UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO TECNOLÓGICO
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

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Synthesis of polybutylene succinate by enzymatic transesterification: A study of kinetic behavior and enzymatic stability in the reuse of immobilized lipase

Florianópolis – Santa Catarina
2022

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Dissertação submetida ao Programa de Pós-graduação em Engenharia Química da Universidade Federal de Santa Catarina para a obtenção do título de mestre em Engenharia Química
Orientador: Prof. Bruno Francisco Oechsler, Dr.
Coorientador: Prof. Pedro Henrique Hermes Araújo, Dr.

Florianópolis – Santa Catarina
2022

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Guckert, Fernando Elias
Synthesis of polybutylene succinate by enzymatic transesterification: A study of kinetic behavior and enzymatic stability in the reuse of immobilized lipase / Fernando Elias Guckert ; orientador, Bruno Francisco Oechsler, coorientador, Pedro Henrique Hermes de Araújo, 2022.
153 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, Centro Tecnológico, Programa de Pós-Graduação em Engenharia Química, Florianópolis, 2022.

Inclui referências.

1. Engenharia Química. 2. Polybutylene Succinate . 3. Enzymatic Polycondensation. 4. Kinetic Behavior. 5. Reuse of immobilized biocatalyst (Novozyme 435, N435). I. Oechsler, Bruno Francisco. II. Araújo, Pedro Henrique Hermes de . III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Engenharia Química. IV. Título.

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

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Prof. Bruno Francisco Oechsler, Dr.

Orientador

Florianópolis – Santa Catarina, 2022

Este trabalho é dedicado a todas as pessoas que fazem parte da minha vida, amo vocês!

AGRADECIMENTOS

Nesse momento, gostaria de direcionar grande parte dos meus agradecimentos a todos que fizeram parte da minha caminhada até aqui, saibam que os frutos colhidos também são de vocês. Agradeço a vocês por fazerem parte da minha vida, principalmente pelo apoio e por entenderem os momentos de ausência em datas especiais. Amo todos vocês!

Sou grato a todos que conheci durante essa jornada, principalmente as amizades que construí. Obrigado pelos momentos de reflexão, carinho e aconselhamentos. Vocês sempre serão os melhores! Desejo muito sucesso a vocês! Gostaria de direcionar agradecimentos especiais a Clara, minha companheira de guerra no laboratório e conselheira nos momentos difíceis. Você foi fundamental nesse processo! Também gostaria de deixar meu muito obrigado a Daniela, pelo apoio nos momentos de dificuldade e também pelos momentos de descontração. Você é excepcional!

Gostaria também de simbolizar gratidão aos meus orientadores, professores Bruno e Pedro. Obrigado pelo apoio e aconselhamento durante essa jornada. Vocês foram (e são) maravilhosos! Com toda certeza, o suporte fornecido foi fundamental para alcançar os resultados aqui apresentados. Ainda, não poderia esquecer da professora Claudia, por toda sabedoria e conhecimentos. Seu apoio é ímpar, e agradeço por sua contribuição! Ainda, devo direcionar meu mais sincero muito obrigado a professora Débora. Além de todo subsídio fornecido, você foi fundamental em minha formação como pesquisador. Tenho prazer enorme em poder dizer que trabalhei com você. Gratidão pelo apoio e conhecimento partilhado.

Agradeço a UFSC pela oportunidade de desenvolvimento profissional e também a CAPES, pelo suporte financeiro.

Por fim, deixo meus sinceros cumprimentos aos que contribuíram para o desenvolvimento do trabalho. Mesmo que de forma indireta, toda a ajuda serviu como suporte para alcançarmos os resultados aqui apresentados. Muito obrigado a todos!

Conceptual diagram

Synthesis of polybutylene succinate by enzymatic transesterification: A study of kinetic behavior and enzymatic stability in the reuse of immobilized lipase

What?

Enzymatic synthesis of Polybutylene Succinate (PBS) using N435, both in bulk and in solution (with diphenyl ether, DE).

Why?

- Growing interest in the development of biotechnological processes to obtain sustainable polymers;
- Description of the synthesis behavior for future exploration in commercial/industrial processes;
- Reuse of the immobilized biocatalyst, aiming at greater employability in future processes at large scale.

State of the art

- No studies were found addressing the kinetic behavior of PBS synthesis catalyzed by immobilized lipase;
- Absence of studies describing the stability and reuse of the immobilized biocatalyst in new PBS production cycles.

Hypotheses

- Obtaining the kinetic behavior of biocatalyzed PBS synthesis in bulk and solution systems;
- Improvements in the synthesis process and in the catalytic stability of N435 when comparing the bulk system with the solution system (due to the high viscosity of the reaction medium).

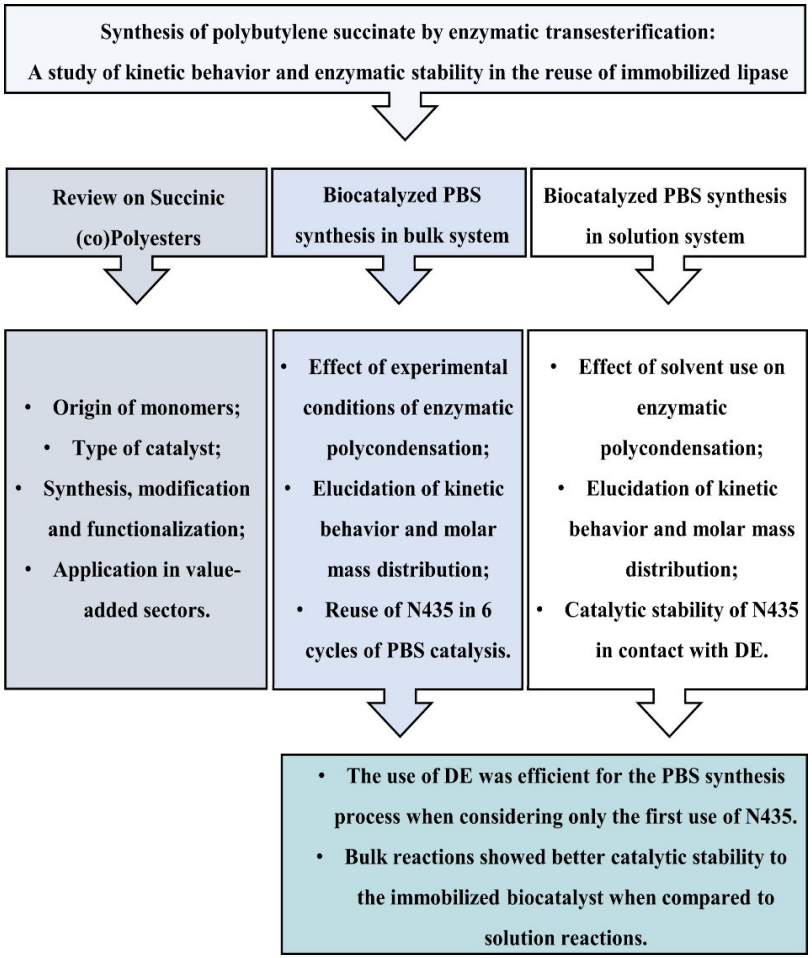
Which steps?

For both bulk and solution systems:

- Evaluation of the operational conditions of synthesis;
- Recovery of reaction by-products to investigate the kinetic behavior;
- Description of properties presented by the polymer products;
- Recovery of the immobilized biocatalyst for application in new production cycles;
- Evaluation of the catalytic stability between the reuse cycles of the immobilized biocatalyst.

Expected results

- Description of the kinetic behavior, both via the recovery of by-products and the polymeric products obtained;
- Description of the stability of N435 in new PBS production cycles, comparing which synthesis system provides greater catalytic stability for the PBS synthesis process.



RESUMO

Neste trabalho, as condições de síntese enzimática (bulk e solução) de PBS, seguido do reuso da lipase Novozym 435 (N435) em novos ciclos de síntese foram investigadas, com ênfase na descrição do comportamento cinético, atividade enzimática e distribuições de massas molares dos polímeros. Para as reações bulk, foram utilizados 0,1 mol de cada monômero (DS e BDO), sob agitação de 400 rpm e baixa pressão (0,1 atm), avaliando-se o efeito das concentrações de lipase N435 (5, 10 e 20 m/m%) e temperatura (60 a 100 °C). Para as reações em solução, as condições foram similares, avaliando-se a adição de 5 e 50 m/m% de DE no meio reacional. A quantidade de N435 foi fixada em 10 m/m%, avaliando-se as temperaturas de 70, 80 e 90 °C. Em cada sistema, foram selecionadas as melhores condições de síntese para a análise do reuso da N435. As reações em massa apresentaram limitações difusionais das cadeias até o sítio ativo da enzima imobilizada, devido às altas viscosidades do meio nas temperaturas investigadas. Nas reações bulk, a condição de melhor desempenho foi com 10 m/m% de N435 a 90 °C. O tempo de reação foi de 90 minutos, obtendo-se 8,08 g de subprodutos (sendo 9,20 o valor estequiométrico) e M_w de 4.000 $g.mol^{-1}$. Nesta condição, o procedimento de reuso apresentou 4 ciclos com boa estabilidade enzimática. A atividade inicial da N435 foi 32,4 $U.g^{-1}$, decaindo para 3,8 $U.g^{-1}$ ao final do reuso. Além disso, testes indicaram entupimento dos poros do suporte enzimático por cadeias poliméricas após o reuso, contribuindo para o decaimento da atividade enzimática. Para o sistema em solução, maiores taxas de remoção de etanol foram observadas com 5 m/m% de DE. Limitações viscosimétricas ainda foram observadas nesta condição, decaindo com o aumento das temperaturas de reação. Utilizando-se 50 m/m% de DE, foram observados maiores tempos de reação, devido à menor viscosidade gerada pelo solvente. Porém, quanto maior a diluição, menores taxas de reação foram obtidas. Dessa forma, a condição de 5 m/m% de DE apresentou destaque, com valores de M_w entre 2.000 e 3.350 $g.mol^{-1}$, sendo aplicada aos procedimentos de reuso, na temperatura de 70 °C (condição ótima de operação da N435), em reações de 60 minutos. Na presença do DE, os resultados indicaram perda significativa de atividade, onde a atividade enzimática inicial foi 31,1 $U.g^{-1}$, decaindo para 4,5 $U.g^{-1}$ após o primeiro ciclo de uso, e 1,9 $U.g^{-1}$ ao fim de 3 ciclos. Um grande decaimento no rendimento em massa do polímero foi observado, em que o valor inicial foi 74,9 %, decaindo para 16,9 % no 3° ciclo de reuso. Testes adicionais sobre os efeitos do DE em contato com a N435 indicaram interações no suporte da enzima, causando maior porosidade, além da lixiviação da enzima adsorvida no suporte, justificando a perda de atividade da N435. Comparando-se os resultados de reuso da N435, o sistema bulk foi mais eficiente, com maior estabilidade e eficiência catalítica, enquanto que o uso do difenil éter causou perdas na atividade e eficiência catalítica de N435.

Palavras-chave: Polibutileno Succinato; Policondensação Enzimática; Cinética; Reuso do biocatalisador imobilizado.

RESUMO EXPANDIDO

Introdução

Devido ao desenvolvimento tecnológico e industrial, materiais poliméricos vêm sendo aplicados nos mais variados segmentos, como automotivo, agrícola, alimentício e farmacêutico. Com tamanha versatilidade, grandes quantidades desses materiais são produzidas, estimando-se em 400 milhões de toneladas em 2020, em sua maioria de origem fóssil. Dessa forma, são gerados problemas ambientais, seja pela extração de petróleo ou pela ampla deposição desses materiais em locais inadequados, causando degradação ambiental. Assim, esta problemática leva a busca por materiais poliméricos mais sustentáveis. Diante de tais problemas, o Polibutileno Succinato (PBS) apresenta-se como uma alternativa biodegradável, em que seus constituintes podem ser produzidos através de processos biotecnológicos. Remetendo-se aos processos industriais de produção, a síntese de PBS ocorre principalmente aplicando-se catalisadores de origem metálica, como acetatos de manganês, zinco, titânio e óxidos de ferro, em altas temperaturas e sob vácuo. Tais catalisadores possuem baixa seletividade, podendo gerar produtos indesejados durante a reação. Ainda, é constatada a deposição de resíduos metálicos dos catalisadores nos materiais poliméricos produzidos, o que pode ser desfavorável, dependendo de sua aplicação, devido à sua toxicidade. Dessa forma, outras fontes de catalisadores devem ser estudadas, destacando-se os processos catalíticos biotecnológicos, com o uso de enzimas. Os processos de síntese enzimática apresentam diversas vantagens, tais como: alta especificidade entre reagentes e produtos, temperaturas mais baixas e ausência de metais tóxicos em sua composição, além da possibilidade de reaproveitamento, no caso de enzimas imobilizadas (sendo um exemplo a N435). Até onde foi pesquisado, não foram encontrados trabalhos abordando a reutilização de biocatalisadores na síntese de PBS (ou (co)polímeros succínicos). Além disso, não foram encontrados trabalhos na literatura sobre o comportamento cinético e crescimento das cadeias em processos de policondensação enzimática. Dessa forma, a análise do comportamento das distribuições de massa molar e da cinética da reação, juntamente com a estabilidade enzimática por meio de ciclos de reutilização, é de grande importância para estudos de viabilidade técnico-econômica e escalonamento de reatores de policondensação enzimática, como forma de viabilizar futuros trabalhos envolvendo a produção industrial de PBS.

Objetivos

O objetivo geral do trabalho está relacionado à investigação do comportamento cinético da produção de PBS através da transesterificação entre o dietilsuccinato (DS) e 1,4-butanodiol (BDO) catalisada por lipase imobilizada (N435). Além disso, foram investigados o desempenho da enzima em teses de recuperação e reuso em novos ciclos de síntese do polímero, com ênfase na análise da atividade e estabilizade enzimática durante os ciclos de reaproveitamento e das distribuições de massas molares dos polímeros produzidos, em reações livres de solvente como em solução. Para isso, os objetivos específicos foram definidos para avaliar as melhores condições de síntese, seguido da reutilização da N435 em novos ciclos de síntese nas condições selecionadas, acompanhando-se o comportamento cinético, a estabilidade biocatalítica e as distribuições de massas molares dos produtos poliméricos. Além disso, o efeito da presença do solvente (difenileter, DE) nas reações em solução foi investigado, avaliando-se a atividade e estabilidade enzimática, assim como as

distribuições de massas molares dos produtos obtidos durante os ciclos de reuso do biocatalisador.

Metodologia

Durante as reações, o comportamento cinético foi avaliado, recuperando-se os subprodutos (etanol) e pesando a massa obtida, a cada 5 minutos da reação. Para as reações em massa, foram utilizados 0,1 mol de cada monômero (DS e BDO), sob agitação de aproximadamente 400 rpm e vácuo de 0,1 atm. Nestas reações, o efeito das concentrações de N435 (5, 10 e 20 wt%) e temperatura (60 a 100 °C) foram avaliadas no processo de síntese do PBS. O término das reações foi estabelecido quando não era mais possível homogeneizar o meio com a agitação magnética. Após a síntese, os produtos poliméricos foram separados da N435 por solubilização em clorofórmio e filtração, onde o biocatalisador foi lavado com clorofórmio novamente e ambos os produtos e enzima (PBS e N435) foram levados para secagem em estufa de convecção forçada. Para as reações em solução, as quantidades de monômeros, agitação e vácuo foram as mesmas do sistema bulk, avaliando-se a adição de 5 e 50 wt% de DE no meio reacional. A quantidade de N435 foi fixada em 10 wt%, avaliando-se as temperaturas de 70, 80 e 90 °C. O procedimento de purificação consistiu na separação via solubilização da solução polimérica contendo a enzima em clorofórmio, seguido por filtração, em que o biocatalisador foi lavado novamente e levado para secagem. Os produtos poliméricos foram precipitados com etanol gelado, seguido de filtração para separação das cadeias poliméricas precipitadas, levadas em seguida à estuda de convecção forçada. O procedimento de reuso consistiu, após a recuperação, em aplicar-se novamente o biocatalisador imobilizado em novos ciclos de síntese, monitorando-se a atividade enzimática e eficiência catalítica via recolhimento dos subprodutos da reação, nas condições escolhidas para cada sistema de síntese. As caracterizações dos produtos poliméricos consistiram em determinar as distribuições de massas molares e suas respectivas médias, permitindo a análise da dispersão dos produtos formados através dos processos de síntese. Para o sistema em massa, o biocatalisador foi submetido a análise de fisiossorção em nitrogênio, para avaliar possíveis entupimentos no suporte e, para o sistema em solução, foram avaliados os efeitos do solvente na estabilidade da N435 via Microscopia Eletrônica de Varredura (para a estrutura do suporte) e Microscopia Confocal a Laser (para avaliar a quantidade de enzima no suporte da N435).

Resultados e discussão

Através do sistema em massa, foram obtidos valores de massa molar média entre 2.000 e 4.000 $g.mol^{-1}$ (M_w). Os tempos de reação variaram entre 25 e 90 minutos, justificados por limitações difusionais relacionadas a altas viscosidades no meio reacional. O comportamento cinético foi descrito através da recuperação do subproduto da reação, obtendo-se valores entre 7,45 e 8,26 g, sendo que o valor estequiométrico é de 9,20 g. As reações de policondensação enzimática foram conduzidas em temperaturas amenas (60-90 °C), devido à desativação do biocatalisador por desnaturação das proteínas em temperaturas mais elevadas. Dessa forma, os produtos poliméricos produzidos geralmente estão abaixo da temperatura limite de pré-fusão (83,6 °C), de tal modo que a reação é controlada pela difusão das cadeias poliméricas através do suporte poroso até o sítio ativo da enzima. Assim, a temperatura mostra ser um dos fatores limitantes para o processo de síntese. Com relação ao objetivo de selecionar as melhores condições operacionais da síntese, a quantidade de 10 w/w% de N435, operando a

temperatura de 90 °C, apresentou destaque. O tempo de duração foi de 90 minutos, obtendo-se 8,08 g de subprodutos, M_w de 4.000 e M_n de 1.400 g.mol^{-1}, sendo a condição aplicada ao reuso do biocatalisador. O procedimento de reutilização apresentou 4 ciclos com alta atividade enzimática. Foram observados ainda mais 2 ciclos de reuso para produção de PBS antes da completa desativação enzimática. A atividade enzimática inicial da N435 foi determinada, sendo 32,4 U.g^{-1}, passando a ser 3,8 U.g-1 ao final do reuso (atividade residual de 13,3%). Ainda referindo-se à eficiência catalítica, a análise de fisiossorção em nitrogênio foi realizada na N435 após o reuso, indicando entupimento dos poros do suporte do biocatalisador por cadeias poliméricas, contribuindo para o decaimento da atividade enzimática através do processo de reuso. Ao analisar a quantidade de subprodutos obtidos, 3,52 g de EtOH foram recuperados no 7° ciclo, (apresentando perda de 56,4%). Além disso, as massas molares obtidas ficaram abaixo dos valores representados pelos materiais de calibração da técnica de aferição de massas molares médias (580 g.mol^{-1}, via GPC), observando-se a predominância de dímeros e trímeros. Observando-se as distribuições de massas molares, nota-se uma tendência de formação de distribuições bimodais no decorrer dos ciclos, com o aumento da fração de cadeias poliméricas com menor comprimento, corroborando com o decaimento da atividade enzimática da N435 com o processo de reuso. Para o sistema em solução, os polímeros sintetizados apresentaram massas molares entre 2.250 e 3.300 g.mol^{-1} (M_w). Os tempos de reação foram de 60 e 90 minutos. Utilizando 5 wt% de DE no meio reacional, maiores taxas de recuperação de etanol foram observadas a 80 e 90°C. Os valores de massa molar ficaram entre 2500 e 3300 g.mol^{-1} (M_w), e os valores de subprodutos recolhidos ao final da reação entre 7,39 e 8,18 g. Limitações viscosimétricas ainda foram observadas na adição de 5 wt% de DE, decaindo com o aumento das temperaturas de reação. Utilizando-se 50 wt% de DE, maiores tempos de reação foram observados, devido à menor viscosidade do meio gerada pelo adição solvente. Porém, quanto maior a diluição, menores taxas de reação são observadas, indicando que o excesso de solvente afeta a atividade enzimática. Foram obtidas massa molares entre 2.200 e 2.850 g.mol^{-1} (M_w), com valores de subprodutos entre 7,39 e 8,19 g. Dessa forma, a condição de 5 wt% de DE apresentou destaque, sendo aplicada aos procedimentos de reuso, na temperatura de 70 °C (temperatura ótima de operação da N435), em reações com duração de 60 minutos. Com o reaproveitamento da N435, não foi possível realizar novos ciclos com alta eficiência catalítica, onde a atividade enzimática inicial foi determinada em 31,1 U.g^{-1}, decaindo para 4,5 U.g^{-1} após o primeiro ciclo de uso, obtendo-se valor de 1,9 U.g^{-1} ao fim do processo de reutilização (sendo realizados 3 ciclos de reuso, com atividade residual de 6,5% ao final do processo). Um grande decaimento no rendimento em massa dos produtos poliméricos foi apresentado, com valor inicial de 74,9%, decaindo para 16,9% no 3° ciclo do reaproveitamento do biocatalisador. Investigações realizadas com relação aos efeitos do DE em contato com a N435 (usando MEV) indicaram interações entre o solvente e o suporte da enzima, causando formação de maior porosidade na estrutura do suporte. Em relação à quantidade de enzima adsorvida (usando CLSM), foram observados menores quantidades de enzimas imobilizadas no suporte, devido à perda da atividade enzimática da N435 em contato com o solvente.

Conclusão

Reações de polimerização enzimática são fortemente afetadas pela temperatura, devido à alta viscosidade do meio. Como característica, estas reações apresentam temperaturas operacionais mais baixas, relacionadas à estabilidade catalítica da enzima imobilizada. Assim,

operando em temperaturas abaixo da condição limite de pré-fusão, o meio reacional apresenta maior viscosidade, causando problemas de difusão das cadeias poliméricas através do suporte poroso até o sítio ativo da enzima. A partir dos resultados das reações em massa, quando há um aumento na temperatura reacional, observam-se maiores taxas de reação de PBS, devido às menores viscosidades do meio reacional. A adição de baixas quantidades de solvente possibilitou a obtenção de maiores taxas de remoção de subproduto devido à menor viscosidade do meio reacional. No entanto, não foram observadas diferenças significativas nas massas molares médias e quantidades de etanol coletado ao final da reação, indicando que as reações são controladas pela difusão interna. O uso do solvente em excesso apresentou uma diminuição nas taxas de remoção de etanol, indicando que o solvente pode afetar a atividade enzimática da N435. Ao relacionar os procedimentos de reuso da N435 em novos ciclos para a síntese de PBS, o sistema em massa se mostrou mais eficiente, possibilitando maior estabilidade do biocatalisador. O uso de DE no sistema de síntese proposto se mostrou eficiente em apenas um ciclo de catálise, enquanto as reações em massa apresentaram 4 ciclos com boa atividade enzimática na produção de PBS.

Palavras-chave: Polibutileno Succinato; Policondensação Enzimática; Cinética; Reuso da enzima.

ABSTRACT

In this work, the conditions of enzymatic synthesis (in mass and solution) of PBS, followed by the reuse of the commercial immobilized lipase Novozyme 435 (N435) in new synthesis cycles were investigated, with emphasis on the description of the kinetic behaviour, enzymatic activity and molar mass distributions of the polymers. For the bulk reactions, 0.1 mol of each monomer (DS and BDO) were used, under agitation of 400 rpm and low pressure (0.1 atm), evaluating the effect of the concentrations of lipase N435 (5, 10 and 20 wt%) and temperature (60 to 100 °C). For the reactions in solution, the conditions were similar, evaluating the addition of 5 and 50 wt% DE in the reaction medium. The amount of N435 was fixed at 10 wt%, evaluating the temperatures of 70, 80 and 90 °C. In each system, the best synthesis conditions were selected for the analysis of N435 reuse. The bulk reactions showed diffusion limitations from the chains to the active site of the immobilized enzyme, due to the high viscosities of the medium at the investigated temperatures. In the bulk reactions, the best performance condition was with 10 wt% N435 at 90 °C. The reaction time was 90 minutes, obtaining 8.08 g of by-products (9.20 being the stoichiometric value) and M_w of 4,000 $g.mol^{-1}$. In this condition, the reuse procedure presented 4 cycles with good enzyme stability. The initial activity of N435 was 32.4 $U.g^{-1}$, decreasing to 3.8 $U.g^{-1}$ at the end of reuse. In addition, tests indicated clogging of the pores of the enzymatic support by polymeric chains after reuse, contributing to the decay of enzymatic activity. For the solution system, higher ethanol removal rates were observed with 5 wt% DE. Viscometric limitations were still observed in this condition, decreasing with increasing reaction temperatures. Using 50 wt% DE, longer reaction times were observed, due to the lower viscosity generated by the solvent. However, the higher the dilution, the lower reaction rates were observed. Thus, the condition of 5 wt% DE stood out, with Mw values between 2,500 and 3,350 $g.mol^{-1}$, being applied to the reuse procedures, at the temperature of 70 °C (optimal operating condition of the N435), in reactions of 60 minutes. In the presence of DE, the results indicated a significant loss of activity, where the initial enzyme activity was 31.1 $U.g^{-1}$, decreasing to 4.5 $U.g^{-1}$ after the first cycle of use, and 1.9 $U.g^{-1}$ at the end of three cycles. A large decrease in the mass yield of the polymer was observed, in which the initial value was 74.9 %, decreasing to 16.9% in the 3rd reuse cycle. Additional tests on the effects of DE in contact with N435 indicated interactions in the support of the enzyme, causing greater porosity, in addition to leaching of the enzyme adsorbed on the support, justifying the loss of activity of N435 Comparing the results of reuse of N435, the bulk system was more efficient, with greater stability and catalytic efficiency, while the use of diphenyl ether caused losses in the activity and catalytic efficiency of N435.

Keywords: Polybutylene Succinate; Enzymatic Polycondensation; Kinetic Behavior; Reuse of immobilized biocatalyst

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BDO	1,4-Butanediol
BET	Brunauer, Emmett and Taller Methods
Bi	Bismuth
BJH	Barrett, Joyner and Halenda Methods
CALB	*Candida antarctica* Lipase B
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CE	Circular Economy
CLSM	Confocal Laser Microscopy
Đ	Polydispersion Index
DAPI	4,6-diamidino-2-phenylindole
DE	Diphenyl Ether
DPn	Degree of Polymerization
DS	Diethylsuccinate
DSC	Differential Exploratory Calorimetry
EA	Enzymatic Activity
EA_f	Enzymatic Activity at the End of Use
EA_I	Enzymatic Before Participating in Catalysis
EtOH	Ethanol
FT-IR	Fourier-Transform Infrared Spectroscopy
GBL	γ-Butyrolactone
GPC	Gel Permeation Chromatography
HEPBP	2-hydro-4-(2,3-epoxypropoxy)benzophenone
Hf	Hafnium
HPLC	High Performance Liquid Chromatography
MK	Make-up Procedure
M_n	Number Average Molecular Weight
MSC	Mesenchymal Stem Cells
M_w	Weight Average Molecular Weight
N435	Novozym N435
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance
AO	Oleic Acid
PBS-co-BM	Polybutylene Succinate-co-malate
PBSmTESn	Polybutylene/triethylene Succinate
PBNPGS	Polybutylene Succinate-co-neopentylglycol
PBS	Polybutylene Succinate
PBSA	Polybutylene Succinate-co-adipate
PBSCL	Polybutylene Succinate-co-ε-caprolactone
PBS-DLA	Polybutylene-butylene Succinate Dilinoleate
PBS-DLS	Polybutylene Succinate-dilinoleic Succinate

PBSPS	Polybutylene Succinate-co-propylene Succinate
PBST	Polybutilene Succinato-co-terephthalate
PC	Polycarbonate
PCL	Polycaprolactone
PE	Polyethylene
PES	Polyethylene Succinate
PEST	Polyethylene Succinate-co-terephthalate
PET	Polyethylene Terephthalate
PGS	Polyglycerol Sebacate
PHA	Polyhydrodyalkanoates
PLA	Polylactic Acid
PLLA	Poly-L-lactide
PLLA-co-PMPS-co-PLLA	Poly-L-lactide-b-poly-2-methyl-1,3-propanediyl succinate-b-poly-L-lactide
PP	Polypropylene
OS	Polystyrene
PVC	Polyvinyl Chloride
REA	Residual Enzymatic Activity
AS	Succinic Acid
Sb	Antimony
SEC	Size-exclusion Chromatography
SEM	Scanning Electron Microscopy
Sn	Tin
$Sn(Oct)_2$	Tin (II) 2-ethylhexanoate
Tg	Glass Transition Temperature
TGM2_v2	Transglutaminase Variant 2
THF	Tetrahydrofuran
Ti	Titanium
$Ti(OBu)_4$	Titanium (IV) Butoxide
Tm	Melt temperature
UFSC	Universidade Federal de Santa Catarina
$U.g^{-1}$	Enzyme Activity per Gram
USA	United States of America
UV	Ultraviolet
V_f	Final Titrant Volume
V_i	Initial Titrant Volume
w/w	Mass by Mass
wt%	Percentage by Weight
Zr	Zirconium

SUMMARY

CHAPTER 1

In this chapter, the motivations and objectives of this work are presented.

1 Introduction

Through the growth of the world population and consumption demands, debate on sustainability and the development of Circular Economy (CE) models has been gaining ground. Environmental problems, such as the depletion of non-renewable energy sources and global warming, have been increasingly addressed in issues about the future of life on Earth (GEISSDOERFER et al., 2017). When addressing sustainability and CE, an integration between economic performance, social inclusion, and environmental resilience is aimed, seeking to provide subsidies for the lives of future generations (PIERONI; MCALOONE; PIGOSSO, 2019). Relating such aspects to polymers, many parameters have been debated, as these materials are among the most synthesized products in the world currently (WORTHINGTON; KUCERA; CHALKER, 2017). An important issue is that about 90% of monomers used in polymer production are based on petrochemical processes (DOURADO FERNANDES et al., 2022). Not only the monomers but another factor of wide environmental impact is also related to polymer disposal, which generates great environmental degradation when performed incorrectly (OKUNOLA et al., 2019).

As a way of mitigating the rampant degradation of natural assets, preservation goals have been developed and widely discussed. One can cite the Green Chemistry movement, which establishes 12 principles intending to provide alternatives to the consumption of non-renewable raw materials. Among the main strategies, renewable products and cleaner processes (as the bioprocesses) have been gaining ground (STERNBERG; SEQUERTH; PILLA, 2021). In the particular case of polymers, renewable sources of monomers, synthesis processes that are less aggressive to the environment, alternatives for recycling, as well as the correct disposal of these materials have been encouraged (STERNBERG; SEQUERTH;

PILLA, 2021), aiming at the development of products and processes with less environmental impact.

One of the highlighted polymeric products is polybutene succinate (PBS), which can be produced by biotechnological-based approaches (KAWAGUCHI; OGINO; KONDO, 2017),(DE MATOS COSTA et al., 2020), presenting high degradability (DE MATOS COSTA et al., 2020) and mechanical properties similar to polyolefins (HU et al., 2020). Therefore, PBS appears as a prominent alternative in the search for products less aggressive to the environment. Currently, in industrial processes, about 10 to 15 kton is already produced per year (JIANG; LOOS, 2016), being applied in different fields, such as agriculture, packaging and engineering (HU et al., 2019), in addition to standing out in the biomedical sectors (RAFIQAH et al., 2021). Regarding the application of PBS in biomedical sectors, Gigli et al. (2016) indicate the application of those materials for the development of films for tissue engineering, scaffolding for cells and carrying active principles, among other possibilities. Fabbri et al. (2018) also describe that such materials present easy synthesis strategies, tunable biodegradation, and biocompatibility, being favorable characteristics when related to the need to develop new polymeric materials in areas of added value.

