CLEONICE APARECIDA SALGADO

APLICAÇÃO DE MICRORGANISMOS LIPOLÍTICOS EM ALIMENTOS E NA BIODEGRADAÇÃO DE POLIURETANOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Maria Cristina Dantas Vanetti

Coorientador: Felipe Alves de Almeida

VIÇOSA - MINAS GERAIS
2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
S164a
2023

Salgado, Cleonice Aparecida, 1990-
Aplicação de microrganismos lipolíticos em alimentos e na biodegradação de poliuretanos / Cleonice Aparecida Salgado. – Viçosa, MG, 2023.
1 tese eletrônica (105 f.): il. (algumas color.).

Texto em português e inglês.
Orientador: Maria Cristina Dantas Vanetti.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Microbiologia, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.255
Modo de acesso: World Wide Web.

1. Lipase. 2. Catálise. 3. Poliuretanas - Biodegradação. 4. Alimentos - Qualidade. I. Vanetti, Maria Cristina Dantas, 1957-. II. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. III. Título.

CDD 22. ed. 572.793

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

CLEONICE APARECIDA SALGADO

APLICAÇÃO DE MICRORGANISMOS LIPOLÍTICOS EM ALIMENTOS E NA BIODEGRADAÇÃO DE POLIURETANOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

APROVADA: 03 de maio de 2023.

Assentimento:

Cleonice Aparecida Salgado
Autora

Maria Cristina Dantas Vanetti
Orientadora

AGRADECIMENTOS

A Deus, que sempre me sustentou na fé, me dando forças nos percalços da vida.

À Universidade Federal de Viçosa e ao Departamento de Microbiologia, pela estrutura e pelas oportunidades de formação acadêmica.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro.

À minha mãe Lourdes, pelo apoio e ajuda durante todos os momentos da minha vida e pela sua fé que me ajuda nesta caminhada.

Ao meu filho Henrique Ailton, que é meu amor incondicional, sendo o meu porto seguro e minha fonte de inspiração que me impulsiona a sempre buscar o melhor.

Ao meu marido Ângelo, pelo companheirismo e apoio. Obrigada por me incentivar a nunca desistir perante as dificuldades.

Aos meus irmãos, Ana, Jacira, Isabel, Lúcia, Silvano, Nelci, Maria e José Arlindo, pelo incentivo em estudar e por serem exemplo de resiliência para mim.

À professora Cristina Vanetti, pela orientação, ensinamentos, atenção, disponibilidade, competência e amizade, que foram imprescindíveis para o êxito deste trabalho.

Ao Felipe, pela coorientação, amizade, ensinamentos, sugestões durante a execução das metodologias e escrita do trabalho.

Ao Júnio, pela amizade, disponibilidade e ajuda nas análises de bioinformática, com dicas valiosas durante a execução dos experimentos e desenhos de estruturas químicas.

Ao Leandro, pela amizade, conversas aleatórias e ajuda na extração de DNA.

Ao Edvaldo Barros, pela ajuda, ensinamentos, sugestões para o desenvolvimento do trabalho.

À professora Solimar por ter isolado *Serratia liquefaciens*, oportunizando a continuação das pequisas, pelas sugestões e pela disponibilidade em participar da banca.

À Aline Machado de Castro pela solicitude, pelas sugestões e disponibilidade em participar da banca.

Ao Pedro Vidigal, pelos ensinamentos e ajuda na montagem e anotação do genoma.

Aos amigos do Laboratório de Microbiologia de Industrial, Clarissa, Dara, Gabriel, Leonardo, Leandro e Deisy, por todo auxílio, aprendizado e acima de tudo, a amizade.

Aos demais amigos que direta ou indiretamente contribuíram para realização deste trabalho, Bruna, Rafael, Girlã e Bianca (Virologia), Juliana e Nívea (Anaeróbios), Bianca (Fermentados), Isabel e Ramilla (Bioquímica), Hellen (Engenharia Civil).

Ao Núcleo de Microscopia e Microanálise, pelo uso de equipamentos e pelo apoio técnico dos funcionários Gilmar, Cristiane e Carla.

Ao NuBioMol-UFV, pelo uso de equipamentos e análises de dados.

Aos funcionários, Patrícia, Sandra, Gabriel, Letícia, Raquel, Iraci, Emília, Célio e Sr. Paulo, pelo convívio e ajuda durante este período.

Pelo apoio e solicitude de todos, fica minha eterna gratidão!

BIOGRAFIA

Cleonice Aparecida Salgado, filha de Maria de Lourdes da Silveira Salgado e João Pereira Salgado (*in memoriam*), nasceu em Guaraciaba, Estado de Minas Gerais, em 19 de julho de 1990.

Graduou-se em Bioquímica em julho de 2016 pela Universidade Federal de Viçosa (UFV). Em março de 2017, ingressou no Programa de Pós-Graduação em Microbiologia Agrícola da UFV, obtendo o título de mestre em Microbiologia Agrícola em fevereiro de 2019. Em março do mesmo ano, iniciou o curso de doutorado em Microbiologia Agrícola pela mesma instituição.

RESUMO

SALGADO, Cleonice Aparecida, D.Sc., Universidade Federal de Viçosa, maio de 2023. **Aplicação de microrganismos lipolíticos em alimentos e na biodegradação de poliuretanos.** Orientadora: Maria Cristina Dantas Vanetti. Coorientador: Felipe Alves de Almeida.

Os microrganismos são considerados fontes inestimáveis de compostos extracelulares, como enzimas, tornando-os o centro da investigação científica, tanto nos aspectos de biodeterioração em que estão envolvidos, quanto para aplicação biotecnológica. As lipases são enzimas responsáveis principalmente, pela hidrólise dos triacilgliceróis e amplamente exploradas comercialmente. Além da hidrólise, conseguem catalisar a reação reversa, a esterificação e, também reações como transesterificação, interesterificação, acidólise e aminólise. Dese modo, as lipases são reconhecidas como catalisadores promissores no melhoramento da qualidade sensorial de diversos produtos alimentícios além de, biodegradarem diversos substratos. A lipase de *Serratia liquefaciens* L135 foi identificada como uma poliuretanase, ou seja, uma enzima com a capacidade de biodegradar poliuretanos (PU). Este trabalho foi desenvolvido com o objetivo de explorar o potencial desta enzima na indústria de alimentos e na biodegradação de poliuretanos por *S. liquefaciens* isoladamente ou em consórcio microbiano. Para isso buscou-se isolar potenciais biodegradadores de PU do intestino de larvas de *Galleria mellonella* e avaliar o efeito da associação com *S. liquefaciens* na biodegradação de PU *in vitro*. O primeiro capítulo apresenta uma revisão de literatura que abrange as aplicações de lipases em indústrias de laticínios; óleos e gorduras; panificação e confeitaria; carnes; sabores e aromas; e outras indústrias alimentícias. Além disso, aborda as técnicas de fermentação de baixo custo e engenharia de proteínas, como promissoras para produção de lipases microbianas. No segundo capítulo são apresentados resultados de estudos *in silico* e *in vitro* do potencial biodegradador de PU por *S. liquefaciens* L135 e sua poliuretanase. Para o estudo *in silico*, foram construídos monômeros e tetrâmeros de PU para realizar o *docking* molecular com a poliuretanase modelada e validada de *S. liquefaciens*. Foi possível verificar que os PU ligaram ao resíduo de aminoácido S207, que faz parte da tríade catalítica e é responsável pela ação hidrolítica da poliuretanase. Os monômeros apresentaram melhores *scores* de ligações, quando comparados com os tetrâmeros, devido às interações estéricas

repulsivas. Os PU, Impranil® e poli[4,4'-metilenobis(fenilisocianato)-alt-1,4-butanodiol/di(propilenoglicol)/policaprolactona] (PCLMDI), foram usados para as análises *in vitro*. A biodegradação do Impranil® por *S. liquefaciens* e sua poliuretanase foi confirmada em ágar pela formação de halos transparentes. Além disso, foram preparados discos de Impranil® e filmes de PCLMDI e a biodegradação durante o cultivo de *S. liquefaciens* foi avaliada por microscopia eletrônica de varredura (MEV). Foi possível averiguar a formação de rachaduras e poros na superfície dos PU, configurando o processo de biodegradação. No terceiro capítulo, foi feito o isolamento da bactéria *Staphylococcus warneri* do intestino da larva de *G. mellonella*, com potencial de biodegradação de PU. O isolado *S. warneri*, denominado de UFV_01.21, foi avaliado em cultura pura e em consórcio com *S. liquefaciens* L135 para biodegradação de PU e, em ambas condições, o Impranil® foi utilizado como única fonte de carbono. Com seis dias de incubação, suspensões de Impranil® em caldo Luria-Bertani (LB) inoculadas com *S. liquefaciens*, *S. warneri* e com consórcio microbiano, apresentaram 88, 96 e 76% de biodegradação, respectivamente. Tanto nos discos de Impranil®, quanto em filmes de PCLMDI, *S. warneri* em cultura pura e em consórcio com *S. liquefaciens* foi capaz de aderir e formar biofilmes. Por MEV, foi possível confirmar a biodegradação devido a formação de rachaduras, sulcos, poros e rugosidades nas superfícies dos PU avaliados. Esses resultados reforçam o potencial dos microrganismos e suas enzimas para biodegradação de PU.

Palavras-chave: Biocatalisadores. Biodegradação. Consórcio Microbiano. Docking Molecular.

ABSTRACT

SALGADO, Cleonice Aparecida, D.Sc., Universidade Federal de Viçosa, May, 2023. **Application of lipolytic microorganisms in foods and in the biodegradation of polyurethanes.** Adviser: Maria Cristina Dantas Vanetti. Co-adviser: Felipe Alves de Almeida.

Microorganisms are considered invaluable sources of extracellular compounds, such as enzymes, making them the center of scientific investigation, both in the aspects of biodeterioration in which they are involved, and for biotechnological application. Lipases are enzymes mainly responsible for the hydrolysis of triacylglycerols and are widely exploited commercially. In addition to hydrolysis, they can catalyze the reverse reaction, esterification, and also reactions such as transesterification, interesterification, acidolysis and aminolysis. Thus, lipases are recognized as promising catalysts in improving the sensory quality of various food products, in addition to biodegrading various substrates. *Serratia liquefaciens* L135 lipase was identified as a polyurethanase, that is, an enzyme capable of biodegrading polyurethanes (PU). This work was developed with the objective of exploring the potential of this enzyme in the food industry and in biodegradation of polyurethanes by *S. liquefaciens* alone or in microbial consortium. For this, we sought to isolate potential PU biodegraders from the intestine of *Galleria mellonella* larvae and evaluate the effect of the association with *S. liquefaciens* on PU biodegradation in vitro. The first chapter presents a literature review covering the applications of lipases in the dairy industry; oil and fat; baking and confectionery; meat; flavors and aromas; and other food industries. In addition, it discusses low-cost fermentation and protein engineering techniques as promising for the production of microbial lipases. In the second chapter, results of *in silico* and *in vitro* studies of the biodegradation potential of PU by *S. liquefaciens* L135 and its polyurethanase are presented. For the *in silico* study, PU monomers and tetramers were constructed to perform molecular docking with the modeled and validated polyurethanase from *S. liquefaciens*. It was possible to verify that the PU bound to the amino acid residue S207, which is part of the catalytic triad and is responsible for the hydrolytic action of the polyurethanase. Monomers showed better binding scores when compared to tetramers, due to repulsive steric interactions. PU, Impranil® and poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI) were used for *in vitro*

analyses. The biodegradation of Impranil® by *S. liquefaciens* and its polyurethanase was confirmed in agar by the formation of transparent halos. Furthermore, Impranil® disks and PCLMDI films were prepared and the biodegradation during *S. liquefaciens* cultivation was evaluated by scanning electron microscopy (SEM). It was possible to verify the formation of cracks and pores on the surface of the PU, configuring the biodegradation process. In the third chapter, the bacteria *Staphylococcus warneri* was isolated from the intestine of *G. mellonella* larvae, with potential for biodegradation of PU. The isolated *S. warneri*, called UFV_01.21, was evaluated in pure culture and in consortium with *S. liquefaciens* L135 for PU biodegradation and, in both conditions, Impranil® was used as the only carbon source. With six days of incubation, suspensions of Impranil® in Luria-Bertani (LB) broth inoculated with *S. liquefaciens*, *S. warneri* and with microbial consortium showed 88, 96 and 76% of biodegradation, respectively. Both on Impranil® disks and on PCLMDI films, *S. warneri* in pure culture and in consortium with *S. liquefaciens* was able to adhere and form biofilms. By SEM, it was possible to confirm the biodegradation due to the formation of cracks, furrows, pores and roughness on the surfaces of the evaluated PU. These results reinforce the potential of microorganisms and their enzymes to biodegrade PU.

Keywords: Biocatalysts. Biodegradation. Microbial Consortium. Molecular Docking.

SUMÁRIO

1. INTRODUÇÃO GERAL

Os microrganismos e seus metabólitos vêm ganhando mais visibilidade em diferentes aplicações industriais. As enzimas microbianas, em destaque as lipases, são consideradas biocatalisadores promissores para as transformações industriais, por apresentarem versatilidade das reações que catalisam, alta especificidade e estabilidade à diferentes condições. As lipases são consideradas alternativas “verdes” aos métodos químicos e, são usadas em aplicações em diversos setores industriais. A inclusão de lipases de origem microbiana em indústrias alimentícias, como leite; óleos e gorduras; panificação e confeitaria; carnes; sabores e aromas; tem por objetivo obter alimentos mais saborosos, nutritivos, com aromas e texturas agradáveis.

Serratia liquefaciens L135 é uma bactéria psicrotrófica Gram-negativa, que foi reconhecida por secretar uma lipase termorresistente, responsável pela deterioração lipolítica de produtos lactéos. Esta lipase foi identificada como poliuretanase e o seu potencial de biodegradação de poliuretanos (PU) ainda precisa ser explorado. O PU é um polímero plástico muito comum e versátil, possui a durabilidade e a resistência dos metais e a elasticidade da borracha, tornando-se substitutos adequados para metais, plásticos e borracha em diversos produtos. Os avanços tecnológicos levaram a novas formulações de PU e a sua produção aumentou no decorrer dos anos. Consequentemente, os resíduos de PU acumulam-se no meio ambiente e poluem todo ecossistema. Logo, métodos sustentáveis estão sendo explorados para reduzir a contaminação do meio ambiente por este polímero recalcitrante.

A reciclagem biológica, que é a utilização de organismos e/ou enzimas capazes de biodegradar PU, é ambientalmente desejável e está em crescente demanda. Devido a diversidade de estruturas de PU, o substrato Impranil® é usado em estudos de biodegradação, como PU modelo. O Impranil® é uma dispersão branca de baixa viscosidade em água com um teor de sólidos de aproximadamente 40% e com intervalo de fusão de 175-200 °C, que permite a identificação rápida de organismos biodegradadores. No entanto, a biodegradação de PU comerciais, que apresentam estruturas mais cristalinas e hidrofóbicas, demanda a exploração de metodologias inovadoras para alcançar uma biodegradação eficiente.

Larvas de insetos são relatadas por possuírem capacidade de biodegradar plásticos por mastigação e fragmentação digestiva. Logo, estudos da microbiota intestinal de larvas estão em ascensão, pois cientistas acreditam que os

microrganismos presentes no intestino desses animais, são os principais responsáveis pela biodegradação de plásticos. Os plásticos, como os PU, por serem sólidos, estão indisponíveis para potenciais biodegradadores e não são usados diretamente pelos microrganismos, logo são necessárias ações externas para quebra desses polímeros em oligômeros, dímeros e monômeros. Fatores abióticos, como abrasão, moagem e picotação podem ser usados para aumentar a disponibilidade dos polímeros.

Os microrganismos potenciais para biodegração de polímeros possuem enzimas extracelulares, como lipases, esterases, proteases, amidases, ureases, cutinases, lacases, oxigenases, para quebra desses polímeros em produtos menores, que são metabolizados e promovem o crescimento microbiano. Além disso, a capacidade de formar biofilme na superfície do polímero é uma característica desejável, pois diminui a flutuabilidade e a hidrofobicidade dos polímeros. A biodegradação de polímeros é possível de ser observada macro e, ou microscopicamente, pelas mudanças físicas na superfície, como rachaduras, trincas, poros, rugosidades e sulcos.

Apesar dos polímeros plásticos e PU serem dificilmente quebrados em monômeros, estudos recentes mostram a capacidade de microrganismos em biodegradá-los. Um consórcio microbiano otimizado e organizado pode ser intencionalmente formado para realizar a biodegradação de polímeros, uma vez que, a mineralização completa por enzimas e diferentes vias metabólicas pode ser alcançada facilmente por um consórcio microbiano. Assim, encontrar microrganismos em intestinos de larvas, com capacidade de biodegradar polímeros, e explorar o potencial biotecnológico deles em consórcio microbiano pode ser promissor.

Este estudo objetivou i) descrever as principais aplicações industrias de lipases de origem microbiana nas indústrias de alimentos; ii) explorar o potencial de biodegradação de poliuretanos por *S. liquefaciens* L135 e sua poliuretanase e; iii) isolar bactérias biodegradadoras de PU do intestino da larva de *G. mellonella*, e explorar o potencial desta bactéria em biodegradar PU em cultura pura e em consórcio com *S. liquefaciens* L135.

CAPÍTULO 1

Microbial lipases: propitious biocatalysts for the food industry

Review article published in the journal Food Bioscience

Link: https://doi.org/10.1016/j.fbio.2021.101509

Authors: Cleonice Aparecida Salgado[a], Clarissa Isabela Aparecida dos Santos[a], Maria Cristina Dantas Vanetti[a*]

[a] Department of Microbiology, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil; E-mail address: cleonice.salgado@ufv.br; clarissa.aparecida@ufv.br

* Corresponding author: Department of Microbiology, Universidade Federal de Viçosa, Viçosa, MG, Brazil. E-mail address: mvanetti@ufv.br Telephone: +55 31 3612 2445

Highlights

- Microbial lipases are considered promising biocatalysts for industrial application.
- Advances in protein engineering and immobilization techniques increase the use of lipases.
- Agro-industrial residues can be used for both production and lipase immobilization.
- Lipases are of great importance in enhancing the flavor, aroma, and texture of foods.
- The use of microbial lipases in industries represents a safe and promising green tool.

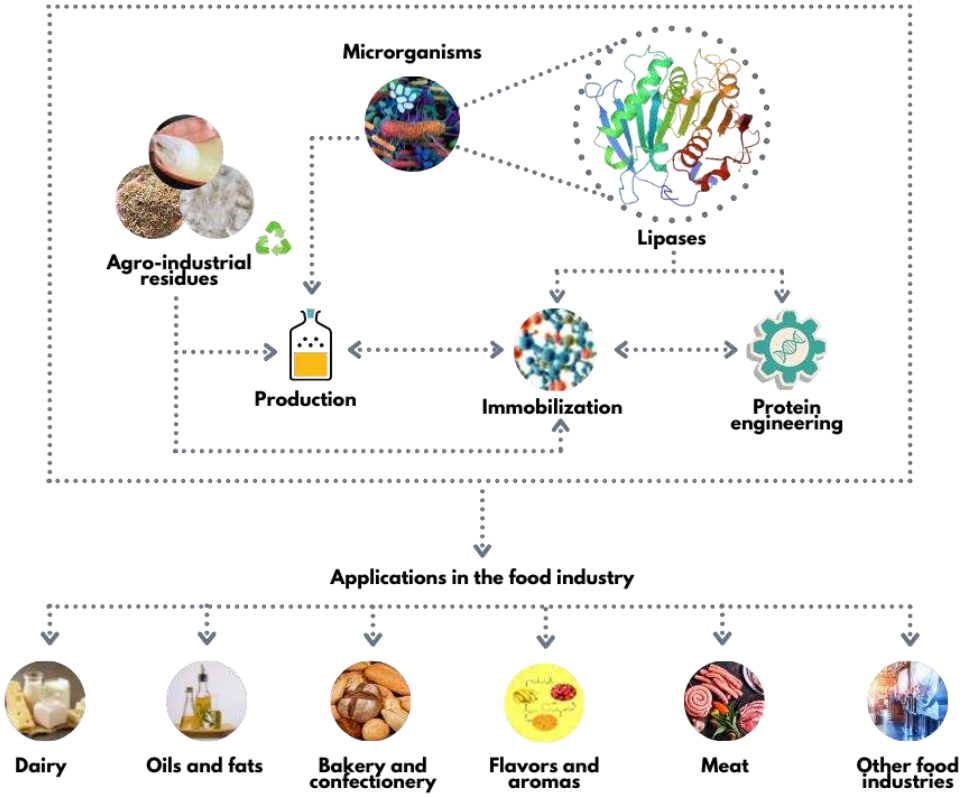
Abstract

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the largest groups of enzymes and are used in various industrial processes. Lipases of microbial origin are currently receiving increased attention for industrial application as microorganisms grow quickly and are easily genetically manipulated. Furthermore, they offer several advantages, such as catalysis of diverse reactions, high specificity, high yields, low energy consumption and reduced processing time and production costs. There is a relentless ongoing effort to optimise the production of microbial lipases for potential application in the food industry. In this context, this review highlights the most promising techniques for producing microbial lipases and the recent applications of these lipases in dairy, oils and fats, bakery and confectionery, meat, flavours and aromas and other food industries. Microbial lipases are normally obtained by fermentation, but the high costs of carbon and nitrogen sources limit the process. To overcome this problem, low-cost agro-industrial residues in the lipase production process are explored. To obtain lipases with high yields and improved characteristics, the technique of protein engineering is described as promising, and the immobilization method that allows the recycling of lipases to improve their catalytic performance is focused. Due to their catalytic properties and versatility, lipases of microbial origin are considered extremely important catalysts in the food industry, meeting the demand for

tastier foods with pleasant aromas and textures. Therefore, microbial lipases are considered safe and sustainable biocatalysts.

Keywords: Microbial Lipases; Food Industry, Agro-Industrial Residue.

Graphical abstract



1. Introduction

1.1 Lipases as biocatalysts

Lipases are recognized as lipolytic enzymes belonging to the serine hydrolase group, responsible for catalysing the hydrolysis of ester-carboxylic bonds of triacylglycerols (TAG) with the release of free fatty acids, diglycerides (DAG), monoglycerides (MAG) and glycerol (Chen et al., 2003; Jaeger et al., 1994; Nagarajan, 2012). Lipases preferentially hydrolyse substrates with long-chain fatty acids greater than 10 carbons. However, lipases can also hydrolyse substrates with short- and intermediate-chain fatty acids (Anthonsen et al., 1995; Chen et al., 2003; Jaeger et al., 1999).

In addition to hydrolysis, in which the nucleophilic agent is a water molecule, lipases can catalyse the reverse reaction, esterification (Borrelli & Trono, 2015). Depending on the transformation of the ester group, other reactions can also be catalysed, such as (1) transesterification, which encompasses reactions with alcohols acting as nucleophiles, for example, glycerolysis; (2) interesterification, which is characterized by exchange between the substituents of two different esters and (3) acidolysis and (4) aminolysis, which, as the names suggest, refer to breakdown by carboxylates and amines as nucleophilic agents, respectively (Alfonso & Gotor, 2004; Farfán et al., 2013; Otera, 1993; Parker & Baker, 1968) (Fig. 1). Due to the versatility of reactions catalysed by lipases, they are considered biotechnologically important biocatalysts, becoming green alternatives to chemical methods, providing safe and invaluable tools for industrial transformations to synthesize natural or synthetic materials with lower energy consumption under moderate reaction conditions (Memarpoor-Yazdi et al., 2017).

Based on specificity, lipases can be grouped into four major types: i) enantioselective: can distinguish enantiomers in a racemic mixture; ii) substrate-specific: selectively act on a specific substrate in a mixture of different materials, facilitating the synthesis of the desired product, iii) regioselective (which are divided into 1,3 regiospecific and 2-regiospecific lipases) and iv) non-specific (Borrelli & Trono, 2015; Verma et al., 2021). The 1,3 regiospecific lipases release fatty acids from positions 1 and 3 of TAG and cannot hydrolyse ester bonds at secondary positions. The 2-regiospecific lipases release fatty acids at the secondary position of TAG,

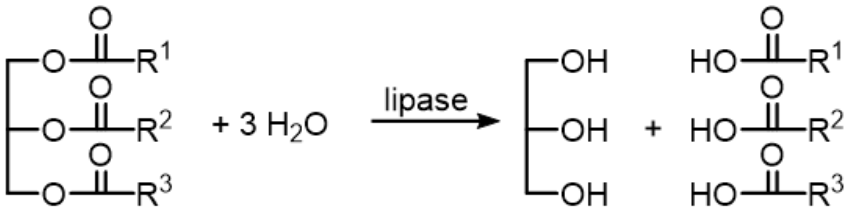
specifically producing 1,3-diacylglycerol. Non-specific lipases catalyse the hydrolysis of TAG into fatty acids and glycerol, with MAG and DAG intermediates, and can hydrolyse the ester group at any position on the substrate (Hari Krishna & Karanth, 2002; Jensen, 1974; Kapoor & Gupta, 2012).

Considering the biotechnological importance of lipases as biocatalysts, this review highlights the production and applications of microbial lipases in the dairy, oil and fat, bakery and confectionery, meat, flavour and aroma and other food industries.

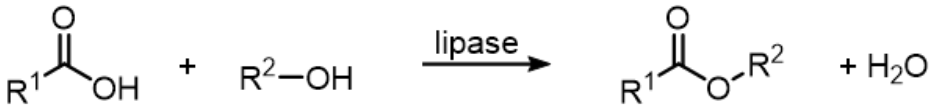
1.2 Production of microbial lipases

Lipases are ubiquitous in plants, animals and microorganisms and vary considerably in their characteristics. Lipases of microbial origin have attracted much attention from industry, due to their catalytic versatility, specificity, high yields, superior stability and greater availability than lipases of animal and vegetable origin (Chandra et al., 2020; Geoffry & Achur, 2018). Microorganisms represent an excellent source of lipases, as they have a high growth rate and low production cost, are more easily genetically manipulated and can regularly supply lipases due to the absence of seasonal fluctuations (Borrelli & Trono, 2015; Hasan et al., 2006). Because of these properties, microbial lipases have wide application in the food, detergent, chemical, pharmaceutical, pulp and paper, bioremediation, cosmetic and other industries (Hasan et al., 2006; Uppada et al., 2017). Lipase-producing microorganisms are generally isolated from environments where lipids and conditions suitable for the action of lipases are present. The isolated microorganisms are evaluated in a solid or liquid media to produce lipases (Geoffry & Achur, 2018), and then the enzyme is characterized and purified. Several factors influence enzyme activity, such as pH; temperature and the presence or absence of solvents, inhibitors and prosthetic groups. Table 1 shows the main characteristics of some of the microbial lipases used in the food industry.

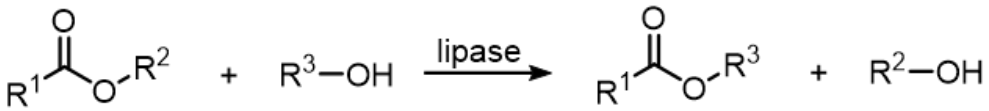
Hydrolysis



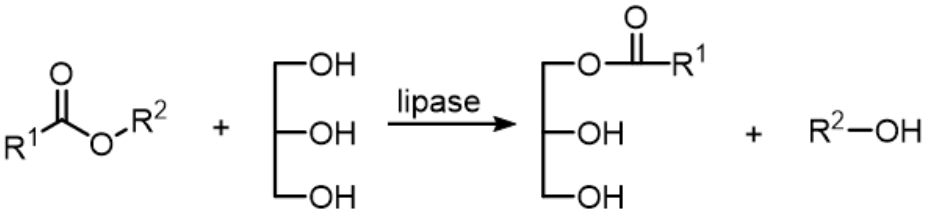
Esterification



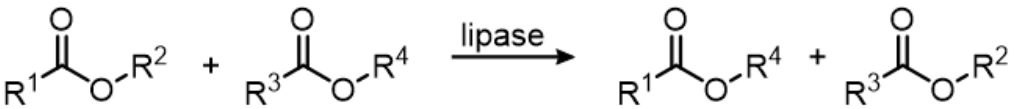
Transesterification



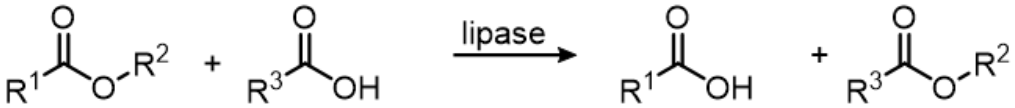
Glycerolysis



Interisterefication



Acidolysis



Aminolysis

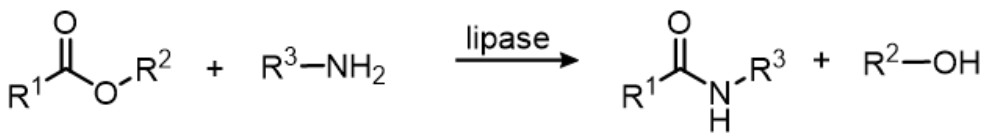


Fig. 1: Reactions catalysed by lipases.

Table 1: Microbial lipases used by the food industry and their main characteristics

Microbial source of lipase	Main characteristics of lipase	Reference
Penicillium cyclopium	Stability at 4 °C at pH values ranging from 5.0 to 10.0	(Huang et al., 2013)
Lactobacillus plantarum	Active on tributyrin and also on other long-chain substrates Optimum temperature: 40 °C Optimum pH: 7.0 Active under NaCl concentrations higher than 20% 40% of activity at refrigeration temperature (5 °C)	(Esteban-Torres et al., 2015)
Lactobacillus plantarum	Lipase immobilized: Optimum temperature: 45 °C Optimum pH: 6.5 Synthesizing different short-chain fatty acid esters	(Uppada et al., 2017)
Rhodothermus marinus	Preferential hydrolysis by esters with short chain fatty acids (<C8). Optimum temperature: 70 ºC Optimum pH: 8.5 Optimum concentration of NaCl: 0.2 M Organic solvent tolerant	(Memarpoor-Yazdi et al., 2017)
Malbranchea cinnamomea	Optimum temperature: 40 °C Optimum pH: 7.5 Specificity towards triglycerides with short and medium-chain fatty acids	(Duan et al., 2019)
Streptomyces violascens	Better regioselectivity at the *sn*-1 and *sn*-3 positions Optimum temperature: 30 ºC Optimum pH: 9,0 Organic solvent tolerant	(Gao et al., 2020)
Cersospora kikuchii	Lipase immobilized: Optimum temperature: 40 ºC Optimum pH: 6,5 Residual activity was high 88.7% after storage at 5 °C for six months.	(Costa-Silva et al., 2021)

The market value of microbial lipases was estimated at US$425.0 million in 2018 and is projected to reach US$590.2 million in 2023 (Chandra et al., 2020). For industrial applications, lipases must be produced on a large scale by fermentation.

