol. *Enzyme and Microbial Technology*, *41*(4), 528–532. https://doi.org/10.1016/j.enzmictec.2007.04.006

Saratale, G. D., & Oh, M. K. (2015). Characterization of poly-3-hydroxybutyrate (PHB) produced from Ralstonia eutropha using an alkali-pretreated biomass feedstock. *International Journal of Biological Macromolecules*, *80*, 627–635. https://doi.org/10.1016/j.ijbiomac.2015.07.034

Sen, K. Y., Hussin, M. H., & Baidurah, S. (2019). Biosynthesis of poly(3-hydroxybutyrate) (PHB) by Cupriavidus necator from various pretreated molasses as carbon source. *Biocatalysis and Agricultural Biotechnology*, *17*, 51–59. https://doi.org/10.1016/j.bcab.2018.11.006

Silva, L. F., Taciro, M. K., Michelin Ramos, M. E., Carter, J. M., Pradella, J. G. C., & Gomez, J. G. C. (2004). Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. *Journal of Industrial Microbiology and Biotechnology*, *31*(6), 245–254. https://doi.org/10.1007/s10295-004-0136-7

Singh, J., Suhag, M., & Dhaka, A. (2015). Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. In *Carbohydrate Polymers* (Vol. 117, pp. 624–631). Elsevier Ltd. https://doi.org/10.1016/j.carbpol.2014.10.012

Sirohi, R., Prakash Pandey, J., Kumar Gaur, V., Gnansounou, E., & Sindhu, R. (2020). Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB). In *Bioresource Technology* (Vol. 311). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2020.123536

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: 7/17/2005*. www.nrel.gov

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2011). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP); Issue Date: April 2008; Revision Date: July 2011 (Version 07-08-2011)*. http://www.nrel.gov/biomass/analytical_procedures.html

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of Extractives in Biomass: Laboratory Analytical Procedure (LAP); Issue Date 7/17/2005*. http://www.nrel.gov/biomass/analytical_procedures.html

Stein, H. H., Berger, L. L., Drackley, J. K., Fahey, G. C., Hernot, D. C., & Parsons, C. M. (2008). Nutritional Properties and Feeding Values of Soybeans and Their Coproducts. *Soybeans: Chemistry, Production, Processing, and Utilization*, 613–660. https://doi.org/10.1016/B978-1-893997-64-6.50021-4

Su, T., Zhao, D., Khodadadi, M., & Len, C. (2020). Lignocellulosic biomass for bioethanol: Recent advances, technology trends, and barriers to industrial development. *Current Opinion in Green and Sustainable Chemistry*, *24*, 56–60. https://doi.org/10.1016/J.COGSC.2020.04.005

Thomas, C. M., Kumar, D., Scheel, R. A., Ramarao, B., & Nomura, C. T. (2022). Production of Medium Chain Length polyhydroxyalkanoate copolymers from agro-industrial waste streams. *Biocatalysis and Agricultural Biotechnology*, 102385. https://doi.org/10.1016/J.BCAB.2022.102385

UNEP. (2018). *Beat plastic pollution*. Visual Feature | Beat Plastic Pollution (unep.org), accessed on 21 August 2021.

Wang, X., Xia, K., Yang, X., & Tang, C. (2019). Growth strategy of microbes on mixed carbon sources. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-09261-3

Wu, D., Wei, Z., Mohamed, T. A., Zheng, G., Qu, F., Wang, F., Zhao, Y., & Song, C. (2022). Lignocellulose biomass bioconversion during composting: Mechanism of action of lignocellulase, pretreatment methods and future perspectives. *Chemosphere*, *286*. https://doi.org/10.1016/j.chemosphere.2021.131635

Xu, H., Li, B., & Mu, X. (2016). Review of Alkali-Based Pretreatment to Enhance Enzymatic Saccharification for Lignocellulosic Biomass Conversion. In *Industrial and Engineering Chemistry Research* (Vol. 55, Issue 32, pp. 8691–8705). American Chemical Society. https://doi.org/10.1021/acs.iecr.6b01907