The conventional industrial process of PBS uses metallic catalysts at high temperatures (DOUKA et al., 2018). Relating to aspects of sustainability, the use of metal-based catalysts is a point to be debated, as it generates environmental problems in their extraction, and may also cause toxicity when adhered to polymeric products formed, restricting the use of polymer in biomedical and pharmaceutical applications (ALBUQUERQUE et al., 2014). In addition, the use of metallic catalysts is usually associated with low catalytic selectivity and the possibility of degradation of the synthesis products when applied at high temperatures (GKOUNTELA et al., 2021). As alternatives, enzymatic catalysis-based polymerization processes have been encouraged (YANG et al., 2012),

(GKOUNTELA et al., 2021), (AZIM et al., 2006). Biocatalysts have some advantages, such as milder reaction conditions and high product selectivity, in addition to forming products free from contamination of toxic metals and the possibility of reusing them (when immobilized) (SEN; PUSKAS, 2015). However, some points still limit the replacement of metallic catalysts by enzymes, such as products with lower molar mass and longer reaction times (AGUIEIRAS et al., 2017), in addition to higher cost processes and added value to the product, requiring further exploration in studies to expand the enzymes viability on a large scale.

Some (technical) aspects of polymer production via enzymatic catalysis should be emphasized, such as problems of solubility/miscibility between the monomers due to lower reaction temperatures (AZIM et al., 2006). In addition, the milder temperature ranges used in enzymatic polymerization (restricted by the denaturation temperature of enzymes) can also lead to high reaction medium viscosity and diffusional limitations (GKOUNTELA; VOUYIOUKA, 2022). In this way, the use of solvents, such as diphenyl ether, can be an interesting alternative, improving the reaction medium homogeneity and the heat remotion rates, due to lower viscosity, in addition to enabling improvements in the physical-chemical properties of materials (DUBÉ; SALEHPOUR, 2014). When relating the use of solvents to environmental issues, it is important to mention that they can be recovered in separation steps, and can be used again (TOZZI et al., 2018), decreasing their aggressiveness toward the environment.

However, based on searches carried out in the literature, some knowledge gaps were detected regarding the enzymatic synthesis of PBS, such as the description of the kinetic behaviour (important for modelling and scaling up of the process), as well as the recovery and reuse of biocatalysts applied in the synthesis (being important issues on the evaluation of the cost involved in production scale-up). In addition, the description of characteristics such as average molar mass and chain distribution of the polymeric products throughout the reuse of

the biocatalyst is of great value, because it describes catalytic efficiency and the effect of enzymatic activity loss on the dispersion of the polymeric chains (uniformity of the length of the products).

Thus, this work aims to describe the kinetic behaviour and characteristics (such as degree of polymerization, DPn, polydispersion index, Đ, and molar mass distribution) of PBS produced via enzymatic polycondensation using Novozym® N435. Still, the conditions for the best synthesis efficiency (in the proposed reaction system) were investigated, followed by the recovery of the biocatalyst, and its reuse in new synthesis processes (until the complete loss of catalytic activity), in bulk and solution reactions. With this, the effect of N435 reuse in the enzymatic synthesis of PBS was evaluated, in addition to verifying which reaction system (bulk or in solution) offers the greatest advantages regarding the reuse of the biocatalyst (greater stability, enzymatic activity and uniformity of the synthesized polymeric products).

To respond these answers, the present work was divided into 5 chapters. The first chapter presents the motivations and objectives of the work (Chapter 1), followed by the theoretical framework involved in the literature review (Chapter 2). Subsequently, the results of bulk (Chapter 3) and solution reactions (Chapter 4) were addressed, to describe the kinetic behaviour and the characteristics of PBS produced through enzymatic transesterification. Chapters 3 and 4 also present the results of lipase N435 reuse, with the later comparison of the results and the main concluding remarks highlighted in the conclusions (Chapter 5). In the end, this work seeks to point out which system has the best efficiency and catalytic stability in the reuse of N435 for PBS synthesis, based on the operational parameters proposed by the study.

1.1 Objectives

1.1.1 General objectives

This work aimed to describe the kinetic behaviour of the synthesis of PBS through enzymatic transesterification of succinic diester and 1,4-butanediol using immobilized lipase (N435) in bulk and solution reactions. Moreover, the recovery and reuse of the biocatalyst in new PBS production cycles were investigated, to evaluate the enzymatic activity and stability in the reuse, as well as the polymerization degree and molar mass distribution of the polymeric materials, both in bulk and solution reactions.

1.1.2 Specific objectives

Among the specific objectives of this work, one can cite:

1. Elaborate a critical literature review of articles addressing PBS and succinic (co)polymers synthesis and applications;
2. Investigate the effect of operation conditions (reaction temperature) and enzyme concentration on the reaction kinetics and molar mass distribution of PBS synthesized via enzymatic bulk polycondensation using lipse N435 Novozyme as biocatalyst.
3. Evaluate the recovering the immobilized biocatalyst N435 and catalyst stability for reuse in PBS enzymatic bulk polymerization;
4. Evaluate the effects of solvent (diphenyl ether) in the enzymatic polycondensation of PBS with Novozym N435 on the reaction kinetics and molar mass distribution;
5. Investigate the recovering the immobilized biocatalyst N435 and catalyst stability and effciency for reuse in PBS enzymatic solution polymerization with diphenyl ether as solvent;

6. Compare the reuse of N435 in bulk and solution PBS enzymatic polycondensation reactions, pointing out catalytic efficiency and consistency of synthesis products between the biocatalyst recyclability processes.

CHAPTER 2

Chapter 2 presents the theoretical structure of the research, focusing on the origin of monomers, the use of enzymes in synthesis and applications of Succinic Polymers, with emphasis on PBS. A review article based on Chapter 2 will be submitted to a scientific journal.

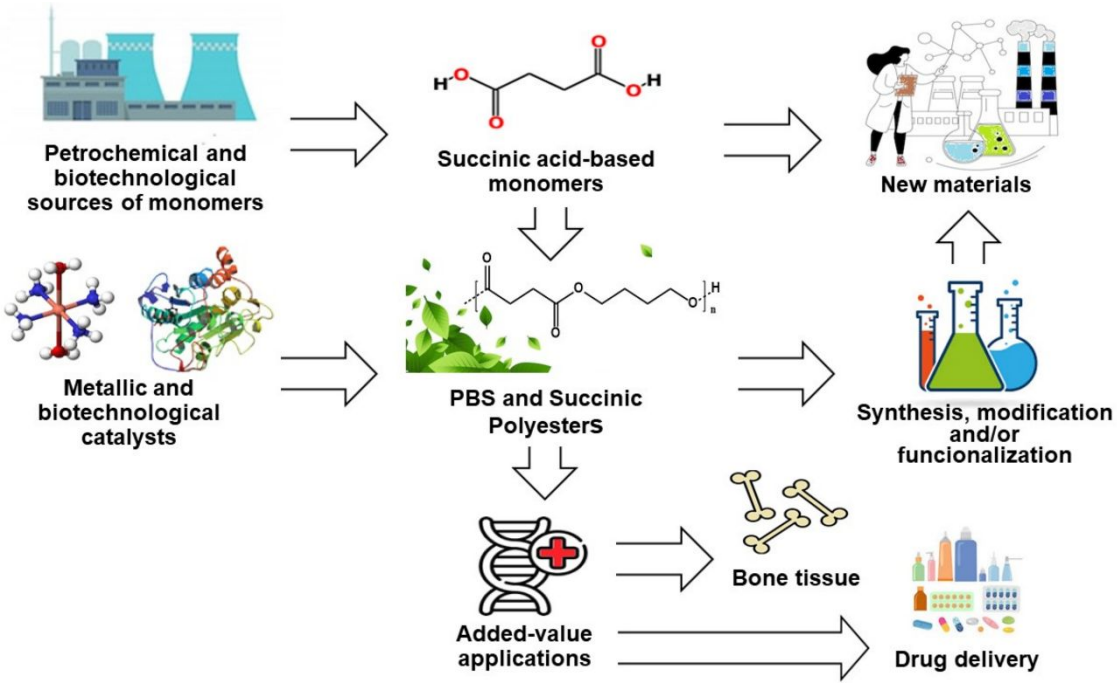
2 Current Status and Perspectives on the Green Synthesis of Succinic Polyesters for Value-Added Applicatins

Abstract

Polybutylene succinate (PBS) and other succinic (co)polyesters are biodegradable polymers with favorable mechanical and thermal properties that find use in many applications. Due to environmental concerns, polymers based on succinic acid (SA) have been gaining attention, as SA can be produced through biotenological processes. Thus, this review aims to highlight the synthesis and characteristics of PBS and other succinic copolyesters, with emphasis in the works employing metallic catalysts and enzymes. In addition, the modification of the macromolecular structure by copolymerization or post-polymerization is also discussed. Currently, metallic catalysts are normally used in the synthesis of these materials, under conditions of high temperatures, which can favor the occurrence of thermal degradation, increasing the dispersion of chain length distributions. Moreover, the incrustation of metallic catalysts in polymeric materials makes their application in biomedical products difficult, due to toxicity requirements. In this context, enzymatic catalysis has been taking up space, offering milder synthesis temperatures, high selectivity and uniformity of synthesized products. This biotechnological route can substitute oligomerization processes with metallic catalysis in future industrial processes, producing materials free from metallic contamination. In addition to production by catalytic routes, trends for future applications of succinic (co)polyesters are presented, with emphasis on the value-added materials sectors.

Keywords: Polybutylene Succinate; Succinic (co)Polyesters; Synthetic Biobased Polymers; Trends in Added-value Applications.

GRAPHICAL ABSTRACT



2.1 Introduction

Present in different fields of human activity, polymers have vast importance in societies and in the world economy with a production of about 320 million tons of synthetic polymers per year (PALETTA et al., 2019). Through technological and industrial development, these materials are applied in the most varied segments, such as automotive (components of the structure of automobiles), pharmaceutical (carrying of drugs), agricultural (dispersion of fertilizers), food (intelligent packaging for conservation of food), among others (XIAO; FONTAINE; BOURBIGOT, 2021). Concomitantly with the growing production and applications of plastic materials, there is a growing concern regarding the environmental aspects of non-biodegradable polymers. Additionally, the most consumed polymers worldwide is derived from petroleum, which is a non-renewable source of carbon (MORALES et al., 2021). According to World Economic Forum (2016), 6% of the extracted fossil oil is destined for the synthesis of polymers, and projections show that the amount will increase to 20% in 2050. Among the synthetic polymers, polyolefins, such as polyethylene (PE) and polypropylene (PP), represent about 62% of world production. These polymers are almost entirely produced from non-renewable fossil derived monomers (OJEDA, 2013), and are non-biodegradable, as the backbone of the main polymer chains are composed of carbon-carbon linkages and the microorganisms in nature are not used to degrade those synthetic macromolecular structures.

Due to these drawbacks and the increasing awareness and prevention of environmental degradation, based on aspects of Green Processes and Green Products, renewable polyesters have been gaining space. In this case, residual organic matter from industrial and food processes is used to produce polymeric raw material (IOANNIDOU et al., 2020). In addition, the production of biodegradable (co)polyesters has been evidenced, due to its polymeric chain being more easily decomposed. In its structure, generally, ester, ether, amine, and amide-type

bonds are present, and the metabolism of microorganisms produces enzymes capable of hydrolyzing polymeric chains with such chemical bonds (COSTA et al., 2014).

Besides, in environmental discussion topics, global agreements aim to reduce greenhouse gas emissions, in addition to the search for raw materials from renewable source. Zhang et al., (2019) indicate, for example, the use of microorganisms for the fixation of atmospheric carbon through the fermentation of biomass in the synthesis of compounds of industrial interest, such as monomers for the production of renewable polymers.

In this scenario, polybutylene succinate (PBS) is a biodegradable alternative, and its constituents (monomers) can be produced through the fermentation of biomasses (KAWAGUCHI; OGINO; KONDO, 2017).

Referring to the synthesis of PBS, the step-growth polymerization process uses succinic acid and/or succinic anhydride, 1,4-butanediol, and related esters, such as diethyl succinate (JIANG; LOOS, 2016), and can also use metal catalysts (DÍAZ; FRANCO; PUIGGALÍ, 2014), and/or enzymatic catalysts (YU et al., 2012). Cinar et al., (2020) indicated that in 2019, 4% of the world production of bioplastics was from PBS, being applied in different fields, such as agriculture, packaging, engineering, and medical articles, due to its good mechanical and thermal properties (HU et al., 2019). Regarding the degradability of PBS both by chemical hydrolysis and enzymatic routes, Dutra et al. (2022) indicated that the chemical degradation is influenced by the temperature and pH, where the increase in temperature in a basic medium (pH 10) favored the degradation. Still, in a test applying different enzymes (amylase, protease, cellulase, cutinase and lipase), the results were more promising, with mass loss values reaching 100 % in 4 weeks (applying cutinase).

PBS is produced mainly in two-step processes (DUTRA; NELE; PINTO, 2018), where low molar mass chains are obtained in the initial step-growth polymerization stage, called oligomerization. In the second stage, named molten state polycondensation, polymer

chains of greater molar mass are formed. The process occurs in two stages because the oligomers, formed in the esterification between diacids and dialcools, result in materials with poor thermomechanical properties for commercial use. Therefore, a later chain growth process is required (transesterification) (BASSYOUNI; JAVAID; UL HASAN, 2017) to obtain products with higher average molar mass, and thus, better thermal stability to be processed by traditional methods of molding and extrusion (XU et al., 2011). Seeking polymeric materials with controlled physicochemical properties, Xu and Guo (2010) described that the addition of comonomers to the PBS synthesis process leads to the production of copolymers with different characteristics, which may have different applicability.

Regarding to oligomerization step, high temperatures (180-280 °C) and metallic catalysts are typically applied in the synthesis processes. High temperatures can allow thermal degradation side reactions, increasing the polydispersion indexes of length size chain distribution (YANG et al., 2012). In addition, metallic catalysts can present low product selectivity (generating unwanted products) and also deposition of toxic metals in the synthesis products, limiting some applications, such as in packaging and products with biomedical and pharmaceutical purposes (ALBUQUERQUE et al., 2014). A promising alternative is the use of lipases as biocatalysts in step-growth polymerization processes for the synthesis of aliphatic polyesters (XU et al., 2008). This type of catalyst enables milder reaction temperatures, high selectivity and products free of metal deposition (YANG et al., 2012), being an alternative to the conventional oligomerization process with metallic catalysts. Moreover, it is an alternative with lower environmental impacts, both in terms of its origin of production and in its aggressiveness to the environment (MONDELLO; SALOMONE, 2020), complying with aspects of processes based on green chemistry.

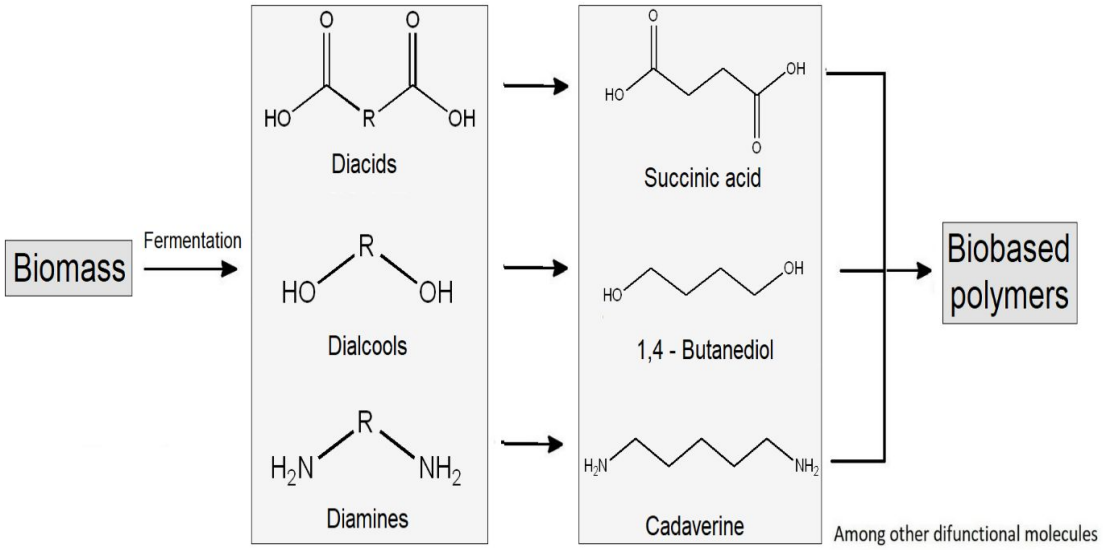
When referring to the physicochemical characteristics of PBS and succinic copolymers (such as biodegradability, good thermal properties, processability and chemical resistance), Rafiqah et al., (2021) pointed them out as promising materials for application in the biomedical area (in addition to other sectors of the industry). Along the same line, Gigli et al., (2016) indicated the possibility of using succinic copolyesters (in the biomedical sector) for making films for tissue engineering, scaffolding for cells and carrying active principles, among other possibilities.

Based on searches carried out in the literature, some review articles addressing PBS and succinic (co)polymers were found (XU; GUO, 2010). Based on these studies, our work seeks to emphasize and elucidate issues related to the synthesis of succinic (co)polymers and the industrial/commercial production of PBS, namely: monomeric production routes and synthesis mechanisms, highlighting renewable processes and obtaining of materials that are less aggressive to the environment. Differently of the most published reviews, this work presents a systematic comparison between operational aspects related to the use of (organo)metallic and biotechnological catalysts, describing properties of the synthesis products and the advantages of lipase-catalyzed PBS synthesis (based on Green Chemistry and environmental preservation). In addition, the main modification and functionalization techniques applied to these materials is discussed, seeking products with controlled physicochemical properties (such as textural properties, degradability, high molar mass, among others). Finally, a discussion about the application of PBS and succinic (co)polymers in value-added sectors will also be presented, relating their properties and future perspectives for these materials.

2.1.1 Bio-Based Monomers and Polymers

Recent studies highlight the production of monomeric raw material by biomass fermentation processes (IOANNIDOU et al., 2020). Different renewable monomers produced by fermentation processes may be further modified by chemical and/or enzymatic routes (KAWAGUCHI; OGINO; KONDO, 2017), as can be seen in Figure 1:

Figure 1: Synthesis of bifunctional monomers by biomass fermentation used as a basis for the production of biobased polymers.



Source: from author

As shown in Figure 1, through the cleavage of glucose and xylose molecules, it is possible to synthesize different monomers of interest for the synthesis of biopolymers, with emphasis on the polycondensation process. As a complement to Figure 1, some monomers that can be obtained (classified by functional groups) in biorefinery processes are presented in Table 1.

Table 1: List of possible monomers produced through biotechnological processes.

Functional Group	Monomer
Carboxylic diacids	Succinic acid (LEE et al., 2022), fumaric acid (LI et al., 2017), malic acid (WEST, 2017), itaconic acid (SCHLEMBACH et al., 2020), and adipic acid (KRUYER et al., 2020).
Amino acids	γ-Aminobutyric (JORGE et al., 2017) acid and 5-aminovaleric acid (SHIN et al., 2016).
Dialcools	1,2-Propanediol (SAXENA et al., 2010) and 1,3-propanediol (ESPINEL-RÍOS; RUIZ-ESPINOZA, 2019).
Diamines	Cadaverine (BHATIA et al., 2015) and putrescine (QIAN; XIA; LEE, 2009).
Aromatic compounds	Caffeic acid (KAWAGUCHI et al., 2017), shikimic acid (ESCALANTE et al., 2010), cinnamic acid (VARGAS-TAH et al., 2015), 3-amino-4-hydroxybenzoic acid (KAWAGUCHI et al., 2015) and p-aminobenzoic acid (AVERESCH; WINTER; KRÖMER, 2016).

Source: from author

By the monomers presented in Table 1, a vast number of bio(co)polymers can be synthesized, and depending on their physical-chemical characteristics, they can be used as

substitutes for polymers obtained from a fossil matrix (XIAO; FONTAINE; BOURBIGOT, 2021). Polylactic acid (PLA), polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) can be cited as examples of these materials (SAMROT et al., 2020). In this scenario, bio-based polyesters have been gaining prominence, as described by Kawaguchi, Ogino and Kondo (2017). The authors emphasize the use of materials of biotechnological origin, combining them with studies on the production of different bio-based monomers and microorganisms involved in the fermentation process. Chinthapalli et al., (2019) forecast the production of about 3.8 million tons of new bio-based polymers by 2025. It is important to note that, as described by Xiang et al., (2021), bio-based polymers are identified as materials of biotechnological origin; not necessarily biodegradable. Biodegradable materials are those that can be degraded by microorganisms, such as bacteria, fungi and naturally occurring algae. Inferring about sustainability concepts, the following sessions will be based on a description of the production of monomeric raw material for the synthesis of biopolymers, pointing out the main routes for their obtention.

2.1.2 Succinic Acid-based Monomers

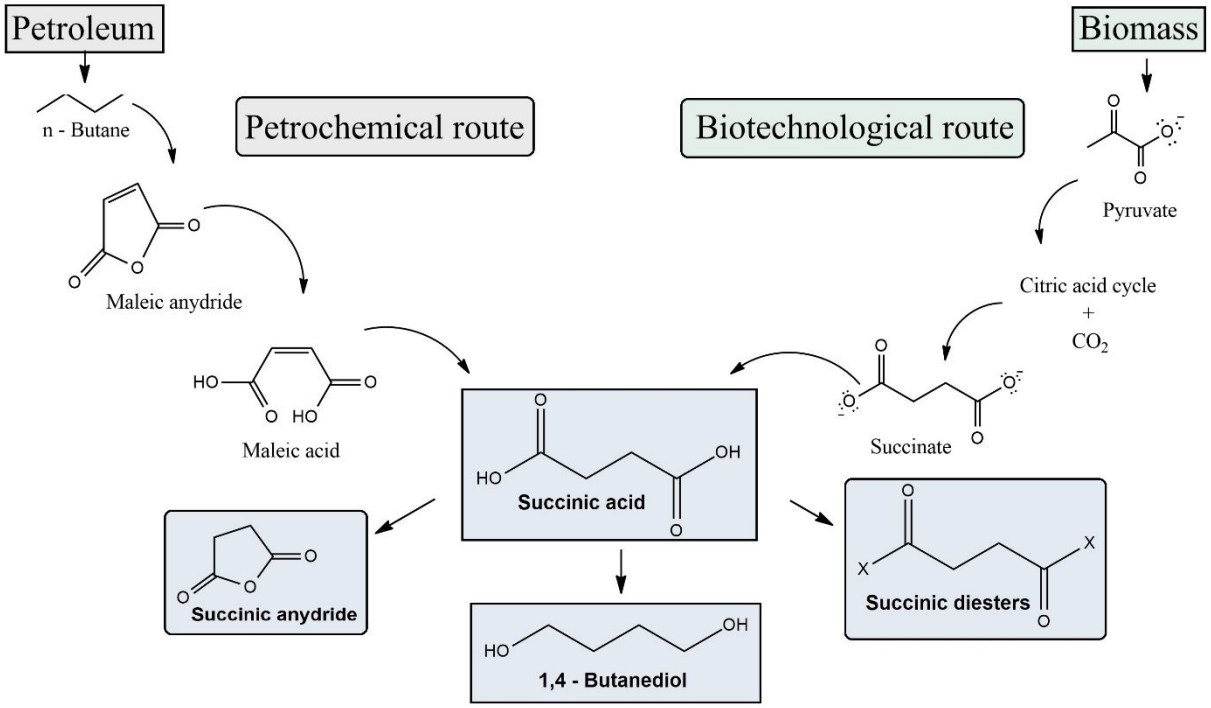
When regarding to renewable constituents from polymer synthesis, succinic acid (SA) has been showing itself increasingly promising, being a precursor to several products of industrial interest (DAI et al., 2020). SA is still considered a route to produce diverse compounds, being considered a building block for secondary products for the chemical, pharmaceutical, food, and agricultural industries (KARTHIK; RATHINAMOORTHY, 2017).

Ladakis et al., (2020) indicate that SA may be used to produce succindiamide, 1,4-diaminobutane, succinonitrile, dimethyl succinate, diethyl succinate, 1,4-butanediol, N-methyl-2-Pyrrolidone, 2-pyrrolidone, tetrahydrofurane, succinic anhydride (and maleic

anhydride) and γ-butyrolactone. In agreement, Ge et al., (2018) indicates the production of 30.000-50.000 tons os SA per year.

In consolidated industrial processes, SA is synthesized by a fossil source, through the oxidation of n-butane or benzene, forming maleic anhydride, followed by hydration to form maleic acid. Finally, maleic acid is converted to SA by the addition of hydrogen (reduction reaction) in π-type bonds between the carbons, by hydrogenation processes (KUMAR; BASAK; JEON, 2020), as described in Figure 2.

Figure 2: Schemes of Biotechnological and Petrochemical routes for the synthesis of SA and derived products for synthesis of biopolymers.



Source: from author

Through biotechnological processes, *Actinobacillus succinogens* and *Basfia succiniciproducens* (among others) are often used for the synthesis of this building block (KAWAGUCHI; OGINO; KONDO, 2017). Zhang et al., (2017) describe that the

fermentation process for SA production involves carbon dioxide fixation (an essential factor to combat environmental problems) in anaerobiosis, through the consumption of biomass, as described in Figure 2. The figure also shows the petrochemical synthesis route of SA, in addition to other precursors of synthesis of polymeric materials described in the following sections.

Also, it is important to mention, according to Börner and Berlin (2012), that malic acid is a compound found in nature, such as in green apples and other green fruits, and can be explored as a building block for different compounds, an example of which is SA.

Dickson et al., (2020) points out an increase in research works and interest in its production by biotechnological processes (ERICKSON; NELSON; WINTERS, 2012). Oliveira (2014) describes that the development of biotechnological processes for SA production has presented interesting alternatives for the synthesis of the compound, providing good yield results in industrial processes. Continuing the description of succinic monomers, notes on succinic anhydride will be presented below.

2.1.3 Succinic Anhydride

When referring to the production of succinic anhydride, Torres et al., (2016) describe that the most used process is the dehydration of succinic acid, at high temperatures, and maleic anhydride can also be used, through hydrogenation processes. Furthermore, they emphasize the use of metal-based catalysts for the processes, which are not always environmentally friendly.

Hong et al., (2011) indicate synthesis routes in which cyclic products such as γ-butyrolactone (GBL) and tetrahydrofuran (THF) can be obtained based on succinic acid and succinic anhydride. In such mechanisms, metal-based catalysts such as alumina, palladium, and ruthenium are applied. Li, Tian and Shi (2010) indicate such products as valuable for the

world market, either as solvents or as monomers for polymer production, in addition to being used as a reaction intermediate for the chemical, pharmaceutical, and food industries.

Wan et al., (2018) indicate succinic anhydride as an important bio-based precursor, produced using biomass in processes that aim environmental conservation, replacing different compounds of fossil origin. Another possible succinic monomer is the succinic diesters, which will be described in the next section.

2.1.4 Succinic Diesters

Succinic diesters, produced from succinic acid (DUDÁŠ et al., 2014) or succinic anhydride (WAN et al., 2018), are precursors of different organic compounds, both for the pharmaceutical and agrochemical industries (JUNG et al., 2003). Dawar, Bhagavan Raju and Ramakrishna (2011) indicate the importance of diesters for the synthesis of polymers, perfumes, and other pharmaceutical intermediates.

As mentioned, both succinic acid and succinic anhydride can have fossil or biotech origins (KAWAGUCHI; OGINO; KONDO, 2017),(KUMAR; BASAK; JEON, 2020), and the esterification reaction is usually performed in the presence of catalysts, such as zirconium oxide, sulfuric acid and tert-butyl methyl ether, in homogeneous or heterogeneous processes (DUDÁŠ et al., 2014), (DAWAR; BHAGAVAN RAJU; RAMAKRISHNA, 2011).

Referring to the production of succinic diesters (DUDÁŠ et al., 2014), (ORJUELA et al., 2012) report the synthesis by reactive distillation processes (using an excess of alcohol), with high yields of diesters. Also, Daviot et al., (2019) present a study of the synthesis of different succinic diesters, both by succinic acid esterification routes and by diethyl succinate transesterification, testing various alcohols. In their conclusions, they indicate that alcohols with less than 6 carbons provide better yields, greater efficiency in esterification processes (compared to the transesterification route) and greater efficiency (yield) of chemical catalysts

compared to enzymatic ones, though the use of these latter is justified by the advantage of requiring lower reaction temperatures. Still based on polymeric raw material, the following section provides information on 1,4-butanediol, a dialcohol widely used in the production of polymers (JIANG; LOOS, 2016).

2.1.5 1,4-Butanediol

Debuissy, Pollet and Avérous (2017) describe that the production of 1,4-butanediol (BDO) is mostly of fossil origin (using maleic anhydride, acetylene, butane, propylene and butadiene), but that the company Genomatica (USA) has already started to commercialize BDO from the hydrogenation of SA since 2007.

Yim et al., (2011) reported the direct production of BDO through metabolic processes of an *Escherichia coli* strain, via fermentation of carbohydrates (glucose, xylose, sucrose, and mixed sugar compounds derived from biomass), obtaining a concentration of BDO in order of 18 gL^{-1} in the biotechnological processes.

Jiang and Loos (2016) pointed out BDO as a building block for polymers, with an annual production of approximately 2.5 million tons. In polymerization processes, it is widely used for the synthesis of polyesters, polyamides, and polyurethanes (DEBUISSY; POLLET; AVÉROUS, 2017), as well as its (co)polymers with biodegradable properties. Based on the subjects presented up to this point, the next topics will show the mechanisms for the synthesis of polyesters, both by esterification routes and by transesterification for the growth of polymeric chains.

2.2 Synthesis of Polyesters

In step-growth polymerization processes, the chain growth occurs in stages, where bifunctional (or polyfunctional) monomers react, through mechanisms involving their

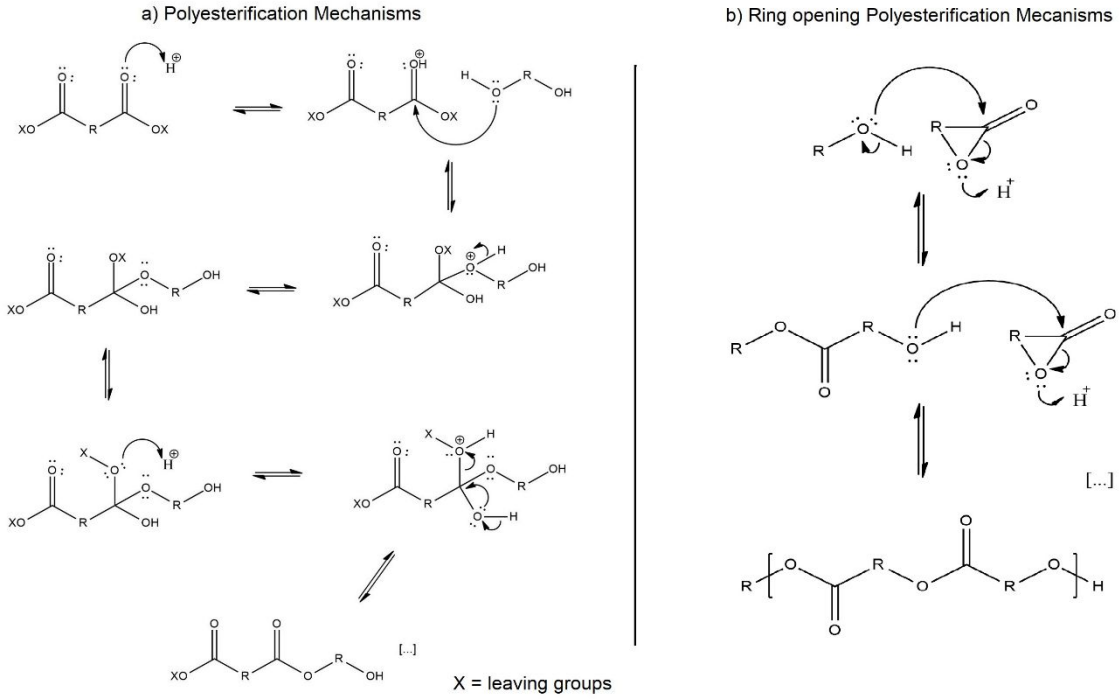
functional groups (BHAT; KANDAGOR, 2014) with polyethylene terephthalate (PET) synthesis as an example.

Within the stepwise growth mechanism, esterification reactions are usually present. Polyesterification route between bifunctional (or polyfunctional) molecules results in the production of oligomers (low molar mass polymer chains, with a degree of polymerization of up to 10 units) and water, which must be removed from the system to improve the reaction yield by shifting the chemical equilibrium (YANG et al., 2012).

Another route is polymerization by transesterification, using compounds based on diacids (such as diesters) and diols, to produce longer chains. In this process, the by-products are low molar mass molecules (such as water, alcohols, halogenated acids, among others), which must also be removed from the system to improve the reaction yield (XU; GUO, 2010). In Figure 3 the polyesterification mechanism is represented generically.