Fermentation technology is the process by which microorganisms grow and metabolize complex substrates by breaking them down into simple compounds and in due course, yield many enzymes and by-products (Fasim et al., 2021). As microbial lipases are mainly extracellular, they can be produced by solid-state fermentation (SSF) or submerged fermentation (SMF). The type of fermentation, SSF or SMF, influences microbial growth and large-scale enzyme production. The main difference between them is the amount of water. In SSF, microorganisms grow on natural or inert solid support materials with a low water activity. SSF is widely used to produce lipases from fungi and yeasts using various simple, inexpensive and abundant agricultural and industrial residues as substrates. Therefore, SSF is an economical alternative for the large-scale production of enzymes that are produced by fungi (Bharathi & Rajalakshmi, 2019; Geoffry & Achur, 2018).

In SMF, microorganisms grow in a liquid medium with high water activity. It has some advantages, such as greater homogeneity of the culture medium and greater ease of controlling variable parameters such as temperature and pH. In addition, recovery and purification of extracellular lipases by SMF are simple, and agro-industrial residue substrates can also be used as carbon and nitrogen sources (Bharathi & Rajalakshmi, 2019; Geoffry & Achur, 2018; Wu et al., 2020).

A general disadvantage of industrial enzyme production, including that of lipases, is the production cost due to expensive sources of carbon and nitrogen, which account for 50% of the total cost of enzyme production. The exploitation of low-cost raw material residues becomes essential when scaling up the production of enzymes from microbial sources (Gaonkar & Furtado, 2021; Singh & Bajaj, 2017). The current emphasis is on the development of new and sustainable methods for industrially important enzyme production to make the whole process commercially cost effective (Sodhi et al., 2022). Residues such as bran, sugarcane bagasse, oil cakes, peels, feathers, whey, crustacean and fish residues, chicken feathers, sweet potato flour and bagasse and others are generated worldwide (El Sheikha & Ray, 2017; Gaonkar & Furtado, 2021). The exploration and valorisation of these residues are considered a widely sustainable approach once contribute to the production of enzymes of microbial origin and reduce production costs and problems associated with their elimination (Gaonkar & Furtado, 2021).

Recent reports exemplify the use of agro-industrial residues as substrates for the production of microbial lipases.Wu et al. (2020) showed the viability of industrial

production of lipase A by *Bacillus subtilis* using glycerol and chicken feather hydrolysate as an inexpensive source of carbon and nitrogen. Knob et al. (2020) isolated lipolytic yeasts from slaughterhouse refrigerator effluent and oil mill effluent. The species identified as *Meyerozyma guilliermondii* was grown in 2% cheese whey at pH 4.0 for 24 hours, with a 6.7-fold increase in lipase production. Cheese whey was also used for lipase production by *Geotrichum candidum* and productivity was increased with the enrichment of the medium with corn steep liquor (Ramos et al., 2021). Gaonkar & Furtado (2021) used different agro-industrial residues for the growth of the archaeon *Haloferax lucentensis* and verified that the use of coconut oil cake under optimized conditions increased lipase production.

The use of free-form lipases obtained from the fermentation process has technical limitations due to difficulty of their recovery for reuse, sensitivity to pH and temperature variations and low operational stability which increases cost. An alternative to circumvent this is to follow protocols of immobilization or encapsulation of the enzyme.

1.3 Lipase immobilization

Immobilization methods have been considered as a promising approach to boost enzyme productivity and operational stability and to facilitate the recycling of enzyme, which decreases cost. The immobilization of enzymes represents a more advantageous configuration since it also improves the separation from reaction mixtures without contaminating the products.

Enzymes can be immobilized by various methods, which can be broadly classified as physical, where there are weak interactions between the support and the enzyme, and chemical, where covalent bonds are formed between the support and the enzyme (Homaei et al., 2013). The physical method involves the adsorption of the enzyme on a support or its imprisonment inside a porous matrix, and interactions between the enzyme and the support are via weaker bonds such as Van der Waals forces and hydrogen-type bonds (Chandra et al., 2020; Girelli et al., 2020). In the chemical method, covalent bonds are usually formed between the support with functional groups present on the protein's surface (Homaei et al., 2013). The only rule is that the covalent bond must not be formed with the enzyme's catalytic activity sites and that the binding reactions must be carried out under mild conditions (Girelli et al., 2020).

Different agro-industrial residues, called lignocellulosic (coconut fibre, corn cob, spent grain, spent coffee, husk, husk ash, straw rice, soybean and wheat bran) and not lignocellulosic by-products (eggshell and eggshell membranes) are materials with great potential for enzymatic immobilization. These highly available residues are underutilized and cause significant environmental problems due to inadequate storage but are of great interest due to their low cost (Girelli et al., 2020). Indeed, these natural polymers display a set of desired attributes and advantages such as nontoxicity, biodegradability, biocompatibility, hydrophilicity, antibacterial properties, physiological inertness, gel formation, heavy metals chelation, and high proteins affinity (Bilal et al., 2021).

Recently, nanostructure supports appear as strong alternatives in the field of enzyme immobilization for the application of industrial nanobiocatalysis, since they provide additional advantageous properties as higher surface areas and ease of recovery and reuse (Oliveira et al., 2021). Different nanomaterials as metal-organic frameworks, carbon nanotubes, silica-based nanoparticles, nanoflowers, nanofibrous membranes, graphene oxide and hybrid nanomaterials have been considered as promising structures for immobilizing lipases. Recent reviews take a broad approach to lipase immobilization in nanomaterials (Bilal et al., 2021; Oliveira et al., 2021; Ismail & Baek, 2020; Liu et al., 2020; Zhong et al., 2020).

Lipase immobilization technology has been used in the food industry for the synthesis of aromatic esters (Costa-Silva et al., 2021; Memarpoor-Yazdi et al., 2017), flavour esters (Narwal et al., 2016; Uppada et al., 2017) and emulsifiers (Bavaro et al., 2020); interesterification of oils and fats (Samoylova et al., 2017; Xie & Zang, 2017); production of margarine (Sellami et al., 2012); addition of value to oils (Binhayeeding et al., 2020) and others. However, according to Coelho & Orlandelli (2021), niche focusing on immobilized lipases remain poorly explored.

1.4 Protein engineering

To produce microbial lipases on an industrial scale, the protein engineering technique is used to obtain enzymes designed with better kinetic properties, greater substrate specificity, greater thermostability, altered pH optima and increased/decreased optimal temperatures, depending on the industrial applications (Verma et al., 2021). Proteins can be engineered by employing various methods;

however, the basis remains recombinant DNA technology: altering the parent amino acid sequence to produce the required pattern. It is generally performed by manipulating the genes from original protein molecules and constructing unique proteins with varied structures, resulting in enhanced performance (Kataria et al., 2021).

Protein engineering methods, when used individually or in combination, mainly with directed evolution and rational design, exhibit a high potential to increase the functionality of existing proteins. This technique has proven an effective tool mainly for the amelioration of enzymes utilized in the food industry (Kataria et al., 2021). Directed evolution involves generating a series of random mutations in the gene that encodes the target protein. Rational design targets specific sites in the gene to make specific changes in the enzyme based on information gained from the protein's sequence, structure and function (Kapoor et al., 2017; Verma et al., 2021). Protein engineering, together with the possibility of immobilizing enzymes on suitable supports, can overcome the limitation of enzyme instability under different conditions, where parameters such as temperature and the presence of solvents and/or reagents and products can cause denaturation of the biocatalyst (Girelli et al., 2020).

Escherichia coli is by far the most used prokaryotic microorganism for recombinant production of lipases, and suitable host organism for bacterial lipases, such as from the genera *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Serratia*, *Burkholderia*, and from metagenomic libraries (Contesini et al., 2020). The first recombinant enzyme approved by the Food and Drug Administration (FDA) of the United States for use in food was bovine chymosin expressed in *E. coli* K-12, for cheese production (Flamm, 1991), and from there, the study and improvement of recombinant enzymes in food manufacturing have been intensified. Protein engineering is a promising technique, and many lipases currently used in food processing are derived from recombinant microorganisms. Table 2 highlights some recombinant lipases approved by the FDA with GRAS (Substances Generally Recognized as Safe) status for use in the food industry.

Gonçalves et al. (2020) used several techniques simultaneously to produce and use lipase, such as protein engineering, the use of agro-industrial residues and enzyme immobilization. The lipase 1 from *Beauveria bassiana* was efficiently expressed in *Aspergillus nidulans* A773. The mutant strain proved to be viable for enzyme secretion with different carbon sources derived from the agro-industrial

process, such as cassava husk, cornmeal, corn syrup, sorghum seed and wheat bran. Finally, the enzyme was immobilized on different hydrophobic supports for the transesterification reaction. An acidic lipase gene from the lipolytic bacteria *Micrococcus luteus* was successfully cloned to the yeast *Pichia pastoris* and utilizing combined codon optimization and optimization of signal sequence for extracellular expression resulted in increased gene expression compared to the previous expression in *E. coli* (Adina et al., 2021).

Table 2: Lipases from recombinant microorganisms based on FDA regulations, GRAS affirmation petitions and intended use

Microbial source of lipase	**GRAS Notice No. / year of closure**	**Intended Use**
Thermomyces lanuginosus	GRN 43 / 2000	Use in dough, baked goods, and the fats and oil industry as a processing aid for the interesterification or hydrolysis of glycerides in brewing beer
Fusarium oxysporum	GRN 75 / 2001	Use in the fats and oils industry, the baking industry, the hydrolysis of lecithin, and the modification of egg yolks
Thermomyces lanuginosus and *Fusarium oxysporum*	GRN 103 / 2002	Use in bakery products, egg yolks, whole eggs, and fats and oils
Candida antarctica	GRN 158 / 2005	Use in the production of triglyceride products
Aspergillus niger	GRN 296 / 2009	Use in baked goods
Pseudomonas fluorescens Biovar I	GRN 462 / 2013	Use in the production of refined triacylglycerol-based oils used in food
Fusarium oxysporum	GRN 631 / 2016	For use in the production of baked goods
Rhizopus oryzae	GRN 783 / 2018 GRN 708 / 2017	For use in the production of cocoa butter substitutes and human milk fat substitutes
Aspergillus tubingensis	GRN 808 / 2019	For use as a processing aid in baking, in brewing processes and cereal manufacturing, in paste production, and potable alcohol production

GRN: GRAS affirmation petitions that have been converted to GRAS notices

2. Application of microbial lipases in food industry

Microbial lipases are widely used during food processing, resulting in desirable changes in sensory quality, such as texture, flavour and aroma. Moreover, the food products obtained may have increased nutritional value. Due to the characteristics of microbial lipases, there is currently interest in a decisive search for them to improve the processing of food products. In this context, we will highlight the most recent studies on microbial lipases for application in the food industry. Fig. 2 presents a summary of the applications of microbial lipases in the food industry, in which the products are obtained from the versatility of reactions that lipases catalyze.

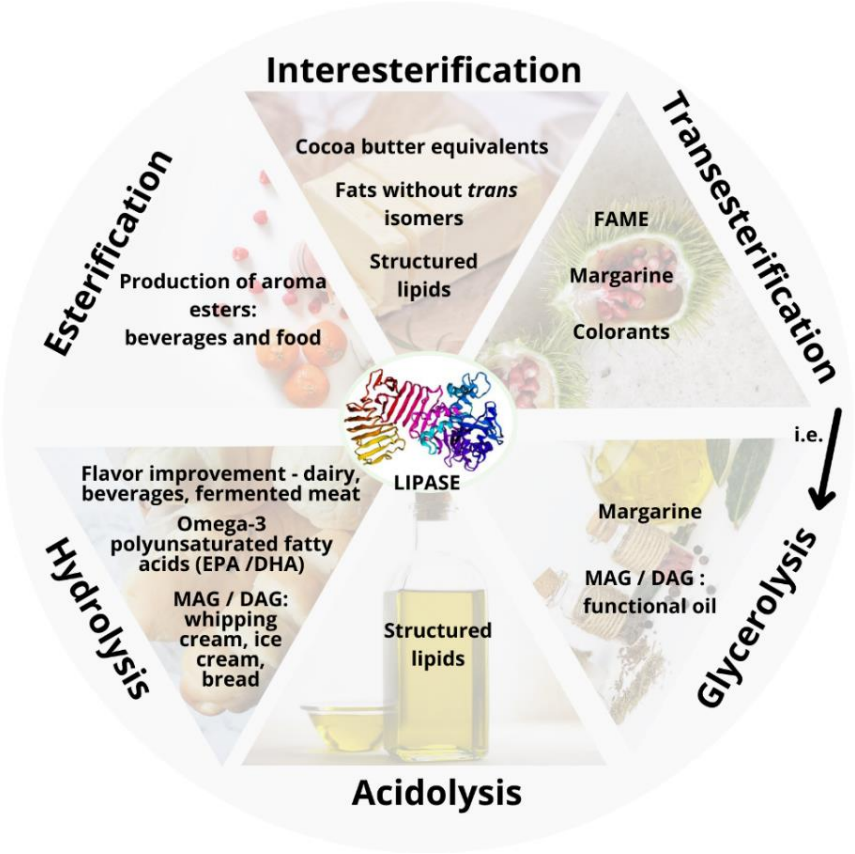


Fig. 2. Mainly important reactions catalysed by lipases and applied in the food industry. FAME = fatty acid methyl esters; DAG = diglycerides; MAG = monoglycerides; EPA = eicosapentaenoic acid; DHA = docosahexanoic acid.

2.1 Dairy industry

Microbial lipases are used in the dairy industry to hydrolyse milk fat, improve yogurt flavour, enhance cheese flavour and lipolysis of butter and cream according to processing needs and accelerate cheese ripening (Jooyandeh et al., 2009;

Memarpoor-Yazdi et al., 2017). Milk fat is the most complex fat found in nature, and more than 400 different fatty acids have already been identified in the TAG present inside milk fat globules (Lopez et al., 2011). According to the fatty acids released, the hydrolysis of TAG can contribute to desirable and undesirable flavours for dairy products. Short-chain fatty acids impart a strong, spicy taste; medium-chain fatty acids play a greater role in soapy flavour formation and long-chain fatty acids contribute little to flavour (Chen et al., 2003; Deeth, 2022; Fox & Stepaniak, 1983; Gupta et al., 2003).

To obtain the desired product, it is essential to choose the correct lipase, as each enzyme has a unique fatty acid release profile. Peng et al. (2014) expressed the lipase Est_p6 from a metagenomic library in *E. coli*, and the enzyme showed high hydrolytic specificity for myristate (C14) and palmitate (C16), which are long-chain fatty acids, concluding that Est_p6 is safe for imparting a distinctive and desirable flavour and odour in milk fat flavour production. Memarpoor-Yazdi et al. (2017) showed that GDSL lipase from *Rhodothermus marinus* cloned and expressed in *E. coli* and recovered by covalent immobilization on chitosan-coated Fe_3O_4 nanoparticles has the potential for hydrolysis of short-chain esters, suggesting its potential to improve dairy products. A lipase gene *McLipB* was cloned from a thermophilic fungus *Malbranchea cinnamomea* and expressed in *Pichia pastoris* (Duan et al., 2019). The produced lipase had high specificity for triglycerides with short and medium-chain fatty acids and had no positional specificity, exhibiting high stability under acidic conditions. McLipB protein may also be a potential candidate for producing milk fat flavour.

Lipases can also play an essential role in the flavour of yogurt. Huang et al. (2020a) used 1,3 regiospecific lipase from *Aspergillus oryzae* (AY30) to exclusively hydrolyse milk fat at positions 1 and 3 of TAG to release a large amount of free fatty acids. The addition of lipase AY30 to the mixed fermentation conducted by *Streptococcus lactis* ACCC 11093 with *Lactobacillus casei* subsp. *rhamnosus* 6013, *Lactobacillus acidophilus* 1.1878 and *Lactobacillus plantarum* DMDL 9010 significantly improved the physicochemical properties of yogurt, especially the total volatile organic acid content, and efficiently improved the quality of yogurt-flavoured bases.

In cheese processing, lipolysis is essential for the development of a characteristic flavour. In ripened cheeses such as Brie, Camembert and Roquefort, *Penicillium* spp. are the essential lipolytic microorganisms. The white mould *Penicillium camemberti* is used for the maturation of soft cheeses, such as Camembert, Brie and Neufchatel (Ropars et al., 2020). Fatty acids produced by lipases of the blue

Penicillium roqueforti mould make a major contribution to the flavour of blue-vein cheeses (Deeth, 2011). Caron et al. (2021) showed that the *P. roqueforti* population strongly impacts cheese quality, appearance and aroma. The populations used for cheesemaking led to bluer cheeses, with a better aroma, probably due to domestication involving the selection of multiple fungal traits by humans seeking to produce the best possible cheeses.

Cheese maturation is an expensive process, and its duration varies according to the type of cheese but contributes to the textural, functional and sensory attributes of the finished product. Karaca & Güven (2018) investigated the supplementation of milk for cheese making with commercial lipase Piccantase A from *Mucor miehei* for 90 days and reported increased lipolysis and acceleration of cheese ripening. Rani & Jagtap (2019) showed that lipase from *Bacillus tequilensis* PR13 reduced the period of maturation of Swiss cheese from 3 to 2 months. This was achieved without compromising the desirable physicochemical properties of the final product. Thus, lipase would result in decreased production costs by reducing the ripening period without affecting the quality characteristics of Swiss cheese. Therefore, the enzyme could be used for the cost-effective production of Swiss cheese. Kendirci et al. (2020) verified that cheeses modified by lipases obtained from *Rhizomucor miehei* and *Candida rugosa* presented a similar flavour to cured white cheeses. It was also observed a 12.5- to 81.9-fold increase in the content of total free fatty acids and an increase in volatile compounds compared with that of cured white cheeses.

2.2 Oil and fat industry

Fat and oil modification is one of the main areas of the food processing industry that requires new economic and green technologies (Hasan et al., 2006). Interesterification can be carried out chemically (using chemical catalysts) or enzymatically (using enzymatic catalysts). Interesterification catalysed either by chemicals or by enzymes leads to the exchange of fatty acids on the glycerol backbone or to a change in the position of fatty acids on the glycerides, resulting in structured lipids with modified physicochemical and nutritional properties. Despite the high cost, enzymatic interesterification offers additional advantages, such as milder processing conditions, fewer by-products and easier product recovery than chemical interesterification (Adhikari et al., 2010; Sivakanthan & Madhujith, 2020). In addition, it

is possible to obtain modified oils and fats with a low content of trans isomers (Samoylova et al., 2016).

Unfortunately, inexpensive natural oils with a high melting point cannot be used directly for edible purposes as they cause the food product to have low plasticity and incomplete melting at body temperature (Samoylova et al., 2017; Xie & Zang, 2017). Interesterification is generally used to customize fat with a range of melting points for different food products and modify crystallization (Adhikari et al., 2010). Samoylova et al. (2017), through enzymatic interesterification catalysed by a lipase immobilized on recombinant thermostable silica of *Geobacillus stearothermophilus* G3, using sunflower oil and hydrogenated soybean oil, verified that this biocatalyst has high stability in the interesterification process. Thus, this biocatalyst is very effective for application in the interesterification of oils with high melting points, such as palm stearin, cacao butter, hydrogenated oils and other oils, which is an advantage to the production process of modified oils. Xie & Zang (2017) also found that *C. rugosa* lipase immobilized via covalent bonds in HAp-γ-Fe_2O_3 nanoparticles showed excellent catalytic performance for the interesterification reaction of soybean oil.

An ecologically sound approach to using relatively low-value biological resources was described by Sellami et al. (2012). Palm stearin and palm olein blends in different ratios were enzymatically transesterified in a solvent-free system using a *Rhizopus oryzae* lipase immobilized onto $CaCO_3$ to produce a suitable fat for margarine formulation. Results indicated that all transesterified blends had lower slip melting points than their non-transesterified counterparts. The rheological analysis showed that margarine prepared with the transesterified blend showed better spreadability than that of control margarine prepared with non-transesterified fat. Another example of the use of transesterification to add value to oils is the conversion of waste frying oil (WCO) into fatty acid methyl esters (FAME). Elhussiny et al. (2020) selected two *Aspergillus* isolates capable of producing lipolytic enzymes and transesterifying WCO into relatively high FAME yields. Binhayeeding et al. (2020) showed that a mixture of lipases from *C. rugosa* and *R. miehei* immobilized on polyhydroxybutyrate was also able to transesterify WCO, producing FAME. In the work by Da Silva et al. (2018), a lipase NS 40116, obtained from genetically modified *Thermomyces lanuginosus* was used to synthesize FAME from chicken abdominal fat, demonstrating high potential in transesterification reactions using this fat as a substrate.

Omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have shown important human-health-promoting biological functions, especially in the development and maintenance of brain function and vision (Castejón & Señoráns, 2020), and has aroused interest in the enrichment of oils using microbial lipases. For example, lipase from *Pseudomonas fluorescens* MTCC 2421 was used to enrich PUFAs from sardine oil, improving the EPA content from 17.8% to 42.5% (Chakraborty & Raj, 2009). Gao et al. (2020) expressed *Streptomyces violascens* OUC-Lipase 6 in *B. subtilis* WB800 under ideal lipase conditions and found that OUC-Lipase 6 could selectively hydrolyse fatty acids on the glyceride backbone, thus improving the contents of DHA and EPA in codfish oil. In a study by Cao et al. (2020), lipase from *Trichosporon* sp. F1-2 also showed potential as a catalyst in enriching EPA and DHA in fish oil. Yang et al. (2021) used the lipase from *Candida cylindracea* to produce omega-3 PUFA from tuna oil. Under ideal conditions, the omega-3 PUFA content in the glyceride fraction of hydrolysed tuna oil increased significantly from 34.30% to 57.7%.

In addition to traditional sources of omega-3, there has been a recent effort to enzymatically enrich oils from vegetable sources, such as chia oil, coconut oil, linseed oil and others. Imanparast et al. (2018) showed that the immobilized lipase from *Actinomadura sediminis* represents a promising biocatalyst for producing PUFA-rich acylglycerols by direct esterification from free fatty acids in linseed oil. Castejón et al. (2019) synthesized omega-3 ethyl esters from chia oil catalysed by a lipase from *T. lanuginosus*. Consequently, omega-3 ethyl esters from chia oil with up to 65% α-linolenic acid were produced. Cipolatti et al. (2021) produced PUFA-enriched edible oils, obtaining lipids containing 181.6 mg of EPA and DHA in coconut oil, with the recombinant *Candida antarctica* lipase, obtained by fermentation in an alternative medium of low cost and immobilized on various particles of mesoporous silica.

Due to the recognition of the benefits of PUFA, PUFA-enriched MAG and DAG are used as emulsifiers and are widely applied in the food industry. Bavaro et al. (2020) established an enzymatic protocol for the continuous production of PUFA-enriched MAG and DAG from hemp seed in a continuous-flow fixed-bed reactor containing lipase from *Pseudomonas cepacia* covalently immobilized on Sepabeads EC-EP. A mixture of MAG and DAG (40% yield, 620 mg) composed of α-linolenic acid (82%) and oleic acid (16%) was obtained in less than 2 h. This bioprocess facilitates recovery of

the different oil components (free fatty acids, MAG and DAG) deriving from oil hydrolysis and the use of lipase in several cycles without any loss of activity.

Currently, the increased interest in improving the structural and functional quality of edible oils is remarkable. Yao et al. (2020a) developed a method to synthesize a functional oil rich in DAG, phytosterol esters and α-linolenic acid, from commercial soybean oil, peanut oil and rapeseed oil, using co-immobilized bi-lipases from *C. rugosa* and *T. lanuginosus*, on polydopamine-modified Fe_3O_4 surfaces. Three kinds of functional oils have been produced with high DAG, phytosterol esters and α-linolenic acid content (≥30.09%, ≥15.61% and ≥26.45%, respectively). Mota et al. (2020) extracted crude oils from coffee grounds and olive pomace that were used as raw material to produce low-calorie structured lipids by acidolysis with capric acid or interesterification with ethyl caprate, catalysed by 1,3 regiospecific lipase from *R. oryzae* immobilized on magnetic nanoparticles. Nicholson & Marangoni (2021) demonstrated a viable technique for structuring cottonseed and peanut oils into structural fats used in food applications. The non-regiospecific *C. antarctica* lipase B was able to perform glycerolysis, converting liquid oils into structural fats. The product of glycerolysis was used to make margarine with similar plasticity to that of commercial margarine and butter. Gunathilake et al. (2021) used pure concentrates of EPA and DHA to produce MAG and DAG oils through glycerolysis catalysed by the immobilized lipase of *C. antarctica* B. The omega-3 acylglycerols produced by the lipase were used for the fortification and stabilization of extra virgin olive oil using the antioxidant hydroxytyrosyl palmitate, and this blend of oils can be referred to as ‘functional olive oil’ or ‘omega-3 enriched olive oil’.

2.3 Bakery and confectionery industry

In the manufacture of bread, it is necessary to use emulsifiers to improve the dough’s volume, texture and stability. These emulsifiers are detected after bread processing and, therefore, must be described on the bread label. Due to the emergence of the clean label trend in recent years, a part of the strategy of some manufacturers has been to use the enzymatic approach, using lipases that are denatured after processing and therefore do not need to appear on the label (Monié et al., 2021; Sangeetha et al., 2011). Moayedallaie et al. (2010) made comparisons between three categories of commercial lipases and an emulsifier. Both the lipases

and the emulsifier caused a significant increase in bread oven rise and specific volume. Lipases, as catalysts of the hydrolysis reactions of TAG, release MAGs and DAGs, which are considered emulsifiers and are capable of forming inclusion complexes with the amylose contained in the flour, improving the specific volume and firmness of the bread (Monié et al., 2021; Purhagen et al., 2011).

Huang et al. (2013) cloned the mono- and diacylglycerol lipase genes from *Penicillium cyclopium* and expressed them in *P. pastoris* strain GS115. The recombinant enzyme was named Lipase GH1 and was more efficient in the synthesis of MAGs and DAGs than was Lipase G50, a similar, commercially available lipase derived from *P. camemberti*, when oleic acid was used as an acyl donor. According to the authors, Lipase GH1 has the potential for food emulsifier preparation.

Milk fat has been used as a functional ingredient in bread making, and Huang et al. (2020b) evaluated the effects of the incorporation of the fungal lipase Lipopan F extracted from the *Fusarium oxysporum* strain in milk fat. As a result of the lipase treatment, the dough structure was strengthened, and the bread volume improved. Furthermore, the lipase treatment reduced the residual water activity, attenuating the hardening process of the bread crumb. Thus, this study demonstrates the potential application of exogenous milk fat treated with microbial lipase to replace synthetic surfactants, allowing for improved product properties and functionality.

The lipases available for bakery applications hydrolyse several lipid structures in flour, resulting in an increase in bread volume. Schaffarczyk et al. (2014) found that digalactosyl diglycerides, monogalactosyl diglycerides and *N*-acyl-phosphatidyl ethanolamine were hydrolysed with concomitant formation of digalactosyl monoglycerides, monogalactosyl monoglycerides and *N*-acyl-lysophosphatidyl ethanolamine by the action of lipase. Changes induced by the lipid fraction of wheat caused increases in bread volume of 56%–58%, depending on the type and concentration of added lipase.

Cocoa butter is the base ingredient for chocolate and other confectionery products and is one of the most expensive fats in the world. The current trends in the market suggest further rises in cocoa butter prices, shortages, strong demand in emerging countries, and profit squeezes for companies in the future. This means that finding a practical alternative is necessary (Ghazani & Marangoni, 2018; Verstringe et al., 2012). Cocoa butter can be replaced by other vegetable fats, and the most important requirement is that they be comparable with respect to melting behaviour

and polymorphism, fatty acid and TAG composition and processing properties (Rohm et al., 2018).

Cocoa butter equivalents was synthesized from a 60:40 (w/w) mixture of a commercial enzymatically synthesized shea stearin and palm mid-fraction, catalysed by a commercially available immobilized fungal lipase, Lipozyme RM IM (Ghazani & Marangoni, 2018). The result showed that it is possible to synthesize cocoa butter equivalents with molecular structure and physical properties that resemble cocoa butter very closely. This was achieved in a two-step solvent-free reaction, with minimal downstream processing. Huang et al. (2021) prepared cocoa butter equivalents by enzymatic interesterification from the palm mid-fraction with stearic acid, using 1,3 regiospecific lipase from *R. miehei*, immobilized on a macroporous anion-exchange resin. They investigated the reaction process parameters and compared the physicochemical properties of the product with those of cocoa butter, concluding that the product obtained can be used as an excellent alternative to cocoa butter. Additionally, lipases are widely used in the chocolate industry to enhance the flavour of toffees, milk chocolate, caramels and buttercreams; decrease excess sweetness and improve the buttery character of caramels and toffees (Negi, 2019).

2.4 Meat industry

Lipases are also explored in the meat industry. In fermented meat products, which are appreciated by consumers for their sensory traits, lipid hydrolysis and oxidation in muscle and fat tissues are important processes that contribute to the development of the fermented flavour (Chen et al., 2017). In fermented meat products, lipase activity originates from a starter culture or from endogenous enzymes of fat cells and muscle fibres (Gandemer, 2002).

Lipases are active during the fermentation and maturation steps of sausage making. Chen et al. (2017) evaluated the role of several single strains or mixed strains (*Pediococcus pentosaceus*, *Lactobacillus curvatus*, *Lactobacillus sakei* and *Staphylococcus xylosus*) in lipolysis and lipid oxidation in Harbin sausages. The free-fatty-acid contents of both muscle and fat tissues were higher in the inoculated sausages than in the non-inoculated control, especially with mixed strains. The results demonstrate that Harbin dry sausage can be inoculated with a starter culture mixture

of *P. pentosaceus*, *L. curvatus* and *S. xylosus* to promote lipid hydrolysis, inhibit lipid autoxidation and improve fermented flavour development.

Xiao et al. (2020) evaluated the effects of *L. plantarum* R2 and *S. xylosus* A2 inoculation on the microbial community, lipolysis, proteolysis and volatile compounds in Chinese dry fermented sausages. The results showed that the total contents of free fatty acids and free amino acids were increased by inoculation of starter cultures, especially a mixed culture. Thus, flavour development in the inoculated dry fermented sausages was attributed to improvements in microbiological quality.

Lipases are used to remove excess fat in the meat and fish processing industries to produce leaner meat. Developing techniques for deep processing of by-products of animal slaughter, such as bones, will have important social and economic benefits while promoting the production of a coordinated and sustainable source, which is desirable for eco-friendly development of the slaughter industry (Yao et al., 2020b). Lipase pre-treatment of bone improved the enzymolytic efficiency of alkaline protease by significantly decreasing the lipid content and changing the surface structure and surface element content, promoting attachment to the sample (Yao et al., 2020b).