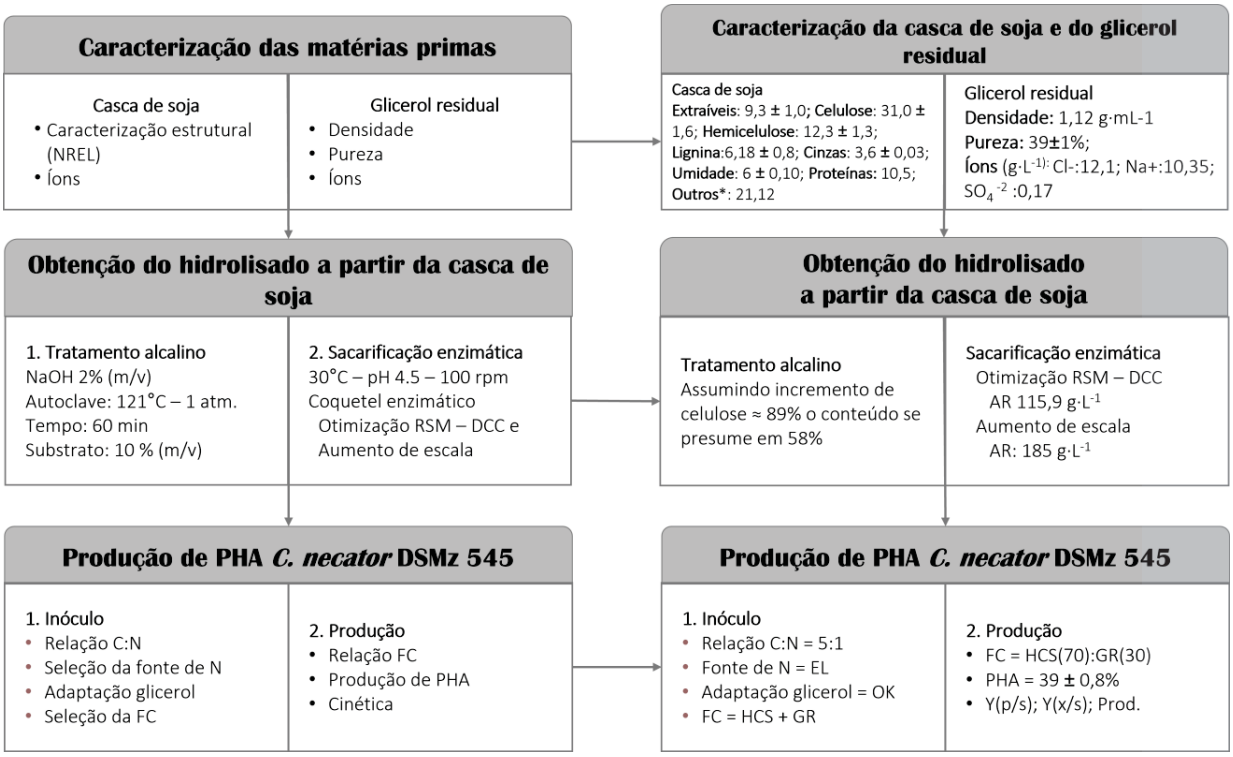
Yang, N., Li, Y., Xing, F., Wang, X., Li, X., Li, L., Yang, J., Wang, Y., & Zhang, M. (2021). Composition and structural characterization of pectin in micropropagated and conventional plants of Premma puberula Pamp. *Carbohydrate Polymers*, *260*. https://doi.org/10.1016/j.carbpol.2021.117711

Yoo, J., Alavi, S., Vadlani, P., & Amanor-Boadu, V. (2011). Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. *Bioresource Technology*, *102*(16), 7583–7590. https://doi.org/10.1016/j.biortech.2011.04.092

4. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Mediante o desenvolvimento desde trabalho, constatou-se a viabilidade para implementar a casca de soja e o glicerol residual como matérias primas de baixo custo para a produção de polihidroxialcanoatos, moléculas de médio a alto valor agregado. Na Figura 13 apresenta-se um resumo dos resultados obtidos.

FIGURA 13 – Resumo de resultados.



Fonte: A autora (2022)

Com o hidrolisado produzido e com o glicerol residual, o microrganismo *Cupriavidus necator DSMz 545* efetuou a fermentação em cultivo em batelada em escala de bancada (inóculo 10% (v/v), 30 °C, 150 rpm, pH 6-7, 96 h) obtendo um acúmulo máximo de 39% de polihidroxialcanoatos.

A composição da casca de soja implementada neste trabalho correspondeu às seguintes frações: celulose 31,0±1,6%; hemicelulose 12,3±1,3%; lignina 6,18±0,8%; proteínas10,5%, extrativos 9,3± 1,0%; cinzas 3,6±0,03%; umidade 6,0±0,10%; outros 21,22%.