Also, ring-opening reactions can be used for the polymerization process, employing cyclic monomers. In this mechanism, catalysts are typically used to improve the spontaneity of ring-opening reactions, and the absence of by-products formation is the main advantage of this polymerization strategy (XIAO; FONTAINE; BOURBIGOT, 2021). In Figure 3 a generic mechanism for polyesters synthesized via ring opening is displayed.

Figure 3: Generic mechanisms of polyesterification, represented by nucleophilic attacks on carbonyl compounds and ring-opening.



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However, it is important to emphasize that polymerization processes can employ catalysts, aiming to reduce the activation energy, where the chemical route uses catalysts of metallic origin (DÍAZ; FRANCO; PUIGGALÍ, 2014) and the biotechnological route uses enzymes (YANG et al., 2012). In the following session, aspects of the use of enzymes, particularly esterases, in the production of polyesters will be discussed.

2.2.1 Enzymatic Polyesterification

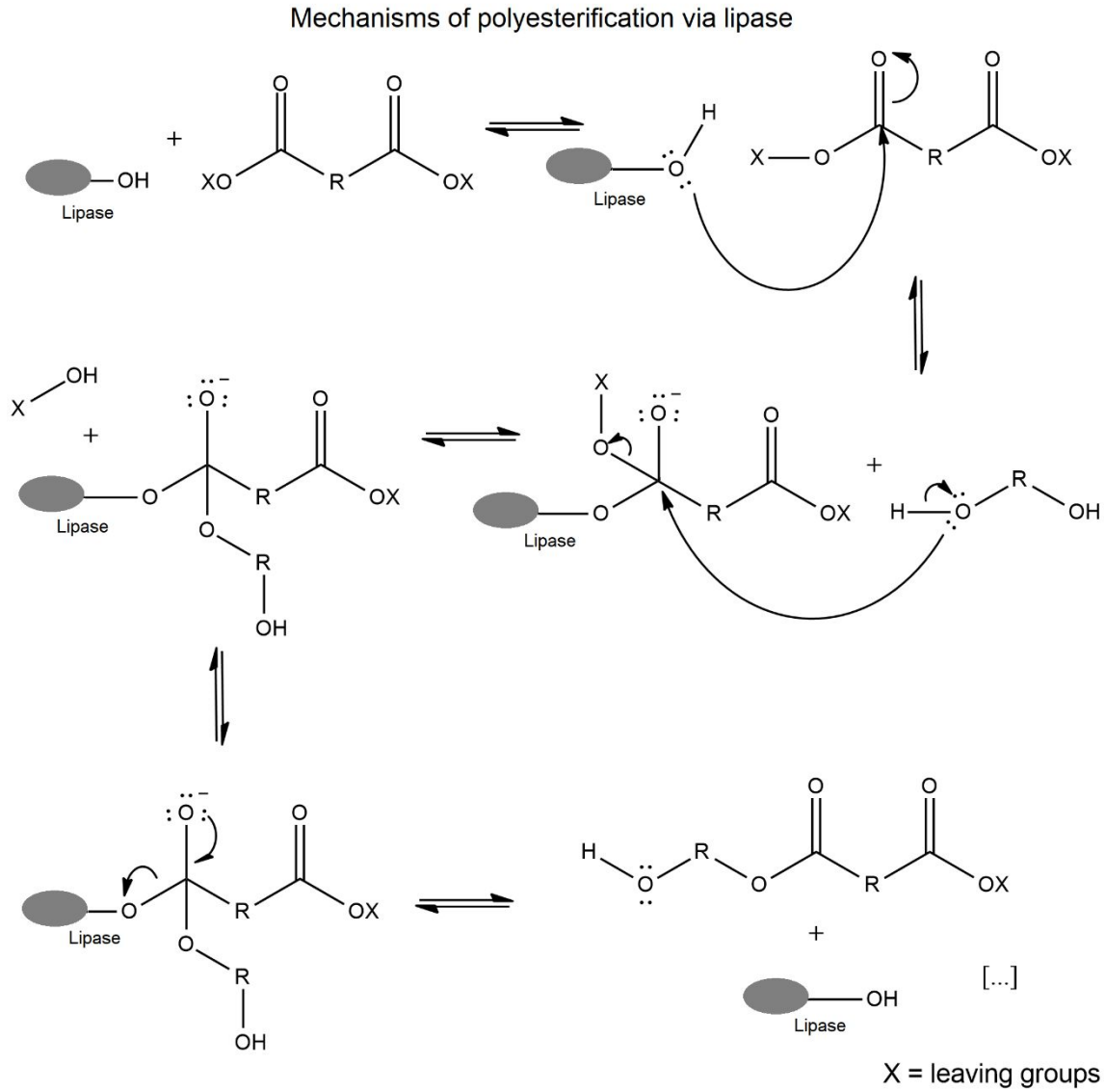
As presented by Yu et al., (2012), polycondensation reactions (including the polyesterification reactions), are commonly conducted at high temperatures (180-280 ºC), which can generate undesirable products, such as olefins and acids. Additionally, these reactions can be performed in presence of catalysts of metallic origin, such as manganese

acetates, zinc, calcium, cobalt and magnesium, antimony oxide, and titanium oxides. One of the disadvantages of using these catalysts is their low catalytic selectivity, in addition to possible depositions in the synthesized material, which can be unfavourable depending on its application, especially in the biomedical area (toxicity) (ALBUQUERQUE et al., 2014).

Thus, enzymatic catalysts are an alternative, due to high selectivity regarding substrates and products, in addition to allowing milder reaction temperatures (YANG et al., 2012), as well as the possibility of reuse, in case of immobilized enzymes (SEN; PUSKAS, 2015).

Stergiou et al., (2013) point to the mechanism of (trans)esterification by lipase, where they indicate that initially, the carboxylic group (described as acyl group in the study) forms an intermediate carboxyl-enzyme complex with lipase, releasing water. Then, the connection of the alcohol (hydroxyl group) to this carboxyl-enzyme complex is introduced, forming the lipase-ester complex, followed by the detachment of the ester from the enzyme's catalytic centre, enabling new catalysis, as can be seen in Figure 4.

Figure 4: Generic mechanisms of polyesterification through the use of lipase.



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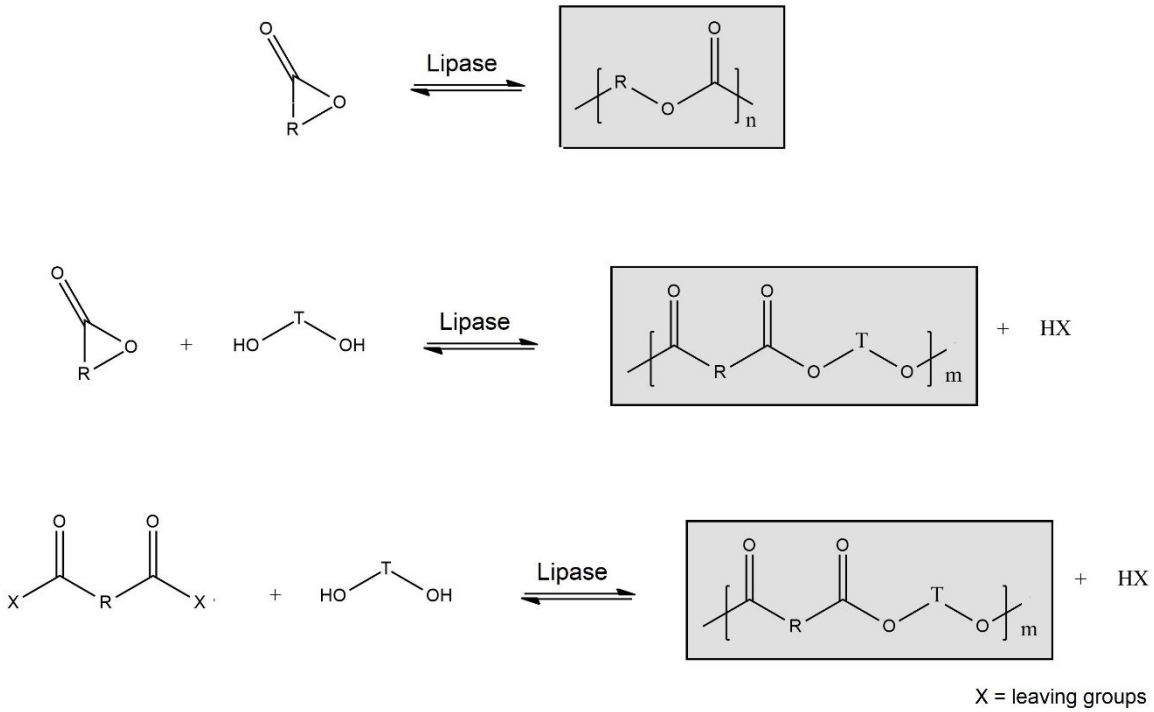
In enzymatic polyesterification systems, Novozym 435 (N435) is a biocatalyst widely used, comprised of lipases from *Candida antarctica* (CALB) immobilized in Lewatit VPOC 1600 macroporous resin (GKOUNTELA et al., 2021), through adsorption processes via interfacial activation with the polymeric support (ORTIZ et al., 2019). CALB belongs to lipases class, which catalyzes the hydrolysis of fatty acid esters in aqueous systems. It is

stable and has good catalytic activity in organic solvent systems, which allows its use in polymerization reactions (YU et al., 2012), as can be seen in Figure 5.

Figure 5: Generic representation of possible enzymatic polyesterification routes via ring-opening and polycondensation.



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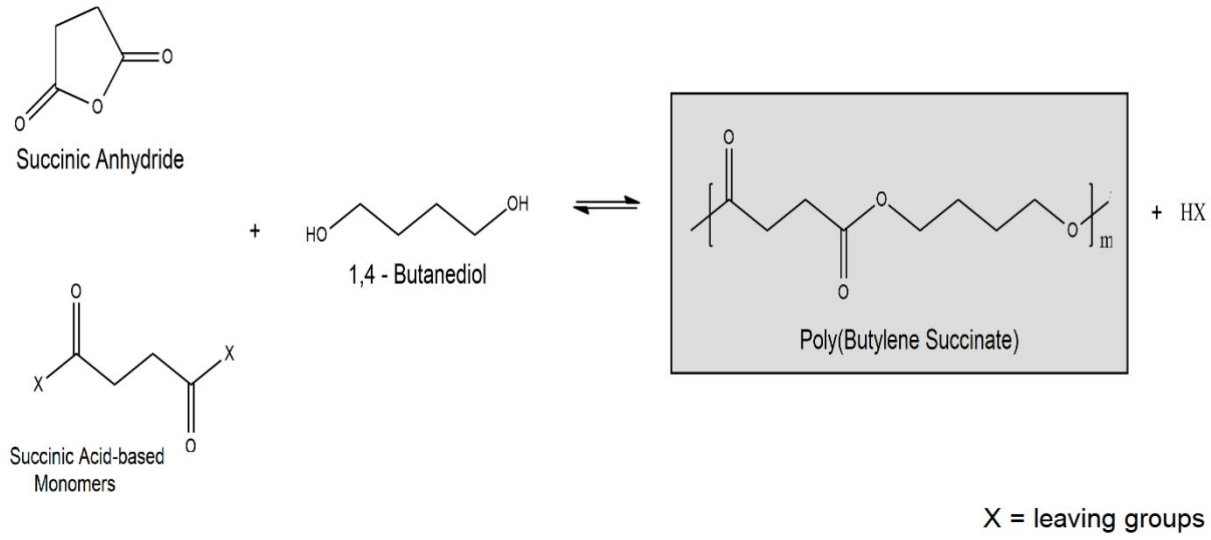
As seen in Figure 5, lipases enable two main catalytic routes: synthesis of polyester via ring-opening (mainly lactones); and polycondensation, allowing polymerization between diacids, diols, and their respective esters (YU et al., 2012). It is also important to note that through their catalytic activity, lipases enable transesterification reactions (YU et al., 2012), which is an important route for the growth of the polymer chain. Also, as indicated by Guckert et al. (2022), the study of catalytic stability and reaction kinetics in biocatalyzed processes is of great importance, for better understanding of process performance and the possibility of reusing the biocatalyst (when immobilized).

The following section will present considerations about polybutylene succinate (PBS), involving aspects such as synthesis (including enzymatic synthesis), applications, as well as major trends and challenges.

2.3 Polybutylene Succinate (PBS)

Polybutylene succinate (PBS) can be obtained using SA-based monomers (such as succinic anhydride and related esters), and BDO (YU et al., 2012), according to the polymerization routes described in Figure 6.

Figure 6: Representation of the routes for the synthesis of PBS, either by succinic anhydride or by linear monomers based on SA.



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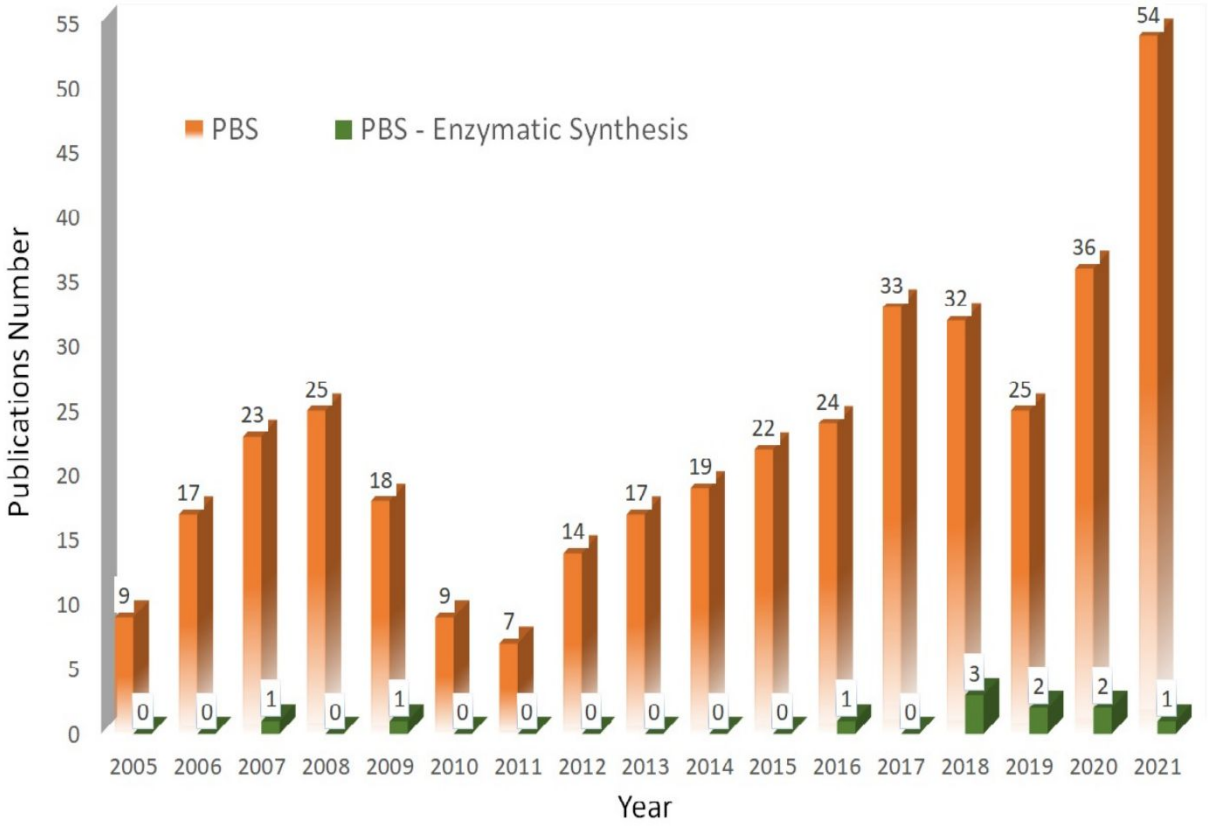
Regarding the physical-chemical characteristics, PBS is an aliphatic and whitish polyester. de Matos Costa et al., (2020) pointed out that the mechanical properties of PBS are similar to those of conventional polyolefins, with moderate hardness and good resistance to stiffness and traction. Zhang et al., (2012) described that the material has a density of 1.25

g.mL^{-1}, a melting temperature of 115 °C, and a glass transition temperature of -30 °C, with a degree of crystallinity between 65-71 % (GKOUNTELA et al., 2021). It is classified as thermoplastic and can be moulded in its molten state (MIZUNO et al., 2015). Still, its mechanical properties allow good processability by different methods, such as injection moulding, film extrusion, flat and split wires, filament, among others, with promising application in polymers for engineering, agriculture, and medical articles (HU et al., 2020). It is important to note that biotechnological processes for obtaining polymers, as well as in the synthesis of PBS, have been gaining space in the academic environment, as pointed out by Hu et al., (2020) and summarized in Figure 7.

Figure 7: Evolution of the number of publications on PBS and PBS synthesized with biocatalysts obtained by searching the Scopus database, with the keywords polybutylene succinate and polybutylene succinate (for publications involving PBS) and enzymatic synthesis (for publications involving biocatalyzed PBS), in the period from 2005 to 2021



Source: from author

Figure 7 shows the number of publications in the Scopus database, between 2005 and 2021, through the search filtered only on keywords related to the publications using the terms polybutylene succinate (numbers symbolized in the orange columns) and polybutylene succinate and enzymatic synthesis (numbers symbolized in the greenish columns). It is noteworthy that in the search, synthesis and/or application works were not distinguished, the objective was to verify the number of publications involving PBS, in the period described, as a way to describe the trend of studying the material. As noted, there is an increase in the number of publications on PBS; and publications on the enzymatic synthesis of PBS have been mildly developed in recent years.

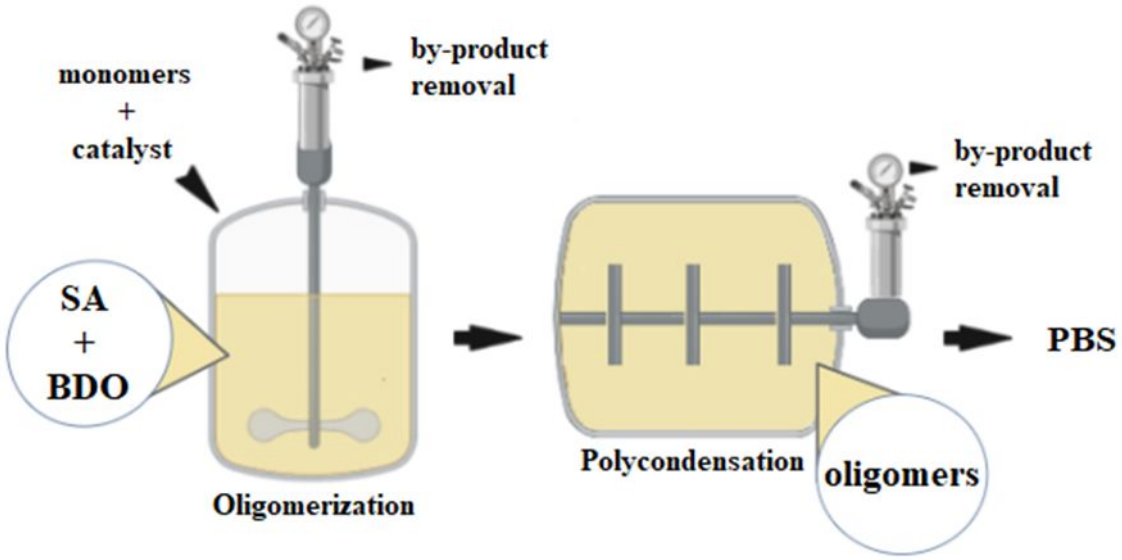
However, Hu et al., (2020) described that increased interest in PBS is associated with good degradability and sustainable origin of the monomers. Mizuno et al., (2015) indicated that ester-type bonds can be chemically cleaved in water, in addition to describing that stereochemistry, the flexibility of molecular chains, and degree of crystallinity are decisive in the biodegradability of the material, also showing good performance when recycled. Rizzarelli and Carroccio (2009) point out that PBS is one of the most attractive biodegradable polyesters, with mechanical properties similar to PET. Industrially, it is produced in stages, which will be presented below.

2.3.1 PBS production

In commercial processes the synthesis of PBS occurs mainly in two steps (stages), being: [a] Oligomerization, occurring in the first stages of the synthesis process, where the molecules of the monomers are (trans)esterified, forming chains of low molecular mass, called oligomers. In this stage, the viscosity of the reaction medium is low, and agitation allows contact between the functional groups (NISOLI; DOHERTY; MALONE, 2004); and

[b] polycondensation in the molten state, where oligomerization products undergo transesterification reactions, to increase the polymer chains. This process does not use solvents nor toxic components and is conducted under vacuum, at high temperatures, between the Tg and melting temperature of the oligomers, allowing the mobility of the chains in the amorphous domains of the material, to reach functional groups and, thus, the condensation between them increases the molar mass (MENDES et al., 2014). Figure 08 demonstrates this process in a simplified way.

Figure 8: Representation of industrial PBS synthesis, through the oligomerization step (formation of short-length chains) and subsequent polycondensation to increase the polymeric chains.



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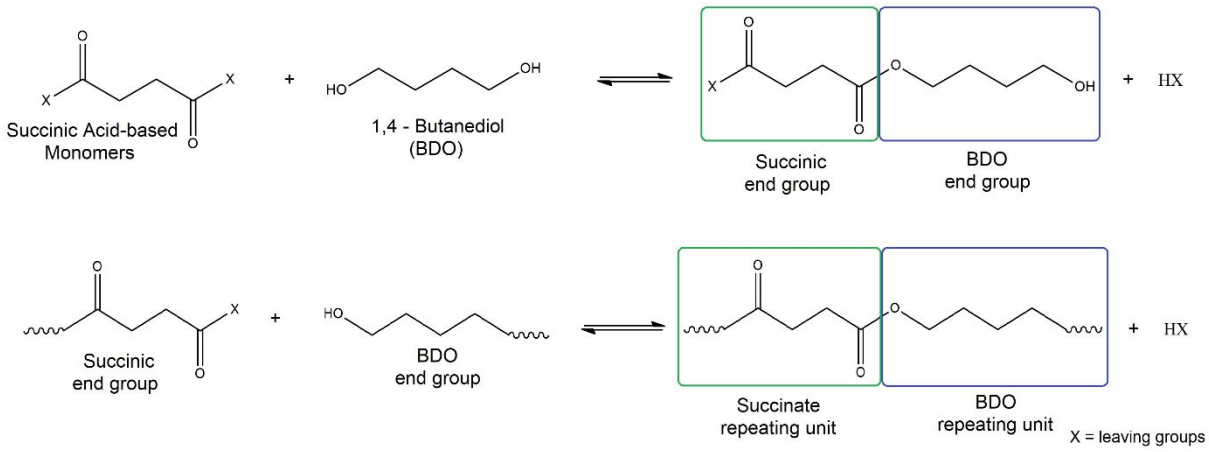
In their work, Rafiqah et al., (2021) informs that since 2003, Mitsubishi Chemicals installed an industrial site with a production capacity of 3000 tons/year. Also, these authors mentioned other companies, such as Hexing Chemical (Anhui, China) producing 10,000 tons annually, and Xinfu Pharmaceutical (Hangzhou, China) announcing the construction of the

largest continuous PBS production line in the world, with an annual capacity of 20,000 tons. It is also worth mentioning that Showa High Polymer, under the Bionolle trademark, installed a semi-commercial plant where, through chain extension methods using diisocyanate, a product with average molar mass ranging from $4x10^4$ to $1x10^6$ $g.mol^{-1}$ was produced (XU; GUO, 2010).

In the synthesis of PBS, in addition to polymeric chains, low molar mass by-products (such as water molecules, alcohols, among others) are formed, where their removal favors the formation of new esterification/transesterifications between the functional groups of the monomers/polymer chains (YU et al., 2012). Thus, Douka et al., (2018) indicate two main methods for the removal of by-products such as the application of vacuum (decreases the boiling point of the constituents of the system) and the use of molecular sieves (by-product is adsorbed on active sites of the material). Additionally, this work points to a strong trend towards the use of vacuum (mainly in reactions with enzymatic catalyst), in addition to considering the possibility of combining both methods. Figure 9 depicts PBS synthesis, describing chain growth by the reaction between terminal functional groups:

Figure 9: Representation of PBS synthesis through the reaction between terminal functional groups.



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Regarding the esterification processes, Gkountela et al., (2021) describe (along with considerations based on other acidic and alcoholic bifunctional molecules) that the equimolar ratio between the reagents is ideal for synthesis (resulting in the highest conversion of diacids). The excess of diol (1:4) did not significantly affect the formation of the esters, but the excess of acid acted as an inhibitor of the reaction, still indicating that the same result must happen with SA and BDO.

Emphasizing the industrial process, Douka et al., (2018) indicate the use of catalysts and elevated temperatures to increase the average molar mass of the products (due to polycondensation reactions having characteristics of the slow growth of the polymer chains). Gkountela et al., (2021) point out that (organo)metallic catalysts (e.g. titanium tert-butoxide, tin (II) 2-ethylhexanoate, antimony oxide (III), among others) coupled with the use of high reaction temperatures (close to 200ºC) can generate undesirable reactions in PBS production, such as deposition of metals in the formed products (causing yellowing of the resin by the use of titanium-based catalysts), dehydration of BDO (when using some solvents such as tetrahydrofuran) and sublimation of the formed oligomers (causing loss of material). Still, Gkountela et al., (2021) report that reactions catalyzed with materials of metallic origin can generate products adjacent to those expected, due to their low catalytic selectivity.

Referring to the use of metal-based catalysts and the average molar mass of PBS, Ferreira et al., (2015) synthesized PBS with the two-step method, where SA or succinic anhydride and BDO (equimolar quantities) were used for direct esterification (oligomerization), through a system with a nitrogen purge, electromagnetic agitation, and heating (135 ºC), with a duration of 6 h. Products obtained from SA as monomer presented better results in terms of weight-average molar mass (M_w) of 5.57 $x10^3$ to 8.67 $x10^3$ $g.mol^{-1}$ against 2.29 $x10^3$ to 4.11$x10^3$ $g.mol^{-1}$ of polymers synthesized from succinic anhydride. In the

second stage, for chain growth, catalysts were used in the transesterification process, where antimony oxide (III) (Sb_2O_3), tin (II) 2-ethylhexanoate ($SnOct_2$), and titanium (IV) butoxide (Ti (OBu) $_4$) were tested, at a temperature between 200 and 250 ºC, lasting 12 h. The percentage of catalyst used was 0.1 to 0.3% (wt). The highest molar mass was reached with $Ti(OBu)_4$, being 1.90 $x10^4$ $g.mol^{-1}$ (at 200 ºC and 0.3% concentration (wt)), which the authors (FERREIRA et al., 2015) indicate to be an M_w similar to those obtained in industrial processes for commercialization. Furthermore, Jacquel et al., (2011) used the oligomers produced between SA, BDO, and different metallic catalysts (with a concentration of 300 ppm, and efficiency indicated as Ti ≫ Zr ~ Sn> Hf> Sb> Bi), vacuum (for water removal), reaction temperature of 230 ºC and 2 h, to produce PBS resins with M_w of 2.14 $x10^4$ to 5.29 $x10^4$ $g.mol^{-1}$.

An environmentally-benign catalytic alternative for the production of PBS is the use of enzymes, due to high selectivity, recyclability, and non-toxicity, in addition to polymerization conditions under milder temperatures (approximately 70 ºC). Aspects such as toxicity are very important when taking into account the application of the formed product, where the presence of metals can be harmful, especially in sectors of packaging and biomedical articles (DEBUISSY; POLLET; AVÉROUS, 2017). Furthermore, the disposal of metallic catalysts must also be taken into account, due to the toxicity that can lead to the environment (YU et al., 2012). Yu et al., (2012) indicated the use of CALB as a catalyst, as it is a lipase (being catalytic in ester-type bonds) and has good stability in organic solvents in PBS production. Also, the use of enzymes in the synthesis process corroborates aspects of the Green Process and Green Products, described as processes that aim at sustainability and reduction of environmental impacts involved in the production process (MONDELLO; SALOMONE, 2020). The longer reaction times is the main disadvantage of the use of

enzymatic catalysis when compared to polymerization processes with organometallic catalysts (AGUIEIRAS et al., 2017).

In enzymatic processes, Gkountela et al., (2021) used diethyl succinate and BDO (equimolar), N435 (10% wt of monomers), toluene, or isooctane (2 (solvent): 1 (monomers) w/w), stirring at 180 rpm, temperatures between 40 and 60 ºC and vacuum (for water removal). The oligomerization time was in the order of 24 h, resulting in polymeric resins with Mw values between 1.000 to 2.800 $g.mol^{-1}$ (calculated based on NMR). The oligomerization products were taken to a transesterification process, to increase the size of polymer chains and the thermal properties of the final products. Transesterification reaction occurred in melted state, under a nitrogen atmosphere, temperature between 80 and 90 ºC, for 26 h, resulting in PBS resins with viscosimetric molar masses between 3.5 $x10^3$ and 5.5 $x10^3$ $g.mol^{-1}$, with a growth rate of 82.94 g/(mol.h). Azim et al., (2008) also used diethyl succinate and BDO (equimolar), besides N435 (10% wt of monomers) for PBS synthesis. The solvents evaluated were diphenyl ether, dodecane, and diglyme, with an addition of 200% (wt) concerning the monomers, the reaction system was magnetically stirred, with temperatures between 60 and 90 ºC, and pressure reduction after the 2 h reaction, lasting 72 h. The M_w values obtained were 3.8 $x10^4$ $g.mol^{-1}$ (GPC).

Regarding the monomers used in the enzymatic production of PBS, Azim et al., (2006) indicate that SA has solubility problems in homogeneous systems, with phase separation at middle temperatures, where the application of diethyl succinate (related ester) is advantageous due to its higher solubility in the reaction medium. Another alternative is the use of solvents for solubilization of SA. Jiang et al., (2013) pointed to diphenyl ether as a potential solvent and Gkountela et al., (2021) corroborated the information. In addition, Ren et al., (2015) performed the synthesis of PBS using toluene, to dilute the reaction system and recover the catalyst (lipase) by filtration. Gkountela et al., (2021) used toluene and isooctane,

justified by the low boiling point, to recover the polymer after the synthesis. The authors also conclude that toluene led to better results, mainly in terms of recovery of the final synthesized product.

Based on the considerations presented so far, the synthesis conditions of PBS were compiled in detail in Table 2, comparing the processes that use metal catalysts and enzymatic catalysts.

Table 2: Comparison between PBS production processes using metallic and enzymatic catalysts.

Process Parameters	**Metallic Catalysts**		**Enzymes Catalysts**	
Monomer types and molar ratio	SA or Succinium Anhydride and BDO; 1:1	SA and BDO; 1:1.05	Diethyl Succinate and BDO; 1:1	diethyl succinate and BDO; 1:1
Catalyst concentration	0.1% (mol) (transesterification); (Sb_2O_3, $SnOct_2$ and $Ti(OBu)_4$)	300 ppm (testing different metallic bases, showing the efficiency:Ti ≫ Zr ~ Sn> Hf> Sb> Bi)	10% wt (N435)	10% wt (N435)
Solvents	None	None	toluene or isooctane;	diphenyl ether, dodecane, or digline
Reaction temperature (ºC)	135 (oligomerization); 150 (transesterification)	230	40 to 60 (oligomerization); 80 to 90 (transesterification)	60 to 90
Reaction time (h)	6 (oligomerization); 12 (transesterification).	2	1 to 16 in the oligomerization and 8 to 24 in the transesterification process	72
Agitation (rpm)	Informs only that electromagnetic agitation was used	150	180	Informs only that electromagnetic agitation was used
By-product removal	Vacuum	Vacuum	Vacuum	Vacuum
Mw ($g.mol^{-1}$)	Average value of 17 $x10^3$ after the transesterification step (between reactions)	21.4 $x10^3$ to 52.9 $x10^3$	1 $x10^3$ to 2.8 $x10^3$ (oligomerization); 3.5 $x10^3$ to 5.5 $x10^3$ (transesterification)	3.8 $x10^3$
Polydispersion Index (Đ)	Average value of 8.3 (between reactions)	1.9 to 3.3	Uninformed	1.39
Source	(FERREIRA et al., 2015)	(JACQUEL et al., 2011)	(GKOUNTELA et al., 2021)	(AZIM et al., 2006)

Source: from author

It may be noted in Table 2 those enzymatic reactions allow for milder operating temperatures, as well as being free of contamination by metal deposition. As a counterpart, reactions containing metallic catalysts can be performed in shorter times and result in higher M_w values.