2.5 Flavour and aroma industry

Flavour esters that possess an aromatic ring in their molecular structure are also known as aromatic esters. These esters are widely found in nature (fruits and plants), and both synthetic (i.e. via chemical) and natural routes (i.e. via direct extraction from nature or via biotechnology) are suitable for their biocatalysis. From the industrial point of view, they are the most economical approach to reaching final green products with no toxicity and no harm to human health (Sá et al., 2017). The synthesis of different short-chain fatty acid esters for use as flavoring agents in the food industry is promissing. Xiao et al. (2015) demonstrated that lipase B from immobilized *C. antarctica* could carry out the esterification reaction of acetoin and fatty acids to synthesize fatty acid esters of acetoin, which are used in food and beverages to enhance the flavors of products. Narwal et al. (2016) immobilized lipase from *Bacillus aerius* and verified the esterification capacity of acetic acid and isoamyl alcohol, and the lipase catalysed the esterification with a yield of about 68% under optimized reaction conditions. The product was identified as isoamyl acetate, which has a pear or banana flavor and is widely used in the food, beverage, cosmetic and

pharmaceutical industries. Uppada et al. (2017) explored esterification efficacy of the immobilized lipase from *L. plantarum* in synthesizing different short chain fatty acid esters which find use as flavoring agents in food industry.

Costa-Silva et al. (2021) combined spouted bed drying with conventional immobilization to increase the activity and stability of the lipase from *Cercospora kikuchii*. The lipase was immobilized in agricultural by-products known as 'green' supports such as rice husks, corn straw, green coconut fibre or sugarcane bagasse through covalent bonding. The rice husk system was used as a biocatalyst for aroma production by esterification of butyric acid to butyl butyrate, with a yield of 95%, reaching a concentration of 15.2 g/L of butyl butyrate, a fruity aroma widely used in the food industry. Oliveira et al. (2017) optimized the lipase yield of *Aspergillus ibericus* in agro-industrial residues and presented an environmental friendly strategy to naturally produce an aroma ester (butyl decanoate) in a solvent-free system, with application in the food industry, while adding value to the agro-industrial residues. Memarpoor-Yazdi et al. (2017) verified that the GDSL lipase from *R. marinus* exhibited a high potential for synthesizing short-chain esters and methyl acetate, suggesting its potential for aromatic compound synthesis.

Chen et al. (2021) proposed a two-step enzymatic catalysis method to modify goat milk fat, with the objective of generating a pleasant aroma to improve the flavour of goat milk products. First, the milk fat was hydrolysed to release free short-chain fatty acids as substrates, and then esterification was carried out to synthesize esters with pleasant notes. Lipase produced from *T. lanuginosus*, proved to be efficient in catalysing the hydrolysis of goat milk fat.

2.6 Other food industries

Recently, other sectors of the food industry have used microbial lipases as essential components for industrial application. Sofian-Seng et al. (2017) reported that direct incorporation of a lipase derived from *R. miehei* into palm oil-in-water emulsion formulations produced a combination of predominantly oleic and palmitic fatty acid and MAG fractions. When applied as part of the processing pathway for whipping cream and ice cream, controlled emulsion lipolysis has been shown to allow structure-function properties comparable to the use of commercial MAG.

The annatto is one of the most used natural colorants in the food industry, and bixin is one of the components responsible for the yellow-orange colour of the annatto extract. The structure of bixin is hydrophobic, which limits its application in water-based food products. To increase the solubility of bixin in water, it could be by attaching a hydrophilic molecule to this pigment. For this, Jahangiri et al. (2018) immobilized the lipase from *C. antarctica* to catalyse the transesterification reaction between bixin and sorbitol, forming a product that will have superior hydrophilic properties to the alcohol group of sorbitol.

Esteban-Torres et al. (2015) cloned the *lp_3562* gene from *L. plantarum* encoding a putative esterase/lipase and expressed it in *E. coli* BL21 (DE3), and the overproduced enzyme Lp_3562 showed biochemical properties that make it attractive for use in food fermentations. Park et al. (2020) produced ricinoleic acid vanillic ester through transesterification of vanillic acid and castor oil, mediated by recombinant *Proteus vulgaris* K80 lipase expressed in *E. coli* BL21 (DE3) cell and lipase acrylic resin from *C. antarctica*. This multifunctional compound showed potent antioxidant and antibacterial activity against Gram-positive bacteria and food spoilage microorganisms and can be used as an important biomaterial in the food and cosmetics industry. The production of a new maltoheptaose-based sugar ester with an excellent emulsifying property was proposed by Nguyen et al. (2021), the ester synthesis reaction was better when catalysed by lipase B from *C. antarctica*.

In this review, several studies of lipases produced by microorganisms were reported, and with this, many lipases with specific characteristics and improved properties were highlighted. Most of the studies are recent and have shown that microbial lipases are responsible for a range of improvements in foods, including flavour and aroma improvements in dairy products; cheese ripening; structured lipid synthesis; PUFA-enriched and functional oils; cocoa butter equivalents; improvements in the texture and volume of bread; synthesis of emulsifiers, aromatics and flavour esters and others.

3. Future directions

Microbial lipases have great potential in the food industry due to their properties, such as specificity, versatility of the reactions they catalyse and varied stability in relation to the presence of solvents, temperatures and pH, making them excellent

biocatalysts. Due to its catalytic properties, it is possible to find or target a suitable lipase for each industrial application, allowing reproducible, efficient and devoid of undesirable by-products catalysis conditions, with lower environmental impacts and greener results.

Despite the numerous studies that focused on the use of lipases for application in food, few of them reached a final product with commercial importance. Therefore, the application of these enzymes in the food industry requires scientists to make a greater effort to obtain enzymes that are commercial and recognized as safe. Advances in more sustainable, economical and advantageous methods such as the use of agro-industrial residues for the production and immobilization of lipases, combined with protein engineering, provide the creation of biocatalysts with improved properties, bringing benefits to industrial processes, which include: impact reduction environment, improved catalytic resources, reusability and more sustainable processes. A promising field for future research is the integration of different techniques to develop desired characteristics in lipases, with low cost production, making them excellent biocatalysts in industrial processes. Recombinant lipases are highly promising and some have been recognized with GRAS status and introduced in several food sectors, showing that application of lipases tends to increase and will be expanded in the coming years. The large-scale production of lipases for application in food industries is hampered by their high cost, and for this reason, extensive research using low-cost agro-industrial residues is aimed at reducing this expense. Furthermore, lipase immobilization strategies are growing rapidly and these by-products can also be used for this purpose, allowing for cheaper, more efficient and more reusable reaction conditions. It is foreseeable that in the coming years, the focus of production and immobilization of enzymes will be on the reuse of agro-industrial residues, since they are considered sustainable and safe. In the near future, the large-scale application of enzymes, such as lipases, in foods will certainly make food products with better sensory characteristics and, consequently, more acceptable to consumers and with greater added value for the products, leading to an increase in financial return for manufacturers.

4. Conclusions

Microbial lipases are more advantageous over other natural sources of lipases, so their importance for application in food industries tends to increase and, for this, scientists are looking for simple and low-cost methods. Throughout this review, the main forms of production and immobilization of lipases were shown and the recent uses of microbial lipases in some food industries were highlighted. The use of lipases for food improvement is considered a more sustainable approach as it replaces traditional chemical methods and, therefore, it can be considered a green and promising tool to perform various reactions in food production. Finally, it is expected that in the coming years, the exploration of this biotechnological resource will be expanded due to the several advantages that microbial lipases have.

Declaration of Competing Interests

No conflict of interest exists.

Acknowledgments

The authors thank the financial support from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Universidade Federal de Viçosa (UFV).

Author contributions:

Salgado, C. A.: Conceptualization, Visualization and Writing - Original Draft. **Dos Santos, C. I. A.:** Conceptualization, and Writing - Original Draft. **Vanetti, M. C.D.:** Conceptualization, Supervision and Writing - Review & Editing.

References

Adhikari, P., Zhu, X. M., Gautam, A., Shin, J. A., Hu, J. N., Lee, J. H., Akoh, C. C., & Lee, K. T. (2010). Scaled-up production of zero-*trans* margarine fat using pine nut oil and palm stearin. *Food Chemistry*, *119*(4), 1332–1338. https://doi.org/10.1016/j.foodchem.2009.09.009

Adina, S. R., Suwanto, A., Meryandini, A., & Puspitasari, E. (2021). Expression of novel acidic lipase from *Micrococcus luteus* in *Pichia pastoris* and its application in transesterification. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 0–10. https://doi.org/10.1186/s43141-021-00155-w

Alfonso, I., & Gotor, V. (2004). Biocatalytic and biomimetic aminolysis reactions: Useful tools for selective transformations on polyfunctional substrates. *Chemical Society Reviews*, *33*(4), 201–209. https://doi.org/10.1039/b307785n

Anthonsen, H. W., Baptista, A., Drabløs, F., Martel, P., Petersen, S. B., Sebastião, M., & Vaz, L. (1995). Lipases and esterases: A review of their sequences, structure and evolution. *Biotechnology Annual Review*, *1*, 315-371. https://doi.org/10.1016/S1387-2656(08)70056-5

Bavaro, T., Benucci, I., Pedrali, A., Marrubini, G., Esti, M., Terreni, M., Massolini, G., & Ubiali, D. (2020). Lipase-mediated hydrolysis of hempseed oil in a packed-bed reactor and in-line purification of PUFA as mono- and diacylglycerols. *Food and Bioproducts Processing*, *123*, 345–353. https://doi.org/10.1016/j.fbp.2020.07.009

Bharathi, D., & Rajalakshmi, G. (2019). Microbial lipases: An overview of screening, production and purification. *Biocatalysis and Agricultural Biotechnology*, *22*(August), 101368. https://doi.org/10.1016/j.bcab.2019.101368

Bilal, M., Fernandes, C. D., Mehmood, T., Nadeem, F., Tabassam, Q., & Ferreira, L. F. R. (2021). Immobilized lipases-based nano-biocatalytic systems — A versatile platform with incredible biotechnological potential. *International Journal of Biological Macromolecules*, *175*, 108–122. https://doi.org/10.1016/j.ijbiomac.2021.02.010

Binhayeeding, N., Klomklao, S., Prasertsan, P., & Sangkharak, K. (2020). Improvement of biodiesel production using waste cooking oil and applying single and mixed immobilised lipases on polyhydroxyalkanoate. *Renewable Energy*, *162*, 1819–1827. https://doi.org/10.1016/j.renene.2020.10.009

Borrelli, G. M., & Trono, D. (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International Journal of Molecular Sciences, 16*(9), 20774–20840. https://doi.org/10.3390/ijms160920774

Cao, X., Liao, L., & Feng, F. (2020). Purification and characterization of an extracellular lipase from *Trichosporon* sp. and its application in enrichment of omega-3 polyunsaturated fatty acids. *LWT*, *118* (April 2019), 108692. https://doi.org/10.1016/j.lwt.2019.108692

Caron, T., Piver, M. Le, Péron, A.-C., Lieben, P., Lavigne, R., Brunel, S., Roueyre, D., Place, M., Bonnarme, P., Giraud, T., Branca, A., Landaud, S., & Chassard, C. (2021). Strong effect of *Penicillium roqueforti* populations on volatile and metabolic compounds responsible for aromas, flavor and texture in blue cheeses. *International Journal of Food Microbiology*, *March*, 109174. https://doi.org/10.1016/j.ijfoodmicro.2021.109174

Castejón, N., Moreno-Pérez, S., Silveira, E. A., Lorente, G. F., Guisán, J. M., & Señoráns, F. J. (2019). Synthesis of omega-3 ethyl esters from chia oil catalyzed by polyethylene glycol-modified lipases with improved stability. *Food Chemistry*, *271*(July 2018), 433–439. https://doi.org/10.1016/j.foodchem.2018.07.215

Castejón, N., & Señoráns, F. J. (2020). Enzymatic modification to produce health-promoting lipids from fish oil, algae and other new omega-3 sources: A review. *New Biotechnology*, *57*(July), 45-54. https://doi.org/10.1016/j.nbt.2020.02.006

Chakraborty, K., & Paul Raj, R. (2009). Selective enrichment of n-3 polyunsaturated fatty acids with C18-C20 acyl chain length from sardine oil using *Pseudomonas fluorescens* MTCC 2421 lipase. *Food Chemistry*, *114*(1), 142–150. https://doi.org/10.1016/j.foodchem.2008.09.029

Chandra, P., Enespa, Singh, R., & Arora, P. K. (2020). Microbial lipases and their industrial applications: A comprehensive review. *Microbial Cell Factories*, *19*(1), 1-42. https://doi.org/10.1186/s12934-020-01428-8

Chen, L., Daniel, R. M., & Coolbear, T. (2003). Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal*, *13*(4), 255–275. https://doi.org/10.1016/S0958-6946(02)00171-1

Chen, Q., Kong, B., Han, Q., Xia, X., & Xu, L. (2017). The role of bacterial fermentation in lipolysis and lipid oxidation in Harbin dry sausages and its flavour development. *LWT*, *77*, 389-396. https://doi.org/10.1016/j.lwt.2016.11.075

Chen, X., Wang, J., Stevenson, R. J., Ang, X., Peng, Y., & Quek, S. Y. (2021). Lipase-catalyzed modification of milk fat: A promising way to alter flavor notes of goat milk products. *LWT*, *145*(June), 111286. https://doi.org/10.1016/j.lwt.2021.111286

Cipolatti, E. P., Rios, N. S., Sousa, J. S., Robert, J. de M., Da Silva, A. A. T., Pinto, M. C. C., Simas, A. B. C., Vilarrasa-García, E., Fernandez-Lafuente, R., Gonçalves, L. R. B., Freire, D. M. G., & Manoel, E. A. (2021). Synthesis of lipase/silica biocatalysts through the immobilization of CALB on porous SBA-15 and their application on the resolution of pharmaceutical derivatives and on nutraceutical enrichment of natural oil. *Molecular Catalysis*, *505*(April), 111529. https://doi.org/10.1016/j.mcat.2021.111529

Coelho, A. L. S., & Orlandelli, R. C. (2021). Immobilized microbial lipases in the food industry: a systematic literature review. *Critical Reviews in Food Science and Nutrition*, *61*(10), 1689–1703. https://doi.org/10.1080/10408398.2020.1764489

Contesini F.J., Davanço, M. G., Borin, G. P., Vanegas, K. G., Cirino, J. P. G., Melo, R. R., Mortensen, U. H., Hildén, K., Campos, D. R., & Carvalho, P. O. (2020). Advances in recombinant lipases: production, engineering, immobilization and application in the pharmaceutical industry. *Catalysts 10,* 1-33. https://doi.org/10.3390/catal10091032

Costa-Silva, T. A., Carvalho, A. K. F., Souza, C. R. F., De Castro, H. F., Bachmann, L., Said, S., & Oliveira, W. P. (2021). Immobilized enzyme-driven value enhancement of lignocellulosic-based agricultural byproducts: Application in aroma synthesis. *Journal of Cleaner Production*, *284*(February), 124728. https://doi.org/10.1016/j.jclepro.2020.124728

Da Silva, J. R. P., Nürnberg, A. J., Da Costa, F. P., Zenevicz, M. C., Lerin, L. A., Zanetti, M., Valério, A., de Oliveira, J. V., Ninow, J. L., & De Oliveira, D. (2018). Lipase NS40116 as catalyst for enzymatic transesterification of abdominal chicken fat as substrate. *Bioresource Technology Reports*, *4*, 214–217. https://doi.org/10.1016/j.biteb.2018.11.005

De Oliveira, A. L. B., Cavalcante, F. T. T., Moreira, K. S., Monteiro, R. R. C., Rocha, T. G., Souza, J. E. S., da Fonseca, A. M., Lopes, A. A. S., Guimarães, A. P., de Lima, R. K. C., de Souza, M. C. M., & dos Santos, J. C. S. (2021). Lipases immobilized onto nanomaterials as biocatalysts in biodiesel production: Scientific context, challenges, and opportunities. *Revista Virtual de Quimica*, *13*(4), 875–

891. https://doi.org/10.21577/1984-6835.20210019
Deeth, H.C. (2011). Milk Lipids | Lipolysis and Hydrolytic Rancidity. *Encyclopedia of Dairy Sciences*, 721–726. https://doi.org/10.1016/B978-0-12-374407-4.00343-5
Deeth, H. C. (2022). Enzymes Exogenous to Milk in Dairy Technology: Lipases. *Encyclopedia of Dairy Sciences*, 640–647. https://doi.org/10.1016/B978-0-12-818766-1.00225-7
Duan, X., Xiang, M., Wang, L., Yan, Q., Yang, S., & Jiang, Z. (2019). Biochemical characterization of a novel lipase from *Malbranchea cinnamomea* suitable for production of lipolyzed milkfat flavor and biodegradation of phthalate esters. *Food Chemistry*, *297*(May), 124925. https://doi.org/10.1016/j.foodchem.2019.05.199
El Sheikha, A. F., & Ray, R. C. (2017). Potential impacts of bioprocessing of sweet potato: Review. *Critical Reviews in Food Science and Nutrition*, *57*(3), 455–471. https://doi.org/10.1080/10408398.2014.960909
Elhussiny, N. I., Khattab, A. E. N. A., El-Refai, H. A., Mohamed, S. S., Shetaia, Y. M., & Amin, H. A. (2020). Assessment of waste frying oil transesterification capacities of local isolated Aspergilli species and mutants. *Mycoscience*, *61*(3), 136–144. https://doi.org/10.1016/j.myc.2020.01.003
Esteban-Torres, M., Mancheño, J. M., de las Rivas, B., & Muñoz, R. (2015). Characterization of a halotolerant lipase from the lactic acid bacteria *Lactobacillus plantarum* useful in food fermentations. *LWT*, *60*(1), 246–252. https://doi.org/10.1016/j.lwt.2014.05.063
Farfán, M., Villalón, M. J., Ortíz, M. E., Nieto, S., & Bouchon, P. (2013). The effect of interesterification on the bioavailability of fatty acids in structured lipids. *Food Chemistry*, *139*(1–4), 571–577. https://doi.org/10.1016/j.foodchem.2013.01.024
Fasim, A., More, V. S., & More, S. S. (2021). Large-scale production of enzymes for biotechnology uses. *Current Opinion in Biotechnology*, *69*, 68–76. https://doi.org/10.1016/j.copbio.2020.12.002
Flamm, E. L. (1991). How FDA approved chymosin: a case history. *Bio/Technology*, *9*(4), 349-351.
Fox, P. F., & Stepaniak, L. (1983). Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas fluorescens* strain AFT 36. *Journal of Dairy Research*, *50*(1), 77-89. https://doi.org/10.1017/S0022029900032544
Gandemer, G. (2002). Lipids in muscles and adipose tissues , changes during processing and sensory properties of meat products. *Meat science*, *62*, 309–321. https://doi.org/10.1016/S0309-1740(02)00128-6
Gao, K., Chu, W., Sun, J., & Mao, X. (2020). Identification of an alkaline lipase capable of better enrichment of EPA than DHA due to fatty acids selectivity and regioselectivity. *Food Chemistry*, *330*(5), 127225. https://doi.org/10.1016/j.foodchem.2020.127225
Gaonkar, S. K., & Furtado, I. J. (2021). Valorization of low-cost agro-wastes residues for the maximum production of protease and lipase haloextremozymes by *Haloferax lucentensis* GUBF-2 MG076078. *Process Biochemistry*, *101*(November 2020), 72–88. https://doi.org/10.1016/j.procbio.2020.10.019
Geoffry, K., & Achur, R. N. (2018). Screening and production of lipase from fungal organisms. *Biocatalysis and Agricultural Biotechnology*, *14*(April), 241-253. https://doi.org/10.1016/j.bcab.2018.03.009
Ghazani, S. M., & Marangoni, A. G. (2018). Facile lipase-catalyzed synthesis of a chocolate fat mimetic. *Scientific Reports*, *8*(1), 1–18. https://doi.org/10.1038/s41598-018-33600-x

Girelli, A. M., Astolfi, M. L., & Scuto, F. R. (2020). Agro-industrial wastes as potential carriers for enzyme immobilization: A review. *Chemosphere*, *244*(April), 125368. https://doi.org/10.1016/j.chemosphere.2019.125368

Gonçalves, E. C. S., Pérez, M. M., Vici, A. C., Salgado, J. C. S., Rocha, M. S., De Almeida, P. Z., Infante, J. da C., Scarcella, A. S. A., De Lucas, R. C., Vieira, A. T., De Faria, A. M., Batista, A. C. F., & Polizeli, M. de L. T. M. (2020). Potential biodiesel production from Brazilian plant oils and spent coffee grounds by *Beauveria bassiana* lipase 1 expressed in *Aspergillus nidulans* A773 using different agroindustry inputs. *Journal of Cleaner Production*, *256*(May), 120513. https://doi.org/10.1016/j.jclepro.2020.120513

Gunathilake, T., Akanbi, T. O., & Barrow, C. J. (2021). Lipase-produced omega-3 acylglycerols for the fortification and stabilization of extra virgin olive oil using hydroxytyrosyl palmitate. *Future Foods*, *4*(December), 100045. https://doi.org/10.1016/j.fufo.2021.100045

Gupta, R., Rathi, P., & Bradoo, S. (2003). Lipase Mediated Upgradation of Dietary Fats and Oils. *Critical Reviews in Food Science and Nutrition*, *43*(6), 635–644. https://doi.org/10.1080/10408690390251147

Hari Krishna, S., & Karanth, N. G. (2002). Lipases and lipase-catalyzed esterification reactions in nonaqueous media. *Catalysis Reviews - Science and Engineering*, *44*(4), 499–591. https://doi.org/10.1081/CR-120015481

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*, *39*(2), 235–251. https://doi.org/10.1016/j.enzmictec.2005.10.016

Homaei, A. A., Sariri, R., Vianello, F., & Stevanato, R. (2013). Enzyme immobilization: An update. *Journal of Chemical Biology*, 6(4), 185–205. https://doi.org/10.1007/s12154-013-0102-9

Huang, J., Yang, Z., Guan, F., Zhang, S., Cui, D., Guan, G., & Li, Y. (2013). A novel mono- and diacylglycerol lipase highly expressed in *Pichia pastoris* and its application for food emulsifier preparation. *Process Biochemistry*, *48*(12), 1899–1904. https://doi.org/10.1016/j.procbio.2013.08.021

Huang, Y. Y., Yu, J. J., Zhou, Q. Y., Sun, L. N., Liu, D. M., & Liang, M. H. (2020a). Preparation of yogurt-flavored bases by mixed lactic acid bacteria with the addition of lipase. *LWT, 131*(September), 109577. https://doi.org/10.1016/j.lwt.2020.109577

Huang, Z., Guo, Z., Xie, D., Cao, Z., Chen, L., Wang, H., Jiang, L., & Shen, Q. (2021). *Rhizomucor miehei* lipase-catalysed synthesis of cocoa butter equivalent from palm mid-fraction and stearic acid: Characteristics and feasibility as cocoa butter alternative. *Food Chemistry*, *343*(May), 128407. https://doi.org/10.1016/j.foodchem.2020.128407

Huang, Z., Brennan, C. S., Zheng, H., Mohan, M. S., Stipkovits, L., Liu, W., Kulasiri, D., Guan, W., Zhao, H., & Liu, J. (2020b). The effects of fungal lipase-treated milk lipids on bread making. *LWT, 128*(June), 109455. https://doi.org/10.1016/j.lwt.2020.109455

Imanparast, S., Hamedi, J., & Faramarzi, M. A. (2018). Enzymatic esterification of acylglycerols rich in omega-3 from flaxseed oil by an immobilized solvent-tolerant lipase from *Actinomadura sediminis* UTMC 2870 isolated from oil-contaminated soil. *Food Chemistry*, *245*(April), 934–942. https://doi.org/10.1016/j.foodchem.2017.11.080

Ismail, A. R., & Baek, K. (2020). International Journal of Biological Macromolecules Lipase immobilization with support materials , preparation techniques , and

applications : Present and future aspects. *International Journal of Biological Macromolecules*, *163*, 1624–1639. https://doi.org/10.1016/j.ijbiomac.2020.09.021

Jaeger, K.-E., Dijkstra, B. W., & Reetz, M. T. (1999). Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology, 53*(1), 315-351. https://doi.org/10.1146/annurev.micro.53.1.315

Jaeger, K.-E., Ransac, S., Dijkstra, B. W., Colson, C., Heuvel, M. V., & Misset, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, 15(1), 29-63. https://doi.org/10.1111/j.1574-6976.1994.tb00121.x

Jahangiri, A., Møller, A. H., Danielsen, M., Madsen, B., Joernsgaard, B., Vaerbak, S., Adlercreutz, P., & Dalsgaard, T. K. (2018). Hydrophilization of bixin by lipase-catalyzed transesterification with sorbitol. *Food Chemistry*, *268*(December), 203–209. https://doi.org/10.1016/j.foodchem.2018.06.085

Jensen, R. G. (1974). Characteristics of the lipase from the mold, *Geotrichum candidum*: A review. *Lipids*, *9*(3), 149–157. https://doi.org/10.1007/BF02532686

Jooyandeh, H., Kaur, A., & Minhas, K. S. (2009). Lipases in dairy industry: A review. *Journal of Food Science and Technology*, *46*(3), 181–189.

Kapoor, M., & Gupta, M. N. (2012). Lipase promiscuity and its biochemical applications. *Process Biochemistry*, *47*(4), 555–569. https://doi.org/10.1016/j.procbio.2012.01.011

Kapoor, S., Rafiq, A., & Sharma, S. (2017). Protein engineering and its applications in food industry. *Critical Reviews in Food Science and Nutrition*, *57*(11), 2321–2329. https://doi.org/10.1080/10408398.2014.1000481

Karaca, O. B., & Güven, M. (2018). Effects of proteolytic and lipolytic enzyme supplementations on lipolysis and proteolysis characteristics of white cheeses. *Foods*, *7*(8). https://doi.org/10.3390/foods7080125

Kataria, A., Sharma, R., Sharma, S., Singh, B., Kaur, G., & Yakubu, C. M. (2021). Recent applications of bio-engineering principles to modulate the functionality of proteins in food systems. *Trends in Food Science and Technology*, *113*(July), 54-56. https://doi.org/10.1016/j.tifs.2021.04.055

Kendirci, P., Salum, P., Bas, D., & Erbay, Z. (2020). Production of enzyme-modified cheese (EMC) with ripened white cheese flavour: II- effects of lipases. *Food and Bioproducts Processing*, *122*(July), 230–244. https://doi.org/10.1016/j.fbp.2020.05.010

Knob, A., Izidoro, S. C., Lacerda, L. T., Rodrigues, A., & De Lima, V. A. (2020). A novel lipolytic yeast *Meyerozyma guilliermondii*: Efficient and low-cost production of acid and promising feed lipase using cheese whey. *Biocatalysis and Agricultural Biotechnology*, *24*(February), 101565. https://doi.org/10.1016/j.bcab.2020.101565

Liu, J., Ma, R., & Shi, Y. (2020). Recent advances on support materials for lipase immobilization and applicability as biocatalysts in inhibitors screening methods-A review. *Analytica Chimica Acta*, *1101*, 9–22. https://doi.org/10.1016/j.aca.2019.11.073

Lopez, C., Briard-Bion, V., Ménard, O., Beaucher, E., Rousseau, F., Fauquant, J., Leconte, N., & Robert, B. (2011). Fat globules selected from whole milk according to their size: Different compositions and structure of the biomembrane, revealing sphingomyelin-rich domains. *Food Chemistry*, *125*(2), 355–368.