As condições de hidrólise química estabelecidas para o pré-tratamento da casca de soja (NaOH 2% (m/v), durante 60 minutos em autoclave a 120 ◦C e 1 atm., seguida das condições otimizadas de sacarificação enzimática com o coquetel enzimático não comercial, concentração de substrato 62% (m/v), 45 °C, 42h e 100 rpm, permitiram obter um hidrolisado com concentração de AR de 115,9 $g \cdot L^{-1}$, melhorando a concentração obtida no processo de sacarificação sem otimização (≈31 $g \cdot L^{-1}$).

Testou-se a etapa de sacarificação aumentando a escala de processamento (260 mL), utilizando um tanque de 1L (com agitação e temperatura controlada), implementando a concentração do coquetel enzimático não comercial (na concentração previamente otimizada), em pH 4,5, temperatura de 45 °C e tempo de incubação de 42 h, sob operação em batelada alimentada com alimentação de substrato após 14h e 22h para completar uma concentração final do substrato de 38,5%, obtendo um hidrolisado com concentração de açúcares redutores de 185,7 $g \cdot L^{-1,}$ o que corresponde 0,38 g/g_{CS} em rendimento de glucose e a 60% em conversão de celulose em glucose.

A continuação, são propostas algumas alternativas a serem consideradas para trabalhos futuros:

- Sugere-se estabelecer o processo total de uma biorrefinaria com o propósito de valorizar ainda mais o resíduo da casca de soja, incluindo estudos de viabilidade técnica, comercial e financeira.
- Recomenda-se analisar e aproveitar as diferentes frações residuais (líquidas e sólidas) obtidas ao longo do processo de obtenção do hidrolisado (efluente gerado após do tratamento alcalino da casca de soja, presumivelmente rico em ligninas e hemiceluloses e recuperação do NaOH) e o resíduo sólido remanescente após do pré-tratamento enzimático.
- Sugere-se realizar a cinética de liberação de açúcares redutores durante a sacarificação enzimática, com o propósito de perfilar adequadamente o processo.
- Aconselha-se efetuar o processo de otimização das condições de fermentação para a produção de PHA.

- Recomenda-se implementar a produção de PHA em biorreator no sistema de fermentação em batelada alimentada estudando alimentação em duas e três etapas.
- Aponta-se a importância de analisar a inclusão de óleo de soja como fonte de carbono adicional aos substratos já analisados, pois este composto induz a produção de copolímeros, valorizando e ampliando as possíveis aplicações do biopolímero obtido. De igual forma, sugere-se analisar a inclusão de VFAs como precursores de copolímeros.
- Sugere-se explorar opções ambientalmente amigáveis para a recuperação e purificação do PHA obtido.
- Recomenda-se realizar a caracterização físico-química do PHA produzido (MEV, NMR, FTIR, DRX e TG) para definir as possíveis aplicações plausíveis segundo o perfil do biopolímero.

5. REFERÊNCIAS

Albuquerque, P.B.S., Malafaia, C.B. (2018). Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2017.09.026

Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S. (2016). Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. International Journal of Biological Macromolecules. https://doi.org/10.1016/j.ijbiomac.2016.04.069

ANP, 2022. Anuário Estatístico 2021 - Agencia Nacional do Petróleo Gás Natural e Biocombustíveis [WWW Document]. URL https://www.gov.br/anp/pt-br/centrais-de-conteudo/publicacoes/anuario-estatistico/anuario-estatistico-2021

Bittencourt, A.G., Porto de Souza Vandenberghe, L., Valladares-Diestra, K., Wedderhoff Herrmann, L., Fátima Murawski de Mello, A., Sarmiento Vásquez, Z., Grace Karp, S., Ricardo Soccol, C. (2021). Soybean hulls as carbohydrate feedstock for medium to high-value biomolecule production in biorefineries: A review. Bioresour. Technol. 339, 125594. https://doi.org/10.1016/j.biortech.2021.125594

Blunt, W., Sparling, R., Gapes, D.J., Levin, D.B., Cicek, N. (2018). The role of dissolved oxygen content as a modulator of microbial polyhydroxyalkanoate synthesis. World Journal of Microbiology and Biotechnology. https://doi.org/10.1007/s11274-018-2488-6

Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V. (2014). Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Express Polymer Letters 8, 791–808. https://doi.org/10.3144/expresspolymlett.2014.82

El-malek, F.A., Khairy, H., Farag, A., Omar, S. (2020). The sustainability of microbial bioplastics, production and applications. International Journal of Biological Macromolecules 157, 319–328. https://doi.org/10.1016/j.ijbiomac.2020.04.076

Embrapa (2021). Empresa Brasileira de Pesquisa Agropecuária – Embrapa, Ministério da Agricultura, Pecuária e Abastecimento. Soja em números (safra 2020/21)

Fukui, T., Doi, Y., (1998). Efficient production of polyhydroxyalkanoates from plant oils by Alcaligenes eutrophus and its recombinant strain. Applied Microbiology and Biotechnology 49, 333–336.