It is also important to highlight the polydispersion index of the molar masses of the works presented in Table 2. The polydispersion index indicates the scattering/dispersion of the size/mass distribution of polymer chains, where the greater the variation in chain sizes, the greater the polydispersion index. As indicated, syntheses using catalysts of metallic origin present higher values (1.9 and 8.3) compared to those using enzymatic catalysts, where Pellis et al., (2018) describe values between 1.07 and 1.79 in their categorization involving enzymatic polymerization processes.

Thus, as seen above, the use of enzymatic catalysts for PBS synthesis is an attractive alternative, concerning the oligomerization process, due to mild temperature conditions, absence of toxic metals, the possibility of catalyst recovery when immobilized in supports and uniformity of dispersion of the size of chains (polydispersion) (PELLIS et al., 2018). These issues corroborate the objectives proposed by Green Processes, which are environmentally friendly. However, information on succinic polymers will be presented in the next session.

2.4 Succinic Copolymers

Related to the environmental concern, involving depletion of non-renewable resources for polymer production, Xu et al., (2008) indicate a strong tendency to develop biodegradable materials from a biotechnological source, citing aliphatic (co)polyesters as promising candidates. The authors also describe the possibility of producing SA-based copolymers such as poly(ethylene succinate-co-butylene succinate), poly(butylene succinate-co-butylene adipate), poly(butylene succinate-co-hexylene), and poly(propylene succinate) which have

excellent physicochemical properties for different applications. Sun, Jiang, and Qiu (2021) indicate that copolymerization is an efficient technique for improving the physical properties of polymeric materials, through the addition of new diols and/or diacids during the polymer synthesis process.

Li et al., (2006) indicate that the addition of aromatic monomers in the synthesis of copolyesters (such as terephthalic acid) assit to increase the thermal and mechanical resistances of the produced materials, sharing the degradability presented by aliphatic materials. Furthermore, the authors describe a process for the synthesis of poly(butylene succinate-co-terephthalate) (PBST), with molar masses between 10^4 and 10^5 and good biodegradability in materials with lower amounts of terephthalic monomer.

Sun, Jiang, and Qiu (2021) portray that those succinic polymers with short-chain branched comonomers infer impacts on the thermal, mechanical, and crystallization properties of the final materials. Whereas, long-chain branched comonomers can modify the rheological properties and processability of materials. The authors also report that the addition of 1,2-decanediol to the PBS copolymer synthesis process provides the decay in the glass transition, melting point, and crystallization temperatures, as well as the increase in the amount of branching decreases the crystalline domains and helps in the degradability of the material.

Zahir et al., (2020) point out the synthesis and properties of poly(L-lactide)-b-poly(2-methyl-1,3-propanediyl succinate)-b-poly(L-lactide) (PLLA-co-PMPS-co-PLLA), with a glass transition temperature below -20 °C and a melting temperature between 133-159.5°C, in addition to good elongation and enzymatic and seawater degradability.

Wang et al., (2019) describe a copolymer with application in agriculture, based on succinic acid and salicylic acid, the materials with a low content of salicylic acid promoted the stimulus for the growth of vegetables in their degradation, besides presenting good degradability, being ecologically friendly.

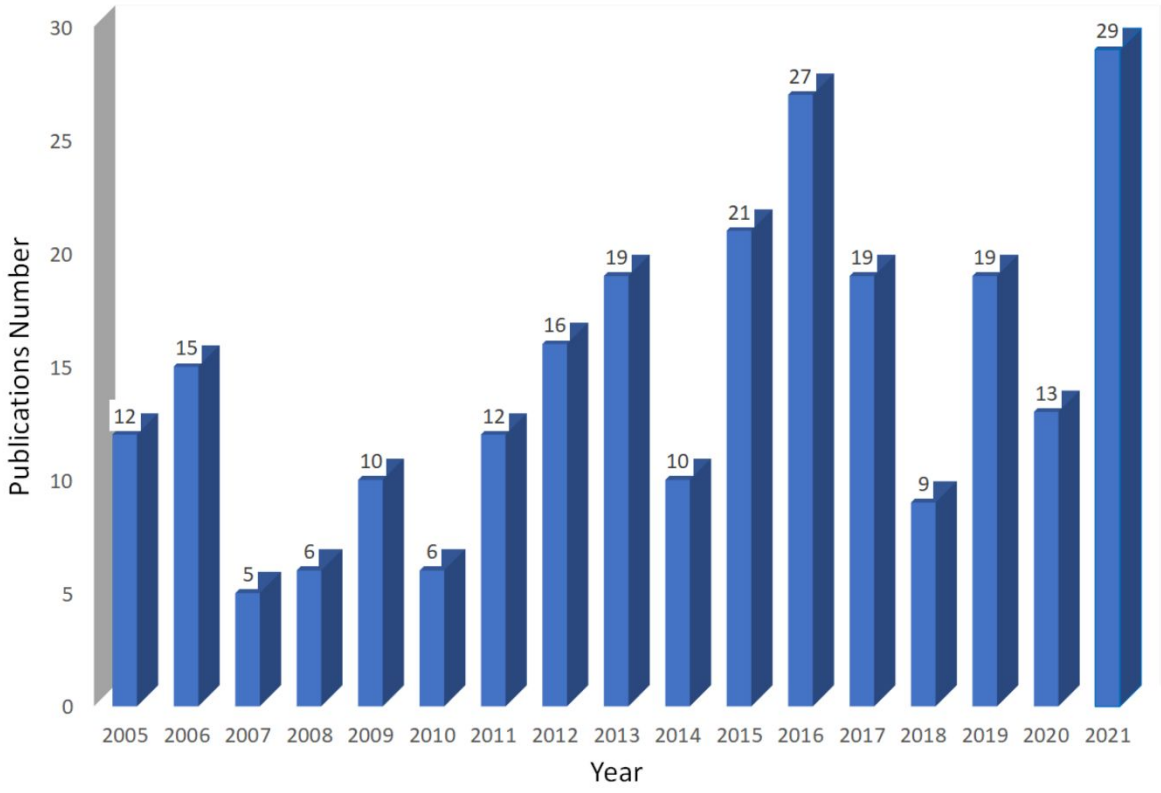
Xu and Guo (2010) specifies that the addition of comonomers in PBS affects the decrease of the crystallinity and melting point of the material, being highly degradable in enzymatic solution, soil, and activated sludge. The authors also indicate that the formation of blends of other polymers as PLA with PBS can improve their physicochemical properties, with good tensile strength and elastic modulus without much loss of ductility.

It is interesting to note that, as described by Tan et al., (2017), a high degradation rate in polymers is not always desirable. In their work, they synthesized a polymer with a domain of 2-hydro-4-(2,3-epoxypropoxy)benzophenone (HEPBP), which is an agent that absorbs photons in the UV range, with a molar mass between 3.5 $x10^3$ and 6.4 $x10^3$ $g.mol^{-1}$. In the accelerated degradation test, the presence of higher amounts of HEPBP leads to a lower degradation rate and a better UV protection effect. The authors also indicate that the melting temperature (Tm) remains practically unchanged, while the crystallization process is favoured with the introduction of HEPBP.

As noted in the previous discussion, the characteristics of each synthesized succinic copolymer depend on the comonomers added to the synthesis route. Thus, as described by Xu and Guo (2010), the thermal behaviour, mechanical properties, and biodegradation rate of PBS can be quite varied, as well as the degradation rate, where aliphatic comonomers result in a higher biodegradation rate, whereas aromatic comonomers lead to a lower one.

To verify the importance of succinic copolymers, Figure 10 lists the publications involving these compounds in the Scopus database, between 2005 and 2021, through the search filtered only on keywords related to the publications using the terms poly and succinate-co-.

Figure 10: Evolution of the number of publications on succinic copolymers obtained by searching the Scopus database, with the keywords poly and succinate-co-, in the period from 2005 to 2021.

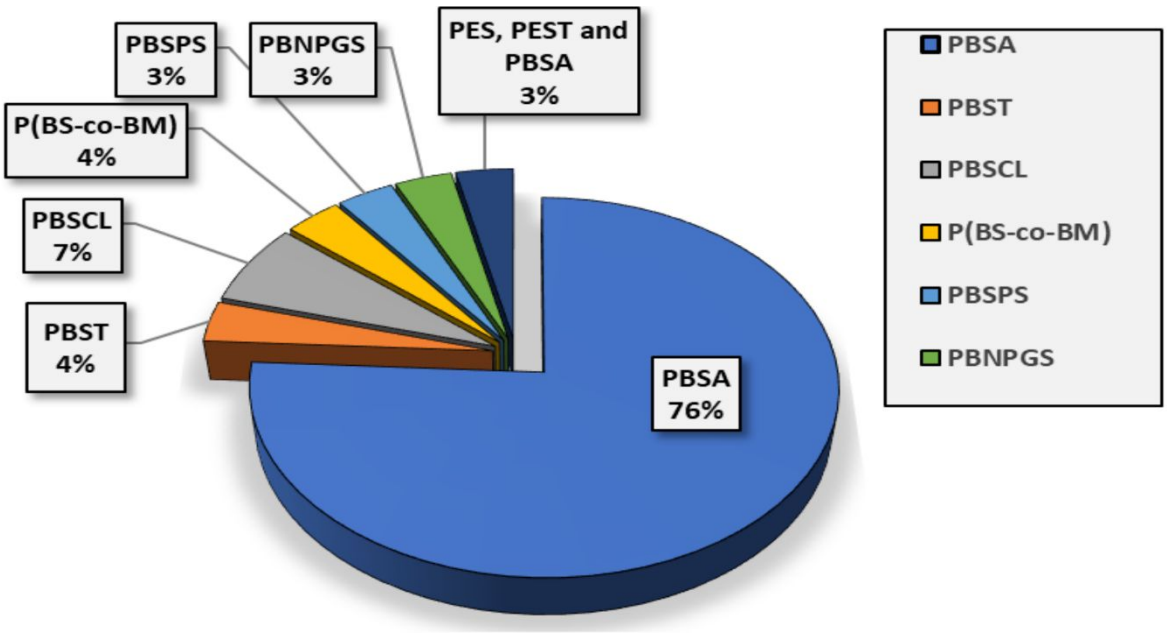


Source: from author

It is noteworthy that in the search, synthesis and/or application works were not differentiated, the objective was to verify the number of publications involving succinic copolymers, in the described period, as a way to describe the trend of studying these

materials. As noted, publication/research values on succinic copolymers have been showing significant growth. Among the 29 articles published in 2021, Figure 11 brings which copolymers are most discussed in these works, with poly(butylene succinate-co-adipate), PBSA being described as the most recurrent by far (76 %):

Figure 11: Percentage representation of scientific works involving succinic copolymers covered in publications in 2021, by searching the Scopus database with the keywords: poly and succinate-co-.



PBSA = poly(butylene succinate-co-adipate); PBST= poly(butylene succinate-co-terephthalate); PBSCL = poly(butylene succinate-co-ε-caprolactone); P(BS-co-BM): poly(butylene succinate-co-malate); PBSPS = poly(butylene succinate-co-propylene succinate); PBNPGS: poly(butylene succinate-co-neopentylglycol); PES = poly(ethylene succinate); PEST= poly(ethylene succinate-co-terephthalate). Table 04 shows the main succinic copolyesters involved in the 2021 publications in the Scopus Database, where most of them used PBSA as the base material.

Source: from author

In the performed search, a new categorization was added, enzymatic synthesis, only on the keywords, which 5 articles were selected. Three of them ((LI et al., 2018), (LI et al., 2019), (WANG et al., 2021)) did not present the enzymatic synthesis, but the degradation of the polymer. One of them (KONDO et al., 2008), described the synthesis process for homopolymers (not fitting the categorization), and Núñez, Muñoz-Guerra and Martínez De

Ilarduya (NÚÑEZ; MUÑOZ-GUERRA; MARTÍNEZ DE ILARDUYA, 2021) described the synthesis of Poly(Butylene succinate-co-ε-caprolactone), through dimethyl succinate, 1,4-butanediol and ε-caprolactone, obtaining stable materials up to 300 °C.

However, the following section will present a categorization about the application of PBS in research studies referenced in the Scopus database, using a selection filter in keywords with the term “Polybutylene Succinate”, in the period from 2015 to 2021 (aiming to screen more current works). The selection filter involved works that tested final products involving PBS in applications related to biomedicine sectors.

2.5 Trends of PBS applications

Regarding the categorization performed, of the 196 presented articles, 41 included PBS applicability in their scope and of these, 11 publications involved specifically biomedical applications. Table 3 presents detailed information on the characteristics of the materials used in biomedical applications of PBS, as a way to indicate the main properties found in the materials involved by the categorization.

From the information presented in Table 3, a greater adherence can be seen in the applications in tissue recovery engineering, with an emphasis on bone tissue engineering. The production of adhesives for cardiac tissue, infection control materials, and encapsulation/adhesion of materials of interest (scaffolding) is also mentioned. In addition, a study was considered on the development of filtering materials for disposable masks, with a view to their use in combating infections (as seen by the SARS-CoV-2 pandemic) (CHOI et al., 2021).

Among the properties of materials categorized in tissue engineering studies, it can be observed that there is great concern with the surface of the product that will be used in the application, in addition to the degradability of the material and cell proliferation. Modification

techniques were used, aiming at the adhesion of the biological material to the application product to achieve the ideal physicochemical properties for applicability, through the formation of blends, processing to improve roughness/porosity or addition of chemical compounds, in addition to the use of sodium hydroxide solutions, O_2 plasma, ozone, and UV light.

Table 3: Description of the characteristics of materials found in PBS biomedical application studies.

Application	Mw ($g.mol^{-1}$)	Type of modification	Degradability study	In vivo study	Compatibility	Morphological study of material/physicochemical characteristics	Source
Support for cell proliferation and differentiation in bone tissue regeneration	$500x10^3$	Chain extension with 1,6-diisocyanatohexane, with the use of salts for scaffolding and coating with fibronectin or laminin as adhesion protein for cell inoculation	Hydrolysis technique in physiological environment simulation, showed mass loss of 100% in 120 days. (Conditions: 37 °C in 0.09% sodium azide and isotonic saline)	Materials were seeded with porcine dental stem cells, showing collagen formation after 20 days, demonstrating tissue formation	Greater proliferation with fibronectin-modified materials	The material had a size of 1x1 cm^2 and porosity of 100 µm, with a rough surface	(ABAY et al., 2016)
Support for the proliferation of human retinal pigment epithelium cells	Uninformed	Materials were processed using solvents, surfactants and sucrose particles as porogen	For 4 weeks, the material showed good stability, with significant losses after 16 weeks (60 %)	Retinal pigment epithelial cells derived from human embryonic stem cells were used, with a significant increase in metabolic activity during the incubation time	On the 1st day of incubation, there were no major changes in metabolic activity, with positive values being observed from the 5th day of incubation	The material was 1x1 cm^2 in size, with a porosity of 3.21 ± 0.37 µm, with a rough surface	(CALEJO et al., 2019)

Support for the proliferation of mesenchymal stem cells derived from the bone marrow of rats translated as tissue transglutaminase variant 2	Uninformed	Blend with PLLA, followed by scaffolding by salt leaching (NaCl)	Uninformed	Mesenchymal stem cells (MSC) were used, evaluating the regeneration of cartilage tissue through the expression of tissue transglutaminase variant 2 (TGM2_v2)	While few cells migrated to the porous structure in the first 14 days, up to the 21st day practically all structures were covered with cells.	Size: 7 × 3 mm^2, showing 47 to 66 % porosity between the formulations	(CALIKOGLU KOYUNCU et al., 2020)
Preparation of membranes for making filters for disposable masks	72.3x10^3	PBS was incorporated into the mask filters	Two degradation techniques were used: enzymatic, with *Thermomyces lanuginosus* lipase decomposed all the material in 7 h, at 50 °C, another in soil decomposition, at room temperature, decomposing the	Not performed because it is a macro protection device not inserted in a live environment	Not performed because it is a macro protection device not inserted in a live environment	Thicknesses from 0.1 to 9.1 g.m^{-2} (author used weight for thickness evaluation). Fiber thickness ranged from 0.51 to 2.25 µm, with pores from 3.5 to 13.1 µm	(CHOI et al., 2021)

			material in 4 weeks				
Control of bacterial infections in wounds	$816x10^3$	Used in the treatment of infections through the production of electrospinned nanofibers	Enzymatic degradation was carried out, with a decay of the mass of approx. 18% in 60 days	Studies were performed in rats on wounds infected with *S. aureus* and *E. coli*	The material showed little efficiency in inhibiting the growth of microorganisms, showing rates close to 40% inhibition	Tg of 115 °C and decomposition at 352 °C. The fibers produced had a diameter between 490 and 632 nm	(HAN et al., 2019)
Support for neural tissue cells.	Uninformed	Blends were produced with PLLA	Degradation was evaluated in DPBS solution (pH 7.4) with constant agitation, showing degradation of almost 18% in 120 days	Schwann cells were used and the greatest cell proliferation was observed for PLLA / PBS structures (3%, p / v, 2:1)	Cells were able to survive in the pores, but showed a decline in the count after 21 days of incubation	The materials presented values between 30 and 36% of their structure, with pores around 280 µm	(KANNECİ ALTINIŞIK et al., 2017)
Repairing damage to bone tissue	20×10^3	Scaffolding with Magnesium Phosphate and Wheat Protein Incorporation	After 12 weeks, the material had a loss of 58.43% in mass, which was favored by the addition of wheat protein, using a Tris-HCl buffer solution (pH =	MC3T3-E1 cells were used, and the modified material showed good cell proliferation	The material showed greater cell proliferation compared to the material composed only by PBS	Flocular structures, 100-500 nm in size	(QUAN-XIANG et al., 2015)

7.4)

Tissue engineering with mouse fibroblast (L929) cell adhesion	Uninformed	The material was treated with NaOH solution, UV, O_2 plasma, and Ozone	Uninformed	Mouse cells were used, the density of generic material was verified as cell growth factor	A greater presence of genetic material was verified after 5h of incubation, except for materials treated with ozone	The materials, after treatment, started to present a rough surface, in addition to greater mechanical resistance	(RIBEIRO et al., 2017)
Releasing active compounds	$67x10^3$	Blends were made with PLA, with the addition of crushed eggshell to form pores	Uninformed	Uninformed	Uninformed	The material presented a porous structure, measuring 0.27µm, with a density of 8.5×10^5 pores / mm^2, with crystalline portions between 23 and 35%	(TARNLERT; TANSIN; JARIYASAKOOLROJ, 2021)
Bone engineering-oriented grafts	Uninformed	Formation of scaffolding through mesoporous calcium-magnesium silicate	Test on simulated body fluid showed constant degradation over time and the rate of mass loss was approximately 3 % per week, and after12	Mouse osteoblastic cells MC3T3-E1 was used, with constant presence in the modified materials	The proliferation rate in the modified materials was significantly higher compared to PBS	The material had well-defined regular mesopores, with an average pore size of approximately 13 nm	(ZHANG et al., 2015)

			weeks it presented approximately 65 % of the initial mass		alone		
Bone graft	Uninformed	Formation of scaffolds with magnesium nitrate and ammonium dihydrogen phosphate and incorporation of wheat protein	Hydrolysis technique in physiological environment simulation, showed a mass loss of 58.43% in 12 weeks	Study carried out through the implantation of the materials in defects in rabbits' femoral, the materials with incorporation of wheat protein led to the best tissue growth results	Good bioactivity in materials that had the incorporation of wheat protein, where the in vivo study stimulated bone growth	The material had macropores from 400 to 600 µm, and also micropores ranging from 10 µm to 20 µm	(ZHAO et al., 2020)

Source: from author

Concerning modification, blends, treatments/physical-chemical transformations on the surface, and adhesion of chemical compounds were observed, aiming at better porosity results and rougher surfaces. By analyzing the behavior of textures and pore size on the surfaces of the materials, pores with varying sizes (without patterns, but with values between 632 nm and 3.21 μm (HAN et al., 2019), (CALEJO et al., 2019)) were observed, as well as formulation of textures with rough behavior, intended to support the adhesion of the material to the interest.

Important aspects about degradation and compatibility in the live environment were also raised. Different material decomposition rates were observed, depending on the characteristics required for the material. Again, a pattern was not observed, but it can be highlighted that the materials involved for bone recovery presented greater resistance to degradation, which is expected. The non-toxicity of the material was reported, being one of the main points for the application.

A study involving the release of active principles through films in sachet format was also highlighted, with pore size measuring on average 0.27 μm, and crystalline portions between 23 and 35 % (TARNLERT; TANSIN; JARIYASAKOOLROJ, 2021). In this work, the porosity was built up by the addition of eggshell solids, for carrying active principles. Another study, Choi et al., (2021), involved the development of efficient filters for particulate material removal, with 97 % efficiency of removal of target materials, pores from 3.5 to 13.1 μm, in addition to a good degradation rate, both in soil and in enzymatic recycling.

In the categorization, it was still prioritized to find some information, such as molar masses and dispersity of the polymeric materials used, and catalyst type. However, this information was only provided in a few studies.

Regarding the average molar masses, the values found were on the scale of 10^4 and 10^5, which is relevant information, because the length of the chains interferes in the

degradation, morphology, surface functionalities, and interaction with active agents, as described by Palacio, Orozco and López (2011).

Finally, it may be concluded that there is a tendency to apply PBS in biomedical areas, for bone tissue recovery materials and as scaffolds for cell materials, observing porosity in the μm or nm range and a rough surface for adhesion of the materials of interest. Still, it is important to highlight the description of information such as molar mass and dispersity and applied catalysts, as they are linked to degradation and toxicity of materials intended for application in biotechnological environments of biomedicine. Referring to succinic polymers, the following section presents information on the synthesis and physicochemical characteristics of succinic copolymers.

2.6 Biomedical applications and modification techniques applied to succinic copolyesters

When referring to succinic copolymers, Fabbri et al., (2018) point out the succinic copolymers as being widely applicable to the biomedical market, presenting easy synthesis strategies, tunable biodegradation, and biocompatibility. Furthermore, there is a tendency for the application of succinic copolymers in drug delivery systems and tissue engineering.

Gigli et al., (2016) point out that the main reasons for the synthesis of succinic copolyesters are to adjust the thermal and mechanical behavior and the biodegradation rate of the final products depending on the desirable application, where catalysts of either metallic or biotech origin may be used. In addition, the authors describe that the main applied techniques are copolycondensation (using comonomers with different functional groups, producing materials of random monomeric composition) and reactive blends, producing multiblock materials (using polymerized chains), with different functional groups, such as ether and

thioether, where the synthesis time is fundamental to determine the length of the blocks present in the polymeric chains.

Referring to polymer functionalization, Tallawi et al., (2015) describe two strategies for covalent functionalization of chains: [i] functionalization of monomers, as a pre-polymerization step, and [ii] functionalization of polymer chains after the polymerization reaction. The authors indicate that these processes involve reactive groups, both in the monomer and in the polymer chain, aiming to modify the properties of the final products. They also describe that post-polymerization functionalization processes can be specific, targeting directed or non-specific functional groups. This latter case can result in the loss of the molecule's bioactivity and also in unwanted reactions, such as chain degradation and cross-linking.

Liverani et al., (2016) synthesized poly(butylene succinate-dilinoleic succinate) (PBS-DLS), which underwent electrospinning and subsequent functionalization with 1-ethyl-3-(3-dimethylaminopropyl) hydrochloride and N-hydroxysuccinimide, for application as soft tissue. FT-IR analysis was used to prove the functionalization and *in vitro* degradation studies indicated a decay of 20% in mass in one week with immersion in phosphate-buffered saline, indicating good degradability.

Fabbri et al., (2018) synthesized poly(butylene/triethylene succinate), P(BSmTESn), using dexamethasone to assess the release of the synthesized compound. In the copolyester synthesis process, $Ti(OBu)_4$ was used as a catalyst in bulk reactions. Next, nanoparticles containing the polymer and the drug were prepared, with a size of 320 ± 40 nm. In their tests, the P(BSmTESn) nanoparticles displayed faster release results than those of PBS, having smaller crystalline domains and greater hydrophilicity, in addition to greater encapsulation efficiency.

Tallawi et al., (2015) developed poly(butylene-butylene succinate dilinoleate) (PBS-DLA) with the addition of poly(glycerol sebacate) (PGS) for the application of cardiac patches. The fiber had a narrow diameter distribution, with improved hydrophilicity in samples with a higher amount of PBS-DLA, in addition to a good elastic mode. Furthermore, a toxicity test was performed on C2C12 myoblast cells, indicating that it is non-toxic, in addition to having good compatibility with rat cardiomyocytes.

In their studies, Zabihi et al., (2019) proposed the synthesis of poly(glycerol-co-succinate) nanogels with either N435 or $Sn(Oct)_2$ as catalysts to deliver active principles that were tested in dermal tissues. The simultaneous encapsulation of Red Nile and Tacrolimus was carried out after synthesis. The presence of succinic segments improved the hydrophobicity of the polymer, with tunable degradability due to ester bonding, and showed no toxicity. Regarding the delivery of the encapsulates, the particles remained in the upper part of the tissue, being a desirable point to avoid problems such as sensitization.

Finally, poly(butylene succinate/diglycollate) was produced by Gigli et al., (2013), through reactive and processed blends to obtain 0.2 mm thick films. The structure of the material presented blocks with well-defined lengths, in addition to good elasticity and deformation properties, where the authors indicate the potential of the material for the development in tissue engineering.

2.7 Final Considerations

In this review, considerations about the synthesis and applications of PBS and succinic copolyesters were addressed. In the production of these materials, catalysts are applied to optimize the synthesis, using materials of metallic origin and biotechnological origin, mainly

lipases (hydrolytic enzymes), presenting advantages and disadvantages of each type of catalytic material.

PBS and most of the succinic copolyesters (depending on comonomers) are indicated as biodegradable, with mechanical and thermal characteristics favorable to application in the most diverse sectors of product development and industry. Succinic copolymers are produced aiming at differentiated physicochemical characteristics, depending on the selected comonomer and its application. When compared to PBS, improvements in the properties of the materials, both in the resistance and in the degradability of the formed copolymers are observed.

Regarding the physicochemical properties discussed in the studies, it is of great interest to describe the average molar masses, polydispersion index, and catalysts used since these characteristics are linked to degradability, morphology, functionalization and interaction between the compounds of interest. However, these properties are little mentioned and explored in the application works. Thus, more studies relating physicochemical properties with applications of PBS, would be of great value for the development of new materials.

Concerning the application of PBS and succinic copolymers in the biomedical areas, there is a trend towards the use in tissue engineering, such as in the regeneration of bone tissue and the transport of materials of interest, using techniques for material modification and/or functionalization.

As perspectives for future works, researches involving the use of enzymatic catalysts for polymer synthesis should be more explored, with an emphasis on expanding the synthesis scale (in addition to the reuse and technical-economic analysis of the use of biotechnological catalysts) and production of new polymeric materials, characterizing their physicochemical properties, in addition to the use and development of modification and/or functionalization

techniques. Also, following aspects of Green Process and Green Products, it is of great value to use solvents from renewable sources that do not harm the environment, as a way of developing more sustainable processes with less environmental impact.

CHAPTER 3

Chapter 3 presents the results obtained by the synthesis of PBS catalyzed by N435 in the bulk reactions, describing kinetic aspects of the synthesis, distribution of polymeric chains and reuse of the biocatalyst. A research article based on Chapter 3 has been accepted for publication in the European Polymer Journal and can be accessed at:

(https://doi.org/10.1016/j.eurpolymj.2022.111573).

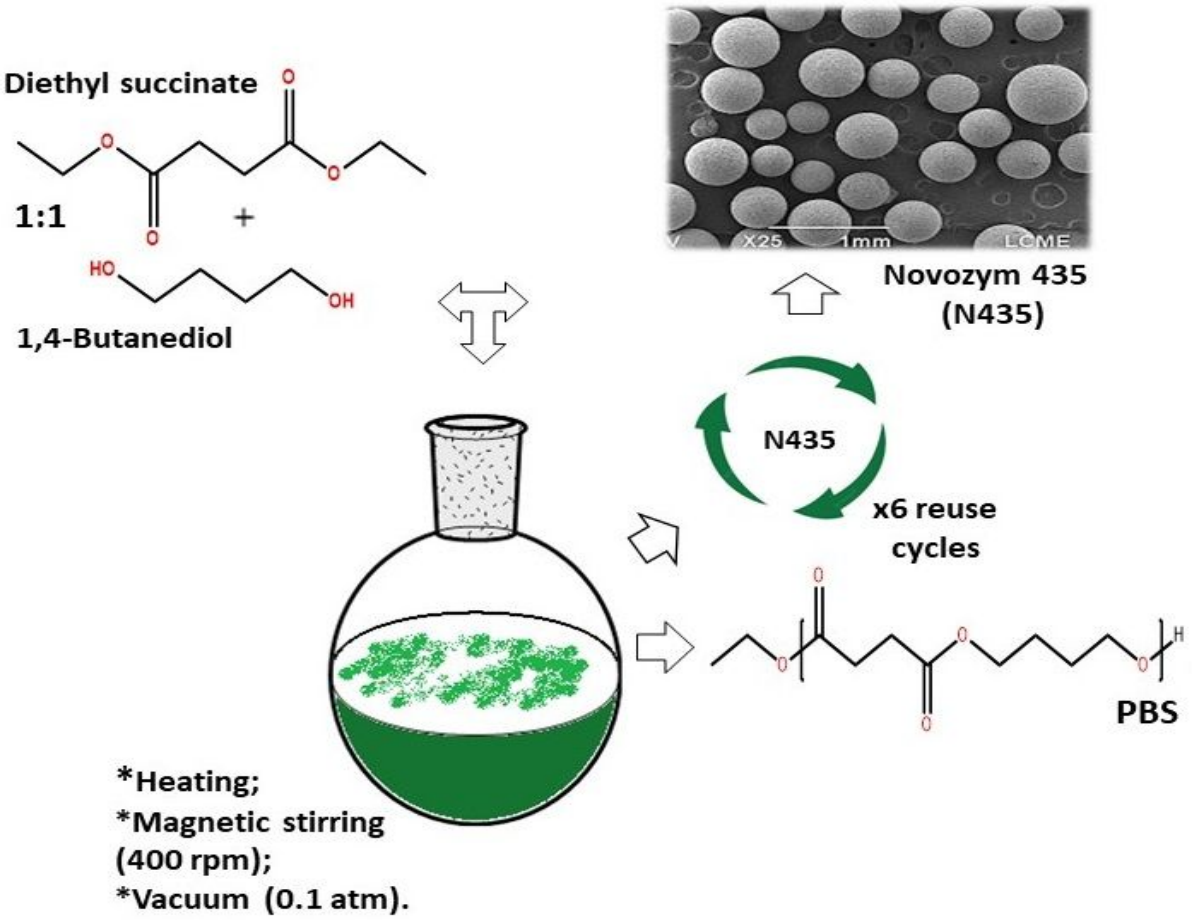
3 Synthesis of Polybutylene succinate via Lipase-Catalyzed Transesterification: Enzyme Stability, Reuse and PBS properties in Bulk Polycondensations

Abstract

Environmental problems involving the inappropriate disposal of non-biodegradable polymers and the production of petrochemical monomers have stimulated the interest in the development of renewable and biodegradable polymers. Among those, polybutylene succinate (PBS) has been highlighted, as it is a biodegradable polyester, synthesized from monomers that can be originated from fermentation processes. Moreover, regarding the application of biocompatible and non-toxic polymers in pharmaceutical and biomedical areas, enzymes can be considered an interesting alternative when compared to metallic catalysts. Therefore, this work aimed to investigate the lipase-catalyzed bulk polycondensation of diethyl succinate and 1,4-butanediol and to evaluate the molar mass distribution of the PBS produced. The recovery of the biocatalyst for application in reuse cycles was also performed. Preliminary tests indicated an enzyme concentration of 10% by mass (regarding the monomers) and a synthesis temperature of 90 °C as the best-operating conditions, in terms of weight of collected by-product (related to the conversion of the functional groups) and the molar masses (M_w: 4,000 $g.mol^{-1}$), in reactions that lasted 90 minutes. These conditions were used in the subsequent recycling tests of the biocatalyst. The reuse of Novozym 435 allowed for 6 cycles, with good catalytic activity in the first 4 cycles. Moreover, the loss of enzyme activity in the last cycles was recovered through enzyme partial replacement (addition of 25 % of fresh enzyme to 75 % of used one). The promising results of enzymatic polycondensations performed in bulk aim to contribute to the development of not only more environmentally friendly polymers but also the use of greener reaction conditions.