Memarpoor-Yazdi, M., Karbalaei-Heidari, H. R., & Khajeh, K. (2017). Production of the renewable extremophile lipase: Valuable biocatalyst with potential usage in

food industry. *Food and Bioproducts Processing*, *102*(March), 153–166. https://doi.org/10.1016/j.fbp.2016.12.015

Moayedallaie, S., Mirzaei, M., & Paterson, J. (2010). Bread improvers: Comparison of a range of lipases with a traditional emulsifier. *Food Chemistry*, *122*(3), 495–499. https://doi.org/10.1016/j.foodchem.2009.10.033

Monié, A., David, A., Clemens, K., Malet-Martino, M., Balayssac, S., Perez, E., Franceschi, S., Crepin, M., & Delample, M. (2021). Enzymatic hydrolysis of rapeseed oil with a non-GMO lipase: A strategy to substitute mono- and diglycerides of fatty acids and improve the softness of sponge cakes. *LWT*, *137*(February), 110405. https://doi.org/10.1016/j.lwt.2020.110405

Mota, D. A., Rajan, D., Heinzl, G. C., Osório, N. M., Gominho, J., Krause, L. C., Soares, C. M. F., Nampoothiri, K. M., Sukumaran, R. K., & Ferreira-Dias, S. (2020). Production of low-calorie structured lipids from spent coffee grounds or olive pomace crude oils catalyzed by immobilized lipase in magnetic nanoparticles. *Bioresource Technology*, *307*(July), 123223. https://doi.org/10.1016/j.biortech.2020.123223

Nagarajan, S. (2012). New tools for exploring "Old friends-microbial lipases". *Applied Biochemistry and Biotechnology*, *168*(5), 1163–1196. https://doi.org/10.1007/s12010-012-9849-7

Narwal, S. K., Saun, N. K., Dogra, P., & Gupta, R. (2016). Green synthesis of isoamyl acetate via silica immobilized novel thermophilic lipase from *Bacillus aerius*. *Russian Journal of Bioorganic Chemistry*, *42*(1), 69–73. https://doi.org/10.1134/S1068162016010118

Negi, S. (2019). Lipases: A Promising Tool for Food Industry. *Green Bio-processes* (pp. 181–198). Springer, Singapore. https://doi.org/10.1007/978-981-13-3263-0_10

Nguyen, P. C., Nguyen, M. T. T., Kim, J. H., Hong, S. T., Kim, H. L., & Park, J. T. (2021). A novel maltoheptaose-based sugar ester having excellent emulsifying properties and optimization of its lipase-catalyzed synthesis. *Food Chemistry*, *352*(August), 129358. https://doi.org/10.1016/j.foodchem.2021.129358

Nicholson, R. A., & Marangoni, A. G. (2021). Lipase-catalyzed glycerolysis extended to the conversion of a variety of edible oils into structural fats. *Current Research in Food Science*, *4*(February), 163–174. https://doi.org/10.1016/j.crfs.2021.03.005

Oliveira, F., Souza, C. E., Peclat, V. R. O. L., Salgado, J. M., Ribeiro, B. D., Coelho, M. A. Z., Venâncio, A., & Belo, I. (2017). Optimization of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions. *Food and Bioproducts Processing*, *102*(March), 268–277. https://doi.org/10.1016/j.fbp.2017.01.007

Otera, J. (1993). Transesterification. *Chemical Reviews, 93*(4), 1449-1470. https://doi.org/10.1021/cr00020a004

Park, C. G., Kim, J. J., & Kim, H. K. (2020). Lipase-mediated synthesis of ricinoleic acid vanillyl ester and evaluation of antioxidant and antibacterial activity. *Enzyme and Microbial Technology*, *133*(February), 109454. https://doi.org/10.1016/j.enzmictec.2019.109454

Parker, V. D., & Baker, A. W. (1968). The mechanism of ester acidolysis. *Chemical Communications (London)*, *873*(12), 691. https://doi.org/10.1039/C19680000691

Peng, Q., Wang, X., Shang, M., Huang, J., Guan, G., Li, Y., & Shi, B. (2014). Isolation of a novel alkaline-stable lipase from a metagenomic library and its specific application for milkfat flavor production. *Microbial Cell Factories*, *13*(1),

1–9. https://doi.org/10.1186/1475-2859-13-1
Purhagen, J. K., Sjöö, M. E., & Eliasson, A. C. (2011). Starch affecting anti-staling agents and their function in freestanding and pan-baked bread. *Food Hydrocolloids*, *25*(7), 1656–1666. https://doi.org/10.1016/j.foodhyd.2011.03.004
Ramos, P. R., Kamimura, E. S., Pires, N. A. M., Maldonado, R. R., & de Oliveira, A. L. (2021). Esterification reaction in SC-CO_2 catalyzed by lipase produced with corn steep liquor and Minas Frescal cheese whey. *Bioresource Technology Reports*, *14*(June), 100670. https://doi.org/10.1016/j.biteb.2021.100670
Rani, S., & Jagtap, S. (2019). Acceleration of Swiss cheese ripening by microbial lipase without affecting its quality characteristics. *Journal of Food Science and Technology*, *56*(1), 497–506. https://doi.org/10.1007/s13197-018-3482-6
Rohm, H., Schäper, C., & Zahn, S. (2018). Interesterified fats in chocolate and bakery products: A concise review. *LWT*, *87*(January), 379–384. https://doi.org/10.1016/j.lwt.2017.08.076
Ropars, J., Didiot, E., De la Vega, R. C. R., Bennetot, B., Coton, M., Poirier, E., Coton, E., Snirc, A., Le Prieur, S., & Giraud, T. (2020). Domestication of the emblematic white cheese-making fungus *Penicillium camemberti* and its diversification into two varieties. *Current Biology*, *30*(22), 4441-4453. https://doi.org/10.1016/j.cub.2020.08.082
Sá, A. G. A., Meneses, A. C., Araújo, P. H. H., & Oliveira, D. (2017). A review on enzymatic synthesis of aromatic esters used as flavor ingredients for food, cosmetics and pharmaceuticals industries. *Trends in Food Science and Technology*, *69*, 95-105. https://doi.org/10.1016/j.tifs.2017.09.004
Samoylova, Y. V., Piligaev, A. V., Sorokina, K. N., & Parmon, V. N. (2017). Enzymatic interesterification of sunflower oil and hydrogenated soybean oil with the immobilized bacterial recombinant lipase from *Geobacillus stearothermophilus* G3. *Catalysis in Industry*, *9*(1), 62–70. https://doi.org/10.1134/S2070050417010123
Samoylova, Y. V., Sorokina, K. N., & Parmon, V. N. (2016). Prospects for application of enzymatic interesterification of oils in the production of modified fats. *Catalysis in Industry*, *8*(4), 348–353. https://doi.org/10.1134/S2070050416040097
Sangeetha, R., Arulpandi, I., & Geetha, A. (2011). Bacterial lipases as potential industrial biocatalysts: An overview. *Research Journal of Microbiology*, *6*(1), 1-24. https://doi.org/10.3923/jm.2011.1.24
Schaffarczyk, M., Østdal, H., & Koehler, P. (2014). Lipases in wheat breadmaking: Analysis and functional effects of lipid reaction products. *Journal of Agricultural and Food Chemistry*, *62*(32), 8229–8237. https://doi.org/10.1021/jf5022434
Sellami, M., Ghamgui, H., Frikha, F., Gargouri, Y., & Miled, N. (2012). Enzymatic transesterification of palm stearin and olein blends to produce zero-trans margarine fat. *BMC Biotechnology*, *12*(48), 1-8. https://doi.org/10.1186/1472-6750-12-48
Singh, S., & Bajaj, B. K. (2017). Agroindustrial/forestry residues as substrates for production of thermoactive alkaline protease from *Bacillus licheniformis* K-3 having multifaceted hydrolytic potential. *Waste and Biomass Valorization*, *8*(2), 453–462. https://doi.org/10.1007/s12649-016-9577-2
Sivakanthan, S., & Madhujith, T. (2020). Current trends in applications of enzymatic interesterification of fats and oils: A review. *LWT*, *132*(October), 109880. https://doi.org/10.1016/j.lwt.2020.109880
Sodhi, A. S., Sharma, N., Bhatia, S., Verma, A., Soni, S., & Batra, N. (2022). Insights on sustainable approaches for production and applications of value added

products. *Chemosphere*, *286*(P1), 131623. https://doi.org/10.1016/j.chemosphere.2021.131623

Sofian-Seng, N. S., Golding, M., Goh, K., Janssen, P., & Lee, S. J. (2017). *In situ* enzymatic synthesis of polar lipid emulsifiers in the preparation and stabilisation of aerated food emulsions. *Food Biophysics*, *12*(3), 323–338. https://doi.org/10.1007/s11483-017-9488-1

Uppada, S. R., Akula, M., Bhattacharya, A., & Dutta, J. R. (2017). Immobilized lipase from *Lactobacillus plantarum* in meat degradation and synthesis of flavor esters. *Journal of Genetic Engineering and Biotechnology*, *15*(2), 331–334. https://doi.org/10.1016/j.jgeb.2017.07.008

Verma, S., Meghwanshi, G. K., & Kumar, R. (2021). Current perspectives for microbial lipases from extremophiles and metagenomics. *Biochimie*, *182*(March), 23–36. https://doi.org/10.1016/j.biochi.2020.12.027

Verstringe, S., De Clercq, N., Nguyen, T. M., Kadivar, S., & Dewettinck, K. (2012). Enzymatic and other modification techniques to produce cocoa butter alternatives. *Cocoa Butter and Related Compounds*.(pp. 443-474). https://doi.org/10.1016/B978-0-9830791-2-5.50021-9

Wu, F., Ma, J., Cha, Y., Lu, D., Li, Z., Zhuo, M., Luo, X., Li, S., & Zhu, M. (2020). Using inexpensive substrate to achieve high-level lipase A secretion by *Bacillus subtilis* through signal peptide and promoter screening. *Process Biochemistry*, *99*(December), 202–210. https://doi.org/10.1016/j.procbio.2020.08.010

Xiao, Z., Hou, X., Lyu, X., Zhao, J. yi, Xi, L., Li, J., & Lu, J. R. (2015). Enzymatic synthesis of aroma acetoin fatty acid esters by immobilized *Candida antarctica* lipase B. *Biotechnology Letters*, *37*(8), 1671–1677. https://doi.org/10.1007/s10529-015-1834-0

Xiao, Y., Liu, Y., Chen, C., Xie, T., & Li, P. (2020). Effect of *Lactobacillus plantarum* and *Staphylococcus xylosus* on flavour development and bacterial communities in Chinese dry fermented sausages. *Food Research International*, *135*(December 2019), 109247. https://doi.org/10.1016/j.foodres.2020.109247

Xie, W., & Zang, X. (2017). Covalent immobilization of lipase onto aminopropyl-functionalized hydroxyapatite-encapsulated-γ-Fe_2O_3 nanoparticles: A magnetic biocatalyst for interesterification of soybean oil. *Food Chemistry*, *227*(July), 397–403. https://doi.org/10.1016/j.foodchem.2017.01.082

Yang, Z., Jin, W., Cheng, X., Dong, Z., Chang, M., & Wang, X. (2021). Enzymatic enrichment of n-3 polyunsaturated fatty acid glycerides by selective hydrolysis. *Food Chemistry*, *346*(June), 128743. https://doi.org/10.1016/j.foodchem.2020.128743

Yao, G., Wang, X., Yang, M., Chen, F., Ling, Y., Liu, T., Xing, S., Yao, M., & Zhang, F. (2020a). Co-immobilization of bi-lipases on magnetic nanoparticles as an efficient catalyst for synthesis of functional oil rich in diacylglycerols, phytosterol esters and α-linolenic acid. *LWT*, *129*(July), 109522. https://doi.org/10.1016/j.lwt.2020.109522

Yao, Y., Wang, M., Liu, Y., Han, L., & Liu, X. (2020b). Insights into the improvement of the enzymatic hydrolysis of bovine bone protein using lipase pretreatment. *Food Chemistry*, *302*(July 2019), 125199. https://doi.org/10.1016/j.foodchem.2019.125199

Zhong, L., Feng, Y., Wang, G., Wang, Z., Bilal, M., Lv, H., Jia, S., & Cui, J. (2020). Production and use of immobilized lipases in/on nanomaterials: A review from the waste to biodiesel production. *International Journal of Biological Macromolecules*, *152*, 207–222. https://doi.org/10.1016/j.ijbiomac.2020.02.258

CAPÍTULO 2

Biodegradation of polyurethanes by *Serratia liquefaciens* L135 and its polyurethanase: *in silico* and *in vitro* analyses

Research article submitted to the journal Environmental Pollution

Authors: Cleonice Aparecida Salgado[1], Júnio Gonçalves Silva[2], Felipe Alves de Almeida[3], Maria Cristina Dantas Vanetti[1*]

[1]Department of Microbiology, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil; E-mail address: cleonice.salgado@ufv.br; mvanetti@ufv.br;
[2]Department of Chemistry, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil; E-mail address: junio.ufv@gmail.com;
[3]Instituto de Laticínios Cândido Tostes (ILCT), Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Juiz de Fora, MG, Brazil; E-mail address: felipe.almeida@epamig.br;

[*]Corresponding author: Department of Microbiology, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil. E-mail address: mvanetti@ufv.br; Telephone: +55 31 3612 2445.

Highlights

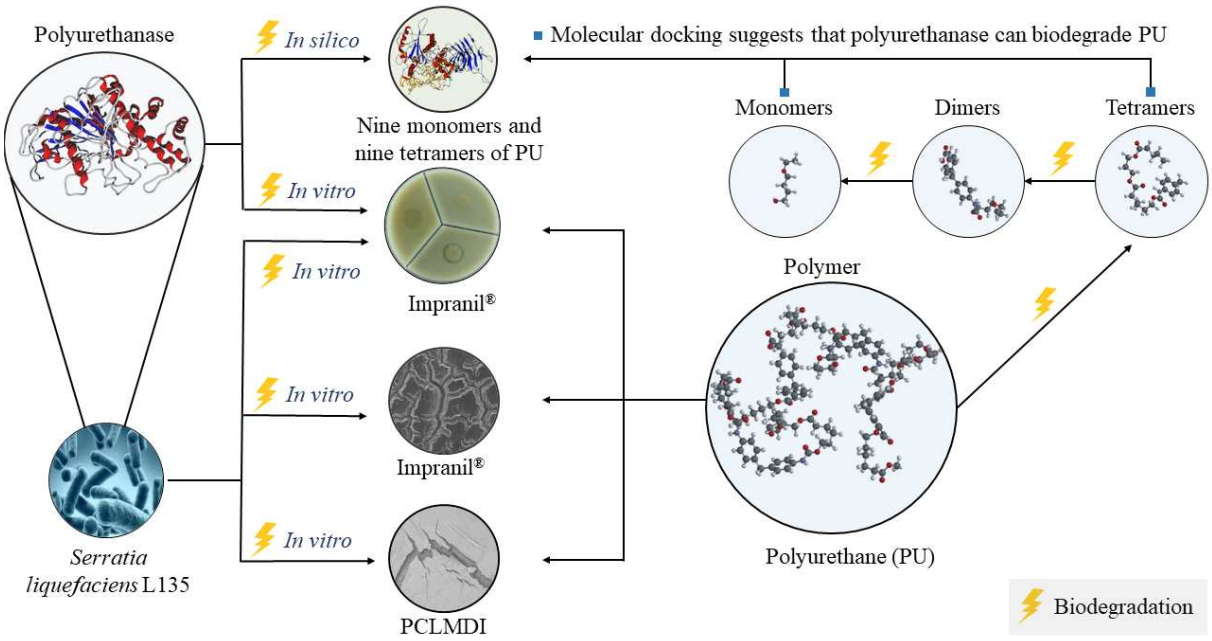
- *In silico*, the polyurethanase showed high binding energy with commercial polyurethanes.
- *In vitro*, S*. liquefaciens* and its polyurethanase biodegrade Impranil®.
- *S. liquefaciens* forms a biofilm on polyurethane films.
- The formation of pores and cracks in the polyurethane films were observed by eletronic microscopy.

Abstract

Polyurethanes (PU) are found in many everyday products and their disposal leads to environmental accumulation. Therefore, there is an urgent need to develop ecologically sustainable techniques to biodegrade and recycle this recalcitrant polymer and replace traditional methods that form harmful by-products. *Serratia liquefaciens* L135 secretes a polyurethanase with lipase activity, and this study explores the biodegradation of PU by this bacterium and its enzyme through *in silico* and *in vitro* analyses. PU monomers and tetramers were constructed *in silico* and tested with modeled and validated structure of the polyurethanase from *S. liquefaciens*. The molecular docking study showed that all PU monomers showed favorable interactions with polyurethanase (values of binding energy between -84.75 and -121.71 kcal mol^{-1}), including PU poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI). Due to repulsive steric interactions, tetramers showed less favorable interactions (values between 24.26 and -45.50 kcal mol^{-1}). *In vitro* analyses evaluated the biodegradation of PU: Impranil® and PCLMDI; the latter showed high binding energy with this polyurethanase *in silico*. Initially, the biodegradation of Impranil® by *S. liquefaciens* and its partially purified polyurethanase was confirmed in agar by forming a transparent halo. Impranil® disks inoculated with *S. liquefaciens* and incubated at 30 °C for six days showed rupture of the PU structure, possibly due to the formation of cracks visualized by scanning electron microscopy (SEM). PCLMDI films were also biodegraded by *S. liquefaciens* after 60 days of incubation, with the formation of pores and cracks visualized by SEM. This biodegradation may have occurred due to the action of polyurethanase produced by this bacterium. This work provides essential information on the potential of *S. liquefaciens* to biodegrade PU through *in silico* analyses combined with *in vitro*.

Keywords: Biofilm; Lipase; Molecular Docking; Polymer; Impranil.

Graphical abstract



1. Introduction

Polyurethanes (PU) were discovered by the German chemist Dr. Otto Bayer and his collaborators in 1937 through the polyaddition reaction between polyols and diisocyanates (Barnoy et al., 2017; Easley et al., 2023; Magnin et al., 2020; Xie et al., 2019). PU is a group of highly versatile polymers due to the range of chemical reagents that can be used in their synthesis, resulting in a multitude of structures with different properties in terms of thermal insulation, conductance, resistance, and elasticity (Cregut et al., 2013; Desai et al., 2023; Magnin et al., 2020). Thus, PU are found in various everyday products, such as tires, gaskets, bumpers, fibers, plastic foam, synthetic leather, jackets, adhesives, paints, sponges, cushions, rubber goods, automobiles, refrigerator insulation, among others (Akindoyo et al., 2016; Banik et al., 2023; Khan et al., 2017).

Due to its wide range of applications, PU is the sixth most used polymer in the world, with worldwide production of 18 million tons in 2016, and the global demand for PU is predicted to reach 22.5 million tons by 2024 (Kemona and Piotrowska, 2016; Simón et al., 2018; Tran and Lee, 2023), making it an environmental problem. As an example of the environmental impact of PU, Turner and Lau (2016) found that out of 70 fragments of foamed plastic collected on a sandy beach in England, 39 were characterized as PU. As PU waste accumulates in the environment, methods are needed to biodegrade and recycle this recalcitrant polymer. Conventional techniques such as burning and landfilling are inadequate due to the harmful by-products formed (Jaiswal et al., 2020). Therefore, there is an urgent need to develop ecologically sustainable techniques to replace traditional methods. Using microorganisms and enzymes to biodegrade PU can be an ecological and effective alternative for environmental decontamination.

The chemical composition of PU influences its physical, mechanical, and chemical properties, as well as its microbial and enzymatic biodegradation. In general, PU are composed of rigid and flexible segments. The rigid segment comprises a wide variety of isocyanate-based components and short-chain extenders, which provide hardness and mechanical strength. The flexible segment mainly consists of the long part of a polyol, such as a polyester or a polyether, which imparts absorption, elasticity, and elongation traits (Bhavsar et al., 2023; Cregut et al., 2013; Magnin et al., 2020; Spence and Plehiers, 2022). The flexible segments do not form crystals or have low

crystallinity, being the part most susceptible to biodegradation. PU polyester is more biodegradable than PU polyether due to the presence of ester groups, which are more prone to attack by microbial enzymes (Bhavsar et al., 2023; Rajan and Vijayalakshmi, 2023).

Bacterial species known to biodegrade polyester PU include *Pseudomonas chlororaphis* (Howard et al., 1999), *Pseudomonas fluorescens* (Vega et al., 1999), *Bacillus subtilis* (Rowe and Howard, 2002), *Bacillus pumilus* (Nair and Kumar, 2007), *Pseudomonas aeruginosa* (Mukherjee et al., 2011), *Acinetobacter gerneri* P7 (Howard et al., 2012), *Pseudomonas putida* A12 (Peng et al., 2014), *Pseudomonas protegens* Pf-5 (Nadeau et al., 2021), and *Serratia* HY-72 (Kim et al., 2022). The biodegradability of polyester PU was also found in different species of fungi, such as *Pestalotiopsis microspora* (Russell et al., 2011), *Aspergillus flavus* (Mathur and Prasad, 2012), *Aspergillus fumigatus*, *Cladosporium cladosporioidese*, *Penicillium chrysogenum*, (Álvarez-Barragán et al., 2016), *Aspergillus tubingensis* (Khan et al., 2017), *Aspergillus* sp. S45 (Osman et al., 2018), among others. These studies show the efforts of scientists to find a microorganism that is effective in the biodegradation of PU.

A strategy for the biodegradation of PU can be through the adhesion and growth of microorganisms, forming biofilms on the surface of plastics, called “plastisphere” (Du et al., 2022; Gaylarde et al., 2023). Bacteria can adhere to the surface of the polymer by extracellular appendages and by producing extracellular polymeric substances (EPS), consequently forming biofilms on the surface of PU (Berne et al., 2018; Koo et al., 2017; Li et al., 2021). After biofilm formation, biofragmentation is the next step of microbial biodegradation, which includes the catalytic action of microbial enzymes on plastic polymers (Jaiswal et al., 2020). Enzymes of bacterial and fungal origin, such as lipases, esterases, proteases, ureases, cutinases, and laccases, are described as capable of biodegrading polymers (Di Bisceglie et al., 2022; Flores-Castañón et al., 2022; Kim et al., 2022; Mansouri and Benslama, 2022; Nair and Kumar, 2007; Rowe and Howard, 2002; Shin et al., 2021; Silva et al., 2007). The production of enzymes, colonization, and persistence of microorganisms on the PU surface alter its physical, chemical, and mechanical properties, contributing to its biodegradation (Jaiswal et al., 2020).

Enzymes play a vital role in biodegradation, so investigations of enzyme-substrate interactions help understand the molecular mechanisms of biodegradation. The *in silico* approach, using molecular docking, allows the analysis of hydrophobic,

electrostatic, and hydrogen bonding interactions between an enzyme and a ligand, selecting the best substrate conformation within the enzyme, according to the score values (Liu et al., 2018). Molecular docking is a fast, inexpensive, and promising computational technique based on several algorithms designed to carry out the target docking process with ligands, widely used in many areas of research (Sarkar et al., 2023). Molecular docking can be used to predict the susceptibility, conformation, position, and orientation of the substrate within the active site, allowing to evaluate of its binding affinity and energy affinity as pre-experiments to indicate whether the enzyme is a good candidate for environmental bioremediation (Flores-Castañón et al., 2022; Santacruz-Juárez et al., 2021). Sonnendecker et al. (2022) determined by molecular docking the amino acid residues of a polyester hydrolase (PHL7) that contribute to the binding affinity to polyethylene terephthalate (PET) and also to its biodegradation efficiency. With advances in computational analysis, molecular docking with polymers can inform, guide, and complement *in vitro* studies. However, there are many challenges to combining theory with reality (Gartner and Jayaraman, 2019; Liang et al., 2018).

Our research group previously identified a polyurethanase with lipase activity secreted by *Serratia liquefaciens* L135. The modeled and validated structure of this polyurethanase was able to bond to urethane by molecular docking (Salgado et al., 2021). However, further analyses are needed to evaluate and validate the potential for PU biodegradation by this enzyme and the producing microorganism. Thus, this study aimed to perform *in silico* analyses to demonstrate the interactions between polyurethanase and different PU. Furthermore, to evaluate *in vitro* the ability of *S. liquefaciens* and its polyurethanase to biodegrade Impranil®, a model substrate for microorganisms capable of biodegrading PU, in addition to a commercial PU, which showed high binding energy with this polyurethanase *in silico*.

2. Materials and methods

2.1 *In silico* construction of polyurethanes (PU)

Eight diisocyanates and the diol precursor, polybutylene adipate (PBA), a polyester derived from the reaction of adipic acid with butanediol, were used to construct PU monomers and tetramers (Table 1, Figs. 1A and 1B). In addition, the

commercial PU monomer and tetramer, poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI; Sigma-Aldrich, St. Louis, USA) (Supplementary Fig. 1), were also constructed (Fig. 1B). The 3D structures of the PU (*.mol* format) were constructed and optimized using Spartan'14 (Version 1.1.8, Wavefunction, Inc., Irvine, CA, 2014) and had their loads calculated by the semi-empirical RM1 method (Rocha et al., 2006).

Table 1. List of diisocyanates, acronym of PU monomers and tetramers

Diisocyanate	**PU monomer**	**PU tetramer**
Bis(4-isocyanatophenyl)methane	MDI	t-MDI
1,6-diisocyanatohexane	HDI	t-HDI
2,6-diisocyanato-1-methylbenzene	2,6TDI	t-2,6TDI
2,4-diisocyanato-1-methylbenzene	2,4TDI	t-2,4TDI
4,4'-diisocyanato-3,3'-dimethyl-1,1'-biphenyl	TODI	t-TODI
Bis(4-isocyanatocyclohexyl)methane	HMDI	t-HMDI
1,2-bis(isocyanatomethyl)benzene	BDI	t-BDI
1,2-bis(isocyanatomethyl)cyclohexane	CDI	t-CDI
Bis(4-isocyanatophenyl)methane	PCLMDI	t-PCLMDI

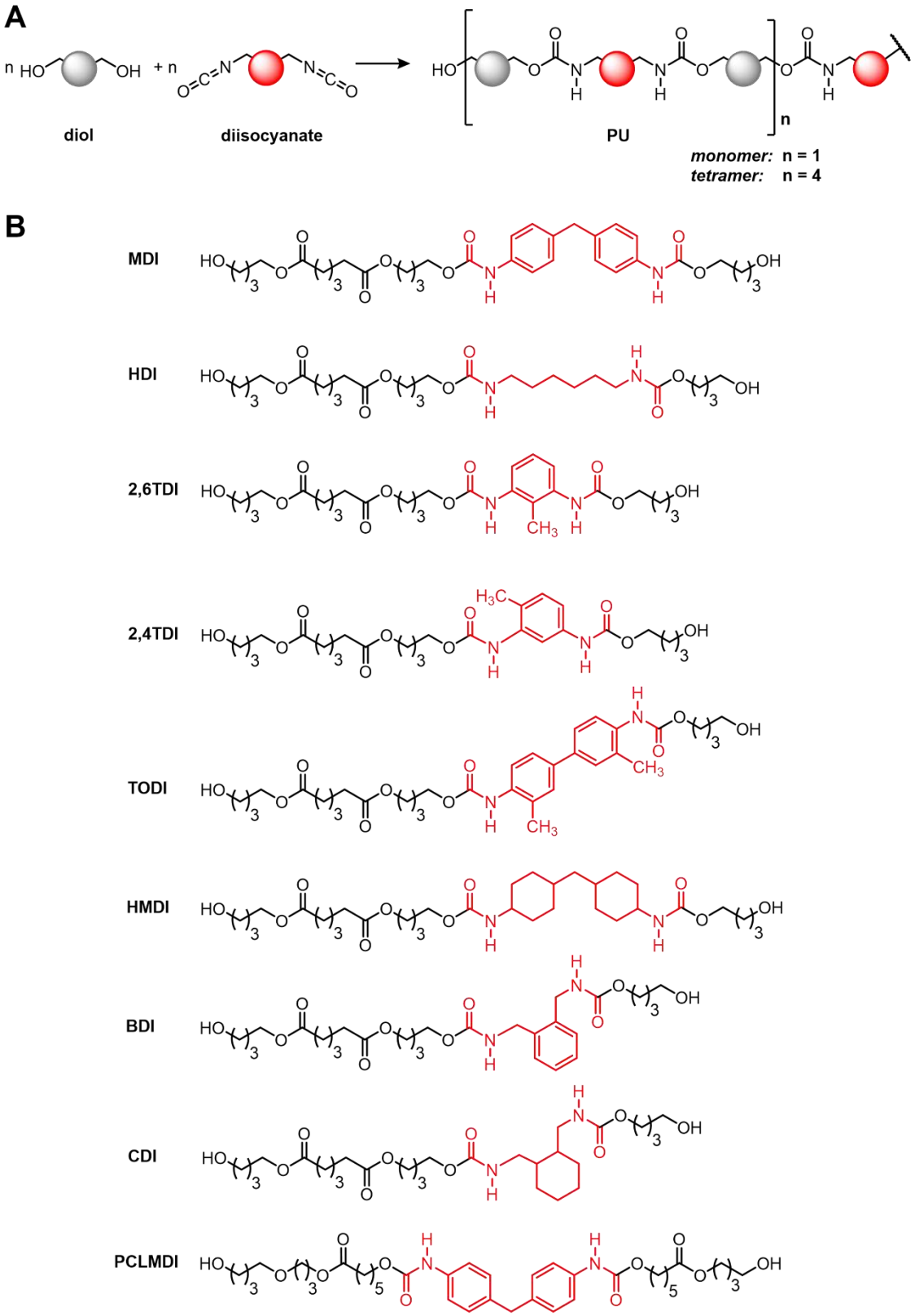


Figure 1. Schematic representation of the *in silico* construction of polyurethanes (PU) monomers and tetramers starting from diisocyanates and diols (**A**). Chemical structures of PU monomers constructed *in silico* and analyzed by molecular docking. Diisocyanates were highlighted in red (**B**).

2.2 Molecular docking

Molecular docking was performed between the nine monomers and nine tetramers of PU constructed *in silico* in this study (Fig. 1B) with the three-dimensional (3D) structure modeled and validated of polyurethanase from *S. liquefaciens* L135 by Salgado et al. (2021), using the Molegro Virtual Docker® software (Thomsen and Christensen, 2006). These PU monomers and tetramers (*.mol* format) and the 3D structure of this enzyme were loaded into the Molegro Virtual Docker® software. The catalytic site was restricted to a sphere with a radius of 30 Å around the enzymatic cavity responsible for the catalytic activity (Fig. 2). All residues within this sphere have been adjusted to be flexible. The influence of water molecules was also considered during molecular docking. As a result of the stochastic nature of the anchoring algorithm, five runs were performed for each PU monomer or tetramer, and 10 poses were analyzed (Guimarães, 2013; Silva et al., 2023). The more negative the values of binding energy and hydrogen bonding, the stronger the bonds.

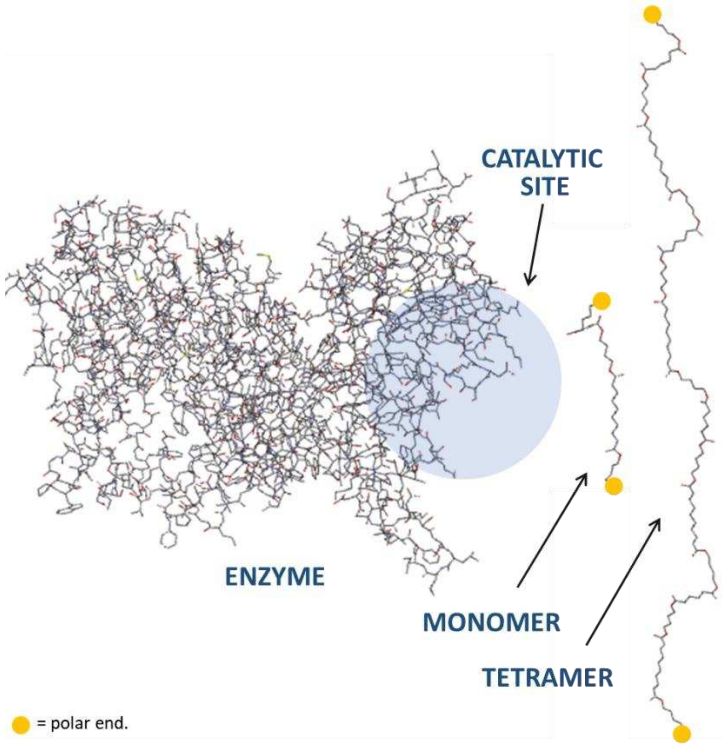


Figure 2. Comparison, in real proportion, between the size of polyurethanase from *S. liquefaciens* L135, catalytic site, polyurethane (PU) monomer and tetramer of HDI (HDI and t-HDI, respectively). The catalytic site was restricted to a sphere with a radius of 30 Å around the enzymatic cavity responsible for the catalytic activity with all amino acid residues adjusted to be flexible.

2.3 Bacterial strain and culture conditions

S. liquefaciens L135 was isolated from Brazilian sample of cold raw cow's milk (Machado et al., 2015) and was recognized to secrete a polyurethanase with lipase activity (Salgado et al., 2021). This isolate was stored at -80 °C in Luria Bertani (LB) broth (Sigma-Aldrich, St. Louis, USA) with 20% (v/v) sterilized glycerol. Before each experiment, *S. liquefaciens* was cultured in LB broth at 30 °C for 18 h. This culture was used as an inoculum for the experiments below and contained approximately 10^9 colony-forming units per milliliter (CFU/mL).

2.4 Evaluation of Impranil® biodegradation by *S. liquefaciens* and its polyurethanase

In a Petri dish with LB agar containing 1% (w/v) of Impranil®-DLN (Impranil®; Covestro, Germany) were placed 10 µL of *S. liquefaciens* culture, 100 µL of partially purified polyurethanase from *S. liquefaciens* according to Salgado et al. (2021), and 10 µL of the *Escherichia coli* culture (negative control), separately. This plate was incubated at 30 °C for six days, and then the biodegradation capacity of this PU was evaluated by forming a transparent halo.