Goyal, S., Hernández, N.B., Cochran, E.W. (2021). An update on the future prospects of glycerol polymers. Polym. Int. https://doi.org/10.1002/pi.6209

Huong, K.H., Azuraini, M.J., Aziz, N.A., Amirul, A.A.A. (2017). Pilot scale production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) biopolymers with high molecular weight and elastomeric properties. Journal of Bioscience and Bioengineering 124, 76–83. https://doi.org/10.1016/j.jbiosc.2017.02.003

Jiang, G., Hill, D.J., Kowalczuk, M., Johnston, B., Adamus, G., Irorere, V., Radecka, I. (2016). Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. International Journal of Molecular Sciences. https://doi.org/10.3390/ijms17071157

Ju, J., Dexin, W., Heo, S., Kim, M., Seo, J., Kim, Y., Kim, D., Kang, S., Kim, C., Oh, B. (2020). Enhancement of 1,3-propanediol production from industrial by-product by Lactobacillus reuteri CH53. Microb. Cell Fact. 19. https://doi.org/https://doi.org/10.1186/s12934-019-1275-x

Keshavarz, T., & Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. In Current Opinion in Microbiology (Vol. 13, Issue 3, pp. 321–326). https://doi.org/10.1016/j.mib.2010.02.006

Koller, M., Reiterer, A., Braunegg, G., Salerno, A., Muhr, A. (2013). Polyhydroxyalkanoates: Biodegradable Polymeric Materials from Renewable Resources. Materials and technology 47

Kosseva, M. R., & Rusbandi, E. (2018). Trends in the biomanufacture of polyhydroxyalkanoates with focus on downstream processing. In International Journal of Biological Macromolecules (Vol. 107, Issue PartA, pp. 762–778). Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2017.09.054

Mohandas, S.P., Balan, L., Jayanath, G., Anoop, B.S., Philip, R., Cubelio, S.S., Bright Singh, I.S. (2018). Biosynthesis and characterization of polyhydroxyalkanoate from marine Bacillus cereus MCCB 281 utilizing glycerol as carbon source. International Journal of Biological Macromolecules 119, 380–392. https://doi.org/10.1016/j.ijbiomac.2018.07.044

Niju, M., Swathika, M., Balajii. (2020). Chapter 10 - Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production, in: Yousuf, A., Pirozzi, D., Filomena, S. (Eds.), Lignocellulosic Biomass to Liquid Biofuels. Academic Press, pp. 301–324.

Poirier'·, Y., Nawrath, C., Somerville, C. (1995). Production of Polyhydroxyalkanoates, a Family of Biodegradable Plastics and Elastomers, in Bacteria and Plants.

Raman, A.A.A., Tan, H.W., Buthiyappan, A. (2019). Two-Step Purification of Glycerol as a Value Added by Product From the Biodiesel Production Process. Front. Chem. 7, 1–9. https://doi.org/10.3389/fchem.2019.00774

Raza, Z.A., Abid, S., Banat, I.M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. International Biodeterioration and Biodegradation. https://doi.org/10.1016/j.ibiod.2017.10.001

Sagong, H.Y., Son, H.F., Choi, S.Y., Lee, S.Y., Kim, K.J. (2018). Structural Insights into Polyhydroxyalkanoates Biosynthesis. Trends in Biochemical Sciences. https://doi.org/10.1016/j.tibs.2018.08.005

Sirohi, R., Prakash Pandey, J., Kumar Gaur, V., Gnansounou, E., Sindhu, R. (2020). Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB). Bioresource Technology. https://doi.org/10.1016/j.biortech.2020.123536

Sudesh, K., Abe, H., Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters.

Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M., Shah, S. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants — A review. Biotechnology Advances 25, 148–175. https://doi.org/10.1016/J.BIOTECHADV.2006.11.007

Tokiwa, Y., Calabia, B.P., Ugwu, C.U., Aiba, S. (2009). Biodegradability of plastics. International Journal of Molecular Sciences. https://doi.org/10.3390/ijms10093722