Keywords: Polybutylene succinate, Lipase; Enzyme reuse, Kinetic evaluation.

Graphical Abstract



3.1 Introduction

Due to the technological and industrial development, polymeric materials have been applied in the most varied segments, such as automotive, agricultural, food and pharmacological (XIAO; FONTAINE; BOURBIGOT, 2021). With such versatility, a large amount of these materials is produced, estimated at 400 million tons in 2020, mostly base on fossil source (DOURADO FERNANDES et al., 2022). In this way, environmental problems are generated, either by petroleum extraction or by the wide deposition of these materials in inappropriate places, generating environmental degradation. This situation has led to the search for more sutanable polymeric materials.

Considering such problems, poly(butylene succinate) (PBS) can be a biodegradable alternative, where its constituents may be produced through the fermentation of biomasses (KAWAGUCHI; OGINO; KONDO, 2017), presenting physicochemical characteristics similar to conventional polyolefins (such as polyethylene and polypropylene) (DE MATOS COSTA et al., 2020). Also, referring to the synthesis of PBS, the polymerization process can employ succinic acid and/or succinic anhydride, 1,4-butanediol, in addition to their related diesters such as diethyl succinate (JIANG; LOOS, 2016), where such polymerization processes present annual production of 10 to 15 ktons per year of PBS (JIANG; LOOS, 2016a).

In industrial processes, the synthesis of PBS occurs mainly in two stages, namely: 1) oligomerization, forming low molar mass chains through esterifications/transesterifications of the monomers, called oligomers; and 2) molten polycondensation, where oligomerization products undergo transesterification reactions, to increase the length of the polymer chains (NISOLI; DOHERTY; MALONE, 2004). In addition, catalysts of metallic origin such as manganese, zinc, titanium, calcium, cobalt and magnesium acetates, antimony oxide and iron

oxides, high temperatures and vacuum are applied to remove the by-products of the reaction, favouring the growth of the chains (DOUKA et al., 2018), (YU et al., 2012). These catalysts have low selectivity and can generate unwanted products during the reaction. Yet, it is also possible the deposition of catalysts in synthesized polymeric materials, which may be unfavorable depending on their application, mainly in the biomedical area due to their toxicity (ALBUQUERQUE et al., 2014). Thus, the toxicity present in metallic-based catalysts can limit the application of polymers obtained with such catalysts in food packaging and controlled drug delivery, with the need to search for other catalytic processes, such as those of biotechnological origin by the use of enzymes.

Biotechnological catalysts, such as peroxidases, aldosales, peptidase, lipases (KOBAYASHI, 2012), among others, have already been applied in polymerization processes. In polymerization processes, lipases have catalytic activity in ring-opening (POLLONI et al., 2018) and (trans)esterification reactions, allowing polymerization between cyclic esters (mainly lactones), diacids, diols and their respective diesters (YANG et al., 2012). Through enzymatic polymerization, different polymeric materials can be produced, such as PBS (AZIM et al., 2006), poly(butylene succinate-co-ε-caprolactone) (NÚÑEZ; MUÑOZ-GUERRA; MARTÍNEZ DE ILARDUYA, 2021) and poly(butylene succinate-co-terephthalate) (LI et al., 2006). Xu et al., (2008) indicate a strong trend in the development of materials in this segment.

Enzymatic synthesis processes have several advantages such as the high specificity of reactants and products, lower temperatures (YANG et al., 2012), and absence of heavy metals (PELLIS et al., 2018), in addition to the possibility of reuse, in the case of immobilized enzymes such as Novozym 435 (N435) (SEN; PUSKAS, 2015), helping to attend several of the 12 Principles of Green Chemistry. Based on the fact that enzymatic reactions are

performed at milder temperatures, solubility problems are seen when SA and BDO are applied. Azim et al. (2006) describe that the low solubility of SA in BDO provided low molar mass products (oligomers), with the need to apply DS instead of SA to form homogeneous systems when biocatalysts are applied.

To the best of our knowledge, there is an absence of works concerned with the reuse of biocatalysts in the synthesis of PBS (or succinic (co)polymers). Some studies were found with other polymers, such as by Nasr et al., (2020) producing polymers through 1,6-hexanediol and diethyl adipate with N435, obtaining 3 recycles; and Poojari, Beemat and Clarson (2013) which produced poly(caprolactone) (PCL), getting 10 reuse cycles with N435. Two other works focused on the reuse of N435 for polymer synthesis through ring-opening were also listed (ORTIZ et al., 2019). Moreover, there is no published work in the open literature about the kinetic behaviour and growth of the chains in enzymatic polycondensation processes to the best of our knowledge. Particularly, the analysis of kinetic behaviour of molar mass distributions and subproducts remotion, coupled with enzyme stability through reuse cycles is widely important for the modelling, design, technical-economical feasibility studies and scale-up of enzymatic polycondensation reactors.

Through the possibility of reuse of N435 in production processes (GKOUNTELA et al., 2020) this study aimed to investigate the best conditions for PBS synthesis via enzymatic bulk polycondensation, the possibility of recovering the immobilized biocatalyst for reuse in the synthesis process, and elucidate the kinetic behaviour of subproducts remotion and the molar mass distributions obtained by N435-catalyzed PBS synthesis.

3.2 Material and Methods

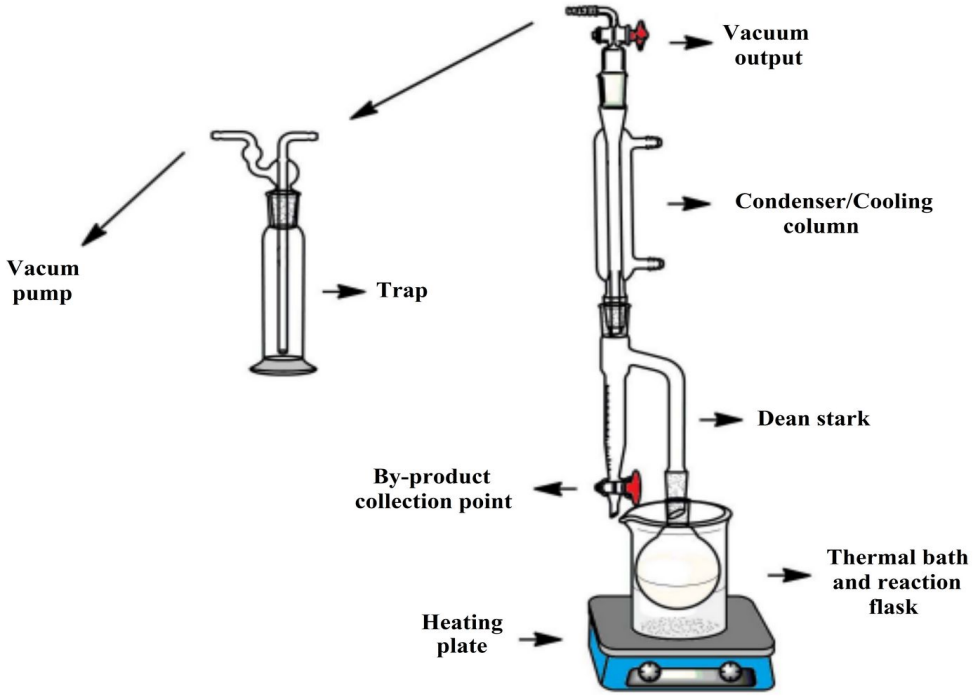
3.2.1 Chemicals and Enzyme

Novozym® 435 immobilized lipase from *Candida antarctica* was kindly donated by Novozymes. Diethyl succinate (DS, purity ≥ 99 %), 1,4-butanediol (BDO, purity ≥ 99 %), sodium hydroxide (NaOH, 99 % minimum purity) and deuterated chloroform anhydrous (99.8 atom %D, with purity ≥ 99 %) were purchased from Sigma-Aldrich. Chloroform (minimum content of 99.8%) was acquired from Dinâmica. Chloroform (HPLC grade, 99.8 % purity) was bought from J.T.Baker. Oleic acid (OA, purity ≥ 99 %), was obtained from Synth, and ethanol (EtOH, purity 95 %) from Neon. All components were used as received without further purification or modification.

3.2.2 Experimental Unit

The experimental unit used for the enzymatic polycondensation reactions in bulk is composed of a heating and magnetic stirring plate (C-MAG HS 7, IKA), thermal bath, reaction flask, dean stark, condenser column (cooled by a thermocryosthatic bath, MQBTC99-2, Microquímica), vacuum output, trap and vacuum pump (I2P-740/SCN, Indutec), arranged in a vertical system. It is important to emphasize that the experimental unit was based on the use of vacuum to remove by-products of enzymatic polycondensations, promoting the shift of the chemical equilibrium of the reaction aiming to increase in the molar mass/length of the polymeric chains (DOUKA et al., 2018). Figure 12 illustrates the experimental unit used in the synthesis processes.

Figure 12: Representation of the experimental unit used for the enzymatic polycondensation reactions in bulk with collection of by-products.



Source: from author

3.2.3 Enzymatic Polycondensation in Bulk

For PBS synthesis, equimolar proportions of the monomers were used (GKOUNTELA et al., 2021). Thus, 0.1 mol of DS (17.42 g) and 0.1 mol of BDO (9.01 g) were weighted for each synthesis procedure, in bulk reactions. Also, a magnetic stirring, approximately 400 rpm, and vacuum, 0.1 atm, were applied right from the start-up of the reaction. The condensation column was adjusted to 5 °C to recover the by-product (ethanol), which was collected and weighed every 5 min, to monitor the kinetic behaviour of each experimental run. Additionally, the molar mass distributions of PBS samples were measured at the end of the reaction. To verify the best conditions for the synthesis process, the effect of enzyme concentration and temperature was evaluated. Regarding the amount of N435, 5, 10 and 20 wt % (based on the total mass of the monomers) were tested at 70 °C. Next, the influence of the

temperature on the reaction kinetics was evaluated, varying from 60 to 100 °C, for a fixed enzyme concentration (chosen from the initial runs). Reaction times ranged from 25 to 90 min. The criterion for determining the end of the reaction for each experimental condition was based on the high viscosity of the medium, making it impossible to continue with homogenization by magnetic stirring.

3.2.4 Purification of Reaction Products

After each synthesis procedure, chloroform was added to the reaction medium to solubilize the chains and allow the separation from N435 by vacuum filtration. Then, the system was heated again on a heating plate with agitation at 200 rpm and 65 °C, for solvent evaporation, maintained until the volume of the medium remained constant. Finally, the samples were taken to a forced convection oven (DL – AF, De Leo) and left for 48 h at a temperature of 60 °C to certify the total evaporation of solvents.

3.2.5 Enzyme Reuse

After the filtration procedure to separate the polymeric material and N435, the biocatalyst retained on the filter paper was washed 3 more times with chloroform and taken to the forced convection oven to dry for 24 h at 60 °C. After the procedure, N435 was used in a new cycle of enzymatic polycondensation. The conditions chosen for the reuse of the enzyme were the same as those reported in section 3.2.3, with 10 wt% of N435 and at 90 °C.

Lipase activity was evaluated before the first polycondensation and after the last reuse cycle. The method consists of the esterification of OA and EtOH, from measures of acid concentration before and after the test. Thus, 0.045 mol (12.771 g) of OA, 0.045 mol (2.071 g) of EtOH, and 0.013% w/w (0.195 g) of N435 were added to a magnetic stirring system, at a

temperature of 40 °C, for 40 min. Samples from time zero and 40 min (0.150 µL of each, diluted in 20 mL of EtOH) were titrated with NaOH solution (0.05 mol.L^{-1}) to determine the acid concentration. Thus, using Equation 1, the enzyme activity was determined, in U.g^{-1} (capacity to react 1 µmol of monomer molecules per min for each gram of enzyme).

$$EA\left(U.g^{-1}\right)=\frac{\left(V_I-V_f\right)\times\left[NaOH\right]\times 10^3}{m\times t}, \qquad \textbf{Equation 1}$$

where:

V_I = ¿ initial titrant volume (mL);
V_f = ¿ final titrant volume, (mL);
$[NaOH]$ = ¿ Concentration of the NaOH solution (mol.L^{-1});
m = ¿ mass of N435 (g);
t = ¿ time (min).

Regarding enzymatic activity and stability, when performing the reuse of the biocatalyst, the kinetic of by-product collected mass and molar mass distributions of PBS samples at the reaction end were also measured to verify the performance of lipase through reuse cycles in the polycondensation. Thus, when the by-products amounts presented significant loss values, the enzymatic activity of N435 was measured, evaluating the catalytic capacity of the immobilized biocatalyst according to the previously prescribed procedure, and calculated using Equation 1. After measuring the enzymatic activity before the procedures and after the reuse cycles, the residual enzymatic activity (REA) was calculated using Equation 2:

$$REA\left(\%\right)=\frac{EA\,f}{EA\,i}x\,100, \qquad \textbf{Equation 2}$$

where:

EAf = ¿ Enzymatic activity at the end of use ($U.g^{-1}$);
EAi = ¿ Enzyme activity before participating in catalysis ($U.g^{-1}$);

To evaluate the diffusional limitations due to the clogging of the pores in the N435 support during reuse, nitrogen adsorption tests were performed, using the Brunauer, Emmett and Taller (BET) and Barrett, Joyner and Halenda (BJH) methods. With the tests, the surface area, average diameter, volume and distribution of pores of the immobilized biocatalyst will be evaluated before and after the recycling process. The desorption of gases was carried out at a temperature of 140 °C for 24 h. The equipment used was Autosorb-1, Quantachrome Instruments.

At the end of the last reuse cycle, N435 was partially replaced, thus 1.98 g (75% of the total mass) of the biocatalyst that had already been applied in the previous reuse cycles were added to 0.66 g (25 % of the total mass) of the fresh enzyme, to verify the behaviour of enzymatic polycondensation in a make-up procedure (MK).

3.2.6 Polymers Characterization

3.2.6.1 Thermal Properties

DSC (Jade-DSC equipment, Perkin Elmer) was used for the thermal behaviour analysis of the polymeric materials produced. Approximately 10 mg of PBS sample was heated at $10°C.min^{-1}$, from an initial temperature of -60 °C up to 200 °C. Nitrogen gas was used at a flow rate of 45 $mL.min^{-1}$. The results are presented in the annexes.

3.2.6.2 Molar Mass Distribution and Averages

Number (M_n) and weight (M_w) average molar masses, degree of polymerization (DP_n), molar-mass dispersity (Đ) and molar mass distributions were raised through the technique of Gel Permeation Chromatography (GPC). Samples preparation involved the dissolution of 20 mg of sample in 4 mL of chloroform and the solution formed was filtered with a nylon membrane, with a pore size of 0.45 µm. The equipment used was SHIMADZUL's LC-20AD, equipped with a RID-10A refractive index detector, an automatic injector SIL-20A, a PL gel MiniMIX pre-column (5µm, 50 x 4 mm) followed by two PL gel MiniMIX columns (5µm, 250 x 4.6 mm) in series. Runs were operated at 40 °C and molar masses were determined based on an calibration curve prepared with polystyrene standards (with molar masses ranging from 580 $g.mol^{-1}$ to $9.8x10^6$ $g.mol^{-1}$). Chloroform (HPLC grade) was used as eluent, with a flow rate of 0.3 $mL.min^{-1}$.

For comparison with the average molar mass values obtained via GPC, M_n values were also determined via Nuclear Magnetic Resonance (NMR). Sample preparation consisted of dissolving 5 mg of the polymer in 0.5 mL of deuterated chloroform. H^1 NMR spectroscopy was performed at a Bruker AVANCE system at 200 MHz. The results are presented in the annexes.

3.3 Results and Discussion

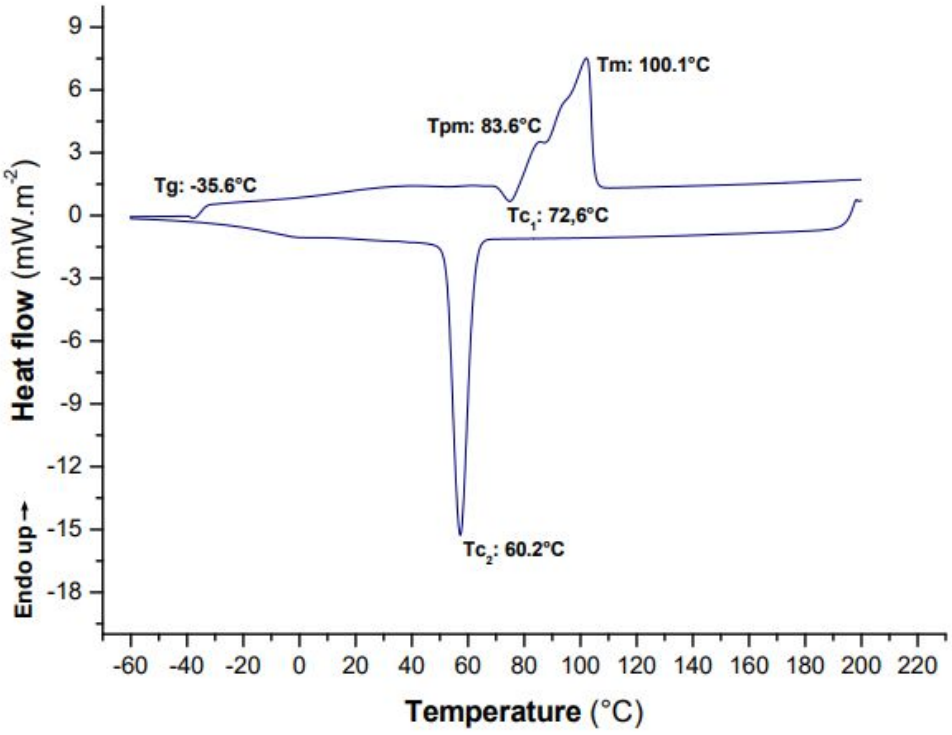
3.3.1 Influence of Reaction Medium Diffusivity on PBS Synthesis via N435

Regarding the kinetics of enzymatic polycondensation reactions, different reaction times were observed, with values between 25 and 90 min. The reactions were ended when the viscosity of the medium no longer allowed homogenization by magnetic stirring. Due to mild temperatures (60-100 °C) used in the enzymatic polycondensation, the thermal

properties of PBS were evaluated as a way to investigate the viscous behaviour of the reaction medium, from the PBS melting temperature (T_m). Through Figure 13, it is observed that PBS samples presented a solid behaviour at temperatures below 83.6 °C (the pre-melting limit temperature being T_{pm}), limiting the diffusion of polymer chains through the porous support to reach the active site of the enzyme. Still, temperatures above this value until a temperature of

Figure 13: DSC thermograms of PBS synthesized via enzymatic polycondensation (10 wt% of N435, at 90 °C) in bulk.

100.1 °C (T_m) showed a mixture of chains in the solid and molten state, being a determining factor for the lipase-catalysed polycondensation, improving diffusion in the reaction medium for a longer time due to the lower viscosities.



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Thus, temperature is a key factor for the enzymatic synthesis of PBS, mainly due to the thermal stability of the enzyme, where values close to 100 °C can cause a loss of activity

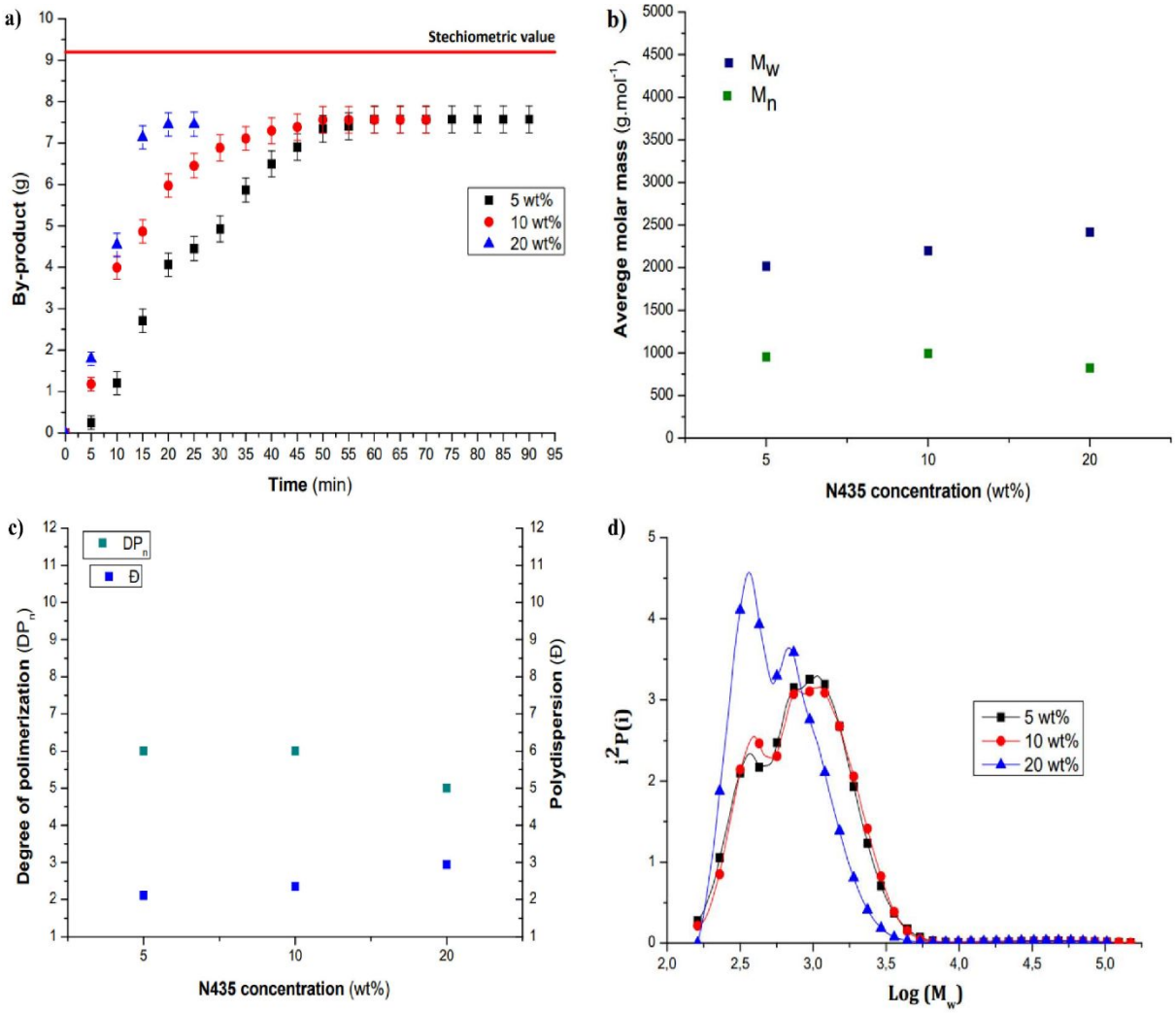
of the biocatalyst by denaturation (KUNDYS et al., 2018). Also, Lerin et al., (2011) reported that the optimal operating temperature of the N435 is 70 °C. However, temperatures below 83.6 °C, in addition to the growth of polymer chains during the reaction, can lead to high viscosities and be a limiting factor for the reaction, justifying the need to verify the ideal temperature for the synthesis.

Although the DSC analyses have not been performed for all samples, eventual changes in the thermal behavior of PBS samples are related to the molar mass distributions. As will be presented in the next sections, the polymerization degree changed between 5-10 in the investigated conditions. Therefore, although some changes could be eventually measured in the thermal behavior of PBS samples in the experimental runs, substantial changes are not expected. Despite slight changes in the thermal behavior of PBS, the main piece of information is that the temperature of enzymatic polycondensation is restricted by enzyme degradation temperature, and this system presents a state transition between solid and molten state polycondensation.

3.3.2 Effects of N435 Concentration

Concerning the proposed synthesis system, under the operational conditions presented in the previous sections, the effect of enzyme concentration (% by weight) on the production of PBS was evaluated at the optimal temperature of enzymatic activity (70 °C). Figure 14 shows the kinetic behaviour of the collected by-product, M_w and M_n, DP_n, $Đ$ and Molar mass distributions of the samples produced.

Figure 14: Effect of N435 concentration during enzymatic polycondensations on: a) Kinetic behaviour of condensed by-product; b) M_w and M_n; c) DP_n and $Đ$; d) Molar mass distributions. Reaction times were 90, 70 and 25 min, respectively, for 5, 10 and 20 wt% of N435.



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As observed in Figure 14a, different reaction times were required to reach the constant final amount of byproduct when the enzyme concentration was changed. Particularly, one can see the following end times: 90, 70, and 25 min, respectively, for 5, 10 and 20 % of enzyme concentration. In these runs, the results of collected ethanol amounts

suggest an increase in reaction rates when the enzyme concentration was increased, as a consequence of higher enzymatic activities. However, despite higher reaction rates with the increased enzyme concentration, one can see that reaction medium viscosity controls the maximum amount of recovered ethanol, which was approximately the same for evaluated conditions. Therefore, the reaction times were limited by the high viscosity of the reaction medium. Regarding the values of M_w and M_n, little variation was observed, confirming that reaction medium viscosity prevents further growth of polymeric chains by diffusional limitations in the porous support of N435, as PBS chains are in a solid-state at 70°C, as discussed based on DSC results. Nasr et al., (2020) also verified the effect of the amount of N435 for the enzymatic polymerization of diethyl adipate and 1,6-hexanediol, at 100 °C, and the variation of the enzyme concentration did not affect the reaction rate of the products in the bulk system.

Regarding the influence of N435 concentration on DP_n values (Figure 14c), one can see values approximately constant following the previous results of average molar masses. The values measured for *Đ* pointing to good uniformity of the chain lengths, where lower *Đ* values are expected for enzymatic reactions due to its high selectivity (PELLIS et al., 2018) compared to PBS synthesis reactions using catalysts of metallic origin, with high values as 8.3, for example (FERREIRA et al., 2015). A slight tendency of increase in the values of *Đ* with the increase of the amount of N453 in the reaction medium is noticed, due to the greater amount of biocatalyst, and consequently, of catalytic active centers.

When referring to the molar mass distributions (Figure 14d), similar values of catalytic efficiency via N435 are presented for the amounts of 5 and 10 % by weight for the synthesis system, corroborating the considerations of Nasr et al., (2020) who used 1 and 10 wt% and described little influence of the amount of biocatalyst for the shown polycondensation system.

However, when increasing the amount of N435 to 20 wt%, there is a rapid increase in the viscosity leading to diffusional limitations of the reaction. Also, as discussed earlier, at the used reaction temperature the PBS produced is in a solid state which causes further diffusional limitation problems. With the greater amount of catalytic sites when 20 wt% of N435 were used, higher reaction rates can be observed as shown by the initial slopes in Figure 13a. As a consequence, there is a substantial increase in the concentration of small polymer chains, since these chains cannot diffuse through the pores of the enzyme and the reaction between the functional groups in the active site of the enzyme does not occur. The similar mass values collected from by-products may be related to mass transfer limitations attributed to the high viscosity of the PBS produced, also linked to the tendency to form more heterogeneous chains with the increase in the amount of biocatalyst. Thus, with the rapid increase in the viscosity of the medium, which can be related either to the greater amount of solids coming from the N435 support and/or to the high production of low molecular weight chains, the reaction quickly stops, limiting the synthesis process with 20 % by weight. So, there is little influence on the synthesis in the amounts of 5 and 10 wt% of N435, with greater diffusional limitations for the value of 20 wt%.

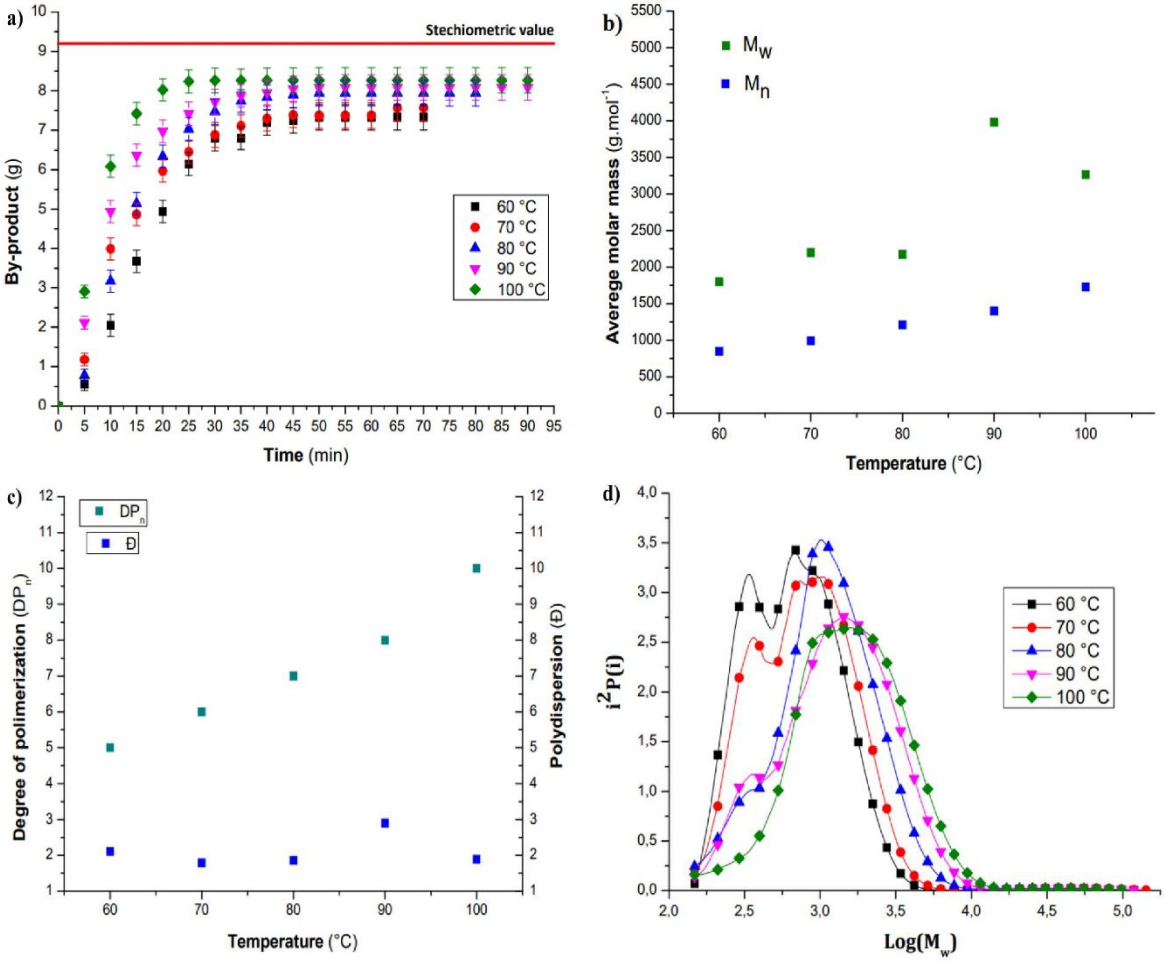
Therefore, through the discussion presented above, the condition of 10 wt% of N435 was selected for further tests, despite the similar values of collected ethanol and average molar masses at the end reaction observed in conditions with 5 and 20 wt% of N435. In this sense, the use of 20 wt% of enzyme presented the disadvantage of high reaction rates which can hinder the temperature control policies in industrial-scale reactors due to high heat release of these reactions, as well as increase the process costs associated with the higher amounts of enzyme. On the other side, the use of lower amounts of enzyme can limit the process feasibility due to higher required reaction times and enzyme activities which may decrease the

enzyme reuse cycles. Moreover, the selection of 10 wt% of N435 for polymer synthesis also corroborates the amount used in other studies (AZIM et al., 2006), (ZHANG et al., 2012).

3.3.3 Influence of Reaction Temperature

After selecting 10 wt% of N435, the temperature that may favour the enzymatic polycondensation was investigated. Thus, the temperatures evaluated were 60, 70, 80, 90 and 100 °C. The results found are shown in Figure 15.

Figure 15: Experimental results of: a) Kinetic behaviour of condensed by-product; b) M_w and M_n; c) DP_n and $Đ$; d) Molar mass distributions of the products obtained through the variation of temperature.