To determine the number of *S. liquefaciens* proteins that possess polyurethanase activity, a zymography was performed and *Escherichia coli* was used as a negative control. The analysis was performed with the supernatant of the cultures according to Peng et al. (2014), with modifications. The Class Five Prestained Multicolor Protein Ladder was used as the molecular weight standard (15- 180 kDa). After electrophoresis, the gel containing 1% Impranil® was washed twice in 50 mM Tris-HCl pH 7.5 with 25% (v/v) isopropanol for 15 min at room temperature. Then, the gel was incubated overnight in 50 mM Tris-HCl pH 7.5 buffer until the appearance of the Impranil® hydrolysis transparent band.

2.5 Preparation and analysis of Impranil® disks inoculated with *S. liquefaciens*

Impranil® was used to prepare disks, according to Nadeau et al. (2021), with modifications. An aliquot of 200 µL of Impranil® was solidified at room temperature in sterile Petri dishes in the shape of a circle with a radius of 1.1 cm. The solidified Impranil® disks were transferred with sterile tweezers to plates containing LB 1.2% agar inoculated with 100 µL of the inoculum of *S. liquefaciens* on the surface. Plates

were incubated at 30 °C for six days. The negative controls consisted of Impranil® disks on LB agar without inoculum.

Changes in the surface morphology of Impranil® disks inoculated or not with *S. liquefaciens* were visually evaluated during six days of incubation and recorded by photographs. In addition, these disks were recovered, washed in 50 mL Falcon® tubes containing 10 mL of 0.85% saline solution, dried in an oven at 37 °C, mounted on stubs, coated with gold using Quorum Q150RS metallizer, and analyzed in scanning electron microscope (SEM) LEO 1430VP (Cambridge, England).

Adhesion and biofilm formation by *S. liquefaciens* on Impranil® disks were also evaluated by enumeration of viable cells. For this, the Impranil® disks inoculated with this bacterium over six days of incubation were removed, washed with 3 mL of 0.85% saline solution, transferred to a microtube containing 1 mL of 0.85% saline solution, and followed by vortexing for 1 min to remove sessile cells. Then, an aliquot of the suspension was taken to prepare serial decimal dilutions, plated on Plate Count Agar (PCA; Acumedia, Lansing, USA) by the microdroplet method (Morton, 2001), and incubated at 30 °C. After 16 h of incubation, the colonies were counted and the results were expressed in CFU per Impranil® disk (CFU / Impranil® disk).

The lipase activity on the Impranil® disk inoculated or not with *S. liquefaciens* over six days of incubation was determined. This disk was added to a microtube containing 1 mL of phosphate buffer (50 mM, pH 8.0), gum arabic (0.1% w/v), and sodium deoxycholate (0.2% w/v). This microtube was mixed using a vortex for 1 min and centrifuged at 10,000 × *g* for 10 min. Aliquots of 135 µL of the supernatant were transferred to a 96-well microplate, added with 15 µL of substrate solution (0.3% (w/v) *p*-nitrophenyl palmitate (*p*-NPP) (Sigma Aldrich, St. Louis, MO, USA) in isopropanol. After 30 min of incubation at 30 °C, absorbance was measured at 410 nm spectrophotometer (Thermo Fisher Scientific, Finland). The negative control consisted of the Impranil® disk without inoculation of *S. liquefaciens*. The standard curve was performed according to Salgado et al. (2021), in which a lipase unit (U) was defined as the amount of enzyme that releases 1.0 µM of *p*-nitrophenol (*p*-NP) per min at pH 8.0 and 30 °C. The results were expressed in lipase unit per Impranil® disk (U / Impranil® disk).

2.6 Preparation and analysis of PCLMDI films inoculated with *S. liquefaciens*

Of the PU tested *in silico*, PCLMDI was analyzed *in vitro*, as it showed high binding energy with polyurethanase from *S. liquefaciens in silico* and is commercially available. The preparation of PCLMDI films was performed according to Shah et al. (2016), with modifications. In a 150 mL Erlenmeyer flask, 2 g of PCLMDI pellets were solubilized in 100 mL of tetrahydrofuran and kept in a desiccator for three days at room temperature. Of this mixture, 10 mL were placed in Petri dishes and left to evaporate in an exhaust cabin until thin, uniform, and transparent PCLMDI films formed. These films were cut into 1 x 1 cm (1 cm^2) sizes and sterilized with 70% ethanol, followed by exposure to ultraviolet radiation for 15 min. Sterility was verified by the absence of turbidity in the LB broth after incubation of film samples submerged in the medium, at 30 °C, for 48 h.

The PCLMDI films with 1 cm^2 were placed in Erlenmeyer flasks containing 50 mL of LB broth inoculated with 500 µL of the inoculum of *S. liquefaciens* and incubated at 30 °C. The inoculated culture medium was changed twice a week for 60 days. The negative control consisted of an LB medium with PCLMDI films without inoculum. After 60 days of incubation, PCLMDI films were analyzed according to item 2.5 by SEM and for adhesion and biofilm formation by *S. liquefaciens*. This last result was expressed in CFU / cm^2 of PCLMDI film.

2.7 Statistical analyses

In vitro analyses were performed in triplicate, with two repetitions. The results were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's test with a significance level of $p < 0.05$. Statistical analyses were performed using GraphPad Prism Software (version 8.4.3; GraphPad Prism Software, USA).

3. Results and discussion

3.1 Polyurethanes (PU) monomers and tetramers construct *in silico*

PU are polymers with different chain sizes, with molecular weights between 200 and 6,000 g mol^{-1} (Shimao, 2001). So far, the works in the literature use monomeric units of PU to perform molecular docking studies of PU and infer the hydrolytic potentials of enzymes (do Canto et al., 2021, 2019; Flores-Castañón et al., 2022). This

is because complex systems like PU require high computational demands for construction, optimization and molecular docking testing.

PU tetramers, or longer chain polymers, should better correlate results from *in silico* studies with *in vitro* ones. Among the factors that can guarantee a better correlation, the size of the chains of tetramers stands out, which that is large enough to represent the real structure of a PU polymer (Fig. 1). In addition, the -OH portions at the ends of the PU chain are at a distance such that they do not interact with the enzyme when interacting with the carbamate nucleus of the polymeric chain, as they should be in real systems. In PU monomers, these ends readily ensure interactions with the enzyme that in real systems should not occur, thus providing good false-positive interaction values (Alicata et al., 2002).

For this purpose, in this work, chemical structures of PU monomers and tetramers were constructed (Fig. 1). It should be emphasized that this is the first work that uses PU tetramers to perform molecular docking, to approximate the structures of these ligands as close as possible to the structures of commercial PU and, consequently, to the expected *in vitro* results.

3.2 PU bond in polyurethanase from *S. liquefaciens* by molecular docking

The active site of polyurethanase from *S. liquefaciens* is composed of the catalytic triad S207, D256, and H314 (Salgado et al., 2021). Therefore, in this molecular docking study, the catalytic site was restricted to a sphere with a radius of 30 Å around this enzymatic cavity responsible for the catalytic activity with all flexible residues (Fig. 2). This study showed that the amino acid residue S207 of polyurethanase and the carbamate and ester groups of all PU were at a distance ranging from 2.0 to 16.2 Å (Table 2). Amino acid residue S207 showed a distance of 6 and 9 Å to the ester and carbamate groups from PCLMDI, respectively (Figs. 3A and 3B), as well as 7 and 9 Å to ester and carbamate groups from t-PCLMDI, respectively (Figs. 3C and 3D). This proximity is essential, as this amino acid residue is responsible for the hydrolytic action of polyurethanase. The proximity of amino acid residues from the enzymes active site to the carbamate and ester groups of the PU forms a stable enzyme-PU complex, making PU susceptible to undergo hydrolysis. On the other hand, PU can interact outside the enzyme's active site with good energy value, thus providing a stable enzyme-PU complex. However, this ligand would not be easily hydrolyzed because it is far from the active site.

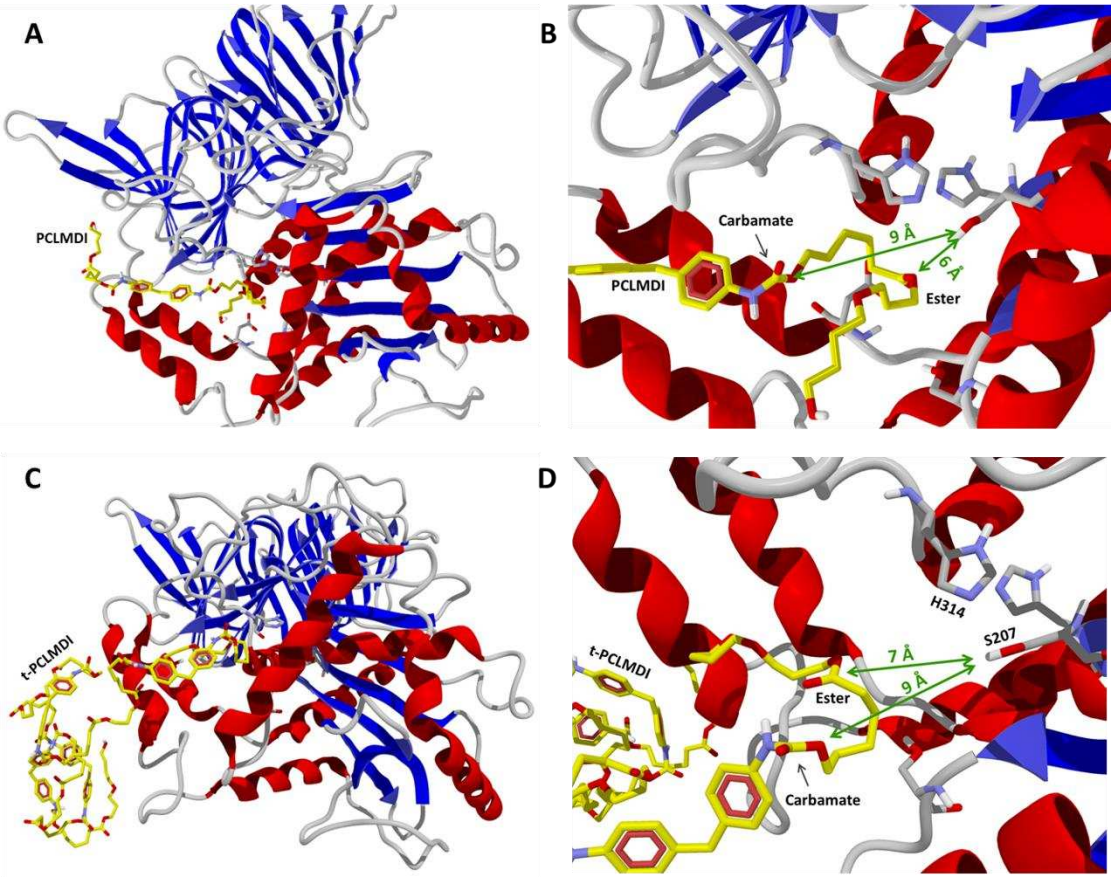


Figure 3. Molecular docking between poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI) monomer (**A** and **B**) and (t-PCLMDI) tetramer (**C** and **D**) with polyurethanase from *S. liquefaciens* L135. Figures **3B** and **3D** represent the ampliation of **3A** and **3C**, respectively.

The *in silico* results also showed that all monomers presented favorable interactions with polyurethanase with values of binding energy ranging from -84.75 to -121.71 kcal mol^{-1}. Tetramers showed less favorable interactions due to repulsive steric interactions due to their long chains, with values of binding energy ranging from 24.26 to -45.50 kcal mol^{-1} (Table 2). The monomers / tetramers that showed the highest binding energies were MDI / t-MDI and PCLMDI / t-PCLMDI, which have the same diisocyanate as precursor (Fig. 1). The higher energies of these PU monomers and tetramers may be due to higher hydrogen bonding interactions with polyurethanase ranging from -9.03 to -3.26 kcal mol^{-1} (Table 2). Do Canto et al. (2019) also showed that the polyurethanases PulA from *P. fluorescens*, PueA, and PueB from *P. chlororaphis* had the highest binding energy values with PU monomer MDI.

Table 2. Values of binding energy and hydrogen bonding of polyurethanase from *S. liquefaciens* L135 with different polyurethane (PU) monomers and tetramers

PU monomer	Binding energy (kcal mol^{-1})	Hydrogen bonding (kcal mol^{-1})	Distance between residue S207 and carbamate group (Å)	Distance between residue S207 and ester group (Å)	PU tetramer	Binding energy (kcal mol^{-1})	Hydrogen bonding (kcal mol^{-1})	Distance between residue S207 and carbamate group (Å)	Distance between residue S207 and ester group (Å)
MDI	-121.49	-9.03	3.9	8.9	t-MDI	-45.50	-5.17	13.6	8.8
HDI	-84.75	-4.45	3.7	12.1	t-HDI	24.26	-5.03	9.8	12.6
2,6TDI	-120.30	-5.00	8.2	9.7	t-2,6TDI	-29.38	0.00	10.4	10.0
2,4TDI	-110.13	-2.69	9.2	5.1	t-2,4TDI	-37.06	-6.66	9.1	5.0
TODI	-107.57	-3.91	2.0	11.0	t-TODI	-19.48	-0.35	10.9	7.3
HMDI	-107.58	-8.16	8.4	11.9	t-HMDI	-15.58	-2.50	11.7	14.4
BDI	-107.06	-3.86	8.7	11.5	t-BDI	-38.29	-0.18	11.7	9.9
CDI	-109.14	-7.89	8.7	11.4	t-CDI	-33.73	0.00	16.2	14.5
PCLMDI	-121.71	-4.15	9.0	6.0	t-PCLMDI	-39.52	-3.26	9.0	7.0

Molecular docking studies also indicated that other ligands, present as environmental pollutants, showed favorable interactions with different enzymes. Shin et al. (2021) showed that the distance between the polycaprolactone ligand and the catalytic residues of an esterase from *Sphingobium chungbukense* DJ77 ranged from 3.0 to 16.7 Å and could be an excellent parameter to estimate its hydrolytic activity. Flores-Castañón et al. (2022) demonstrated that PU monomers interacted with amino acid residues of the active site of microbial cutinases, suggesting that these enzymes can biodegrade PU. Mansouri and Benslama (2022) showed, through molecular docking, values of binding energies and significant molecular interactions between 11 polluting polymers and the active site of the laccase enzyme of *Streptomyces coelicolor*, attesting to the importance of laccase in environmental decontamination. Thus, molecular docking is a helpful tool to explain the enzymes activity to its target.

3.3 *S. liquefaciens* and its polyurethanase biodegrade Impranil®

The biodegradation of model molecules is generally the first step in developing bioremediation or biological recycling processes, as it allows easy identification of efficient biodegrading entities (Magnin et al., 2020). In this sense, Impranil® is a colloidal polyester dispersion with many positive attributes that make it ideal for this initial screening of polyurethanases, as it allows a rapid assessment of the potential for biodegradation by microorganisms and their enzymes. Among these positive attributes are its commercial availability, stability to general hydrolysis between pH 4-8, tolerance to temperatures of up to 80 °C, ability to remain dispersed in a liquid or solid growth medium, and not be cytotoxic (Biffinger et al., 2015).

In a previous study, a polyurethanase from *S. liquefaciens* with lipase activity was identified, and its potential for *in vitro* biodegradation was not explored (Salgado et al., 2021). Here we show that *S. liquefaciens* or its partially purified polyurethanase were able to biodegrade the Impranil® added on LB agar, forming a translucent halo (Fig. 4A). In addition, zymography of the *S. liquefaciens* supernatant using Impranil® as substrate showed that a single transparent band with a size of 65 kDa was able to biodegrade this PU (Fig. 4B). This result confirms the size of the lipase from *S. liquefaciens* that was identified as polyurethanase in our previous study (Salgado et al., 2021). Kim et al. (2022) showed that extracellular lipase from *Serratia* HY-72 presented 95.77% identity with polyurethanase from *S. liquefaciens* L135, as well as the purified lipase presented 65 kDa and was also able to biodegrade Impranil® by

detectable clearance from the suspension. To continue the *in vitro* biodegradation studies of PU, the culture of *S. liquefaciens* was used, instead of its partially purified polyurethanase, since purification is an expensive, time-consuming, and low-yield process.

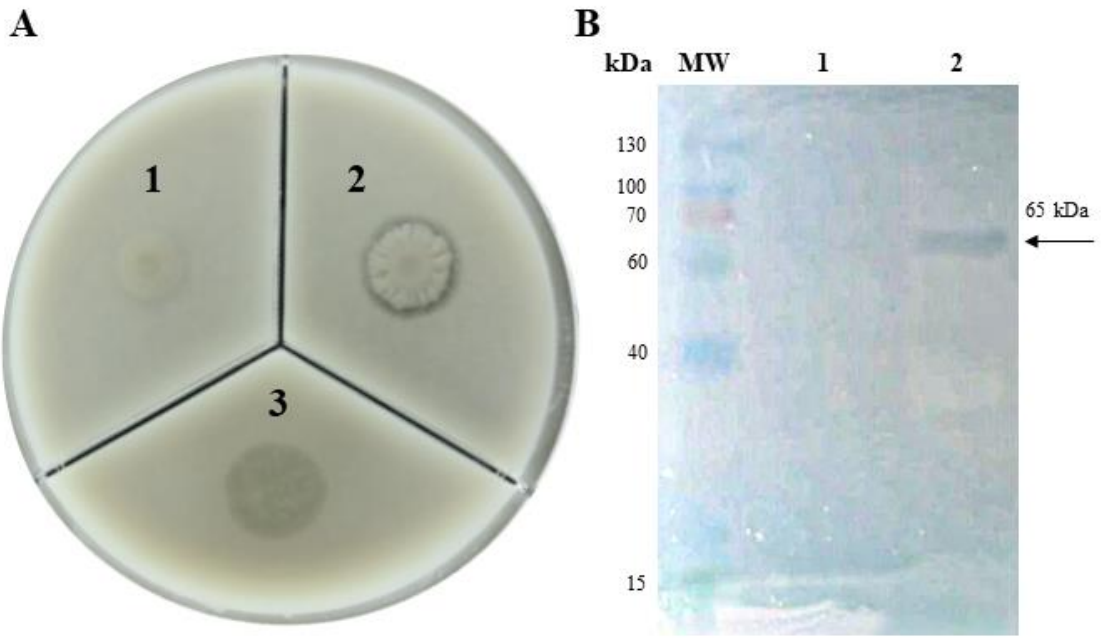


Figure 4. Biodegradation of Impranil® on LB agar at 30 °C for six days: 1 – *Escherichia coli* as negative control, 2 – *Serratia liquefaciens* L135, 3 – polyurethanase partially purified from *Serratia liquefaciens* L135 **(A)**. Zymography of *Serratia liquefaciens* supernatant using Impranil® as substrate: MW – Molecular weight standard, 1 – *Escherichia coli* as negative control, 2 – *Serratia liquefaciens* L135 **(B)**.

Like *S. liquefaciens*, other microorganisms were able to biodegrade Impranil®. *B. pumilus* formed cleaning zones around the colonies in LB agar supplemented with Impranil® 0.3% (w/v) at 37 °C for 18 to 20 h (Nair and Kumar, 2007). *A. gerneri* P7 showed clearing zones on plates containing LB agar supplemented with 1% (w/v) of Impranil® after incubation at 30 °C for 48 h (Howard et al., 2012). *P. putida* A12 took four days to biodegrade 92% of Impranil® at 30 °C (Peng et al., 2014).

3.4 *S. liquefaciens* biodegrades Impranil® disks

In the negative controls, when *S. liquefaciens* was not inoculated, there were no alterations in the macroscopic and microscopic physical structures of the Impranil®

disks during the six days of incubation (Figs. 5A to 5F and Figs. 5M to 5R, respectively). The biodegradation of Impranil® disks was characterized by the increase fragmentation over six days of incubation in LB agar inoculated with *S. liquefaciens* (Figs. 5G to 5L). Images obtained by SEM revealed a greater extent and depth of microscopic cracks in discs over the days of incubation in the presence of *S. liquefaciens* (Figs. 5S to 5Y).

The presence of sessile cells of *S. liquefaciens* on the Impranil® disk confirms adhesion and biofilm formation in this PU (Fig. 6A). The number of sessile cells ranged from 1.9 x 10^9 to 2.7 x 10^{10} CFU / Impranil® disk with no statistical difference over six days of incubation ($p < 0.05$). Furthermore, the lipase activity present in these disks ranged from 313 to 426 U/ Impranil® disk (Fig. 6B), and there was also no statistical difference over the days of incubation ($p < 0.05$). This result shows that adhesion and the formed biofilm remain stable during the incubation period, corroborating the data found by Nadeau et al. (2021), in which the biofilm formed by wild-type *P. protegens* Pf-5 and three mutants on Impranil® disks remained viable for six days, reaching 10^9 to 10^{10} CFU/Impranil® disk. According to Mohanan et al., 2020; Sivan et al., 2006; Tokiwa et al., 2009, the fixation or adhesion of microorganisms to polymeric substrates, followed by the secretion of extracellular enzymes are steps necessary for hydrolysis of the polymer into oligomers, dimers and low molecular weight monomers that are finally assimilated by the microbial cells as a carbon source.

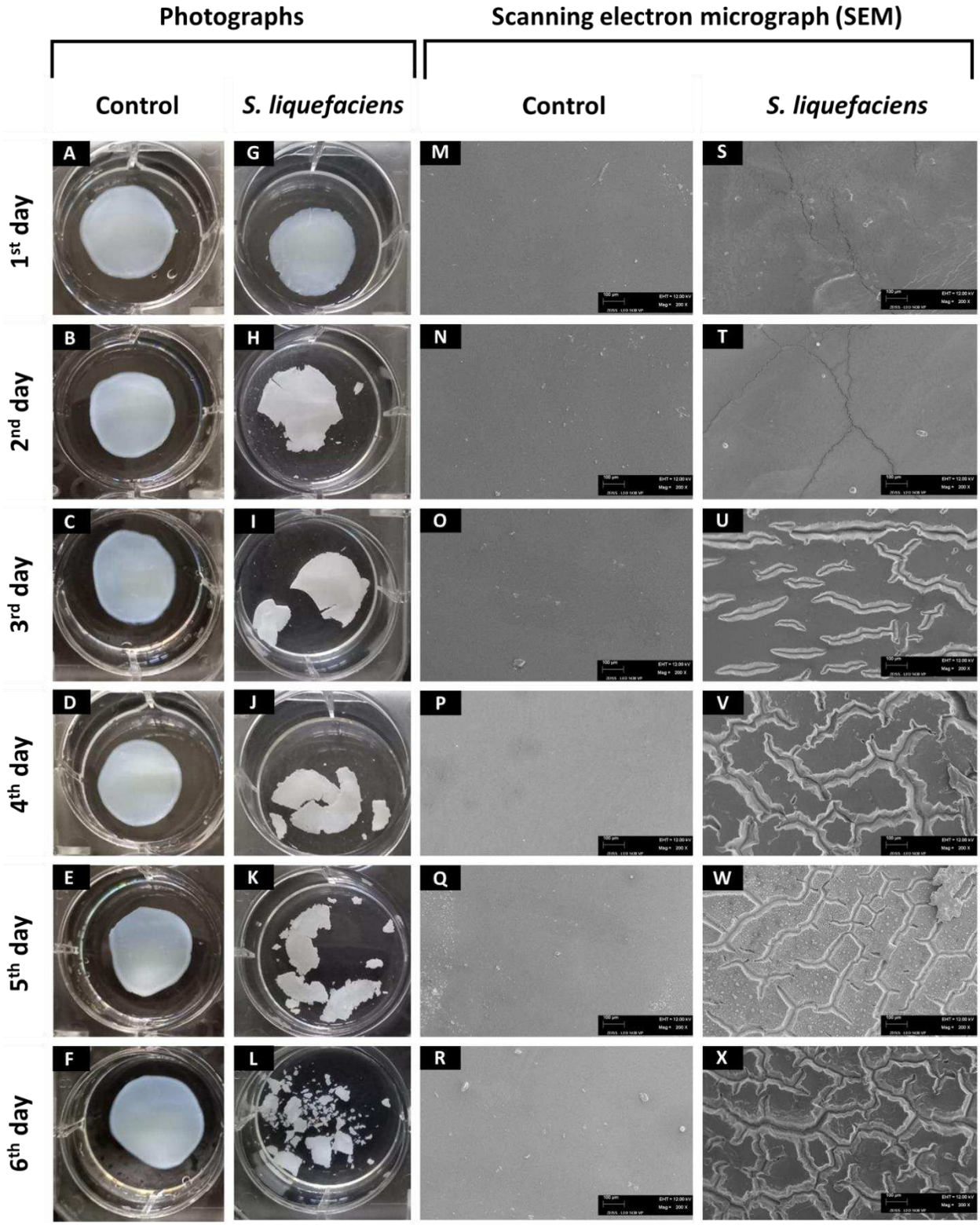


Figure 5. Photographs and scanning electron micrographs (SEM) of Impranil® disks without (**A-F** and **M-R,** respectively) and with (**G-L** and **S-X**, respectively) inoculation of *S. liquefaciens* L135 and incubation at 30 °C for six days.

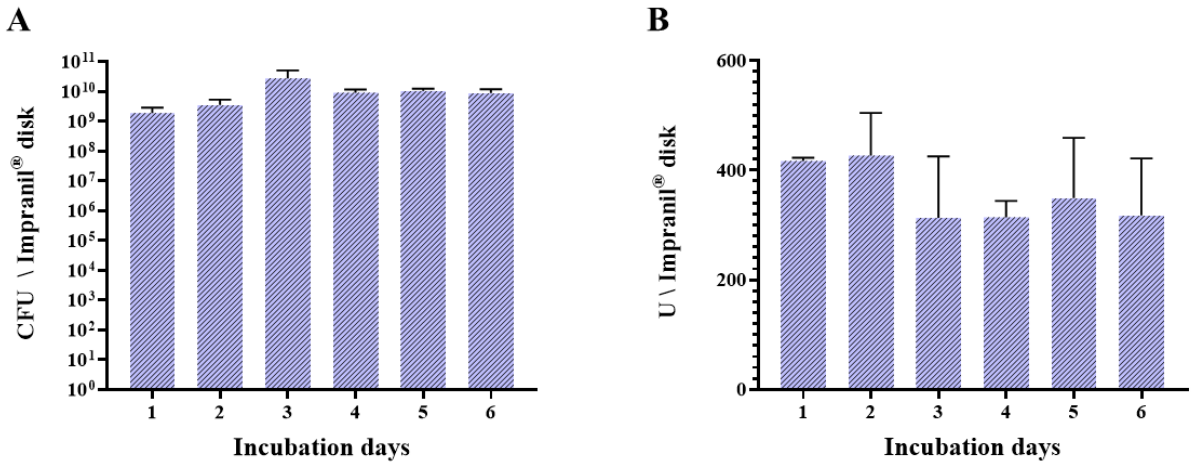


Figure 6. Adhesion and biofilm formation by *S. liquefaciens* L135 (**A**) and lipase activity (**B**) on Impranil® disks during six days of incubation at 30 °C. These results were expressed in colony-forming units (CFU) and lipase unit (U) per Impranil® disk. Error bars indicate standard deviation. There was no statistical difference using a one-way analysis of variance (ANOVA), followed by Tukey's test with a significance level of $p < 0.05$.

3.5 *S. liquefaciens* biodegrades PCLMDI films

Despite being a model substrate to identify microorganisms capable of biodegrading PU, Impranil® is far from faithfully representing the biodegradation of commercial PU. For this, complementary *in vitro* studies were carried out to verify the ability of *S. liquefaciens* to biodegrade commercially available PCLMDI. In the *in silico* analysis, this PU showed high binding energy with the polyurethanase of this bacterium, which can guarantee the stability of the enzyme-ligand complex and also bound to the S207 residue of its active site, showing that it can be biodegraded.

After 60 days of incubation of the PCLMDI films with *S. liquefaciens* it was possible to observe through the SEM analysis the formation of pores and cracks in the PCLMDI films, characterizing the biodegradation process of this recalcitrant polymer (Figs. 7A to 7B). Comparison of these results with those observed in the negative control ensures that the biodegradation process occurred through the action of *S. liquefaciens* (Fig. 7C).

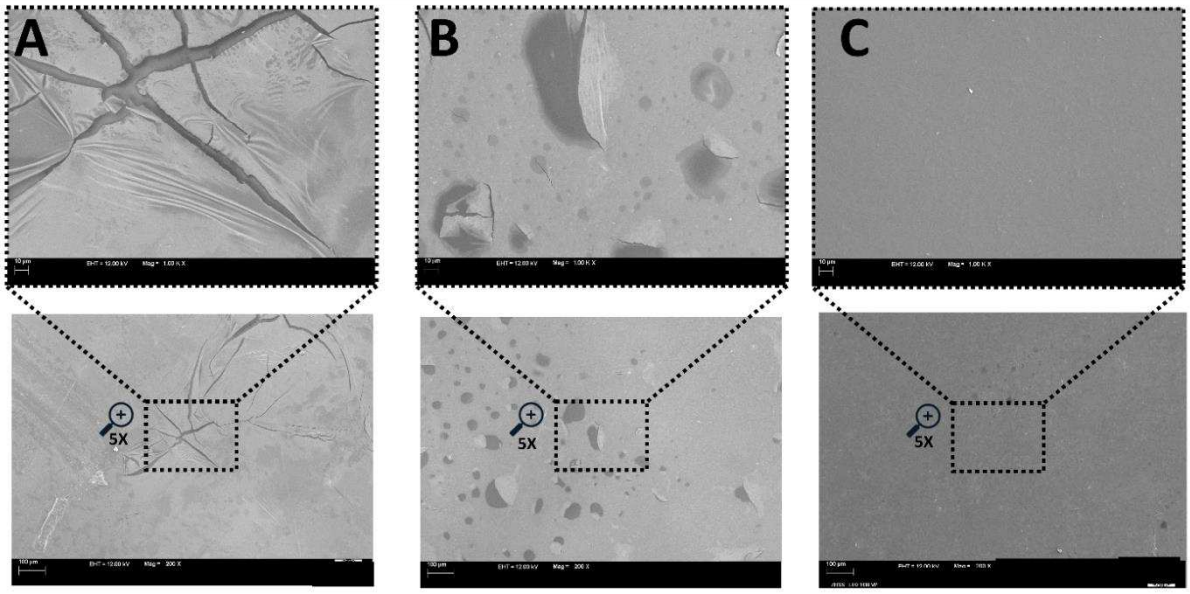


Figure 7. Scanning electron micrographs (SEM) of poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI) films with (**A** and **B**) and without (**C**) inoculation of. *S. liquefaciens* L135 and incubation at 30 °C for 60 days. Top images are five times (5x) magnified from bottom images.