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The results presented in Figure 15 show that reaction temperature has a strong influence on the kinetic behaviour of enzymatic polycondensations. As observed in Figure 15(a), the lower final amounts of condensed by-products measured at 60 and 70 °C are associated with the high reaction medium viscosities due to the solid-state of PBS in these conditions, hindering the diffusion of polymeric chains in the enzymatic support. At temperatures between 80 and 100 °C, one can see higher amounts of condensed by-products associated with lower reaction medium viscosities due to the mixture of chains in the solid and molten state, as discussed based on DSC shown in Figure 13. The synthesis times were: 70 min for temperatures of 60 and 70 °C, 80 min for 80 °C, and 90 min for the values of 90 and 100 °C. The difference in reaction times is also explained based on observations in Figure 13, where temperatures above 83.6 °C already present chains in a molten state, allowing the diffusion of polymeric chains through the support of N435 to the active site of the enzyme, being a factor observed as a determinant in the M_w of the samples produced. Moreover, the increase in temperature favors the reaction rates, presenting greater amounts of by-products measured at higher reaction temperatures (90 and 100°C), in addition to the increase in the values of M_w and M_n, with emphasis on the value of M_w at 90 °C.

Although higher initial ethanol removal rates were observed at 90 and 100 °C with emphasis on results measured at 100 °C, one can see only a slight increase in the final amounts of ethanol collected at 100 °C, followed by some increase in the polymerization degree. Despite the higher by-product removal rates, due to lower viscosities and, therefore, higher external diffusion and by-product removal rates, the final conversion seems controlled by the internal diffusion of polymeric chains and ethanol through the porous support and the enzyme active sites.

In comparison to the M_w value of PBS synthesized with 10 wt% of N435 at 90 °C, 4,000 g.mol^{-1}, Gkountela et al., (2021) reported molar masses from 1,000 to 2,800 g.mol^{-1} in their pre-polymerization step (40 to 60 °C, 1 to 16 h of reaction) and 3,500 to 5,500 g.mol^{-1} in the post-polymerization (80 to 90 °C, 8 to 24 h of reaction), in reactions with DS and BDO (1:1), under vacuum and with 10 wt% N435, in solution reactions with toluene or isooctane. Through the NMR analysis (please see supplementary material for details), the M_n of PBS sample synthesized in this condition was 1,000 g.mol^{-1}, while a value of 1,400 g.mol^{-1} was measured by GPC for the same sample. Based on these results, the polymerization degree changed between 6 and 8. Therefore, the M_n values measured by these techniques can be considered similar, given the uncertainty of both techniques.

Thus, analyzing the M_w values mentioned above and reaction times, the condition of 10 wt% of N435 at 90 °C for PBS enzymatic synthesis presented similar values of molar masses when compared with the ones published by Gkountela et al., (2021), being an alternative for enzymatic synthesis of PBS on a reduced period of time.

Regarding the temperature variation, the DP_n (Figure 15c) is favoured with increasing temperature. The values in average repeat units were: 5, 6, 7, 8, and 10, for 60, 70, 80, 90 and 100 °C, respectively. Pellis et al., (2018) produced different polyesters, one of them being PBS. In 6 h of reaction, they obtained M_w of 1,094 and DP_n of 4.9 (using DS and BDO (1:1), 10 wt% of N435, 85 °C and 1 atm). Gkountela et al., (2021) obtained a DP_n range from 12 to 33 repeat units in solution reactions with isooctane and toluene (2:1 w/w), DS and BDO (1:1), 10 wt% N435, reduced pressure, temperatures from 40 °C (pre-polymerization) to 90 °C (post-polymerization) lasting up to 26 h. It is important to emphasize that the reaction time in solution are longer and favour the chain growth, due to the presence of a solvent that reduces the viscosity of the reaction medium.

Although higher molar masses can be obtained with reaction temperatures above PBS melting temperature (110-115 °C) (ZHANG et al., 2012), the use of these temperatures can substantially affect both enzymatic activity and stability. Particularly, the use of temperatures above 100 °C can lead the biocatalyst degradation due to enzyme denaturation, limiting the possibility of its reuse. It is worth mentioning that the scale-up and development of economically feasible enzymatic polycondensation processes also depend on enzyme stability through reuse cycles. Enzymatic polycondensation can be used as an oligomerization stage followed by polycondensation stages at higher temperatures to increase the molar masses, as typically performed in industrial sites.

In addition, the values presented for *Đ* by the variation of temperature were (Figure 15c): 2.1, 2.3, 1.8, 2.9 and 1.9 for 60, 70, 80, 90 and 100 °C, respectively. Therefore, one can see that the molar-mass dispersity was not affected by the reaction temperature, as these values fluctuate around an average value (with small increase in value of 90 °C), indicating the polycondensation mechanism was not affected by temperature. The mechanism of (poly)transesterification catalyzed by lipase involves the reaction between the lipase-ester complex and a polymeric species with a hydroxyl end group in the active site of the enzyme. When the reaction temperature is below of pre-melting temperature of PBS, the enzymatic reaction advances until the point at which the PBS produced in a solid-state limiting substantially diffusion and the reaction stops due to the absence of mobility of polymeric chains. When the temperatures are above 83.6 °C, the molar masses increase because the polymeric chains are near to the molten state and, therefore, have segmental mobility to diffuse through the porous enzymatic support and react in the active site of the biocatalyst. Also, the values of the molar-mass dispersity found in this work are similar to those seen in other works (1.07 to 1.79 (PELLIS et al., 2018)).

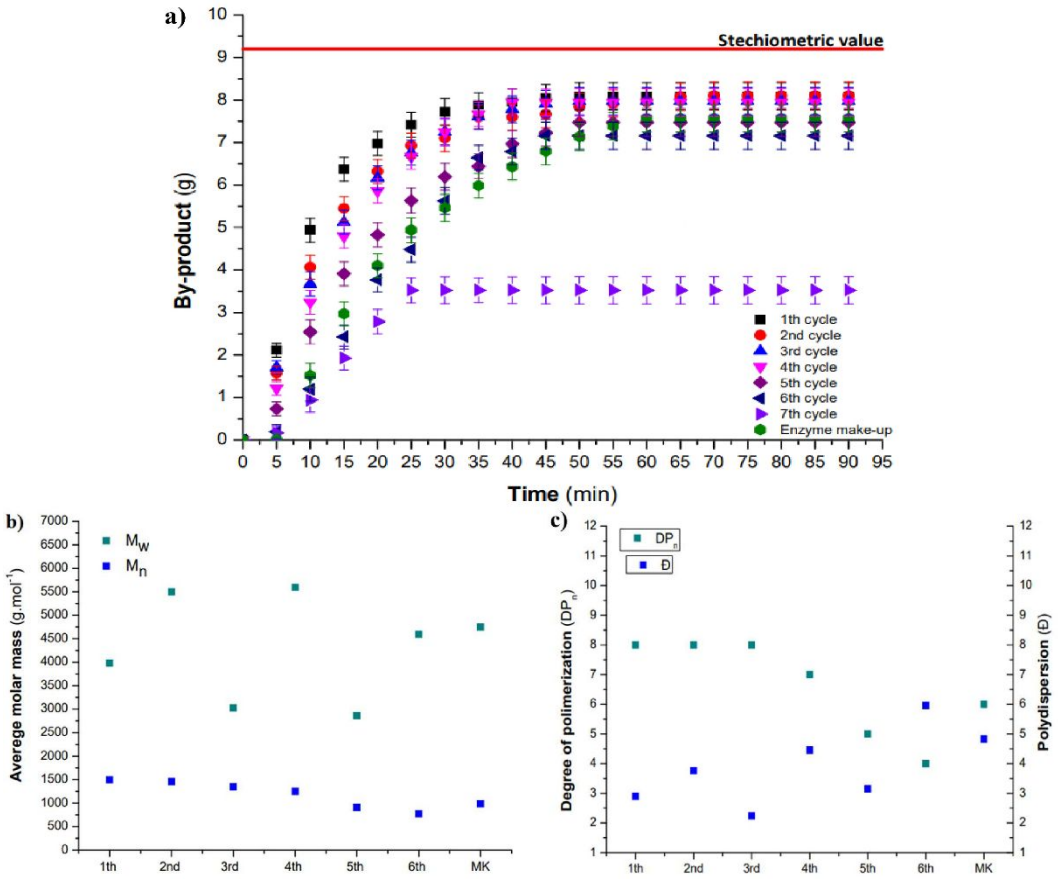
Through the molar mass distributions (Figure 15d), it is possible to verify that with increasing temperature, the polymeric chains present a more uniform size, with evidence at temperatures above 80 °C. Molar mass distributions at 60 and 70 °C presented bimodal peaks associated with polymeric chains of low size, with an increase in the concentration of these chains at 60°C. This peak was less apparent at 80 and 90 °C, while a distribution with monomodal behaviour was observed at 100 °C. It is also possible to corroborate the discussion in Figure 13, as the decrease of low-size chains peak occurred at temperatures above 83.6 °C when the polymeric chains are in a pre-molten state, and can diffuse through the pores and react in the catalytic site of the biocatalyst.

Therefore, the temperature chosen to proceed with the investigation of the reuse of N435 was 90 °C, with 10 wt% of N435, based on results of condensed by-product and molar masses. No studies were found on the stability of enzymatic activity in polymerization systems at a temperature of 90 °C. Only one study of polycondensation via enzymatic transesterification was found, at temperatures of 100 °C, using diethyl adipate and 1,6 - hexanediol as monomers, in 3 cycles of reuse of N435. The following section presents the results of the reuse of the immobilized biocatalyst.

3.3.4 Enzyme Reuse

Based on evaluating the reuse of N435 in different PBS synthesis cycles, the enzymatic activity was evaluated before the first cycle, in addition to monitoring the amounts of by-products in each cycle of use. So, through Equation 1, the initial enzymatic activity was 32.4 $U.g^{-1}$. The value presented was adopted as the maximum value of catalytic activity for the process. Thus, the reuse cycles were performed, where Figure 16 shows the results in each use cycle of N435 as a catalyst synthesis (under the conditions of 10 wt% and 90 °C).

Figure 16: Experimental results of: a) Kinetic behavior of condensed by-product; b) M_w and M_n; c) DP_n and *Đ* of the products obtained through the N435 reuse. Average molar mass, DPn and *Đ* values for the 7th cycle were not included due to their molar mass being lower than the calibration standards used (580 $g.mol^{-1}$). Reaction time weas 90 min.



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As seen in Figure 16a, 6 reuse cycles of N435 were possible, plus the enzyme make-up. By observing the values of by-products collection, it was evidenced the decay of the by-products remotion rates, indicating the gradual loss of activity in the enzyme during reuse. During the first 4 cycles, the amounts of by-products at the reaction end were uniformly close (8.08 g (1st cycle), 8.10 g (2nd cycle), 7.97 g (3rd cycle) and 7.94 g (4th cycle)), decaying in the following recycles (7.47 g (5th cycle), 7.16 g (6th cycle) and 3.52 g (7th cycle)). Thus, concerning the values of by-products obtained in the 7th cycle of N435 use compared to the 1st cycle, a 56.4% decrease was observed in the by-products remotion. After the 7th reuse, the enzymatic activity was measured again, and the value obtained was 3.8 U.g^{-1} (corresponding to a residual activity of 11.7 %), defining the end of the reuse process. Then, the biocatalyst used after the 7th cycle was recycled in a enzyme make-up procedure, with 25% of its mass being replaced by a new enzyme, applied again in a new synthesis cycle. So, an increase in by-product remotion was observed, with values above the 5th reuse cycle, obtaining 7.56 g of by-products (a 114.8% increase compared to the 7th cycle).

According to results shown in Figure 16b, M_w data were not significantly affected in the first 6 reuse cycles, fluctuating close to the initial M_w value, which may indicate that the mass fraction of the longer polymer chains was not altered throughout the reuse cycles. This behaviour is attributed to how the M_w is calculated, being a weighted average that shows the mass value of the polymeric chains (favouring higher mass values). However, the M_n data (being calculated through the arithmetic mean of the mass value of the distribution of polymeric chains) corroborate the kinetic behaviour of by-product remotion, showing greater similarity between the values of the first 4 cycles, followed by the decay of the M_n values in the final cycles, with an increase after the addition of fresh enzyme. This

behaviour indicates an increase in the number fraction of polymeric chains with lower size. The average molar mass values for the 7th cycle were below the standard average molar mass values (580 g.mol^{-1}) used in the GPC analysis. With this, and based on the measured by-product values, it is possible that there are only dimers or trimers produced in the 7th reuse cycle. Thus, when evaluating the values of Mn obtained in the 1st and 6th cycle of reuse, there was a decrease of 48.5 % in the values observed, followed by an increase of 27.7 % after replacement with a new biocatalyst (concerning the 6th cycle M_n).

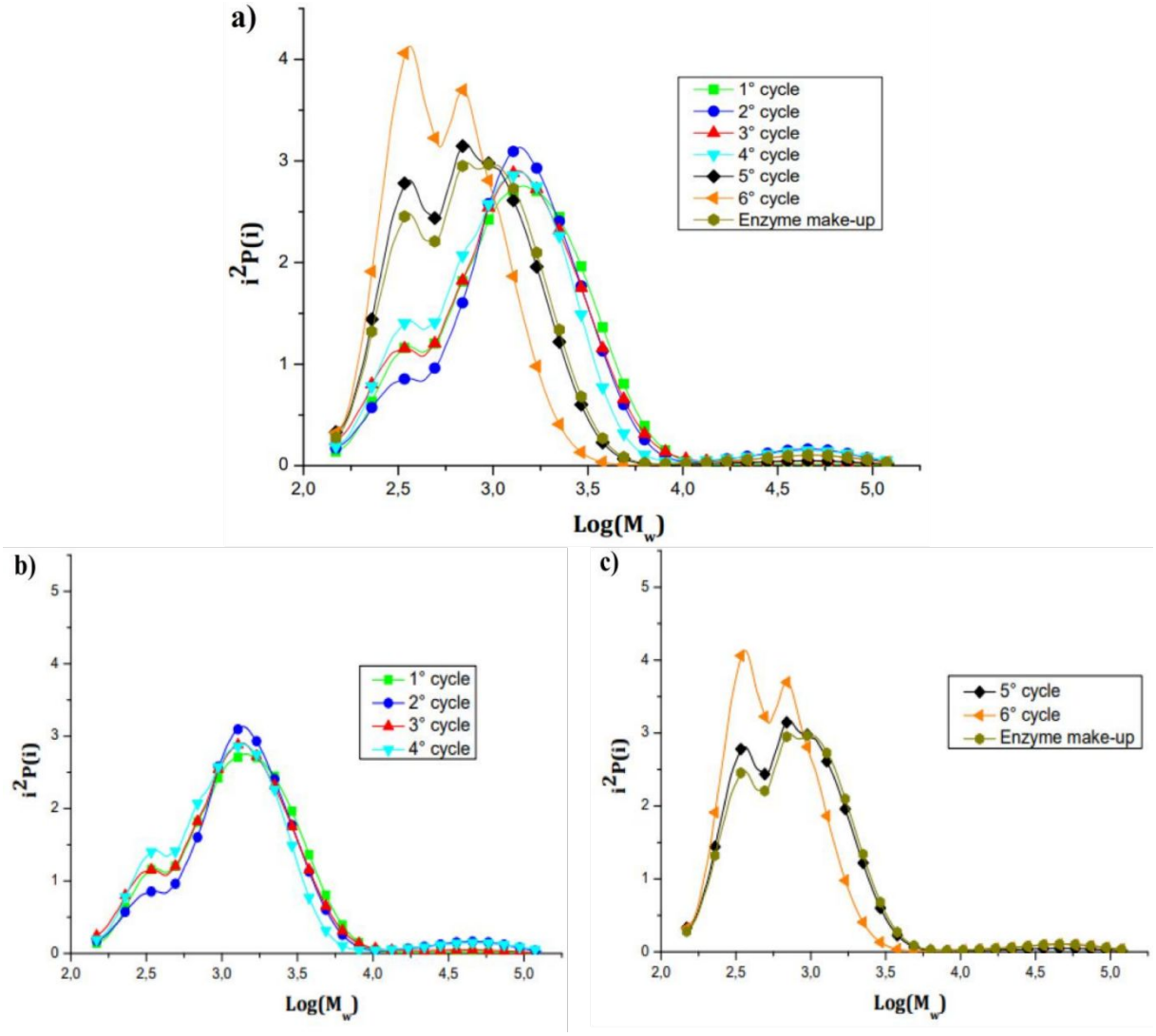
Nasr et al., (2020) also carried out the reuse of N435, but with other monomers, being diethyl adipate and 1,6-hexanediol. In their work, they investigated bulk and solution polycondensations (with diphelinic ether), where the reuse of the bulk system (operated at 100 °C with 10 wt% N435) did not present a significant change in the M_n of the polymeric products during 3 reuse cycles.

The values of DP_n in Figure 16c show that the values of the first 4 cycles are similar, decreasing in the following ones. Enzyme make-up showed an increase in the DPn value, which is important for the process of reusing enzymatic biocatalysts and for the cost of producing polymeric materials on an industrial scale.

Regarding the *Đ* (Figure 16c), there is an upward trend in molar-mass dispersity in the first six reuse cycles, indicating an increase in the dispersion of polymeric chain length formed through the reuse of N435, which can be explained by the decay of the catalytic activity of the biocatalyst (32.4 to 3.8 U.g^{-1} at the end of the last cycle). However, 4 cycles were possible with good reuse efficiency, followed by the decay of the enzymatic activity of N435 in the next 3 cycles, with the recovery of the enzymatic activity when the enzyme make-up was performed.

Figure 17 represents the behavior of the molar mass distributions obtained during the recycling process of the biocatalyst.

Figure 17: Representation of: a) molar mass distributions in N435 reuses; b) molar mass distribution of the first cycles of reuse of N435; c) molar mass distribution of the last N435 cycles.



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As observed, along with the reuse, the synthesis efficiency decreases, indicating the gradual loss of the catalytic activity of N435. The first four cycles showed good catalytic

efficiency (as indicated by the similarity among the molar mass distribution curves of these reactions, Figure 17, kinetic behaviour in Figure 16a, M_n in Figure 16b and DP_n in Figure 16c), with low dispersion of chain length distributions. However, in the following cycles, one can see the appearance of bimodal peaks (important increase in the low molar mass population) and by association with greater polydispersity and lesser DP_n, as seen in Figure 16c. The strong increase of the peak associated with polymeric species with low chain length is possibly a result of the decrease in enzymatic activity through reuse cycles by protein denaturation at 90 °C and clogging of enzyme pores by polymeric chains.

When evaluating the values of specific surface area, there is a decrease in the values obtained, with initial surface area results of 79.6 $m^2.g^{-1}$ decreasing to 38.8 $m^2.g^{-1}$. The pore diameter averages were 461 (initially) to 252 Å at the end of reuse, with maximum volume of desorption pores measured from 0.9162 to 0.2450 $cm^3.g^{-1}$ at the end of the process. Thus, it is observed that even with the cleaning cycles carried out with chloroform between the reuse cycles, polymeric chains obstructed the pores of the support, causing diffusion problems. In this way, problems of percolation of polymeric molecules through the pores of the support to the adsorbed enzyme are noticed, limiting the chain growth and favoring the emergence of the bimodal behavior of the molar mass distributions, as observed in Figure 17. Thus, both the decay of the catalytic activity by denaturation, as by the obstruction of the pores, can cause the loss of the catalytic activity of N435 in the reuse cycles.

However, as an important part of the data, with the make-up with the new N435, part of the system efficiency is recovered, showing chain length distributions similar to the 5^{th} reuse cycle. Then, as expected by the enzymatic activity presented initially and in the 7^{th} cycle (32.4 to 3.8 $U.g^{-1}$), when passing through the reuse cycles, the behaviour of the chains shows a bimodal tendency, with low DP_n values and M_n, Figure 16c) and greater heterogeneity in the

moolar mass distribution of synthesized polymer chains. So, 4 cycles of reuse of N435 for PBS synthesis are indicated, under the conditions presented in this study.

3.4 Conclusions

Based on a bulk system for the production of PBS catalyzed by N435 and the objectives of this work, the best synthesis conditions, kinetic behaviour of the process and properties of the polymeric materials produced were described, also evaluating the reuse of the immobilized biocatalyst. M_w values were obtained between 2,000 and 4,00 $g.mol^{-1}$, varying the amount of biocatalyst between 5, 10 and 20 wt%, at temperatures from 60 to 100 °C. The outstanding conditions were 10 wt% N435, at 90 °C (obtaining M_w of 4,000 $g.mol^{-1}$), with a reaction time of 90 minutes, being the conditions selected for the operation of the biocatalyst's recyclability. The analysis of the reuse process showed that four cycles allowed good results, followed by the decay of the catalytic efficiency of the N435 (a total of 6 cycles plus the enzyme make-up). However, based on the results presented, it is evident that the understanding of the kinetic behaviour in conjunction with the stability of immobilized biocatalysts applied in polymer synthesis is of extreme relevance; nevertheless, is little addressed in studies in the literature. Still, such explorations should be expanded to other synthesis systems via biocatalysis with immobilized enzymes, verifying better synthesis conditions, more efficient production of polymers and evaluating possibilities of greater efficiency in the reuse of N435 and other biocatalysts in polymeric synthesis processes. Such evaluations are of great importance for the modelling, control, design, scale-up and optimization of enzymatic polycondensation processes.

CHAPTER 4

Chapter 4 presents the results obtained for the synthesis of PBS catalyzed by N435 in the bulk and solution reactions, focusing on the effects of solvent use, kinetic aspects of the synthesis, distribution of polymeric chains and reuse of the biocatalyst (for the solution reactions). An research article, to be submitted, was written based on the results presented on Chapter 4.

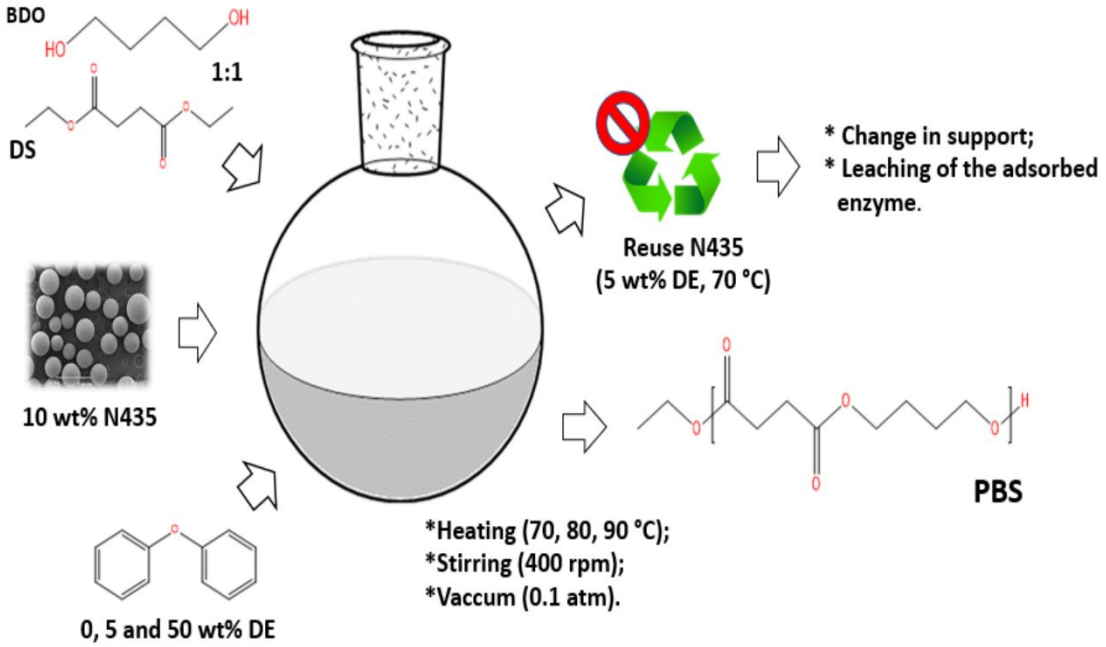
4 Lipase-Catalyzed Solution Polycondensation of butandiol and diethyl succinate: Effect of diphenyl ether concentration, Enzyme Stability, Reuse and PBS molar mass distribution

Abstract

Environmental problems related to polymers and petrochemical source monomers have stimulated the interest in developing renewable and biodegradable polymers. Among these, Polybutylene Succinate (PBS), a biodegradable polyester produced from monomers provided by biotechnological processes, has been highlighted. When referring to production aspects of PBS, biotechnological catalysts have been gaining prominence, due to their high selectivity, milder operating temperature, absence of toxic metals and the possibility of reuse (when immobilized). Therefore, this work seeks to investigate the enzymatic polycondensation in solution for PBS synthesis, using diethyl succinate and 1,4-butanediol as monomers, Novozym 435 as biocatalyst, and diphenyl ether as solvent. The possibility of reusing the immobilized biocatalyst was also addressed. Solvent-free reactions, or containing 5 and 50 wt % of diphenyl ether were tested, at 70, 80 and 90 °C. Characterizations via Gel Permeation Chromatography were realized to evaluate the catalytic efficiency of the process, in addition to monitoring the kinetic behaviour of the synthesis (via by-product collection). The reactions containing 5 wt% of solvent proved to be more advantageous, resulting in M_w between 2,480 and 3,341 $g.mol^{-1}$, in reaction times between 60 and 90 min, being the selected condition to carry out the reuse of the biocatalyst, operating at 70 °C. A pronounced decrease of the enzymatic activity was observed in the N435 reuse cycles, indicating that diphenyl ether affected the stability of the immobilized biocatalyst. Small additions of diphenyl ether presented diffusional advantages when compared to solvent-free processes. However, further explorations are still needed in research to obtain greater stability of the immobilized biocatalyst, providing subsidies for future works aimed at expanding the scale and commercial/industry production of biocatalyzed PBS.

Keywords: Polybutylene succinate (PBS), Lipase; Bulk and Solution Polycondensation; Kinetic evaluation; Enzyme reuse.

Graphical Abstract



4.1 Introduction

Due to the concerns about environmental preservation and the search for materials that are less aggressive to the environment, processes and products based on Green Chemistry principles have been gaining prominence (WORTHINGTON; KUCERA; CHALKER, 2017). When this discussion is addressed on polymers, different issues are emphasized, especially concerning the origin of the monomers, the constituents of the synthesis process and later the destination of their discards (STERNBERG; SEQUERTH; PILLA, 2021). Another factor is related to the large production scale of these materials, which appear among the most synthesized materials on the entire planet (WORTHINGTON; KUCERA; CHALKER, 2017) (aimed at meeting world consumption needs). About 320 million tons of polymers are generated annually (PALETTA et al., 2019), causing environmental problems when their disposal is inappropriate. Still, as discussed by Scholten (2021), applications of polymeric materials prove to be virtually irreplaceable by other materials (such as glass, and steel). In any case, one of the main issues involving environmental degradation is focused on the reuse and recyclability, indicating the awareness of the circular use of these resources, aiming at sustainability.

Currently, polymer production processes are based on non-renewable sources, with about 90% of the monomers coming from petrochemical origin (DOURADO FERNANDES et al., 2022). In addition, a wide discussion of alternative methods of production has been performed, based on environmental preservation and sustainability in the synthesis processes. These aspects aim to meet current environmental demands, with the least possible impact on future generations (DUBÉ; SALEHPOUR, 2014), through the use of renewable inputs and bioprocesses.

Taking into account these considerations, Poly(butylene succinate) (PBS) has been gaining prominence, as its monomers can be produced by fermentation routes (KAWAGUCHI; OGINO; KONDO, 2017). Moreover, PBS presents physicochemical characteristics similar to the main polyolefins (DE MATOS COSTA et al., 2020), in addition to good biodegradability (HU et al., 2020), and wide applicability, such in agriculture, packaging, engineering (HU et al., 2019a) and biomedical sectors (RAFIQAH et al., 2021).

Regarding the works presented in the literature, Gkountela et al., (2021) described that (organo)metallic catalysts are commonly applied in PBS synthesis processes, also pointing out the use of enzymatic catalysts. Particularly, the use of enzymes has advantages over (organo)metallic catalysts, such as high product selectivity, and lower synthesis temperatures. In addition, the use of immobilized lipases in polymeric supports enables its reuse, being a great advantage over non-immobilized catalysts (SEN; PUSKAS, 2015). It is worth also mentioning that catalysts of metallic origin can undergo adsorption in the products, generating toxicity (ALBUQUERQUE et al., 2014), which is undesirable for some applications. However, some disadvantages are listed in enzymatic processes, such as longer synthesis times and lower average molar masses (POSPIECH et al., 2021). As an alternative, a technique already used in the production of high molar mass PBS can also be useful to increase the size of the polymeric chain produced via enzymatic synthesis, in a two-step synthesis route. In these processes, the first step is typically an enzymatic oligomerization (formation of low molar products) (NISOLI; DOHERTY; MALONE, 2004), followed by a polycondensation step in the molten state, for transesterification of the chains and increase of the polymeric chains (MENDES et al., 2014).

By referring to polymer production processes, Dubé and Salehpour (2014) stated that polycondensation reactions in solution have an advantage over bulk ones, due to lower

viscosities, as well as higher heat and mass transfer rates. Furthermore, they indicated that not all polymerization reactions can be conducted in the absence of solvents, and their presence in the reaction medium can benefit the physicochemical properties of the materials. In bulk reactions for the enzymatic synthesis of PBS, Guckert et al., (2022) observed limitations in the diffusibility of polymeric chains, due to the synthesis temperature being lower than the melting temperature of the polymeric material. Thus, as these reactions are typically solid-state polycondensation reactions, the use of solvents can be interesting, as it reduces the viscosity of the medium, favouring both the external and internal mass transfer rates. Fernandes et al. (2022) also indicated that the use of solvents improved the diffusivity of monomers and polymeric species in the reaction medium, allowing the obtaining of products with higher molar mass values. The authors ((Fernandes et al. (2022)) also describe diphenyl ether (DE) as an option with good results concerning higher molecular weights. Although the use of the solvent can be harmful to the environment, its use in industrial plants involves its recovery in separation steps (distillation and pervaporation, for example), and its recycling in the synthesis process for economic reasons (TOZZI et al., 2018). Azim et al., (2006) carried out a study on the effect of solvent on the enzymatic synthesis of PBS, where the use of DE led to the best average molar mass results (M_n: 10,000 $g.mol^{-1}$), followed by diglyme (4,400), bulk (3,300) and dodecane (2,500). Hevilla et al., (2021) corroborates these results, where the use of DE also led to the best molar mass values in enzymatic polymerization reactions.

Several works were published involving the synthesis of PBS via enzymatic catalysis (HEVILLA et al., 2021), (YU et al., 2012), (AZIM et al., 2006). However, some aspects of great relevance still need to be addressed, mainly about the kinetic behaviour of enzymatic polycondensation in bulk and solution, as well as the reuse of the biocatalyst (when immobilized). Thus, this work aimed to elucidate the kinetic behaviour of PBS synthesis

catalysed by Novozyme® 435 (N435) in bulk and solution reactions, as well as evaluate the reuse of the N435, to verify the effect of DE on both the production of PBS and the catalytic stability of immobilized lipase.

4.2 Material and Methods

4.2.1 Chemicals and Enzyme

Novozym® 435 immobilized lipase from Candida antarctica was kindly donated by Novozymes. Diethyl succinate (DS, purity ≥ 99 %), 1,4-butanediol (BDO, purity ≥ 99 %), diphenyl ether (DE, purity ≥ 99 %), sodium hydroxide (NaOH, 99 % minimum purity) and deuterated chloroform anhydrous (99.8 atom %D, with purity ≥ 99 %) were purchased from Sigma-Aldrich. Chloroform (minimum content of 99.8 %) was acquired from Dinâmica. Chloroform (HPLC grade, 99.8 % purity) was bought from J.T.Baker. Oleic acid (OA, purity ≥ 99 %) was obtained from Synth and ethanol (EtOH, purity 95 %) from Neon. All components were used as received without further purification or modification.

4.2.2 Experimental Unit

The experimental unit is composed of a vertically mounted system, containing: a reaction flask, dean stark, condensation column (cooled by thermocryostatic bath, MQBTC99-2, Microquímica), vacuum outlet, trap, vacuum pump (I2P-740/ SCN, Indutec), heating plate with magnetic stirring (C-MAG HS 7, IKA) and thermal bath. The experimental unit was designed based on the use of vacuum to remove polycondensation by-products, shifting of the reaction chemical equilibrium to favour the formation of high molar mass polymeric chains (DOUKA et al., 2018). An illustrative scheme of the experimental unit is provided in the supplementary materials (Figure 26).

4.2.3 PBS production

Bulk and solution reactions were evaluated to elucidate the effect of solvent (DE) on the PBS synthesis catalyzed by N435. Thus, the experimental runs were performed with equimolar proportions of monomers under magnetic stirring of approximately 400 rpm and vacuum (0.1 atm), at different temperatures (70, 80 e 90 °C). Thus, 0.1 mol of DS (17.42 g) and 0.1 mol of BDO (9.01 g) were used in each synthesis. The amount of N435 used was 10 wt% (regarding the total mass of monomers), corresponding to 2.64 g of the biocatalyst. For the reactions in solution, 5 wt% (1.32 g) and 50 wt% (13.21 g) of DE were applied, to enable the decrease in the viscosity of the reaction medium. The condensation column of the experimental unit was set at 5 °C for the condensation of the reaction by-products, which were collected and weighed every 5 min to investigate the kinetic behaviour of each experimental run. Reaction times ranged between 60 and 90 min. The criterion for determining the end of the reaction for each experimental condition was based on the high viscosity of the medium, making it impossible to continue with homogenization by magnetic stirring. Additionally, the molar mass distributions of PBS samples were measured at the end of each reaction.

4.2.4 Purification of Synthesis Products

After each synthesis procedure, chloroform was added to the reaction medium to solubilize the chains and allow the separation of N435 from the polymer solution by vacuum filtration. For bulk reactions, after the enzyme separation, the liquid phase was heated again on a heating plate under stirring (approximately 200 rpm) at 65 °C for solvent evaporation, until the observation of a constant volume. For the reactions in solution, ice-cold EtOH was added to the filtered liquid products, for precipitation of the polymeric chains. Subsequently,

the precipitated polymer was filtered and reserved on filter paper. This procedure is performed due to the high vapour pressure of DE. All samples were taken to a forced convection oven (DL – AF, De Leo), and left for 48 h at 60 °C to certify the complete evaporation of solvents. For the solution reactions, the mass yield was calculated using Equation 3.

$$Yield(\%) = \frac{M_{dp}}{(M_{DS} + M_{BDO}) - M_{EtOH}} \times 100 \qquad \textbf{Equation 3}$$

Where:

M_{dp} = Mass of dried polymer at the end of the reaction (g);
M_{DS} = ¿ Mass of DS used in the reaction (g);
M_{BDO} = ¿ Mass of BDO used in the reaction (g);
M_{EtOH} = Mass of EtOH produced at the end of the reaction (g).

Additional tests on the filtered products in the purification process were carried out to verify the possibility of the presence of oligomers not precipitated with EtOH. Thus, of FT-IR spectra were obtained using a Shimadzu's spectrometer, IR Prestige 21. The sample preparation consisted of applying drops of the filtrate on the KBr tablet and carrying out the analysis after evaporating the solvent. The spectra are presented in the supplementary materials section (Figure 27).

4.2.5 Enzyme Reuse and Stability

After the filtration to separate the polymer solution and the enzyme particles, the biocatalyst retained on the filter paper was washed 3 times with chloroform and dried in a forced convection oven for 24 h at 60 °C. After this procedure, the N435 was used in a new

synthesis cycle. The conditions chosen for the enzyme reuse were 5 wt% of DE at 70 °C and 60 min. Furthermore, it is important to note that the collected by-product was again weighed every 5 min, with the condenser temperature at 5 °C.

Enzymatic activity was evaluated before the use in polycondensation processes and after each reuse cycle. The method consists of the esterification of oleic acid (OA) and ethanol, with acid concentration measurements before and after the test. Thus, 0.045 mol (12.771 g) of OA, 0.045 mol (2.071g) of EtOH, and 0.013 % w/w (0.195 g) of N435 were magnetically stirred at 40 °C, for 40 min. Samples from time zero and 40 min (0.150 μL of each, diluted in 20 mL of EtOH) were titrated with NaOH solution (0.05 $mol.L^{-1}$) to determine the acid concentration. Thus, through Equation 1, the enzyme activity was determined, in $U.g^{-1}$ (capacity to react 1 μmol of monomer molecules per min for each gram of enzyme).

Regarding enzymatic activity and stability, when performing the reuse of the biocatalyst, the kinetic of by-product collected mass and molar mass distributions of PBS samples at the reaction end were also measured to verify the performance of lipase during reuse cycles in this polycondensation. Thus, the complete loss of enzymatic activity was assumed when the amounts of by-products presented a significant decrease, and the residual enzymatic activity (REA) presented values lower than 10 %, as calculated by Equation (2).

To verify the behaviour of enzymatic polycondensation in a make-up procedure (MK) at the end of the last reuse cycle, the biocatalyst was partially replaced, for this 1.98 g (75 % of the total mass) of the N435 that had been used in the reuse cycles was mixed to 0.66 g (25 % of the total mass) of fresh biocatalyst.

Still referring to the enzymatic activity and stability, tests involving the solvent (DE) were carried out, to verify its effect on the enzyme activity. Samples of N435 were left in contact with DE, at 70 °C, stirring at 400 rpm and vacuum, for 60 min, simulating the

conditions of enzymatic transesterification reactions. Then, the biocatalyst was washed 3 times with chloroform and dried in a forced convection oven, as described above. Scanning electron microscopy (SEM) was performed to investigate the morphological characteristics of the N435 support after contact with DE. This analysis was carried out using a JSM microscope – 6390 LV (gold coating and 10 kV voltage), at 25 and 200x magnification. In addition, confocal laser microscopy (CLSM) was analysis was performed to qualitatively verify the amount of enzyme adsorbed to the N435 support before and after contact with DE, using DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes®, USA), as a protein indicator. Fluorescence verification was performed using a DMI6000 B (Leica®) with a 63x objective lens with image processing in the software Leica Application Suite X (LAS X).

4.2.6 Molar Mass Distribution of Polymeric Chains

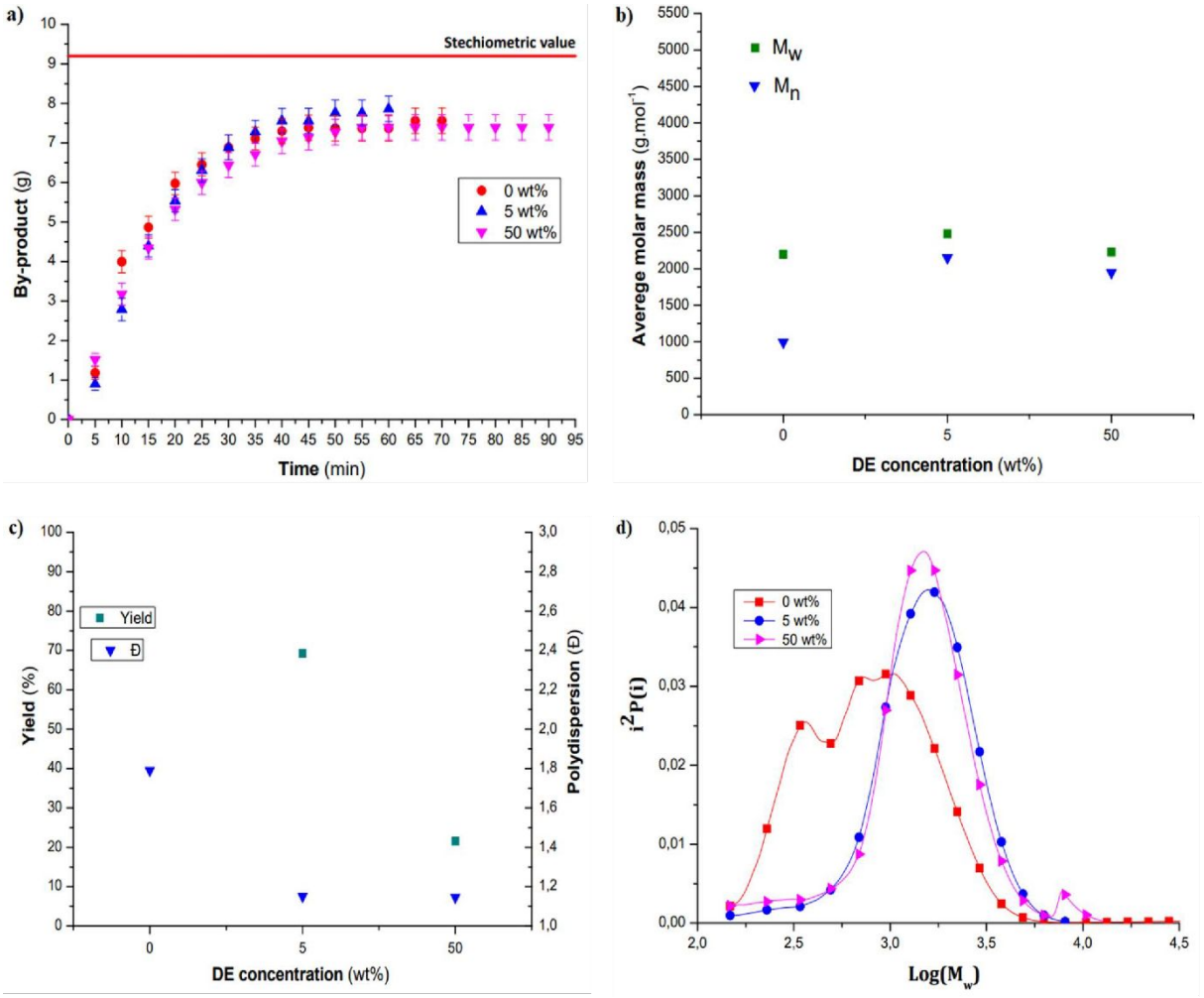
Molar mass distributions, weight average (M_w) and number average molar mass (M_n), and molar-mass dispersity (Đ) were measured by Gel Permeation Chromatography (GPC) at the end of all reactions. Sample preparation involved the dissolution of 20 mg of PBS in 4 mL of chloroform, followed by filtration using a nylon membrane with a pore size of 0.45 µm. The equipment used was SHIMADZUL's LC-20AD, equipped with a RID-10A refractive index detector and automatic injector SIL-20A, coupled with a PL gel MiniMIX pre-column (5µm, 50 x 4 mm) plus two PL gel MiniMIX columns (5µm, 250 x 4.6 mm) in series. Chloroform (HPLC grade) was used as the mobile phase, with a flow rate of 0.3 $mL.min^{-1}$. Runs were operated at 40 °C, and the calibration curve was prepared with polystyrene standards (with molar masses ranging from 580 $g.mol^{-1}$ to $9.8x10^6$ $g.mol^{-1}$).

4.3 Results and Discussion

4.3.1 Effect of Organic Solvent on N435-Catalyzed PBS Synthesis

To evaluate the effect of solvent (DE) on the N435-catalyzed PBS synthesis, bulk and solution reactions (containing 5 and 50 wt% of solvent based on monomer masses) were performed at 70, 80 and 90 °C. Figure 18 presents the following results: a) kinetic behaviour based on collected by-product masses; b) average molar masses (M_w and M_n); c) molar-mass dispersity (Đ) and d) Molar mass distributions of the reactions performed at 70 °C.

Figure 18: Results obtained in the reactions at 70 °C: a) Kinetic behaviour of condensed by-product; b) M_w and M_n; c) *Đ*; d) Molar mass distributions. The synthesis times were 70, 60 and 90 min in 0, 5 and 50 wt% DE, respectively.



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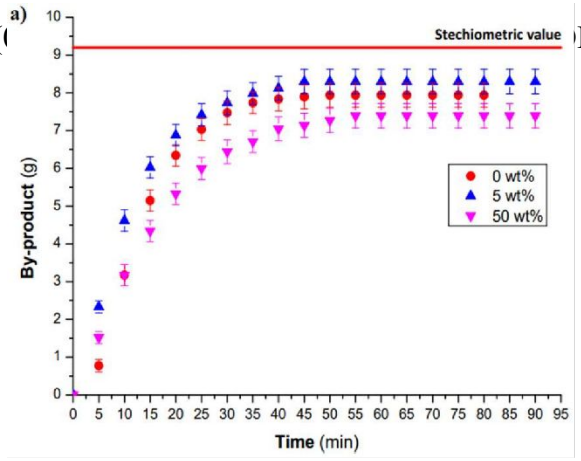
As seen in Figure 18a, the kinetic behaviour of the reactions in terms of by-product formation seems to be similar. And the final amounts of collected by-products were also quite similar: 7.65 (0 wt% DE), 7.86 (5 wt% DE) and 7.39 g (50 wt% DE) for reaction times of, respectively, 70, 60 and 90 min. Here it is important to remember that the criterion for determining the end of each reaction was based on the high viscosity of the medium, making it impossible to continue with homogenization by magnetic stirring, thus shorter reaction times are associated with a faster increase with of the viscosity due to higher reaction rates. In the bulk reaction conducted at 70°C after the consumption of the monomers the diffusivity of growing polymer chains is severely limited as this reaction tempertures is below the pre-melting temperature of PBS (T_{pm}, approximately 83.6 °C), as discussed in the previous work (GUCKERT et al., 2022). Thus, the shorter reaction time required when 5 wt% of DE was used is related to the increase of the mobility of the growing polymer chains. On the other hand, when solvent concentration is increased further to 50 wt% the effect of the lower reactant concentration prevails and a longer reaction time is required until the increase of the viscosity of the reaction medium hinders the stirring of the reaction medium.

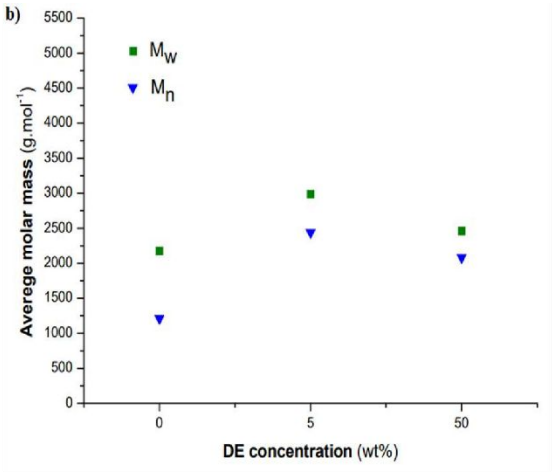
When evaluating average molar masses (Figure 18b), one can see that in the presence of 5 wt% of DE M_w of 2,500 g.mol^{-1} can be achieved. Pellis et al. (PELLIS et al., 2018) reached M_w of 1,094 and M_n of 851 g.mol^{-1}, at 85 °C and 6 h. The results presented are as efficient as those of the work of Pellis et al., also suggesting that shorter reaction times can be applied in the synthesis of PBS through enzymatic transesterification, justifying the importance of elucidating the kinetic behaviour of these reactions. The low molar mass dispersities (Đ in Figure 18c) and narrow distributions (Figure 18d) of the reactions

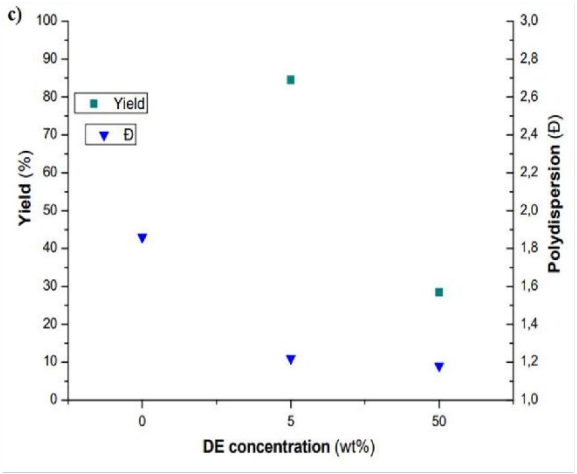
performed in solution are associated with the purification scheme applied to separate the polymer from its solution. When solubilizing the polymeric chains in chloroform, followed by precipitation with ice-cold EtOH, the oligomers (such as dimers and trimers) might remain soluble and being filtered out. As consequence, the measures of molar mass distributions are narrower and shifted to higher values.

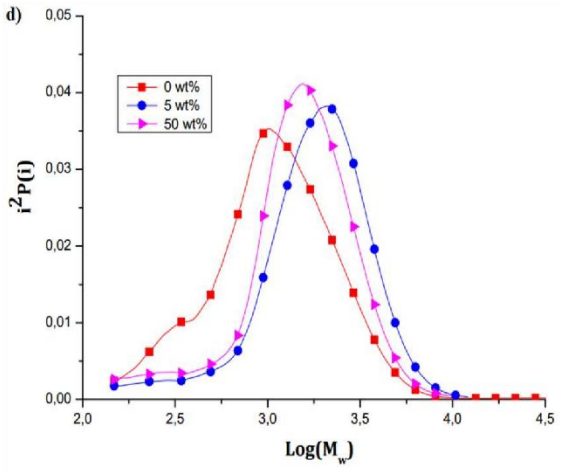
Regarding the mass yield of reactions in solution presented in Figure 18c (being a relevant factor to evaluate possible losses of reaction products in solution), one can see losses in the expected polymer mass values, with values of 30,7 (5 wt% DE) and 78,6 % (50 wt% DE). Such losses may be related to the lower molar mass products that were not recovered in the purification scheme, as previously discussed. Yield values for the reaction containing 5 wt % DE are similar to those determined by Sonseca et al., (2017), which showed yield of 74 %. Next, Figure 19 will present the synthesis information at 80 °C.

Figure 19: Results obtained in the reactions at 80 °C: a) Kinetic behaviour of condensed by-product; b) M_w and M_n; c) Yield and *Đ*; d) Molar mass distributions. Reaction times were 80 (









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When analyzing the kinetic behaviour at 80 °C in Figure 19, it is observed an increase in the by-product amounts of kinetic curves at 5% DE in comparison with the reaction in bulk. The final amounts of ethanol recovered were: 7.93, 8.29 and 7.39 g (0, 5 and 50 wt% DE, respectively). No significant gains were presented in the values of by-products collected in comparison to 70°C. Reaction times were 80 (0 wt% DE) and 90 min (5 and 50 wt% DE), this times are higher than those for the reactions performed at 70°C, due to the effect of reaction temperature on viscosity.

Regarding average molar masses (Figure 19b), the reaction performed with 5 wt% DE presented M_w of 3,000 $g.mol^{-1}$, the small addition of DE to the reaction medium and of temperature increase (from 70 °C in Figure 18 to 80 °C in Figure 19) increases the external diffusion rates (from the bulk phase to the enzyme surface) and internal diffusion rates (through the porous support to enzyme active sites). Gkountela et al. (2021), using isooctane and toluene, obtained M_n between 1,000 and 2,800 $g.mol^{-1}$, in dilutions of 100 wt% (in the pre-polymerization process), lasting 24 h. The results presented here are promising concerning the results presented by Gkountela et al., [22] and can be used in future studies that follow the growth procedure in stages, as described by the authors.

The Đ values again indicated a narrower molar mass distribution across the polymer samples produced by solution polycondensation when compared to the bulk reaction (influenced by the purification scheme), with values of 1.9 (70 °C), 1.2 (80 °C) and 1.3 (90 °C) (Figure 2c). The values are similar to those described in the literature (1.07 to 1.79 (PELLIS et al., 2018)). Regarding the mass yield of reactions in solution presented in Figure 19c, one can see a certain improvement in the values of recovered products in comparison with reactions performed at 70 ºC (Figure 18c), with values of 84.5 (5 wt% DE) and 28.5 (50

wt% DE). A decline in polymerization rates is observed in the presence of 50 wt% of DE, indicating large losses in the enzymatic activity, corroborated by lower by-product amounts and molar masses.

Referring to the molar mass distributions, in Figure 19d, the products of the solution reactions that were recovered in the precipitation process, again a narrower distribution is observed. Products obtained with the synthesis containing 5 wt% of DE presented a tendency of a high amount of polymeric chains with higher molecular weight, indicating higher conversion of the functional groups in the PBS synthesis process. Next, Figure 3 will present the synthesis information at 90 °C.

Figure 20: Results obtained in the reactions at 90 °C: a) Kinetic behaviour of condensed by-product; b) M_w and M_n; c) Yield and *Đ*; d) Molar mass distributions

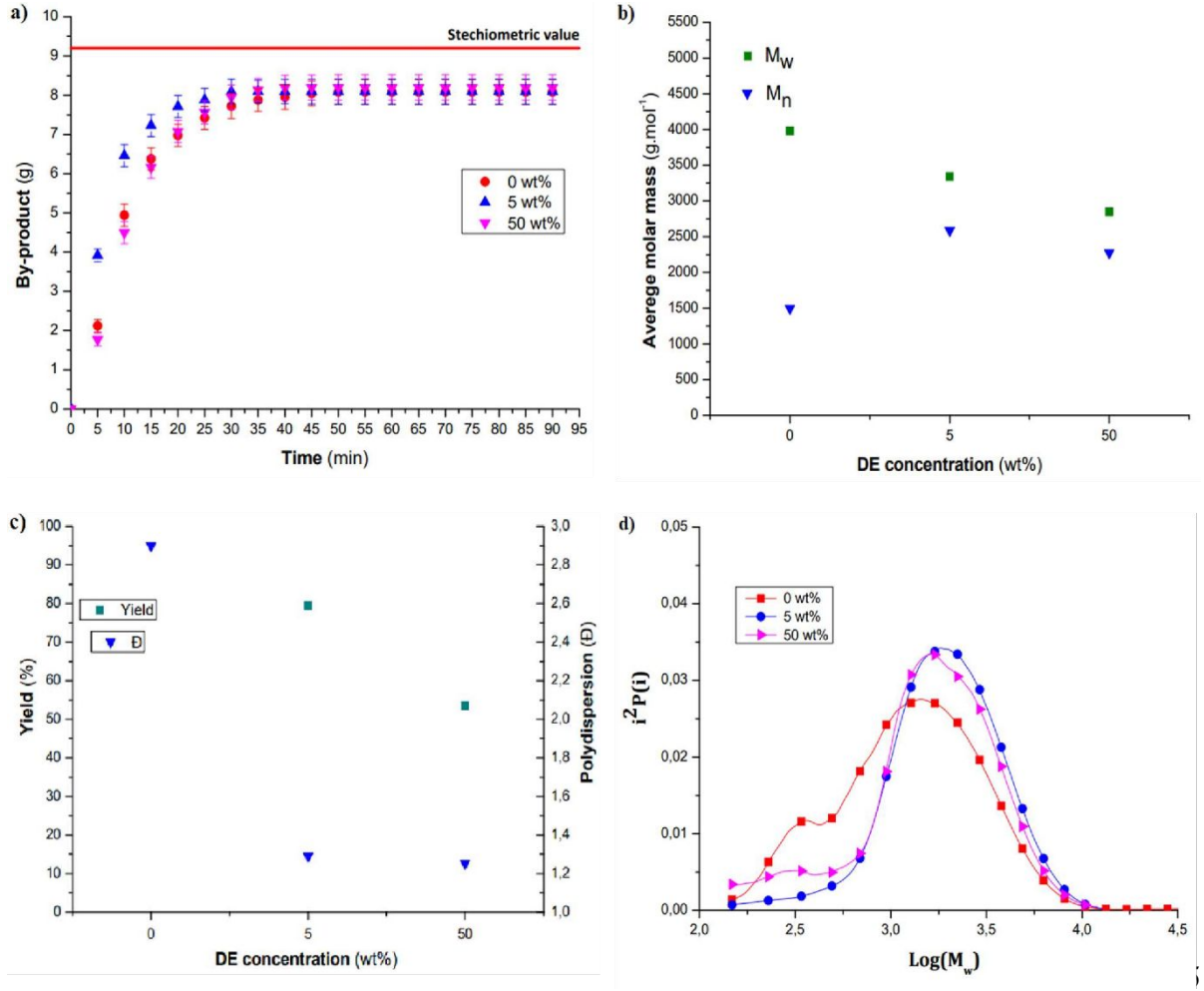

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Figure 20 presents the results of the reactions performed at 90 °C. As can be seen in Figure 20a, during the first 15 min again the reaction performed with of 5 wt% DE led to the formation if the highest by-product amounts. Nevertheless, this difference leveled off during the reactions reaching comparable final values. In addition, the final amounts of collected ethanol were also very similar to values previously measured at 70 and 80 °C, being: 8.08 (0 wt% DE), 8.09 (5 wt% DE) and 8.19 g (50 wt% DE). All syntheses lasted 90 min, due to the decrease in viscosity generated by the increase in temperature above the T_{pm} of PBS (83.6 °C, (GUCKERT et al., 2022)), which facilitated the mass transfer rates.

The values of M_w were 4,000, 3,350 and 2,850; while the M_n were 1,500, 2,600 and 2300 $g.mol^{-1}$ at 0, 5 and 50 wt% DE, respectively (Figure 20b). Azim et al., (2006) obtained M_n values of 4,000 (at 70 °C), 8,000 (80 °C) and 7,000 (90 °C) $g.mol^{-1}$ in reactions with a dilution of 200 wt% DE, lasting 24 h. Reaction time is also an important factor, especially in more dilute systems. As the reaction medium has a lower viscosity, improving the heat and mass transfer rates, longer reaction times can be used. In this case, higher growth of polymeric chains is expected. However, an important task is also to investigate the enzyme stability in the presence of the solvent for longer times, which is often neglected in the works published in the literature.

The yields of the reactions were 79.4 and 53.5 % (at 5 and 50 wt% DE, respectively, Figure 20c). The increase in temperature favoured the reaction rates, producing greater amounts of polymeric chains with higher molar mass when compared to the previous results. Gkountela et al., (2021) describe yields between 25 and 60 % in reactions using isooctane and toluene as solvents, where the current results indicate greater effectiveness in the polymerization process, through greater amounts of polymeric chains of higher molar mass.

The Đ values for the reactions in solution remained low, 1.3 and 1,2 (5 and 50 wt% DE), mainly due to the loss that occurred by the precipitation of the system in solution, with values very close to those presented by Azim et al., (2006), being between 1.1 and 1.6 (also in reactions with DE). Reporting the value of Đ for the solvent-free synthesis, the value presented was higher, 2.9, due to the population of low molar mass polymer and a high molar mass tail (Figure 20d), justifying the increase in M_w in the bulk reaction at 90 °C.

In general, it would be expected that the use of a solvent would offer advantages in comparison to bulk reactions, improving the external diffusion rates of the polymeric chains from bulk phase to enzyme surface as well as the by-product evaporation rates, due to the lower reaction medium viscosity. As observed, the presence of small amounts of the solvent (5 wt%) provided small improvements in the synthesis process, whereas the presence of 50 wt % of DE provided a decay in reaction rates, average molar masses and the mass yield of the products, indicating that solvent excess can affect the enzymatic activity. Additionally, the solvent addition did not provide significant improvements in the final amounts of collected by-products as well as in the average molar masses, indicating that enzymatic transesterification is controlled by the internal diffusion of polymeric species through porous support. Thus, reactions in solution suggested that temperature is the main factor affecting these reactions.

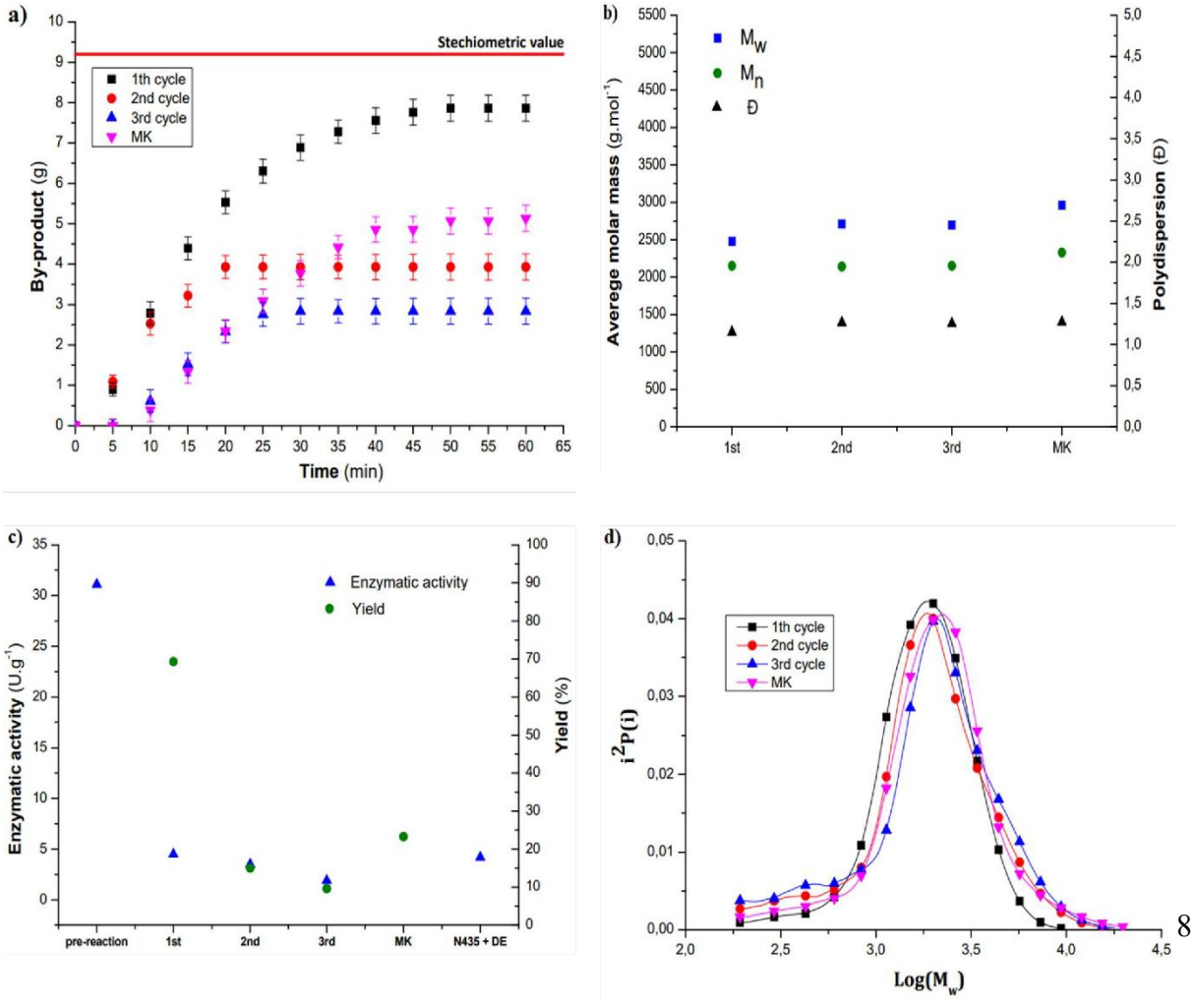
Addressing the possibility of reusing the immobilized biocatalyst (SEN; PUSKAS, 2015), the system with a dilution of 5 wt% DE was selected due to the better results in terms of yield and molar mass when compared with the reactions performed with 50 wt% of DE. The temperature of 70 °C was chosen, as suggested by Lerin et al., (2011) as the optimal temperature for the enzymatic activity of the biocatalyst used in this work. Thus, the results

obtained in the reuse of N435 for the synthesis of PBS in the selected condition are presented in the next section.

4.3.2 Enzyme Reuse

Based on the possibility of reusing N435 in different cycles of PBS synthesis, the enzymatic activity was evaluated before and after each reuse cycle, to monitor the stability and effectiveness of the catalytic process. The initial enzymatic activity was 31.1 $U.g^{-1}$, adopted as the maximum value of catalytic activity for the process. Thus, the reuse cycles were performed and where Figure 21 shows the results in each cycle of using N435 as a catalyst for PBS synthesis (under the conditions of 5 wt% DE, at 70 °C).

Figure 21: Results of reactions with reuse of N435 performed at 70 °C with 5 wt% of DE a) Kinetic behaviour of condensed by-product; b) M_w, M_n and $Đ$; c) Enzymatic activity and Yield; d) Molar mass distributions.