Other authors used SEM to find alterations in the PU surface structure, which configure the biodegradation caused by microbial action. Umare and Chandure (2008) synthesized a PU from MDI and observed that the number of cracks and small holes became deeper with increasing exposure time, possibly due to hydrolytic biodegradation by a lipase from *Rhizopus delemar*. Shah et al. (2016) used MDI polyester PU pellets to synthesize films and observed the formation of pores on their surface after 30 days of incubation with a consortium of *Pseudomonas* and *Bacillus* species. Álvarez-Barragán et al. (2016) observed through SEM images the rupture and loss of integrity of the reticulated cellular structure of PU foam when incubated with fungi. Khan et al. (2017) used polyester PU pellets buried in the ground for four months and showed by SEM a film cracked and biodegraded by *A. tubingensis*. Di Bisceglie et al. (2022) observed by SEM that cutinase from *Humicola insolens* can hydrolyze PU-polyester copolymers with the appearance of deep and branched cracks in the films. Kim et al. (2022) demonstrated that PU foams incubated with *Serratia* HY-72 for 14 days developed cracks, holes, and surface roughness when observed by SEM, as well as the loss of integrity of the reticulated structure in panoramic views.

Polymers of a hydrophobic nature restrict microbial action by inhibiting water absorption, but this can be overcome by biofilm formation (Jaiswal al., 2020). In this study, *S. liquefaciens* could adhere and form biofilm on the hydrophobic surface of PCLMDI films, reaching a population density of 3.3 x 10^6 CFU/cm^2. Sivan et al. (2006) demonstrated that *Rhodococcus ruber* was capable of biodegrading polyethylene (PE) films and could also form biofilms, reaching a density of 7.3 x10^7 CFU/cm^2, with viability even after 60 days of incubation. Other studies have reported the effectiveness of bacterial biofilms in biodegrading other polymers, toxic pesticides, toxic dyes, and heavy metals, emphasizing the potential of biofilms in decontaminating the environment (Dash and Osborne, 2020; Eich et al., 2015; Kantar et al., 2011; Mahto et al., 2022; Mangwani et al., 2014; Tribedi and Sil, 2013).

4. Conclusions

In silico analyzes suggested explanations for the possible enzymatic activities of polyurethanase from *S. liquefaciens* with different PU, including PCLMDI. *In vitro*, this bacterium could biodegrade, adhere and form biofilm in different PU, such as Impranil® and PCLMDI. The polyurethanase from *S. liquefaciens* L135 may be the main, if not the only, responsible for the biodegradation of these PU once this partially purified enzyme was also able to biodegrade Impranil®. Thus, *in silico* analyzes combined with *in vitro* analyzes constitute an essential tool to suggest and explain the biological action of potential microorganisms and enzymes capable of biodegrading PU and, thus, to develop new "green" technologies that effectively reduce the pollution caused by PU and other recalcitrant polymers in the environment.

Declaration of competing interest

None.

Acknowledgments

The authors acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support, and to the Electron Microscopy Center (NMM) from the Universidade Federal de Viçosa (UFV) by tech support.

References

Akindoyo, J.O., Beg, M.D.H., Ghazali, S., Islam, M.R., Jeyaratnam, N., Yuvaraj, A.R., 2016. Polyurethane types, synthesis and applications-a review. RSC Adv. 6, 114453–114482. https://doi.org/10.1039/c6ra14525f

Alicata, R., Montaudo, G., Puglisi, C., Samperi, F., 2002. Influence of chain end groups on the matrix-assisted laser desorption/ionization spectra of polymer blends. Rapid Commun. Mass Spectrom. 16, 248–260. https://doi.org/10.1002/rcm.573

Álvarez-Barragán, J., Domínguez-Malfavón, L., Vargas-Suárez, M., González-Hernández, R., Aguilar-Osorio, G., Loza-Tavera, H., 2016. Biodegradative activities of selected environmental fungi on a polyester polyurethane varnish and polyether polyurethane foams. Appl. Environ. Microbiol. 82, 5225–5235. https://doi.org/10.1128/AEM.01344-16

Banik, J., Chakraborty, D., Rizwan, M., Shaik, A.H., Chandan, M.R., 2023. Review on disposal, recycling and management of waste polyurethane foams: A way ahead. Waste Manag. Res. https://doi.org/10.1177/0734242X221146082

Barnoy, S., Gancz, H., Zhu, Y., Honnold, C.L., Zurawski, D. V., Venkatesan, M.M., 2017. The *Galleria mellonella* larvae as an in vivo model for evaluation of *Shigella* virulence. Gut Microbes 8, 335–350. https://doi.org/10.1080/19490976.2017.1293225

Berne, C., Ellison, C.K., Ducret, A., Brun, Y. V., 2018. Bacterial adhesion at the single-cell level. Nat. Rev. Microbiol. 16, 616–627. https://doi.org/10.1038/s41579-018-0057-5

Bhavsar, P., Bhave, M., Webb, H.K., 2023. Solving the plastic dilemma: the fungal and bacterial biodegradability of polyurethanes. World J. Microbiol. Biotechnol. 39, 1–11. https://doi.org/10.1007/s11274-023-03558-8

Biffinger, J.C., Barlow, D.E., Cockrell, A.L., Cusick, K.D., Hervey, W.J., Fitzgerald, L.A., Nadeau, L.J., Hung, C.S., Crookes-Goodson, W.J., Russell, J.N., 2015. The applicability of Impranil® DLN for gauging the biodegradation of polyurethanes. Polym. Degrad. Stab. 120, 178–185. https://doi.org/10.1016/j.polymdegradstab.2015.06.020

Cregut, M., Bedas, M., Durand, M.J., Thouand, G., 2013. New insights into polyurethane biodegradation and realistic prospects for the development of a

sustainable waste recycling process. Biotechnol. Adv. 31, 1634–1647. https://doi.org/10.1016/j.biotechadv.2013.08.011

Dash, D.M., Osborne, W.J., 2020. Rapid biodegradation and biofilm-mediated bioremoval of organophosphorus pesticides using an indigenous *Kosakonia oryzae* strain -VITPSCQ3 in a Vertical-flow Packed Bed Biofilm Bioreactor. Ecotoxicol. Environ. Saf. 192, 110290. https://doi.org/10.1016/j.ecoenv.2020.110290

Desai, S.M., Sonawane, R.Y., More, A.P., 2023. Thermoplastic polyurethane for three-dimensional printing applications: A review. Polym. Adv. Technol. 1–22. https://doi.org/10.1002/pat.6041

Di Bisceglie, F., Quartinello, F., Vielnascher, R., Guebitz, G.M., Pellis, A., 2022. Cutinase-Catalyzed Polyester-Polyurethane Degradation: Elucidation of the Hydrolysis Mechanism. Polymers (Basel). 14, 411. https://doi.org/10.3390/polym14030411

do Canto, V.P., Thompson, C.E., Netz, P.A., 2021. Computational studies of polyurethanases from *Pseudomonas*. J. Mol. Model. 27, 1–8. https://doi.org/10.1007/s00894-021-04671-x

do Canto, V.P., Thompson, C.E., Netz, P.A., 2019. Polyurethanases: Three-dimensional structures and molecular dynamics simulations of enzymes that degrade polyurethane. J. Mol. Graph. Model. 89, 82–95. https://doi.org/10.1016/j.jmgm.2019.03.001

Du, Y., Liu, X., Dong, X., Yin, Z., 2022. A review on marine plastisphere: biodiversity, formation, and role in degradation. Comput. Struct. Biotechnol. J. 20, 975–988. https://doi.org/10.1016/j.csbj.2022.02.008

Easley, A., Mangano, A., Fors, B.P., 2023. Safer Polyurethane Foams with Cyclic Carbonates. Angew. Chemie Int. Ed. 2–4. https://doi.org/10.1002/anie.202218062

Eich, A., Mildenberger, T., Laforsch, C., Weber, M., 2015. Biofilm and diatom succession on polyethylene (PE) and biodegradable plastic bags in two marine habitats: Early signs of degradation in the pelagic and benthic zone? PLoS One 10, 1–16. https://doi.org/10.1371/journal.pone.0137201

Flores-Castañón, N., Sarkar, S., Banerjee, A., 2022. Structural, functional, and molecular docking analyses of microbial cutinase enzymes against polyurethane monomers. J. Hazard. Mater. Lett. 3. https://doi.org/10.1016/j.hazl.2022.100063

Gartner, T.E., Jayaraman, A., 2019. Modeling and Simulations of Polymers: A Roadmap. Macromolecules 52, 755–786. https://doi.org/10.1021/acs.macromol.8b01836

Gaylarde, C.C., de Almeida, M.P., Neves, C. V., Neto, J.A.B., da Fonseca, E.M., 2023. The Importance of Biofilms on Microplastic Particles in Their Sinking Behavior and the Transfer of Invasive Organisms between Ecosystems. Micro 3, 320–337. https://doi.org/10.3390/micro3010022

Guimarães, A. P. 2013. Estudos por modelagem molecular da enzima timidilato quinase do Variola vírus. Tese (Doutorado em Ciências em Química) - Instituto Militar de Engenharia, Rio de Janeiro.

Howard, G.T., Norton, W.N., Burks, T., 2012. Growth of *Acinetobacter gerneri* P7 on polyurethane and the purification and characterization of a polyurethanase enzyme. Biodegradation 23, 561–573. https://doi.org/10.1007/s10532-011-9533-6

Howard, G.T., Ruiz, C., Hilliard, N.P., 1999. Growth of *Pseudomonas chlororaphis* on a polyester-polyurethane and the purification and characterization of a polyurethanase-esterase enzyme. Int. Biodeterior. Biodegrad. 43, 7–12. https://doi.org/10.1016/S0964-8305(98)00057-2

Jaiswal, S., Sharma, B., Shukla, P., 2020. Integrated approaches in microbial degradation of plastics. Environ. Technol. Innov. 17, 100567. https://doi.org/10.1016/j.eti.2019.100567

Kantar, C., Demiray, H., Dogan, N.M., Dodge, C.J., 2011. Role of microbial exopolymeric substances (EPS) on chromium sorption and transport in heterogeneous subsurface soils: I. Cr(III) complexation with EPS in aqueous solution. Chemosphere 82, 1489–1495. https://doi.org/10.1016/j.chemosphere.2011.01.009

Kemona, A., Piotrowska, M., 2016. Microorganisms Potentially Useful in the 1297–1308.

Khan, S., Nadir, S., Shah, Z.U., Shah, A.A., Karunarathna, S.C., Xu, J., Khan, A., Munir, S., Hasan, F., 2017. Biodegradation of polyester polyurethane by *Aspergillus tubingensis*. Environ. Pollut. 225, 469–480. https://doi.org/10.1016/j.envpol.2017.03.012

Kim, J.H., Choi, S.H., Park, M.G., Park, D.H., Son, K.H., Park, H.Y., 2022. Polyurethane biodegradation by *Serratia* sp. HY-72 isolated from the intestine of

the Asian mantis *Hierodula patellifera*. Front. Microbiol. 13, 1–13. https://doi.org/10.3389/fmicb.2022.1005415

Koo, H., Allan, R.N., Howlin, R.P., Stoodley, P., Hall-Stoodley, L., 2017. Targeting microbial biofilms: Current and prospective therapeutic strategies. Nat. Rev. Microbiol. 15, 740–755. https://doi.org/10.1038/nrmicro.2017.99

Li, X., Chen, D., Xie, S., 2021. Current progress and prospects of organic nanoparticles against bacterial biofilm. Adv. Colloid Interface Sci. 294, 102475. https://doi.org/10.1016/j.cis.2021.102475

Liang, H., Feng, Y., Lu, J., Liu, L., Yang, Z., Luo, Y., Zhang, Y., Zhang, C., 2018. Bio-based cationic waterborne polyurethanes dispersions prepared from different vegetable oils. Ind. Crops Prod. 122, 448–455. https://doi.org/10.1016/j.indcrop.2018.06.006

Liu, Z., Liu, Yujie, Zeng, G., Shao, B., Chen, M., Li, Z., Jiang, Y., Liu, Yang, Zhang, Y., Zhong, H., 2018. Application of molecular docking for the degradation of organic pollutants in the environmental remediation: A review. Chemosphere 203, 139–150. https://doi.org/10.1016/j.chemosphere.2018.03.179

Magnin, A., Pollet, E., Phalip, V., Avérous, L., 2020. Evaluation of biological degradation of polyurethanes. Biotechnol. Adv. 39, 107457. https://doi.org/10.1016/j.biotechadv.2019.107457

Mahto, K.U., Vandana, Priyadarshanee, M., Samantaray, D.P., Das, S., 2022. Bacterial biofilm and extracellular polymeric substances in the treatment of environmental pollutants: Beyond the protective role in survivability. J. Clean. Prod. 379, 134759. https://doi.org/10.1016/j.jclepro.2022.134759

Mangwani, N., Shukla, S.K., Kumari, S., Rao, T.S., Das, S., 2014. Characterization of *Stenotrophomonas acidaminiphila* NCW-702 biofilm for implication in the degradation of polycyclic aromatic hydrocarbons. J. Appl. Microbiol. 117, 1012–1024. https://doi.org/10.1111/jam.12602

Mansouri, N., Benslama, O., 2022. Reprint of: Molecular docking exploration of the degradation activity of some synthetic hydrocarbons polymers by the laccase enzyme of *Streptomyces*. Mater. Today Proc. 53, A1–A4. https://doi.org/10.1016/j.matpr.2022.04.311

Mathur, G., Prasad, R., 2012. Degradation of polyurethane by *Aspergillus flavus* (ITCC 6051) isolated from soil. Appl. Biochem. Biotechnol. 167, 1595–1602. https://doi.org/10.1007/s12010-012-9572-4

Mohanan, N., Montazer, Z., Sharma, P.K., Levin, D.B., 2020. Microbial and Enzymatic Degradation of Synthetic Plastics. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.580709

Morton, R.D., 2001. Aerobic Plate Count, in: Compendium of methods for the microbiological examination of foods, (4th ed.), American Public Health Association-APHA, Washington, 63-67 (Chapter 7).

Mukherjee, K., Tribedi, P., Chowdhury, A., Ray, T., Joardar, A., Giri, S., Sil, A.K., 2011. Isolation of a *Pseudomonas aeruginosa* strain from soil that can degrade polyurethane diol. Biodegradation 22, 377–388. https://doi.org/10.1007/s10532-010-9409-1

Nadeau, L.J., Barlow, D.E., Hung, C.S., Biffinger, J.C., Crouch, A.L., Hollomon, J.M., Ecker, C.D., Russell, J.N., Crookes-Goodson, W.J., 2021. Colonization and degradation of polyurethane coatings by *Pseudomonas protegens* biofilms is promoted by PueA and PueB hydrolases. Int. Biodeterior. Biodegrad. 156. https://doi.org/10.1016/j.ibiod.2020.105121

Nair, S., Kumar, P., 2007. Molecular characterization of a lipase-producing *Bacillus pumilus* strain (NMSN-1d) utilizing colloidal water-dispersible polyurethane. World J. Microbiol. Biotechnol. 23, 1441–1449. https://doi.org/10.1007/s11274-007-9388-5

Osman, M., Satti, S.M., Luqman, A., Hasan, F., Shah, Z., Shah, A.A., 2018. Degradation of polyester polyurethane by *Aspergillus* sp. strain S45 isolated from soil. J. Polym. Environ. 26, 301–310. https://doi.org/10.1007/s10924-017-0954-0

Peng, Y.H., Shih, Y. hsin, Lai, Y.C., Liu, Y.Z., Liu, Y.T., Lin, N.C., 2014. Degradation of polyurethane by bacterium isolated from soil and assessment of polyurethanolytic activity of a *Pseudomonas putida* strain. Environ. Sci. Pollut. Res. 21, 9529–9537. https://doi.org/10.1007/s11356-014-2647-8

Rajan, A., Vijayalakshmi, S., 2023. New insights on polyurethane biodegradation. AIP Conf. Proc. 2690. https://doi.org/10.1063/5.0122262

Rocha, G. B., Freire, R. O., Simas, A. M., & Stewart, J. J., 2006. Rm1: A reparameterization of AM1 for H, C, N, O, P, S, F, Cl, Br, and I. Journal of computational chemistry, 27(10), 1101-1111. https://doi.org/10.1002/jcc.20425

Rowe, L., Howard, G.T., 2002. Growth of *Bacillus subtilis* on polyurethane and the purification and characterization of a polyurethanase-lipase enzyme. Int.

Biodeterior. Biodegrad. https://doi.org/10.1016/S0964-8305(02)00047-1

Russell, J.R., Huang, J., Anand, P., Kucera, K., Sandoval, A.G., Dantzler, K.W., Hickman, D.S., Jee, J., Kimovec, F.M., Koppstein, D., Marks, D.H., Mittermiller, P.A., Núñez, S.J., Santiago, M., Townes, M.A., Vishnevetsky, M., Williams, N.E., Vargas, M.P.N., Boulanger, L.A., Bascom-Slack, C., Strobel, S.A., 2011. Biodegradation of polyester polyurethane by endophytic fungi. Appl. Environ. Microbiol. 77, 6076–6084. https://doi.org/10.1128/AEM.00521-11

Salgado, C.A., Almeida, F.A. de, Barros, E., Baracat-Pereira, M.C., Baglinière, F., Vanetti, M.C.D., 2021. Identification and characterization of a polyurethanase with lipase activity from *Serratia liquefaciens* isolated from cold raw cow's milk. Food Chem. 337, 127954. https://doi.org/10.1016/j.foodchem.2020.127954

Santacruz-Juárez, E., Buendia-Corona, R.E., Ramírez, R.E., Sánchez, C., 2021. Fungal enzymes for the degradation of polyethylene: Molecular docking simulation and biodegradation pathway proposal. J. Hazard. Mater. 411. https://doi.org/10.1016/j.jhazmat.2021.125118

Sarkar, P., Srivastava, V., Ailani, K., 2023. Molecular docking simulation, drug pharmacokinetics and synthesis of carbon nanodots from phytochemicals against isoenzymes of cancer. Mater. Today Proc. 76, 289–302. https://doi.org/10.1016/j.matpr.2022.11.234

Shah, Z., Gulzar, M., Hasan, F., Shah, A.A., 2016. Degradation of polyester polyurethane by an indigenously developed consortium of *Pseudomonas* and *Bacillus* species isolated from soil. Polym. Degrad. Stab. https://doi.org/10.1016/j.polymdegradstab.2016.11.003

Shimao, M., 2001. Biodegradation of plastics Masayuki Shimao. Curr. Opin. Biotechnol. 12, 242–247.

Shin, W.R., Um, H.J., Kim, Y.C., Kim, S.C., Cho, B.K., Ahn, J.Y., Min, J., Kim, Y.H., 2021. Biochemical characterization and molecular docking analysis of novel esterases from *Sphingobium chungbukense* DJ77. Int. J. Biol. Macromol. 168, 403–411. https://doi.org/10.1016/j.ijbiomac.2020.12.077

Silva, C., Araújo, R., Casal, M., Gübitz, G.M., Cavaco-Paulo, A., 2007. Influence of mechanical agitation on cutinases and protease activity towards polyamide substrates. Enzyme Microb. Technol. 40, 1678–1685. https://doi.org/10.1016/j.enzmictec.2006.09.001

Silva, J.G., Borgati, T.F., Lopes, S.M.G., Heise, N., Hoenke, S., Csuk, R., Barbosa,

L.C.A., 2023. New amides derived from sclareolide as anticholinesterase agents. Bioorg. Chem. 130, 106249. https://doi.org/10.1016/j.bioorg.2022.106249

Simón, D., Borreguero, A.M., de Lucas, A., Rodríguez, J.F., 2018. Recycling of polyurethanes from laboratory to industry, a journey towards the sustainability. Waste Manag. 76, 147–171. https://doi.org/10.1016/j.wasman.2018.03.041

Sivan, A., Szanto, M., Pavlov, V., 2006. Biofilm development of the polyethylene-degrading bacterium *Rhodococcus ruber*. Appl. Microbiol. Biotechnol. 72, 346–352. https://doi.org/10.1007/s00253-005-0259-4

Sonnendecker, C., Oeser, J., Richter, P.K., Hille, P., Zhao, Z., Fischer, C., Lippold, H., Blázquez-Sánchez, P., Engelberger, F., Ramírez-Sarmiento, C.A., Oeser, T., Lihanova, Y., Frank, R., Jahnke, H.G., Billig, S., Abel, B., Sträter, N., Matysik, J., Zimmermann, W., 2022. Low carbon footprint recycling of post-consumer PET plastic with a metagenomic polyester hydrolase. ChemSusChem 15. https://doi.org/10.1002/cssc.202101062

Spence, M.W., Plehiers, P.M., 2022. A brief overview of properties and reactions of diisocyanates. Toxicol. Ind. Health 38, 495–499. https://doi.org/10.1177/07482337221090709

Thomsen, R., Christensen, M. H., 2006. Molegro Virtual Docker. Version: 1.0.

Tokiwa, Y., Calabia, B.P., Ugwu, C.U., Aiba, S., 2009. Biodegradability of plastics. Int. J. Mol. Sci. 10, 3722–3742. https://doi.org/10.3390/ijms10093722

Tran, M.H., Lee, E.Y., 2023. Production of polyols and polyurethane from biomass: a review. Environ. Chem. Lett. https://doi.org/10.1007/s10311-023-01592-4

Tribedi, P., Sil, A.K., 2013. Low-density polyethylene degradation by *Pseudomonas* sp. AKS2 biofilm. Environ. Sci. Pollut. Res. 20, 4146–4153. https://doi.org/10.1007/s11356-012-1378-y

Turner, A., Lau, K.S., 2016. Elemental concentrations and bioaccessibilities in beached plastic foam litter, with particular reference to lead in polyurethane. Mar. Pollut. Bull. 112, 265–270. https://doi.org/10.1016/j.marpolbul.2016.08.005

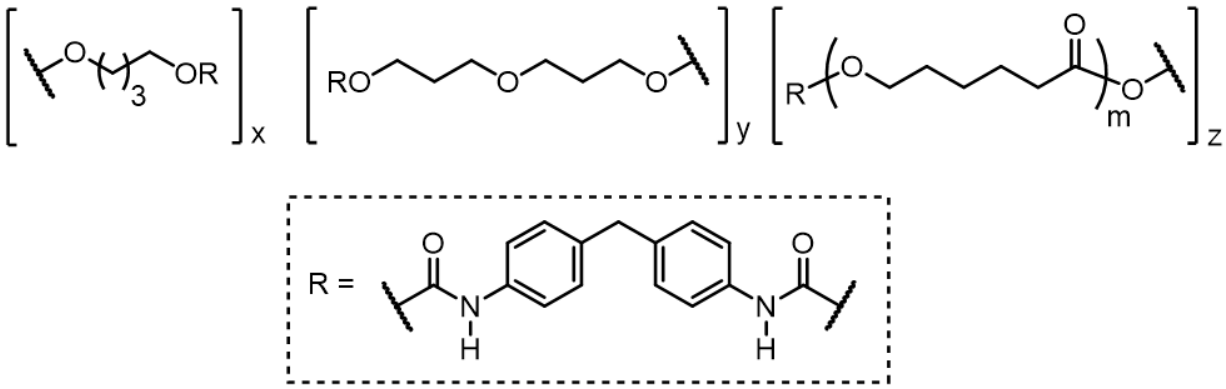
Umare, S.S., Chandure, A.S., 2008. Synthesis, characterization and biodegradation studies of poly(ester urethane)s. Chem. Eng. J. 142, 65–77. https://doi.org/10.1016/j.cej.2007.11.017

Vega, R.E., Main, T., Howard, G.T., 1999. Cloning and expression in *Escherichia coli* of a polyurethane-degrading enzyme from *Pseudomonas fluorescens*. Int. Biodeterior. Biodegrad. 43, 49–55. https://doi.org/10.1016/S0964-

8305(98)00068-7

Xie, F., Zhang, T., Bryant, P., Kurusingal, V., Colwell, J.M., Laycock, B., 2019. Degradation and stabilization of polyurethane elastomers. Prog. Polym. Sci. 90, 211–268. https://doi.org/10.1016/j.progpolymsci.2018.12.003

Supplementary figure caption



Supplementary figure 1. Chemical structure of poly[4,4′-methylenebis(phenyl isocyanate)-alt-1,4-butanediol/di(propylene glycol)/polycaprolactone] (PCLMDI). Its transition temperature (T_g) is -40 °C (DSC) with softening point 85 °C (Vicat, ASTM D 1525) (Sigma-Aldrich, St. Louis, USA).

CAPÍTULO 3

Staphylococcus warneri isolada de *Galleria mellonella* biodegrada poliuretanos em cultura pura e em consórcio com *Serratia liquefaciens*

Resumo

Com o aumento da produção e do uso de poliuretanos (PU) é necessário encontrar técnicas sustentáveis para a remediação da poluição plástica. O uso de microrganismos capazes de biodegradar PU pode ser a solução ambientalmente desejável para o controle desses contaminantes plásticos. Visando contribuir com a descoberta de alternativas para a mitigação de plásticos no ambiente, esse trabalho teve como objetivo explorar o potencial de *Staphylococcus warneri* UFV_01.21, isolado do intestino da larva de *Galleria mellonella*, para a biodegradação de PU em cultura pura e em consórcio microbiano com *Serratia liquefaciens* L135. *S. warneri* foi capaz de utilizar Impranil® PU como única fonte de carbono, tanto em cultura pura, quanto em consórcio. Com seis dias de incubação, a biodegradação do Impranil® em caldo Luria Bertani foi de 96, 88 e 76% e em meio mínimo foi de 58, 54 e 42% por *S. warneri*, *S. liquefaciens* e consórcio, respectivamente. Além disso, *S. warneri* em cultura pura ou consórcio foi capaz de biodegradar, bem como aderir e formar biofilmes nas superfícies de discos de Impranil® e de filmes de poli[4,4'-metilenobis(fenilisocianato)-alt-1,4-butanodiol/di(propilenoglicol)/policaprolactona] (PCLMDI). A biodegradação foi evidenciada por microscopia eletrônica de varredura, pela formação de rachaduras, sulcos, poros e rugosidades nas superfícies dos PU inoculados, tanto com a cultura pura quanto em consórcio microbiano. Logo, esse estudo é o primeiro a mostrar o potencial de *S. warneri* para biodegradação de PU.

Palavras-chave: Biofilme. Biorremediação. Consórcio microbiano. Intestino de larvas. Polímeros.

1 Introdução

Os poliuretanos (PU) são geralmente obtidos por poliadição de diisocianatos e polióis, principalmente poliésteres ou poliéteres, para produzir diferentes estruturas com propriedades físicas únicas (Liu et al., 2021; Magnin et al., 2020). Os PU são materiais leves de alta resistividade, encontrados em vários produtos do uso cotidiano, ocupando o sexto lugar entre os plásticos mais utilizados no mundo (Cregut et al., 2013; Kaur et al., 2022). Com a grande produção e o uso rotineiro dos PU, uma grande quantidade de resíduos de PU também é gerada e uma fração significativa não é reciclada, sendo destinada a aterros sanitários e incineração (Liu et al., 2021). Atualmente, todo ecossistema, incluindo oceanos, água doce, manguezais, solos, florestas, regiões polares e atmosfera, estão ameaçados pela poluição plástica (Jiménez et al., 2021). Logo, um dos maiores desafios ambientais das próximas décadas é a luta contra a poluição plástica (Delangiz et al., 2022; Lau et al., 2020).

A biodegradação utilizando microrganismos e, ou enzimas tornou-se uma alternativa promissora para a reciclagem de plásticos por apresentarem condições de reação amenas e ecologicamente corretas (Delangiz et al., 2022; Liu et al., 2021). A biodegradação de polímeros plásticos por microrganismos envolve as seguintes etapas: biodeterioração, biofragmentação, despolimerização, bioassimilação e biomineralização. Resumidamente, a colonização superficial de polímeros e a formação subsequente de biofilme conduz à biodeterioração, alterando as propriedades físicas, químicas e mecânicas da superfície plástica, diminuindo a flutuabilidade e hidrofobicidade da mesma (Ganesh Kumar et al., 2020; Lobelle e Cunliffe, 2011; Sivan, 2011). Em seguida, o material polimérico é decomposto em oligômeros, dímeros e monômeros por enzimas que desestabilizam o esqueleto de carbono dos polímeros, promovendo a biofragmentação (Magnin et al., 2020; Zeenat et al., 2021). Os monômeros liberados na despolimerização enzimática podem ser facilmente bioassimilados como fonte de carbono e biomineralizados por microrganismos para promover o crescimento microbiano (Mohanan et al., 2020; Shah et al., 2008). Wierckx et al. (2015) acreditam que os resíduos plásticos podem e devem ser estabelecidos como uma nova fonte de carbono de segunda geração para a biotecnologia.

Frente ao problema que os plásticos representam para o ambiente, a busca e a identificação de microrganismos e enzimas eficientes na reciclagem biológica está em ascensão. O isolamento de microrganismos de intestino de larvas de diferentes

espécies animais para a biodegradação plástica parece ser uma estratégia atraente (Goveas et al., 2023). Estudos isolaram bactérias e fungos do intestino de larvas que foram capazes de biodegradar com eficiência poliestireno (PS), polietileno (PE) e PU. *Enterobacter* sp. D1 (Ren et al., 2019) e *Aspergillus flavus* PEDX3 (Zhang et al., 2020) foram isolados do intestino de larvas de *Galleria mellonella* e mostraram capacidade de biodegradar PE. Cassone et al. (2020) cultivaram bactérias intestinais de larvas de *G. mellonella*, utilizando PE como fonte exclusiva de carbono e identificaram *Acinetobacter*, que parece estar envolvida no processo de biodegradação desse polímero. *Pseudomonas aeruginosa* DSM 50071, isolado do intestino de larvas de *Zophobas atratus*, apresentou potencial para a biodegradação de PS (Kim et al., 2020). Os gêneros *Enterococcus* e *Mangrovibacter* foram dominantes em intestino das larvas de *Z. atratus* alimentadas com PU e foram relacionados com a sua biodegradação (Luo et al., 2021). *Acinetobacter* sp., isolado de larvas de *Tribolium castaneum*, pode desempenhar um papel na biodegradação de PS e tem potencial para ser aplicado em estudos sobre a biodegradação de plásticos (Wang et al., 2020). Liu et al. (2022) verificaram o aumento das famílias Enterobacteriaceae e Streptococcaceae no intestino larvas de *Tenebrio molitor*, sugerindo que esses microrganismos podem contribuir para a biodegradação do PU de poliéter. Orts et al. (2023) relataram que bactérias dos gêneros *Lactococcus*, *Pediococcus*, *Enterococcus*, *Paraclostridium*, *Chryseobacterium*, *Kosakonia* e *Pseudomonas*, isoladas de larvas de *T. molitor* podem estar associadas à biodegradação de espumas de PU.