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As seen in Figure 21a, a loss in enzyme catalysis efficiency was observed after the first cycle of using N435. A decrease in EtOH removal rates was evidenced from the lower slope of kinetic curves (Figure 21a), indicating a loss of N435 activity after the first use and after each of the 3 cycles. With the make-up procedure, part of the efficiency was recovered, due to the replacement of 25 wt% of N435 with fresh enzyme. By tracking the by-product values in each cycle (Figure 21a), were recovered: 7.68 g (1st cycle), 3.93 g (2nd cycle, indicating a decrease of 48.8 % concerning the 1st cycle), 2.83 g (3rd cycle) and 5.13 g (MK, indicating an increase of 81.2 % concerning the 3rd cycle). With the values of by-products presented, a decline in enzymatic activity and lower reaction rates are indicated, which may be related to the denaturation and/or leaching of enzymes adsorbed on the polymeric support of N435. When checking the molar masses obtained (Figure 21b), the values did not show significant changes.

The average molar mass values did not change due to the purification technique applied to the reaction products in the solution, as previously discussed. However, the enzymatic activity loss is corroborated by measured yields of the products obtained (which were highly affected) (Figure 21c): 69.3 % (1st cycle), 15.0 % (2nd cycle, indicating a decrease of 78.4 % concerning the 1st cycle), 9.6 % (3rd cycle, indicating a decrease of 86.2 % concerning the 1st cycle) and 23.3 % (MK, indicating an increase of 142,7 % concerning the 3rd cycle). Thus, a loss of catalytic efficiency is observed, from lower polycondensation rates and polymer mass yields.

With the lower enzymatic activities and polycondensation reaction rates, an increase in the oligomers (low molar mass) fraction is expected, as observed in N435 reuse results for bulk reactions (GUCKERT et al., 2022). However, this fraction was not recovered by the

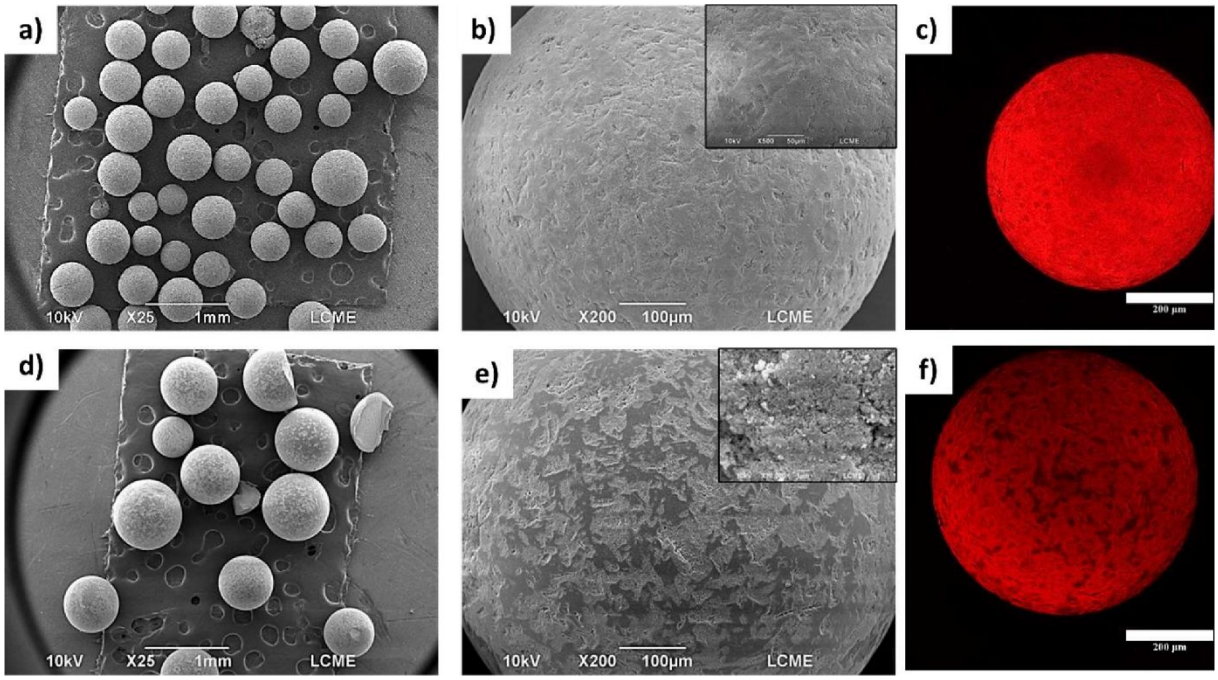
purification technique, and, as only the chains of higher molar mass precipitate with the cold EtOH, the molar masses remained with similar values, as shown in Figure 21b.

Still describing the polymeric products of the reaction, recovered polymers showed high uniformity, with *Đ* values (Figure 21b) of 1.15 (1st), 1.26 (2nd), 1.25 (3rd) and 1.27(MK), which indicated low dispersion between the formed chains, with a narrow monomodal molar mass distributions (Figure 21d).

Regarding the catalytic activity of N435, values were determined after each use, being (Figure 4c): 31.1 $U.g^{-1}$ (before the reaction), 4.5 $U.g^{-1}$ (after the 1st cycle, with a loss of 85.5 % of the activity before the 1st use), 3.5 $U.g^{-1}$ (after 2nd cycle, with loss of 88.7 % of activity before 1st use), 1.9 $U.g^{-1}$ (after 3rd cycle, with loss of 93.9 % of activity before 1st use and REA of 6.1 %). Nasr et al., (2020) also used DE in solution polymerization reactions. Their results indicated that the use of solvents improved the heat transfer, but were more prone to enzymatic degradation and leaching of the support. The authors also showed that the enzyme on the support tends to be more susceptible to leaching in hydrophobic solvents, which can cause losses in catalytic efficiency.

With such indications, the influences of DE on N435 were verified under reuse conditions (leaving the biocatalyst in contact with DE, under stirring at 400 rpm at 70 °C, for 60 min), where the enzymatic activity dropped to 4.2 $U.g^{-1}$ (decrease of 86,5% of the enzymatic activity). In Figure 22, the characteristics of the biocatalyst can be observed before and after contact with DE, where one can see significant changes.

Figure 22: Characteristics of N435 before (a), b) and c)) and after (d), e) and f)) the contact with the DE (60 min at 70 °C and 400 rpm).



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Analyzing the SEM images of the biocatalyst before contact with the solvent (Figures 22a and b), uniform textures are observed on the polymeric support. Through the representation by protein staining, in Figure 22c, a large amount of adsorbed enzyme can be seen, with an intense red hue as an indicator. When comparing the images after contact with the solvent (Figure 22d, e and f), greater porosity is observed in the polymeric support, indicating degradation of the structure, in addition to the decrease in staining intensity by the CLSM test, indicating leaching of the enzymes adsorbed on the support. Therefore, the the information in Figure 22 corroborates the enzymatic activity results , pointing to a tendency of loss of catalytic efficiency of N435, hampering reuse cycles in polymerization processes in

solution reactions using DE. Interestingly, one can see a significant loss of enzymatic activity at 60 min, corroborating that DE is not proper for the longer enzymatic reactions, as well as its use in excess, as presented in the previous results of enzymatic polycondensation with 50% of DE.

4.4 Conclusions

Based on bulk and solution systems for N435-catalyzed PBS synthesis, the kinetic behaviour during the reactions and the properties of final polymeric materials were discussed, in addition to the stability of the biocatalyst in new reaction cycles in solution. The use of 5 wt % of solvent improved the by-product evaporation rates at 80 and 90°C, due to the lower reaction medium viscosity of the medium. However, the use of solvent in excess decreased the by-product evaporation rates, indicating a decrease in enzymatic activity. The measured values of final amounts of by-product and molar masses did not present significant changes, indicating that enzymatic polycondensation is controlled by internal diffusion through porous support. The experimental condition chosen for the reuse of the biocatalyst was 5 wt% DE, at 70 °C. When reusing N435, the use of the solvent affected the immobilized biocatalyst (both in its catalytic activity and in its structural properties), obtaining polymeric products with low mass yield, due to substantial enzymatic activity loss in the 3 performed reuse cycles. Part of the catalytic capacity of the system was recovered when applying the MK procedure, with the partial replacement of the used enzyme by new N435. Thus, the use of DE showed to be efficient for only one catalysis cycle, inhibiting the possibility of reuse of N435 in new reuse cycles for PBS synthesis, as well as its use in excess or longer enzymatic reactions. In this way, the study of the kinetic behaviour in conjunction with the catalytic stability of N435 for polymer synthesis is emphasized, these aspects are rarely addressed in the literature. Also, the

present work expands possibilities of exploration in different synthesis systems, with different biocatalysts and polymeric products, enabling polymerization processes that are less aggressive to the environment, in addition to providing subsidies for future implementation projects in commercial/industrial production systems.

CHAPTER 5

Chapter 5 presents the main conclusions obtained in this study, as well as suggestions for future works.

5 Conclusions

By highlighting the trend of developing materials with physicochemical properties suitable for biomedical applications, succinic polymers stand out. Still, emphasis is given to such materials because they can be obtained through biotechnological processes, contributing to the development of smaller polymers harmful to the environment and to the search for ecologically correct processes. A trend of application of biocatalyzed processes for the production of such polymeric products has been noticed, when the kinetic behavior, the analysis of polymeric products and recyclability of the biocatalyst for the synthesis of PBS, in bulk and solution reactions, were elucidated. For the bulk reactions, the operating condition containing 10 wt% of N435 at 90 °C showed evidence, with M_w values of 4,000 $g.mol^{-1}$, 8.08 g of by-products (with the stoichiometric amount of approximately 9.20 g), in 90 minutes of reaction, being the condition chosen to carry out the reuse of the immobilized biocatalyst. The reuse procedure allowed 4 cycles with high catalytic efficiency, enabling even more 2 cycles of use. The initial value of the EA at the start of reuse was 32.4 $U.g^{-1}$, decreasing to 3.8 $U.g^{-1}$ in the 7^{th} cycle (with an REA of 13.3 %). With the addition of DE in the reaction medium, the amount of 5 wt% DE stood out, with values of 7.86 and 8.09 g of by-products and molar masses between 2,500 and 3,300 $g.mol^{-1}$ (M_w). Comparing these values with the ones measured in bulk reaction, one can see that solvent addition did not provide significant changes in the final amounts of by-product, indicating that enzymatic polycondensation is controlled by internal diffusion and the main changes are provided by temperature increase.

Thus, the addition of 5 wt% of DE to the reaction medium was selected to investigate the reuse of the biocatalyst at 70 °C (the optimal temperature for using N435). The recyclability procedure in the solution system enabled only 1 cycle with high efficiency, followed by the EA decrease (being 31.1 $U.g^{-1}$ before the process, decreasing to 4.5 $U.g^{-1}$ after the first use and 1.9 $U.g^{-1}$ at the end of the process reuse, corresponding to an REA of 6.5%). Three cycles were carried out, where the yield of the polymeric products followed the accentuated decay of the enzymatic activity, being 74.9 % in the 1st, 24.6% in the 2nd and 16.9 % in the 3rd cycle, respectively. Evaluations related to the effect of DE on the catalytic stability of N435 showed changes in the structure of the polymeric support (increased porosity) and a decrease in the amount of adsorbed enzyme (desorption of the biocatalyst). Thus, when comparing the results of efficiency and catalytic stability in the synthesis of PBS produced via enzymatic transesterification, the bulk reactions were highlighted, mainly because they provide greater catalytic stability for the immobilized biocatalyst and a greater number of reuse cycles in processes of synthesis of biocatalyzed PBS.

5.1 Suggestions for future works

- Extend the kinetic behaviour description studies to other enzymatic polycondensation systems;
- Expand the understanding of the reuse of enzymatic catalysts in polycondensation reactions, for other polymeric materials and other types of enzymatic catalysts;
- Study of the modelling of the synthesis process, aiming at expanding the scale of production of biocatalyzed PBS.

Annexes

Annex I: Supplementary material for Chapter 3

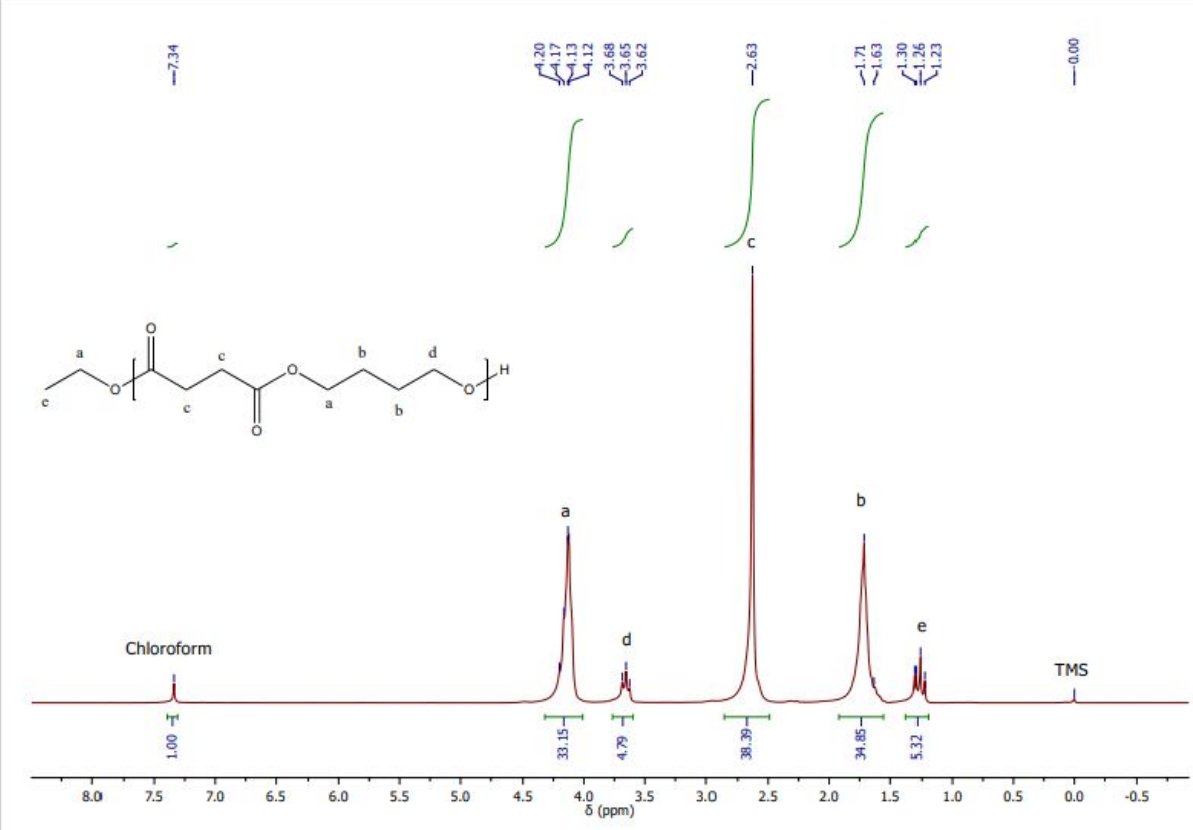
Average molar mass and chemical groups via NMR

^{1}H NMR spectroscopy was performed at a Bruker AVANCE system at 200 MHz. Sample preparation consisted of dissolving 5 mg of sample in 0.5 mL of deuterated chloroform. The objective of using NMR was to describe chemical groups and average molar mass of polymeric samples. Equation 4 was used to determine the numeral average molar mass, and Figure 23 shows the NMR spectrum obtained.

$$M_n = m_{o\,x} \frac{\left(\frac{I\,2.62}{4}\right)}{\frac{\left(\frac{I\,3.69}{2}\right)+\left(\frac{I\,1.30}{3}\right)}{2}} + m_{ends}, \text{ being:} \qquad \textbf{Equation 4}$$

Where:
M_n = numerical average molar mass (g.mol^{-1});
m_o = repeat unit molar mass (172 g.mol^{-1});
$I(1{,}30)$ = value of the sign integral of the methyl hydrogens next to the ester end groups (O-CH_2-C**H**$_3$);
$I(2{,}62)$ = value of the sign integral of the hydrogens that are associated with the methylene protons next to the ester group within the repeating structural unit (-OC-C**H**$_2$C**H**$_2$CO-);
$I(3{,}69)$ = value of the sign integral of the hydrogens close to the final hydroxyl group (-C**H**$_2$-OH);
m_{ends} = molecular mass of the end groups of the chains (218 g.mol^{-1}).

Figure 23: ^{1}H NMR spectrum of PBS synthesized under conditions of 10% N453 and 90°C.



Source: from author

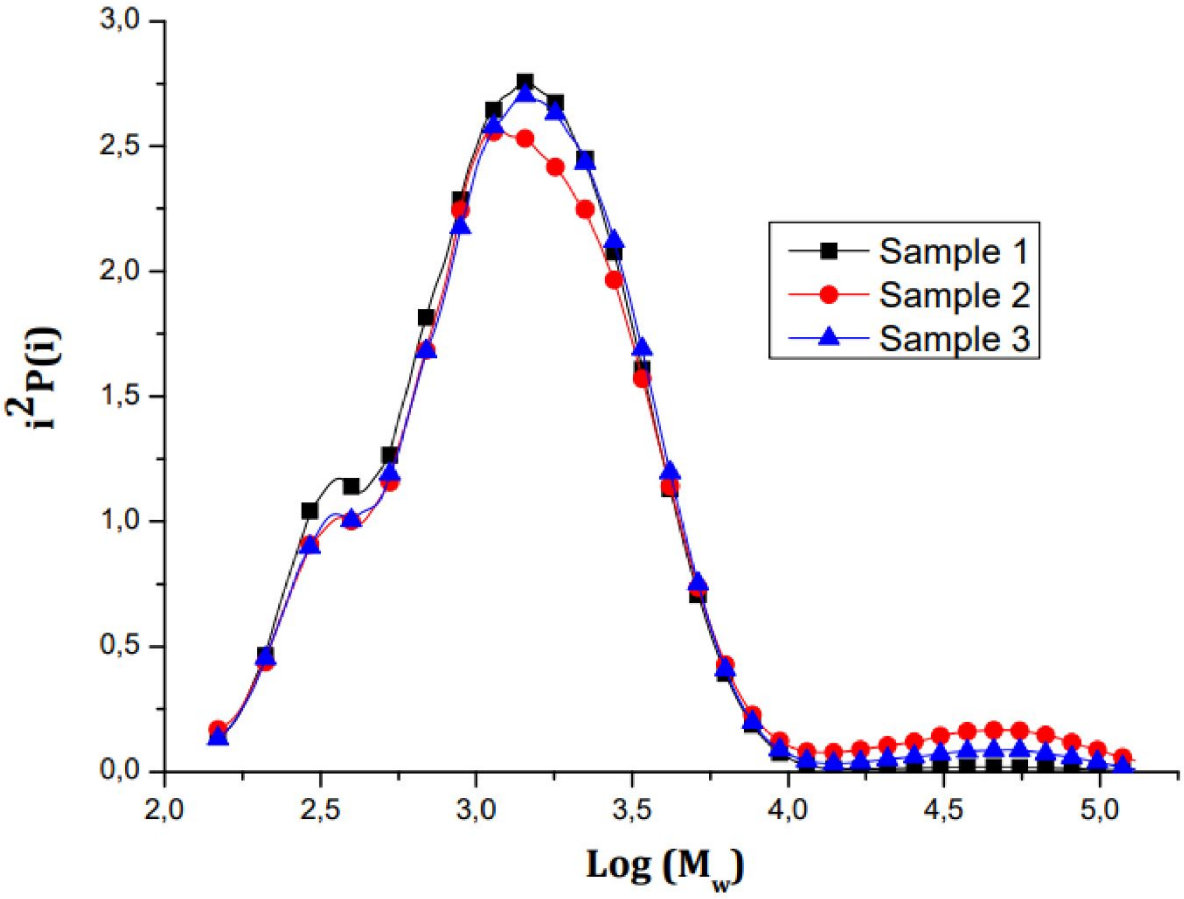
The ^{1}H NMR spectrum is very similar to those presented in the literature (ABDERRAHIM et al., 2015), when the structural composition is given/interpreted by the signs: 1.23 to 1.30: C**H**$_3$CH$_2$O- from the DS (e); 1.63 to 1.71: -O-CH$_2$C**H**$_2$C**H**$_2$CH$_2$-O- from the BDO (b); 2.63: -OC-C**H**$_2$C**H**$_2$CO- from the DS (c); to 3.62 to 3.68: -C**H**$_2$OH- from the BDO (d); 4.12 to 4.20: CH$_3$C**H**$_2$O- from the DS (a).

One of the goals for presenting the NMR spectrum data is the possibility of measuring the M_n by this technique, through Equation 4 (being a comparison to the M_n determined by GPC).

Reproducibility of the synthesis system via distribution of polymeric chains

To verify the reproducibility of chain length distributions of PBS produced by enzymatic polycondensation, samples were synthesized in triplicate, at enzyme concentration of 10 wt% and 90 °C. Figure 24 shows the distribution of polymer chains for the PBS samples produced in triplicates.

Figure 24: Molar mass distribution of samples synthesized in triplicate under conditions of 10 wt% N435 and 90 °C.



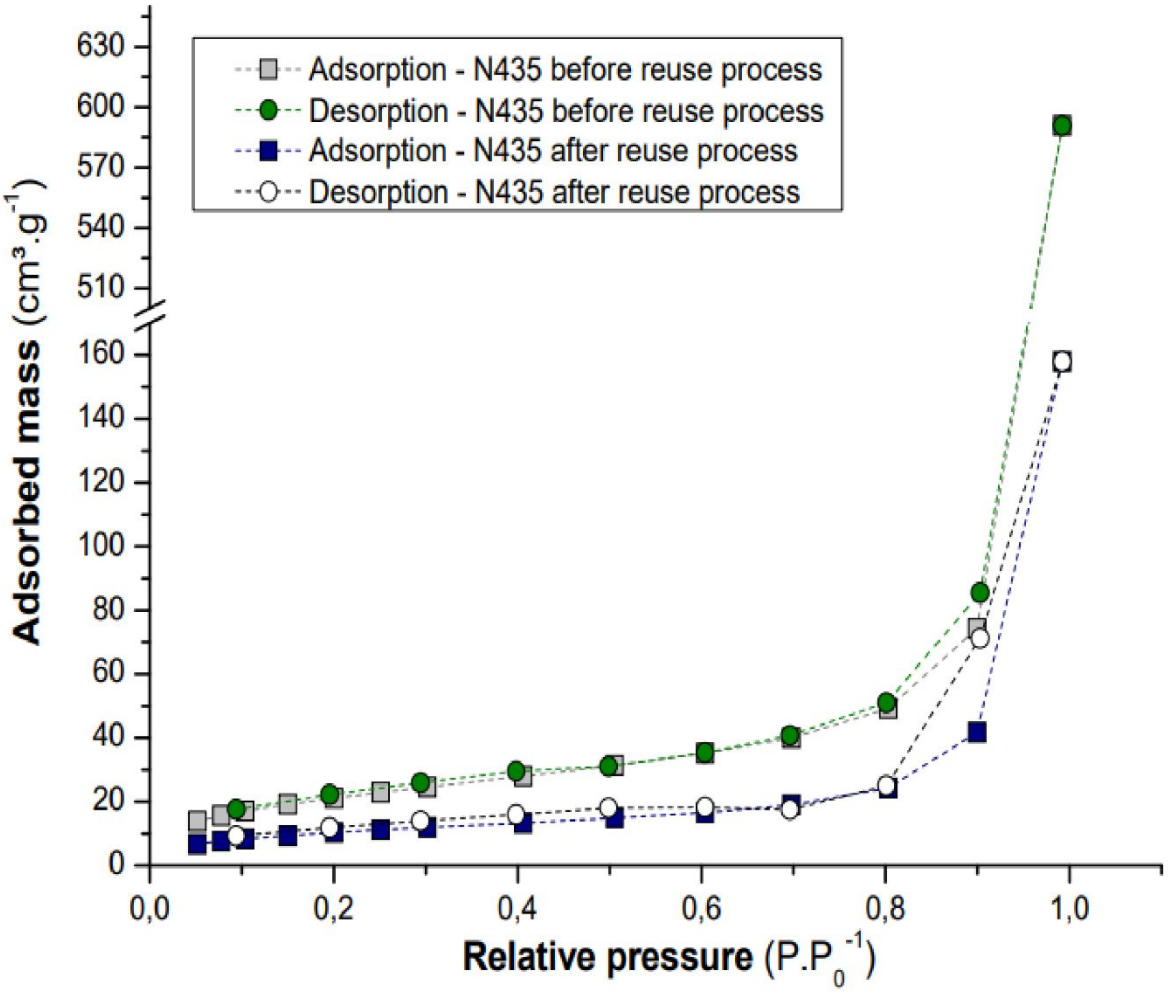
Source: from author

The results in Figure 24 illustrate the good reproducibility of the enzyme system, with similar chain length distributions of the polymer products synthesized in triplicate.

N435 nitrogen adsorption isotherms

To verify the area and pore characteristics of the immobilized biocatalyst before and after the reuse process, tests were carried out with nitrogen adsorption, where Figure 25 represents the results obtained:

Figure 25: Representation of the comparison of nitrogen adsorption isotherms before and after the N435 reuse process



Source: from author

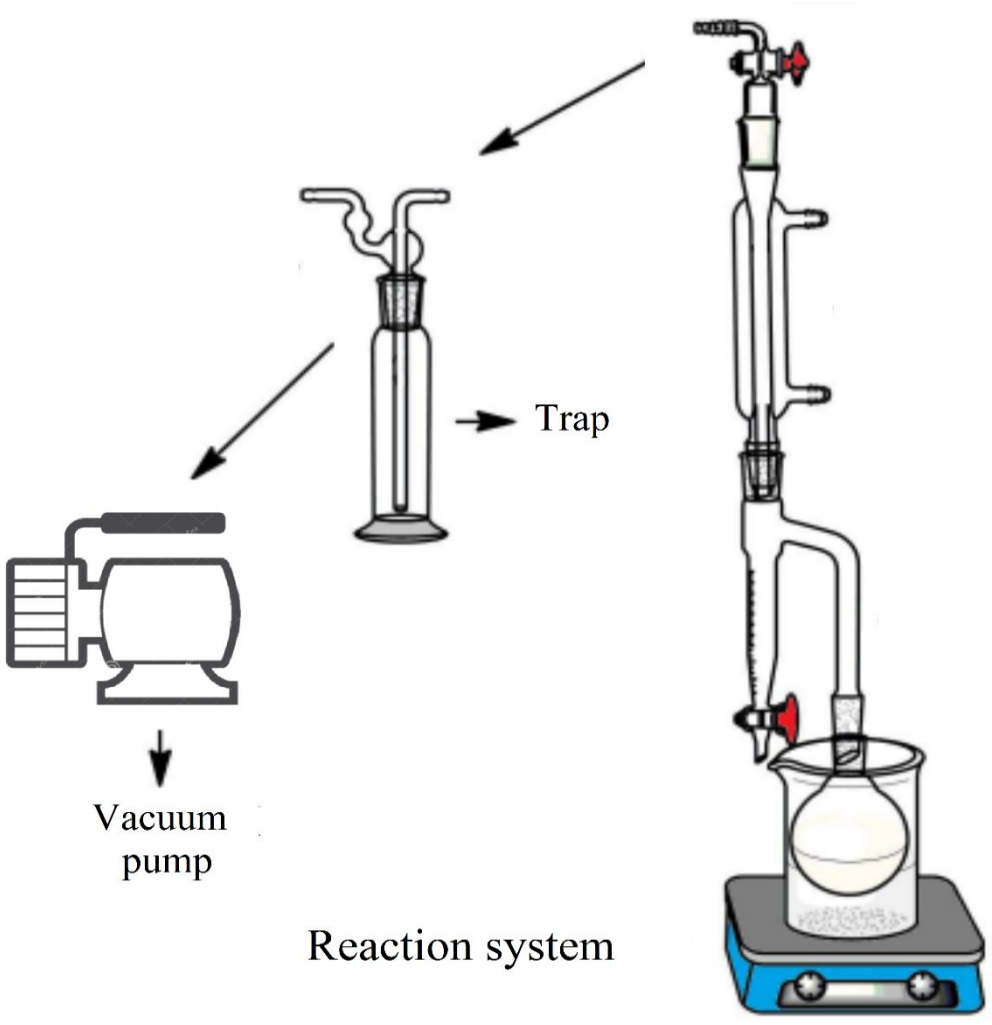
When comparing the nitrogen adsorption values in the isotherms, a decrease in the adsorbed values is observed, indicating obstruction by the polymeric chains synthesized throughout the reuse cycles.

Annex II: Supplementary material for Chapter 4

Experimental unit

The experimental unit used for the enzymatic polycondensation reactions is composed of a heating and magnetic stirring plate (C-MAG HS 7, IKA), thermal bath, reaction flask, dean stark, condenser column (cooled by a thermocryosthatic bath, MQBTC99-2, Microquímica), vacuum output, trap and vacuum pump (I2P-740/SCN, Indutec), arranged in a vertical system. Figure 26 illustrates the experimental unit used in the synthesis processes.

Figure 26: Experimental unit used for syntheses of polymeric material

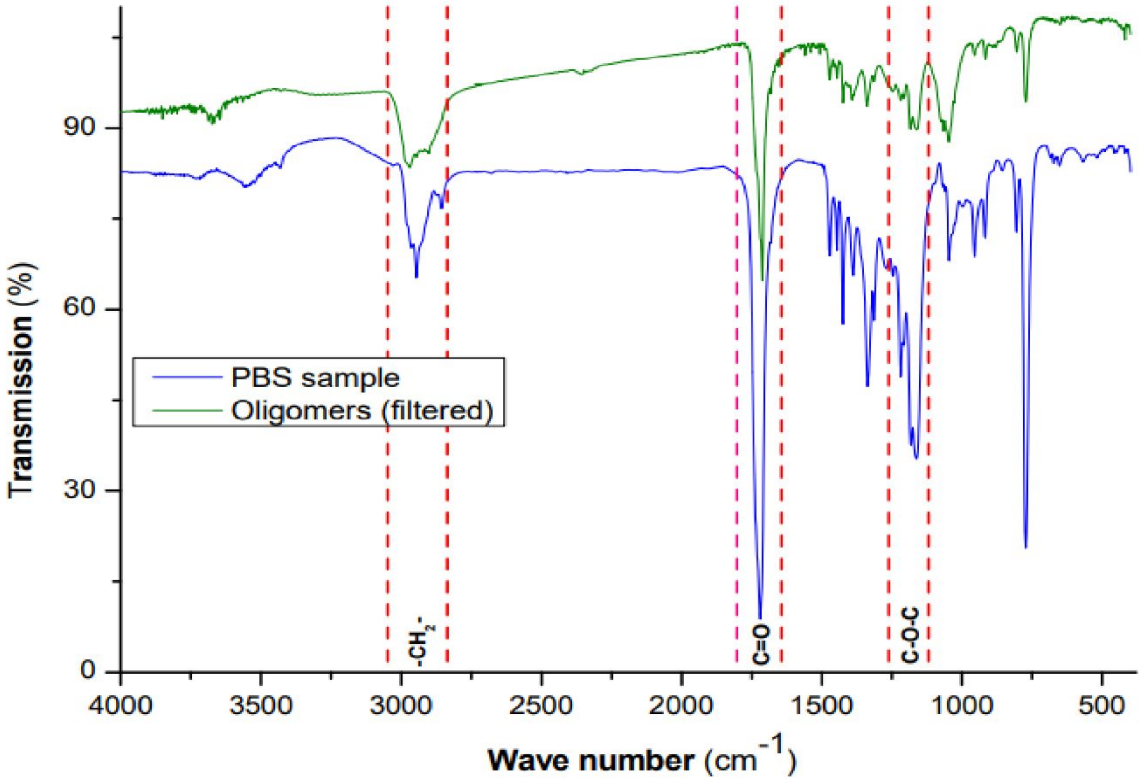


Source: from author

Verification of the presence of oligomers in the ethanolic solution applied in the purification of the system in solution

In order to verify the presence of oligomers in the EtOH used in the purification process of the system in solution, FT-IR tests were performed. Next, Figure 27 describes the spectrum of the polymeric sample produced in a solvent-free system at 90 °C (PBS sample), in addition to the spectrum obtained from the ethanolic solution of the system's filtration in solution.

Figure 27: FT-IR spectrum of PBS and purification products filtered in solution systems, with evidence of non-precipitated oligomers.



Source: from author

As seen in the peaks of 2945 cm^{-1} ($-CH_2$), 1713 cm^{-1} (C=O) and 1144 cm^{-1} (C-O-C), the presence of functional groups characteristic of PBS in the ethanolic solution is visualized, indicating that shorter chains (oligomers) remained soluble in the process of precipitation.

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