Kemona e Piotrowska (2020) acreditam que a biodegradação completa de polímeros maiores geralmente requer a cooperação de vários organismos diferentes. De fato, estudos confirmam que a biodegradação de polímeros pode ser alcançada com formação de um consórcio microbiano. Fernandes et al. (2016) compararam aa biodegradação de um PU termoplástico por *Aspergillus niger* associado ou não a *P. aeruginosa* e os resultados revelaram um efeito sinérgico do consórcio microbiano. Shah et al. (2016) desenvolveram um consórcio de *Bacillus subtilis* MZA-75 e *P. aeruginosa* MZA para a biodegradação de PU de poliéster, e foi encontrada a redução máxima de peso de pedaços de filme de PU em 20 dias na presença do consórcio microbiano. Além disso, o consórcio microbiano resultou em maior produção de dióxido de carbono em razão da alta taxa de biodegradação quando comparado com as culturas

usadas separadamente. Gaytán et al. (2020) isolaram de aterro sanitário e identificaram por análise metagenômica, o consórcio microbiano BP8, composto pelas seguintes bactérias: *Paracoccus* sp., *Chryseobacterium* sp., *Parapedobacter* sp., *Microbacteriaceae bacterium* e *Ochrobactrum intermedium*, que foi capaz de utilizar PU como única fonte de carbono. Esses autores acreditam que consórcio microbiano é uma fonte promissora para mitigar a poluição antrópica por plásticos.

Devido à gravidade da poluição por polímeros plásticos no meio ambiente, a biodegradação desses compostos recalcitrantes por microrganismos e por consórcios microbianos deve ser explorada. Para isso, neste estudo, uma bactéria potencialmente biodegradadora de PU foi isolada do intestino da larva de *G. mellonella*, identificada e avaliada quanto à sua capacidade de biodegradar PU em cultura pura e em consórcio microbiano com *Serratia liquefaciens* L135. O potencial de biodegradação de PU por esta estirpe de *S. liquefaciens* já foi anteriormente explorado em cultura pura (Salgado et al., 2023).

2 Materiais e métodos

2.1 Isolamento de bactérias do intestino da larva de *G. mellonella* com potencial de biodegradação de Impranil®

O intestino da larva de *G. mellonella* foi cedido por Freitas et al. (2022). Em tubos tipo Falcon® de 50 mL, foi inoculado 1 mL da amostra de intestino em 35 mL em caldo Luria Bertani (LB) (Sigma-Aldrich, St. Louis, EUA) suplementado com 5, 1 e 0,1% (m/v) de Impranil (Impranil® DLN; Covestro, Alemanha) e incubado durante sete dias a 30 °C. Subsequente, diluições seriadas foram plaqueadas em ágar LB suplementado com 1% de Impranil®, a fim de isolar bactérias com potencial de biodegradação deste PU. O estoque dos isolados bacterianos obtidos nesta etapa foi feito em caldo LB contendo 20% de glicerol e congelado a -20 °C.

Para os experimentos seguintes, o isolado bacteriano selecionado e *S. liquefaciens* L135 foram reativados em caldo LB, a 30 °C, por 12-16 h e continha, aproximadamente, 10^8 Unidades Formadoras de Colônias por mililitro (UFC/mL).

2.2 Extração de DNA e sequenciamento do genoma completo

O isolado bacteriano selecionado foi cultivado por 16 h a 30 °C e o DNA genômico foi extraído usando o kit de extração Promega Wizard® DNA. A qualidade e

a quantidade de DNA foram avaliadas por eletroforese em gel de agarose 0,8% e em fluorômetro Qubit™ 2.0 (Thermo Fisher Scientific), respectivamente. O sequenciamento do genoma foi realizado pela empresa comercial Novogene (Califórnia, EUA), baseado em *long reads* com a plataforma Pacbio Sequel (Illumina PE150).

2.3 Controle de qualidade dos dados do sequenciamento, montagem e anotação do genoma

O controle de qualidade dos dados do sequenciamento utilizou os relatórios do FASTQC versão 0.11.9 (https://github.com/s-andrews/FastQC). As sequências do adaptador foram detectadas e removidas dos dados do sequenciamento usando a configuração de detecção automática do TrimGalore versão 0.6.7 (Krueger et al., 2021). Em seguida, as *paired reads* foram cortadas para qualidade e filtradas para comprimento usando Trimmomatic versão 0.39 (Bolger et al., 2014) selecionando os seguintes parâmetros: HEADCROP:20, CROP:130, SLIDINGWINDOW:4:20 e MINLEN:100.

O Unicycler versão 0.5.0 (Wick et al., 2017) executou a montagem de novo do genoma usando o método conservador e testando todos os *k-mers* ímpares entre 21 e 127. Em seguida, os *contigs* obtidos foram mesclados em *scaffolds* usando o método "scaffold" do RagTag 2.1.0 (https://github.com/malonge/RagTag) e selecionando um genoma de referência. Este genoma de referência é a sequência com maior cobertura de identidade e alinhamento encontrada pela ferramenta BLASTn do BLAST versão 2.13.0 (Altschul et al., 1990) dentre os genomas de Bacteria (Taxonomy ID: 2) do banco de dados NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nucleotide/), usando um limite de *E-value* de $1e^{-10}$ para selecionar os alinhamentos significativos. Os *scaffolds* foram corrigidos mapeando as leituras pareadas usando o algoritmo BWA-MEM de BWA versão 0.7.17 (Li e Durbin, 2009), classificando os arquivos de mapeamento usando o kit de ferramentas Picard versão 2.26.2 (https://github.com/broadinstitute/picard) e realizando o polimento das sequências usando o Pilon versão 1.24 (Walker et al., 2014), por meio de cinco interações com parâmetros padrão. A montagem foi avaliada pelo *assembly-stats* versão 1.0.1 (https://github.com/sanger-pathogens/assembly-stats), e a cobertura de *scaffolds* foi calculada pelo BBMap versão 38.76 (https://sourceforge.net/projects/bbmap).

O NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018; Li et al., 2021; Tatusova et al., 2016) anotou o genoma durante o processamento de sua submissão ao banco de dados Genbank (https://www.ncbi.nlm.nih.gov/genbank). O servidor *Rapid Annotation Subsystem Technology* (RAST) (https://rast.nmpdr.org/) (Aziz et al., 2008) classificou os genes previstos nos subsistemas do banco de dados SEED (Overbeek et al., 2005). O servidor BAGEL versão 4.0 (Van Heel et al., 2018) identificou as organizações gênicas das bacteriocinas presentes no genoma.

2.4 Atribuição de taxonomia

Comparações genômicas e alinhamentos de sequência reforçaram a atribuição de espécie ao isolado bacteriano. O servidor JSpeciesWS (https://jspecies.ribohost.com/jspeciesws/) (Richter e Rosselló-Móra, 2009) comparou o genoma de *S. warneri* com genomas de referência e calculou dois índices de identidade média de nucleotídeos com base em BLAST (ANIb) e MUMMER (ANIm), usando parâmetros padrão. O servidor Genome-to-Genome Distance Calculator (GGDC) versão 3.0 (https://ggdc.dsmz.de/) (Meier-Kolthoff et al., 2022, 2013) calculou a hibridização digital DNA-DNA (dDDH), usando parâmetros padrão.

2.5 Curvas de crescimento de *S. warneri* e *S. liquefaciens*

Em frascos Erlenmeyer de 250 mL contendo 50 mL de caldo LB, foram inoculados 500 µL das culturas de *S. warneri* e de *S. liquefaciens* L135 preparadas conforme item 2.1, separadamente. Para o consórcio microbiano foram adicionados 250 µL de cada cultura pura. Após 0, 3, 6, 9, 15, 21, 30, 39 e 48 h de incubação a 30 °C, uma alíquota da cultura foi retirada, diluições decimais seriadas foram preparadas e semeadas em ágar padrão para contagem (PCA; Acumedia, Lansing, EUA), pelo método de microgotas (Morton, 2001). Para a determinação da população de cada espécie no consórcio microbiano, além do PCA, foram utilizados o ágar Sal Manitol (KASVI, Brasil) para contagem de *S. warneri* e o ágar MacConkey (KASVI, Brasil) para contagem de *S. liquefaciens*. Após 16 h de incubação a 30 °C, as colônias foram contadas e os resultados em UFC/mL foram convertidos para $\log_{10}$ UFC/mL.

2.6 Determinação do Impranil® como única fonte de carbono

A capacidade das bactérias biodegradadoras de PU em usar o Impranil® como única fonte de carbono foi determinada. O crescimento e a capacidade de

biodegradação foram investigados em meio mínimo (MM; HIMEDIA, Mumbai, Índia) adicionado de 1,2% de ágar e de 0,5% de glicose, 1% Impranil® ou 0,5% de glicose mais 1% Impranil®. As culturas puras de *S. warneri* e *S. liquefaciens* L135 e o consórcio microbiano formado por esses dois isolados foram cultivados por seis dias a 30 °C.

A quantificação da biodegradação de Impranil® foi feita de acordo com Peng et al. (2014) com modificações. Foram utilizados caldo LB e o MM e em tubos de ensaio foram adicionados 10 mL de caldo LB ou de MM suplementado com 0,3% de Impranil®. Os tubos foram inoculados separadamente com 100 µL das culturas puras de *S. warneri* e de *S. liquefaciens*, atingindo 10^5 UFC/mL em cada tubo. Para obter o consórcio microbiano, foram inoculados 50 µL da cultura de *S. warneri* e 50 µL da cultura de *S. liquefaciens*. Os tubos foram incubados à 30 °C e após seis dias de incubação, as amostras foram centrifugadas a 3.000 x *g* por 5 min. O sobrenadante foi utilizado para quantificar a depuração do Impranil®, usando um espectrofotômetro (Thermo Fisher Scientific, Finlândia) a 600 nm. A absorbância encontrada a 600 nm nos ensaios foram comparadas com o controle negativo composto por caldo LB ou MM contendo 0,3% de Impranil®, sem inóculo.

2.7 Preparação e ensaio de biogedração de discos de Impranil®

Impranil® foi utilizado para preparar os discos de acordo com Salgado et al. (2023). Os discos de Impranil® solidificados foram transferidos com pinça esterilizada para placas de Petri contendo ágar LB inoculado com 100 µL da cultura de *S. warneri* na superfície. Para formação do consórcio microbiano foram inoculados 50 µL da cultura de *S. warneri* e 50 µL da cultura de *S. liquefaciens.* As placas foram incubadas a 30 °C durante seis dias. Os controles negativos consistiram em discos de Impranil® em ágar LB sem inóculo.

Alterações na morfologia da superfície de discos de Impranil®, inoculados ou não, foram visualmente avaliadas no segundo, quarto e sexto dia de incubação e registradas por fotografias. Além disso, esses discos foram recuperados, transferidos para tubos Falcon® de 50 mL contendo 10 mL de solução salina 0,85%, secos em estufa a 37 °C, montados em *stubs*, revestidos com ouro usando metalizador Quorum Q150RS e analisados em microscópio eletrônico de varredura (MEV) LEO 1430VP (Cambridge, Inglaterra).

A adesão e a formação de biofilme em discos de Impranil® também foram avaliadas por enumeração de células viáveis. Para isso, os discos de Impranil® inoculados e incubados ao longo de seis dias foram removidos, lavados com 3 mL de solução salina 0,85%, transferidos para um microtubo contendo 1 mL de solução salina 0,85% e agitados em vórtex por 1 min para remover células sésseis. Em seguida, uma alíquota da suspensão foi retirada para preparar diluições decimais seriadas, semeadas em PCA pelo método de microgotas (Morton, 2001). Após 16 h de incubação a 30 °C, as colônias foram contadas e os resultados expressos em UFC por disco de Impranil® (UFC/disco de Impranil®).

2.8 Preparação e ensaio de biodegradação de filmes de PCLMDI

Os filmes do PU comercial poli[4,4'-metilenobis(fenilisocianato)-alt-1,4-butanodiol/di(propilenoglicol)/policaprolactona] (PCLMDI; Sigma-Aldrich, St. Louis, EUA) (Figura suplementar 1), foram preparados de acordo Salgado et al. (2023). Os filmes de PCLMDI com 1 cm^2 foram colocados em frascos Erlenmeyer contendo 50 mL de caldo LB inoculados com 500 µL de células de *S. warneri* e incubados a 30 °C. Para formação do consórcio microbiano foram inoculados 250 µL da cultura de *S. warneri* e 250 µL da cultura de *S. liquefaciens.* O meio de cultura inoculado foi trocado duas vezes por semana, durante 60 dias. O controle negativo consistiu do meio LB com filmes PCLMDI sem inóculo. Após 60 dias de incubação, os filmes de PCLMDI foram analisados por MEV e também quanto à adesão e à formação de biofilme por *S. warneri* em cultura pura e em consórcio conforme item 2.7. O resultado foi expresso em UFC/cm^2 de filme de PCLMDI.

2.9 Análise estatística

Os ensaios foram conduzidos em triplicata, com duas repetições. Os resultados foram analisados por meio de análise de variância ANOVA *one-way*, seguida do teste de Tukey com nível de significância de $p < 0,05$. As análises estatísticas foram realizadas usando o *software* GraphPad Prism (versão 8.4.3; GraphPad Prism Software, EUA).

3 Resultados e discussões

3.1 Microbiota do intestino de larvas de *G. mellonella* biodegrada Impranil®

O intestino de larvas é reconhecido por apresentar bactérias com capacidade de biodegradar polímeros (Li et al., 2020; Yang et al., 2015). A confirmação desta afirmativa foi feita neste estudo quando se observou que bactérias componentes da microbiota do intestino da larva de *G. mellonella* apresentaram nítida capacidade de biodegradar o Impranil® adicionado ao caldo LB nas concentrações de 5, 1 e 0,1% (Figura suplementar 2). Impranil® é uma dispersão de poliéster alifático aniônico, sendo o substrato mais comum para detectar a atividade de biodegradação de PU (Biffinger et al., 2015). Em seguida, isolou-se em ágar LB contendo 1% de Impranil®, bactérias com a capacidade de biodegradar PU. A colônia que apresentou halo transparente ao seu redor foi submetida à purificação, identificação e, posteriormente, avaliada quanto ao potencial de biodegradação de PU.

3.2 *S. warneri* UFV_01.21 foi identificada e seu genoma foi montado e anotado

A bactéria isolada do intestino da larva de *G. mellonella* foi identificada com base no sequenciamento do genoma completo, como sendo pertencente à espécie *Staphylococcus warneri*. O isolado foi nomeado *S. warneri* UFV_01.21 e os números de acesso do BioProject e do BioSample são PRJNA875469 e SAMN30619083, respectivamente. Trata-se de bactéria Gram-positiva, do grupo estafilococos coagulase negativos, pertencente ao filo Firmicutes, classe Bacilli, ordem Bacillales, família Staphylococcaceae e gênero *Staphylococcus*. *S. warneri* é um patógeno oportunista que pode causar várias infecções, especialmente em pacientes com dispositivos protéticos (Karam e Ribeiro, 2022).

As análises dos dados brutos do sequenciamento foram feitas em etapas de controle de qualidade. O comprimento do genoma de *S. warneri* UFV_01.21 foi determinado em 2.544.871 pb, distribuído em 25 *contigs* (22 cromossômicos e três plasmidiais) e um conteúdo médio de GC de 32,7%. Na montagem do genoma foram previstas 2.438 *Coding DNA Sequences* (CDS), 33 genes de tRNA, nove genes de rRNA e um gene tmRNA (Tabela suplementar 1). As características do genoma do *S. warneri* UFV_01.21 e de nove cepas de *S. warneri* foram apresentadas na Tabela suplementar 2.

Com o servidor RAST foi possível prever 1.318 CDS (54,06%) de acordo com a categoria funcional, sendo os mais abundantes: cofatores, vitaminas, grupos prostéticos, pigmentos (192 genes), metabolismo de proteínas (163 genes), aminoácidos e derivados (122 genes), virulência, doença e defesa (88 genes), carboidratos (86 genes), metabolismo de RNA (86 genes) e metabolismo de DNA (75 genes) (Figura 1). Além disso, utilizando o *software* BAGEL foi possível identificar no genoma de *S. warneri*, duas organizações gênicas e três bacteriocinas: uma warnericina-RC e duas delta-lisinas (Figura suplementar 3).

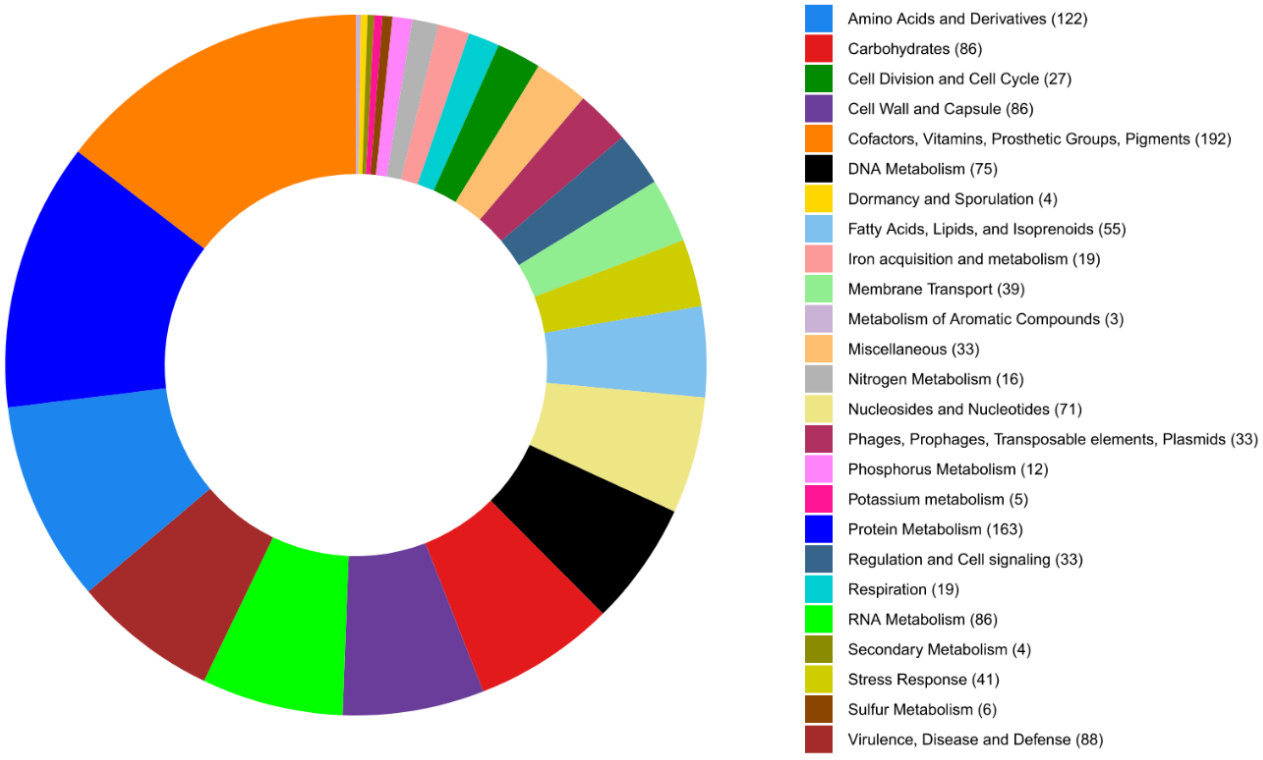


Figura 1. Genes codificadores de proteínas de *Staphylococcus warneri* UFV_01.21 distribuídos em subsistemas. O número entre parenteses representa o número de genes para cada categoria funcional.

O isolado *S. warneri* UFV_01.21 foi comparado a nível genômico com outros genomas de referência da mesma espécie. Para isso, dDDH, ANIb e ANIm foram calculados e os resultados estão apresentados na Tabela 1. A análise genômica comparativa do genoma *S. warneri* UFV_01.21 com outros genomas de *S. warneri* orientou sua classificação taxonômica por meio do cálculo de diferentes índices de

identidade genômica, que foram mais altos do que a pontuação limite de 95~96% de ANI e o valor de corte de 70% de dDDH (Chun et al., 2018; Richter e Rosselló-Móra, 2009).

Tabela 1. Análise genômica comparativa do genoma da estirpe *S. warneri* UFV_01.21, isolada do intestino da larva de *Galleria mellonella*, com outros nove genomas de *S. warneri* para orientação de sua classificação taxonômica através do cálculo de diferentes índices de identidade genômica

GenBank ID	Estirpe	dDDH [P(DDH>=70%)]	ANIb [Coverage]	ANIm [Coverage]
CP049802	Cap 100.1	96,8 [97,61]	99,61 [95,89]	99,63 [98,42]
CP031269	16A	96,8 [97,60]	99,75 [97,23]	99,72 [99,82]
CP033098	SWO	96,4 [97,51]	99,04 [93,06]	99,27 [95,78]
CP038242	GD01	95,9 [97,41]	99,63 [96,77]	99,72 [99,10]
CP003668	SG1	95,7 [97,36]	99,52 [95,78]	99,62 [98,38]
LR134269	NCTC11044	95,6 [97,34]	99,67 [96,48]	99,70 [98,91]
LR134244	NCTC7291	94,7 [97,13]	99,54 [95,37]	99,61 [98,02]
LR134242	NCTC4133	92,4 [96,54]	99,05 [92,78]	99,26 [95,52]
CP061041	WS479	91,7 [96,32]	99,73 [96,89]	99,74 [99,49]

Esse é o primeiro estudo que descreve o isolamento e a identificação de *S. warneri* do intestino da larva de *G. melonella*. Anteriormente, Consuelo et al. (2022) isolaram *S. warneri* (TP8) do intestino da larva de *Acrobasis nuxvorella*, com capacidade de produzir tanase, enzima responsável por hidrolisar ligações éster de taninos. *S. warneri* também já foi isolada de ambientes diversos como da pele de peixes (Musharrafieh et al., 2014), de águas marinhas da Ilha da Trindade, Brasil (Freitas et al., 2020), da pele de humanos (Karam e Ribeiro, 2022), do intestino de humanos e camundongos (Louail et al., 2023).

3.3 *S. warneri* e *S. liquefaciens* crescem em consórcio

Como *S. warneri* possui potencial de produção de bacteriocinas, fez-se necessário a construção de curvas de crescimento em cultura pura e em consórcio microbiano, a fim de verificar a ocorrência de inibição do crescimento quando em co-cultivo. Foi possível observar que *S. warneri* e *S. liquefaciens* em culturas puras, com 48 horas de incubação mantiveram-se na fase estacionária, com aproximadamente 10^8 UFC/mL (Figura 2 A-B). Em consórcio microbiano, o mesmo padrão quantitativo de células foi observado, confirmando que não há inibição das bactérias estudadas quando cultivadas em consórcio (Figura 2C).

Apesar de *S. warneri* UFV_01.21 ter potencial para produção de bacteriocinas, não se observou a inibição do crescimento de *S. liquefaciens* L135. O efeito das bacteriocinas de *S. warneri* sobre diferentes bactérias foi avaliado anteriormente e bactérias do gênero *Legionella* e *Bacillus* foram sensíveis, enquanto que *Enterobacter cloacae, Escherichia coli, Hafnia alvei, Klebsiella pneumoniae, P. aeruginosa* entre outras, são resistentes (Freitas et al., 2020; Verdon et al., 2008).

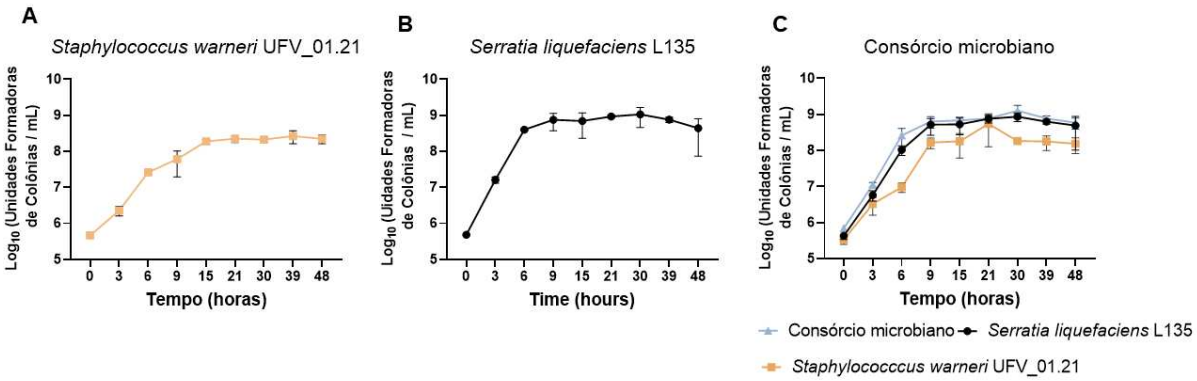


Figura 2. Curvas de crescimento de *Staphylococcus warneri* UFV_01.21 (A), *Serratia liquefaciens* L135 (B) e do consórcio microbiano (C) em caldo LB durante 48 h a 30 °C.

3.4 *S. warneri* e *S. liquefaciens* em cultura pura e em consórcio utilizam Impranil® como única fonte de carbono

Após seis dias de incubação em MM adicionado de glicose foi possível observar o crescimento das culturas puras de *S. warneri* e *S. liquefaciens* e em consórcio microbiano (Figura 3A). A formação de halo translúcido ao redor das áreas de crescimento bacteriano no ágar MM suplementado com Impranil® indicou que *S. warneri* e *S. liquefaciens* em cultura pura e em consórcio, utilizaram o Impranil® como única fonte de carbono (Figura 3B). No entanto, zonas translúcidas não foram observadas quando glicose e Impranil® foram adicionadas concomitantemente ao MM (Figura 3C). Este resultado indica que os dois isolados utilizam preferencialmente a glicose como fonte de carbono e corrobora os resultados encontrados com *Xanthomonas* sp. HY-71 (Kim et al., 2022). Outros microrganismos que utilizam o Impranil® como única fonte de carbono são: a bactéria *P. aeruginosa*, isolada do solo (Mukherjee et al., 2011), o fungo *Cladosporium halotolerans, isolado* do fundo do mar (Zhang et al., 2022) e o fungo *Cladosporium* sp. P7, isolado do solo (Liu et al., 2023).

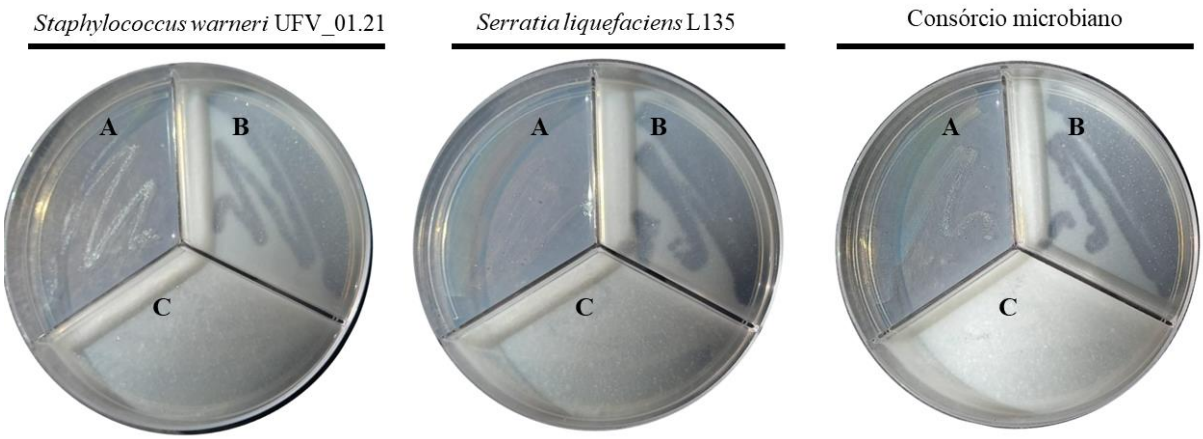


Figura 3. Crescimento de *Staphylococcus warneri* UFV_01.21 e *Serratia liquefaciens* L135, em cultura pura e em consórcio microbiano, durante seis dias de incubação a 30 ºC, em meio mínimo (MM) contendo 0,5% glicose **(A)**, MM contendo 1% Impranil® **(B)** e MM contendo 0,5% glicose mais 1% Impranil® **(C)**. O potencial de biodegradação do PU é demonstrado por zonas translúcidas ao redor das estrias.

A absorbância a 600 nm foi usada como uma medida direta da biodegradação do Impranil® pelas culturas bacterianas puras e em consórcio. O controle negativo (sem inóculo) não apresentou biodegradação e seu valor de absorbância foi usado para comparação com as amostras inoculadas. Com seis dias de incubação, as suspensões de Impranil® em MM inoculadas com *S. warneri*, *S. liquefaciens* e com consórcio microbiano, apresentaram 58, 54 e 42% de biodegradação, respectivamente (Figura 4A). No entanto, em caldo LB, a biodegradação do Impranil® foi de 96, 88, e 76% em razão da atividade de *S. warneri*, *S. liquefaciens* e o consórcio microbiano, respectivamente (Figura 4B). Valores elevados de biodegradação do Impranil® também foram alcançados por outros microrganismos. Fuentes-Jaime et al. (2022) avaliaram a capacidade de *Alicycliphilus denitrificans* BQ1 em biodegradar Impranil® (1,25 mg/mL) em MM e verificaram que, após 2 dias, ocorreu 85% de biodegradação. Khruengsai et al. (2022) demonstraram que o fungo *Embarria clematidis* biodegradou 88,84% de Impranil® (1%), quando utilizado como única fonte de carbono. *Cladosporium* sp. P7 foi capaz de biodegradar 94,5% de Impranil® (1%) em MM, durante quatro dias a 28 °C (Liu et al., 2023). No entanto, os dados apresentados pelos diferentes autores devem ser comparados com cautela, pois foram utilizadas diferentes metodologias.

Os resultados de biodegradação obtidos neste estudo indicaram que a cultura pura de *S. warneri* foi mais eficaz para biodegradação do Impranil® do que em consórcio com *S. liquefaciens*. No entanto, a biodegradação em consórcio também ocorreu, demonstrando que *S. warneri* e *S. liquefaciens* em cultura pura e em consórcio microbiano podem ser uma estratégia promissora para a biorremediação de PU e devem ser exploradas.

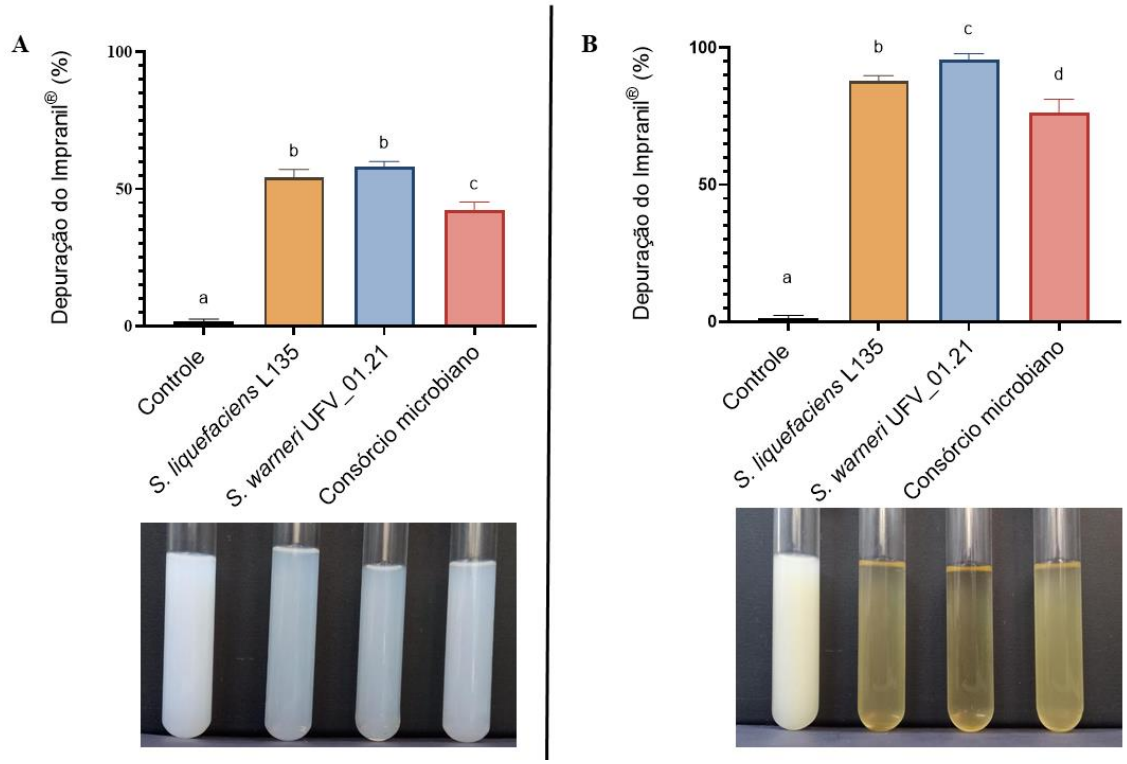


Figura 4. Análise quantitativa da biodegradação de Impranil® em meio mínimo (MM) contendo 0,3% de Impranil® **(A)** e em caldo Luria Bertani (LB) contendo 0,3% de Impranil® **(B)**. A biodegradação foi determinada a 600 nm e o controle negativo (sem inóculo) foi usado para comparar os dados. Letras diferentes em cima das barras de erro indicam uma diferença estatística significativa pelo teste de Tukey ($p < 0,05$) e letras iguais indicam que não há diferença estatística significativa.

3.5 *S. warneri* em cultura pura e em consórcio com *S. liquefaciens* biodegrada discos de Impranil®

Após seis dias de incubação a 30°C, os discos de Impranil® não inoculados e mantidos em ágar LB não apresentaram alterações macro e microscopicamente visíveis na estrutura física (Figuras 5A-C e 6A-C, respectivamente). No entanto, os discos

inoculados com *S. warneri* em cultura pura e em consórcio com *S. liquefaciens* apresentaram alterações como rupturas e formação de vários fragmentos ao longo do período de incubação (Figuras 5D-F e 5G-I, respectivamente). As micrografias obtidas por SEM confirmaram que, no decorrer dos seis dias, as trincas aumentaram e se tornaram mais profundas, causando consequentemente, a ruptura dos discos, tanto na cultura pura, quanto em consórcio microbiano (Figuras 6D-F e 6G-I, respectivamente). A biodegradação de discos de Impranil® por *S. liquefaciens* L135 nas mesmas condições também foi constatada por Salgado et al. (2023) por meio de fotografias e micrografias.

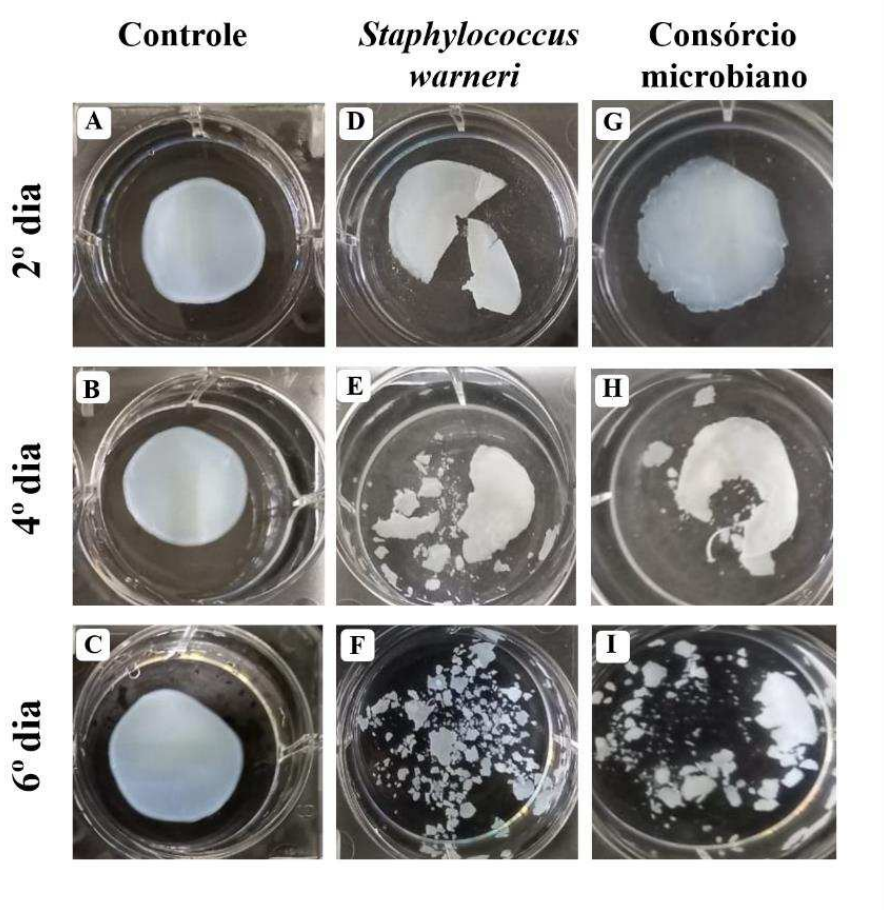


Figura 5. Fotografias dos discos de Impranil® sem inóculo (A-C), com inoculação de *Staphylococcus warneri* UFV_01.21 (D-F), e com inoculação do consórcio microbiano de *Staphylococcus warneri* UFV_01.21 com *Serratia liquefaciens* L135(G-I), com incubação a 30 °C por até seis dias em superfície de ágar Luria Bertani.

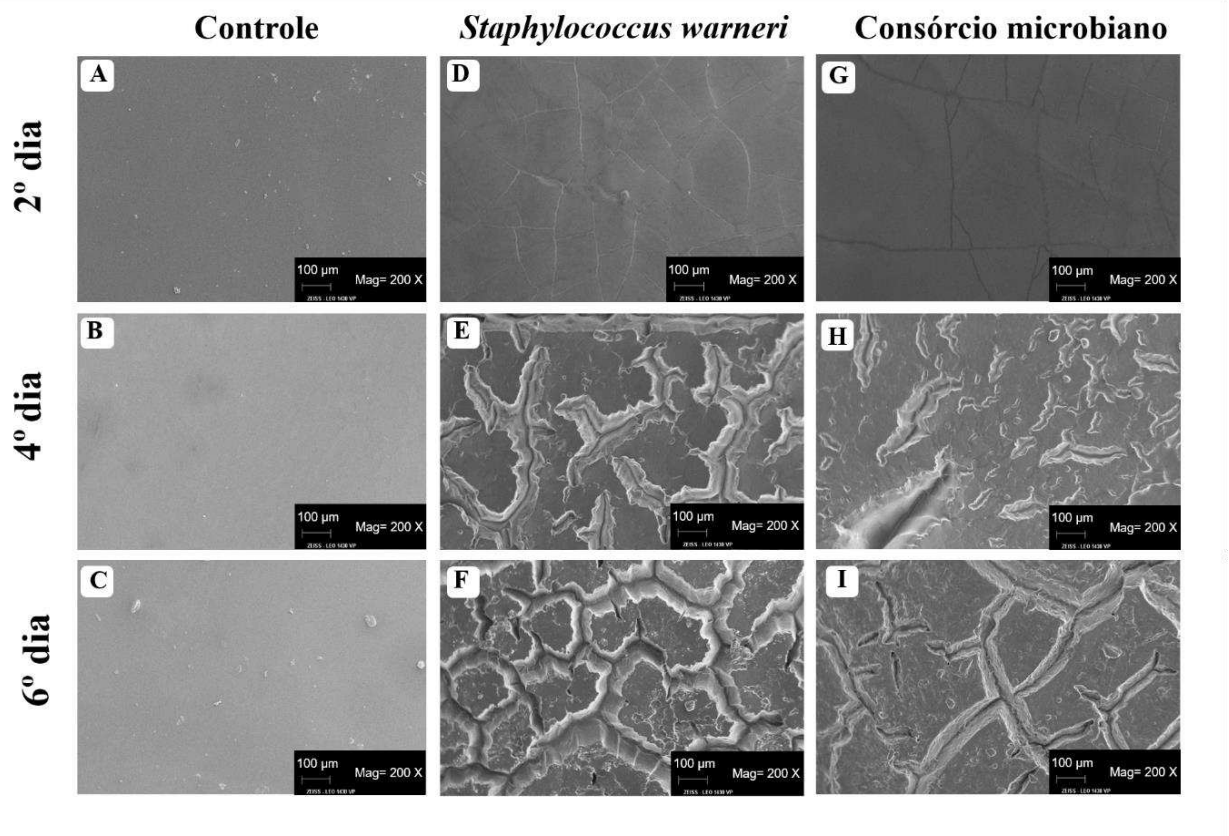


Figura 6. Micrografias eletrônicas de varredura dos discos de Impranil® sem inóculo (A-C), com inoculação de *Staphylococcus warneri* UFV_01.21 (D-F), e com inoculação do consórcio microbiano de *Staphylococcus warneri* UFV_01.21 com *Serratia liquefaciens* L135 (G-I), com incubação a 30 °C por até seis dias em superfície de ágar Luria Bertani.

Durante a biodegradação dos discos de Impranil® foram feitas contagens de células sésseis e foi possível confirmar a adesão e a formação de biofilme na superfície dos discos. O número de células sésseis variou de 1,4 x 10^9 a 1,0 x 10^{10} e 1,5 x 10^9 a 4,9 x 10^9 UFC / disco de Impranil®, em cultura pura e em consórcio, respectivamente, sem diferença estatística em seis dias de incubação (p < 0,05). Esses resultados mostram que o número de células em biofilme permaneceu estáveis durante todo o período de incubação, corroborando com os resultados encontrados por Salgado et al. (2023), no qual as células aderidas e em biofilme de *S. liquefaciens* permaneceram viáveis por seis dias, atingindo 10^9 a 10^{10} UFC/disco de Impranil®. Nadeau et al. (2021) também verificaram que o crescimento de *Pseudomonas protegens* Pf5 e de três mutantes nos discos de Impranil® apresentaram cinética

semelhante e atingiram, após seis de incubação, a densidade populacional de 10^9 a 10^{10} UFC/disco.

3.6 *S. warneri* em cultura pura e em consórcio com *S. liquefaciens* biodegrada filmes de PCLMDI

Após 60 dias de incubação dos filmes de PCLMDI foi possível verificar, por MEV, que o controle negativo (sem inóculo), não apresentou imperfeições, mantendo a superfície lisa e intacta (Figura 7A). No entanto, na presença de *S. warneri* em cultura pura ou em consórcio com *S. liquefaciens*, observou-se a formação de rachaduras, trincas, rugosidades e poros nos filmes de PCLMDI (Figuras 7B e 7C). A biodegradação de filmes de PCLMDI por *S. liquefaciens* L135 nas mesmas condições também foi constatada por Salgado et al. (2023) por meio de micrografias. Shah et al. (2016) também observaram por MEV a formação de rachaduras e poros na superfície de filmes de PU, após 30 dias de incubação com um consórcio formado por *Pseudomonas* e *Bacillus*.

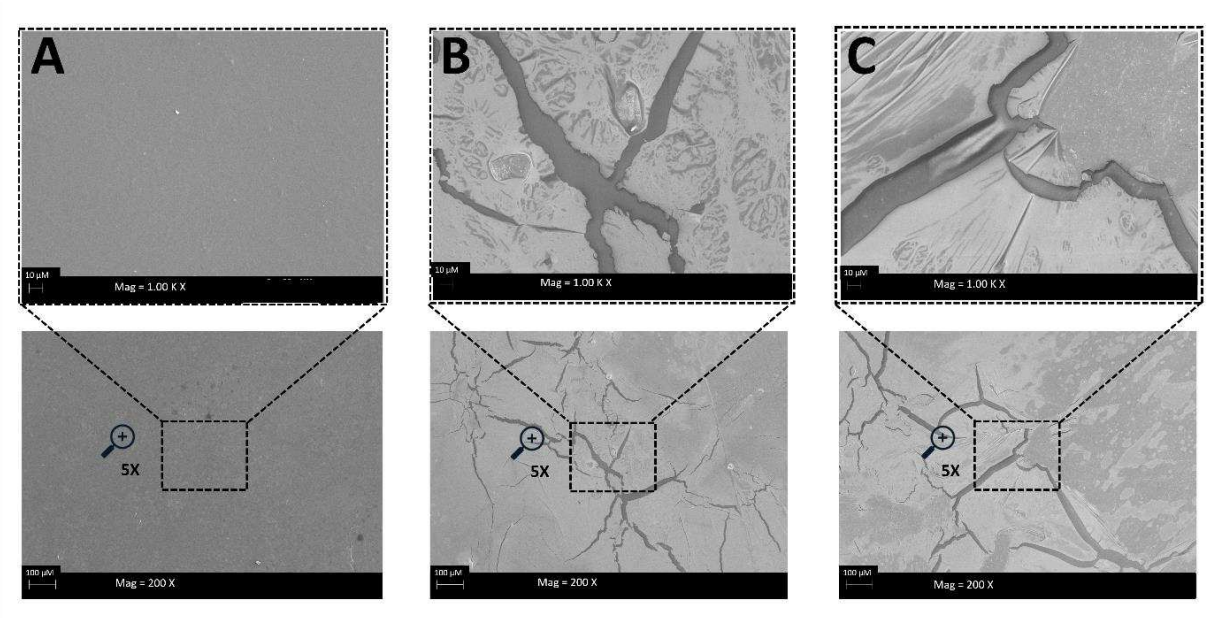


Figura 7. Micrografias eletrônicas de varredura de filmes de poli[4,4'-metilenobis(fenilisocianato)-alt-1,4-butanodiol/di(propilenoglicol)/policaprolactona] (PCLMDI) sem inóculo **(A)**, com inoculação de *Staphylococcus warneri* UFV_01.21 **(B)**, e com consórcio formado por *Staphylococcus warneri* UFV_01.21 com *Serratia liquefaciens* L135 **(C)** incubados a 30 °C por 60 dias em caldo Luria Bertani. As imagens superiores (1000 X) são ampliação (5x) das imagens inferiores (200 X).

As alterações físicas observadas nos filmes de PU são evidências da biodegradação microbiana e podem ser explicadas pela formação de biofilmes à superfície dos filmes. Neste estudo, *S. warneri* mostrou capacidade de aderir e formar biofilme na superfície hidrofóbica de filmes de PCLMDI, atingindo uma densidade populacional de 3,2 x 10^6 UFC/cm^2 em cultura pura e 8,3 x 10^6 UFC/cm^2 em consórcio microbiano. Esses resultados corroboram os apresentados por Salgado et al. (2023), que encontraram uma densidade populacional de 3,3 x 10^6 UFC/cm^2 de *S. liquefaciens*, nas mesmas condições. Segundo Dobretsov (2010), os biofilmes bacterianos em superfícies hidrofóbicas possuem densidade entre 3,0 x 10^6 e 1,3 x 10^7 UFC/m^2, reforçando os resultados encontrados. Devido à natureza hidrofóbica dos PU, a colonização microbiana, seguida da adesão e formação de biofilmes na superfície caracteriza o processo de biodegradação. Kemona e Piotrowska (2020) verificaram alterações macroscópicas na superfície de espumas de PU e a colonização da superfície por diferentes grupos de organismos, como fungos, leveduras e bactérias, incluindo *S. warneri*.

A biodegradação de PU por microrganismos é essencial para a mitigação de contaminação ambiental. No entanto, a capacidade de aderir e crescer em superfícies de PU pode representar um problema para outros setores. Na indústria alimentícia, materiais e superfícies de PU, usados para o processamento de alimentos, estão sujeitos a biodegradação. Fink et al. (2017) mostraram que *Bacillus cereus* forma biofilmes em correias transportadoras de PU usadas na panificação. Na saúde, os PU são utilizados para a produção de dispositivos médicos, incluindo cateteres intravasculares, enxertos vasculares e dispositivos artificiais de assistência ao coração (Francolini et al., 2014). Nestes dispositivos, a formação de biofilmes bacterianos e a sua subsequente biodegradação é indesejável e preocupante. García et al. (2010) verificaram que *Staphylococcus epidermidis* foi capaz de aderir e formar biofilme em cateter intravascular de PU, com 10^6 a 10^7 UFC/cm^2. Werneburg et al. (2023) mostraram que *S. warneri* formou biofilme mais espesso em PU do que em qualquer outro tipo de material usado em dispositivo de esfíncter urinário artificial interno. Esses estudos relataram a capacidade dessas bactérias em aderir e formar biofilmes em PU, mas não exploram a capacidade de biodegradação desse polímero.

Dentro do gênero *Staphylococcus*, *S. epidermidis* modificou as propriedades da superfície de um poliéter PU (Jansen et al., 1991). Entretanto, não há relatos da biodegradação de PU pela espécie *S. warneri*. Portanto, este é o primeiro trabalho

que explora o potencial de biodegradação de PU por esta espécie bacteriana em cultura pura e em consórcio microbiano com *S. liquefaciens*. Apesar do resultado quantitativo mostrar que o consórcio microbiano possui menor eficácia na biodegradação de Impranil® do que em cultura pura, a abordagem do uso de microrganismos para biodegradar PU possui um viés sustentável e pode ser explorado para aumentar a eficiência e otimizar o processo.

4 Conclusões

A biodegradação de polímeros plásticos utilizando microrganismos e, ou suas enzimas possui um viés sustentável e está em ascensão. Nesse estudo, foi relatado pela primeira vez, o potencial de *S. warneri*, isolado do intestino da larva *G. mellonella*, de biodegradação dos PU: Impranil® e PCLMDI. O isolado em cultura pura e em consórcio com *S. liquefaciens* foi capaz de aderir e formar biofilmes nas superfícies desses polímeros, conduzindo o processo de biodegradação. A prospecção de bactérias em intestinos de larvas deve ser explorada em busca de microrganismos promissores para mitigar a poluição ambiental por PU.

Dados suplementares

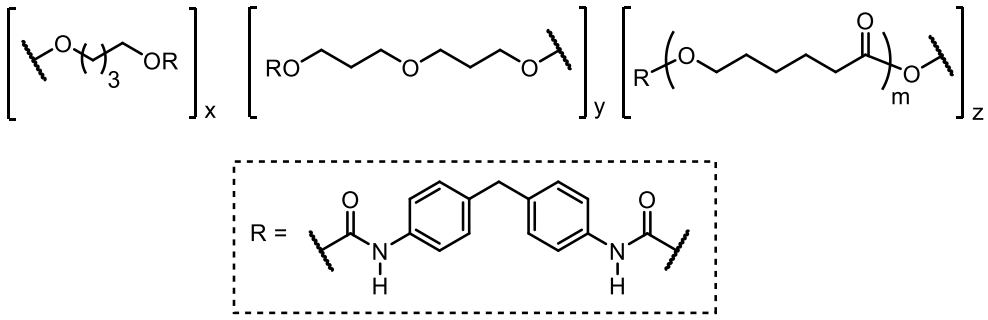


Figura suplementar 1. Estrutura química do poli[4,4'-metilenobis(fenilisocianato)-alt-1,4-butanodiol/di(propilenoglicol)/policaprolactona] (PCLMDI) poliuretano. Sua temperatura de transição (Tg) é de -40 °C (DSC) com ponto de amolecimento de 85 °C (Vicat, ASTM D 1525).

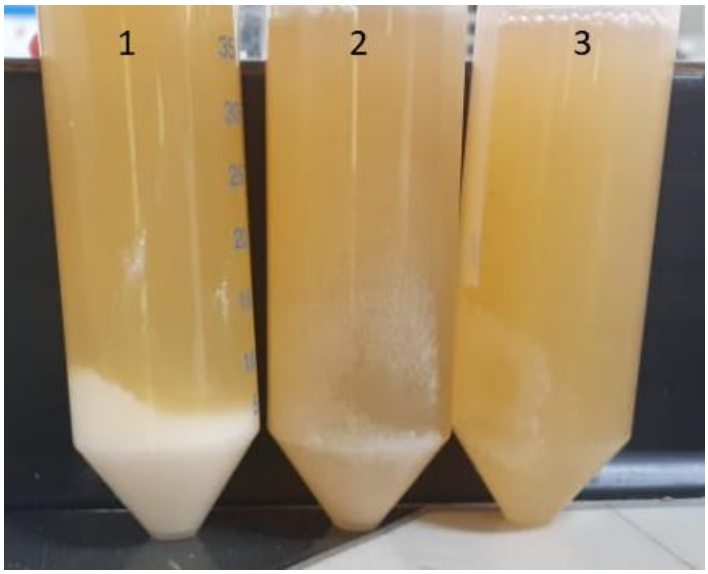


Figura suplementar 2. Hidrólise de Impranil® pela microbiota presente no intestino de *Galleria mellonella*. Tubos 1, 2 e 3 contêm caldo LB suplementado com 5, 1 e 0,1% de Impranil®, respectivamente. A precipitação nos tubos indica a biodegradação do Impranil®.

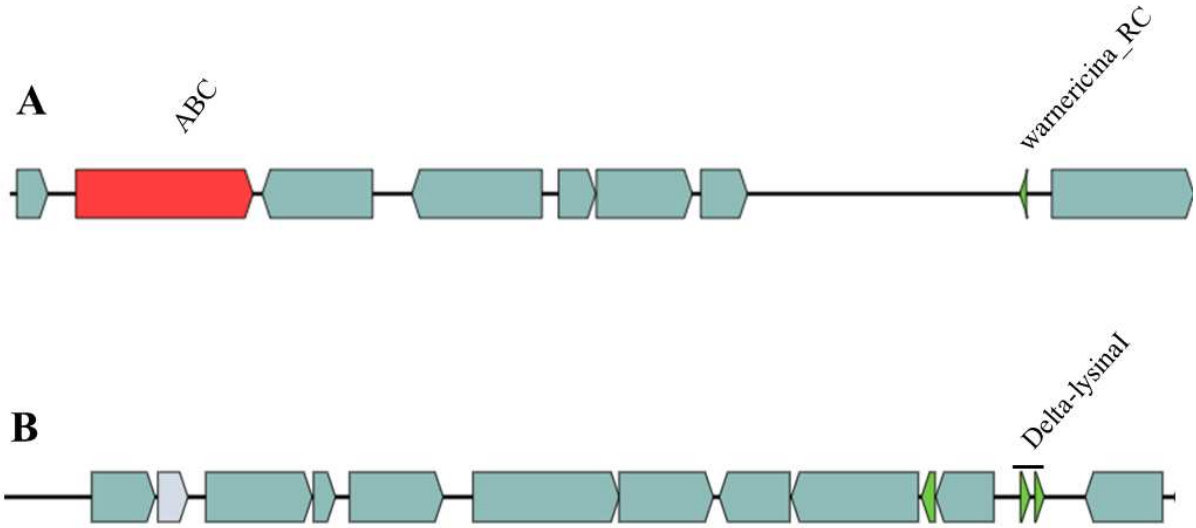


Figura suplementar 3. Representações esquemáticas dos *clusters* de genes putativos para a biossíntese de warnericina_RC **(A)** e das duas delta-lisinas **(B)** identificados no genoma do *Staphylococcus warneri* UFV_01.21 utilizando o *software* BAGEL.

Tabela suplementar 1. Atributos gerais da montagem e anotação do genoma da estirpe *S. warneri* UFV_01.21.

Atributos	**Cromossomo**	**Plasmídeo**
MONTAGEM		
Número de sequencias	22	3
N50 (bp)	2,428,834	10,831
Média (bp)	114,774	6,614
Sequência mais longa (bp)	2,428,834	10,831
Tamanho total (bp)	2,525,029	19,842
G+C (%)	32,7	30,3
ANOTAÇÃO		
Sequências codificadoras de DNA (CDS)	2,438	21
rRNA genes	9	-
tRNA genes	33	-
tmRNA genes	1	-
ncRNA genes	3	-
Completude: BUSCO (%) (ordem dos bacilos; n:450)	Completo e cópia única: 98 Faltando: 2	-

Tabela suplementar 2. Características do genoma de *S. warneri* UFV_01.21 e de nove estirpes de *Staphylococcus warneri*

GenBank ID	Estirpe	Size (Mb)	GC (%)	CDS	rRNA	tRNA
-	UFV_01.21	2,52	32,70	2,438	9	33
CP049802	Cap 100.1	2,47	32,69	2,328	12	63
CP031269	16A	2,77	32,61	2,435	19	61
CP033098	SWO	2,57	32,57	2,443	16	63
CP038242	GD01	2,54	32,73	2,391	19	63
CP003668	SG1	2,56	32,65	2,429	16	60
LR134269	NCTC11044	2,43	32,80	2,239	19	63
LR134244	NCTC7291	2,45	32,80	2,262	19	63
LR134242	NCTC4133	2,43	32,80	2,228	19	63
CP061041	WS479	2,56	32,84	2,405	19	63

Referências bibliográficas

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Aziz, R.K., Bartels, D., Best, A.A., Dejongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., Mcneil, L.K., Paarmann, D., Paczian, T., Parrello, B., PUch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST Server : Rapid Annotations using Subsystems Technology. BMC Genomics 9, 1–15. https://doi.org/10.1186/1471-2164-9-75

Biffinger, J.C., Barlow, D.E., Cockrell, A.L., Cusick, K.D., Hervey, W.J., Fitzgerald, L.A., Nadeau, L.J., Hung, C.S., Crookes-Goodson, W.J., Russell, J.N., 2015. The applicability of Impranil® DLN for gauging the biodegradation of polyurethanes. Polym. Degrad. Stab. 120, 178–185. https://doi.org/10.1016/j.polymdegradstab.2015.06.020

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Cassone, B.J., Grove, H.C., Elebute, O., Villanueva, S.M.P., LeMoine, C.M.R., 2020. Role of the intestinal microbiome in low-density polyethylene degradation by caterpillar larvae of the greater wax moth, *Galleria mellonella*. Proc. R. Soc. B Biol. Sci. 287, 9–11. https://doi.org/10.1098/rspb.2020.0112

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D.R., da Costa, M.S., Rooney, A.P., Yi, H., Xu, X.W., De Meyer, S., Trujillo, M.E., 2018. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int. J. Syst. Evol. Microbiol. 68, 461–466. https://doi.org/10.1099/ijsem.0.002516

Consuelo, G.C.M., Vargas-Arispuro, I., Luis Martínez-Carrillo, J., Pérez-Morales, R., Ángel Martínez-Téllez, M., Aispuro-Hernández, E., Arellano-Gil, M., Castro-Espinoza, L., 2022. The gut bacteria symbionts from the monophagous insect *Acrobasis nuxvorella* produce tannase for the digestion of *Carya illinoinensis* tannins. J. Asia. Pac. Entomol. 25, 4–10. https://doi.org/10.1016/j.aspen.2022.102005

Cregut, M., Bedas, M., Durand, M.J., Thouand, G., 2013. New insights into polyurethane biodegradation and realistic prospects for the development of a sustainable waste recycling process. Biotechnol. Adv. 31, 1634–1647. https://doi.org/10.1016/j.biotechadv.2013.08.011

Delangiz, N., Aliyar, S., Pashapoor, N., Nobaharan, K., Asgari Lajayer, B., Rodríguez-Couto, S., 2022. Can polymer-degrading microorganisms solve the bottleneck of plastics' environmental challenges? Chemosphere 294, 133709. https://doi.org/10.1016/j.chemosphere.2022.133709

Dobretsov, S., 2010. Marine Biofilms. Biofouling 123–136. https://doi.org/10.1002/9781444315462.ch9

Fernandes, I.P., Barbosa, M., Amaral, J.S., Pinto, V., Rodrigues, J.L., Ferreira, M.J., Barreiro, M.F., 2016. Biobased additives as biodegradability enhancers with application in TPU-based footwear components. J. Renew. Mater. 4, 47–56. https://doi.org/10.7569/JRM.2015.634126

Fink, R., Oder, M., Stražar, E., Filip, S., 2017. Efficacy of cleaning methods for the removal of *Bacillus cereus* biofilm from polyurethane conveyor belts in bakeries. Food Control 80, 267–272. https://doi.org/10.1016/j.foodcont.2017.05.009

Francolini, I., Donelli, G., Vuotto, C., Baroncini, F.A., Stoodley, P., Taresco, V., Martinelli, A., D'Ilario, L., Piozzi, A., 2014. Antifouling polyurethanes to fight device-related staphylococcal infections: Synthesis, characterization, and antibiofilm efficacy. Pathog. Dis. 70, 401–407. https://doi.org/10.1111/2049-632X.12155

Freitas, F.D.S., Marcus, P., Vidigal, P., Tatiane, ·, Siqueira, P., De Barros, M., Marcos, ·, Tótola, R., 2020. The draft genome of *Staphylococcus warneri* TRPF4, a bacteriocin producer with potent activity against the causative agent of Legionnaires' Disease. 3 Biotech 10, 232. https://doi.org/10.1007/s13205-020-02231-3

Freitas, L.L., Carneiro, D.G., Oliveira, G.S., Vanetti, M.C.D., 2022. N-acyl-homoserine lactone produced by *Rahnella inusitata* isolated from the gut of *Galleria mellonella* influences *Salmonella* phenotypes. Brazilian J. Microbiol. 53, 819–829. https://doi.org/10.1007/s42770-022-00681-w

Fuentes-Jaime, J., Vargas-Suárez, M., Cruz-Gómez, M.J., Loza-Tavera, H., 2022. Concerted action of extracellular and cytoplasmic esterase and urethane-cleaving activities during Impranil biodegradation by *Alicycliphilus denitrificans* BQ1. Biodegradation 33, 389–406. https://doi.org/10.1007/s10532-022-09989-8

Ganesh Kumar, A., Anjana K., Hinduja, M., Sujitha, M., Dharani, G., 2020. Review on plastic wastes in marine environment – Biodegradation and biotechnological solutions. Mar. Pollut. Bull. 150, 110733. https://doi.org/10.1016/j.marpolbul.2019.110733

García, I., Conejo, M. del C., Ojeda, A., Rodríguez-Baño, J., Pascual, A., 2010. A dynamic in vitro model for evaluating antimicrobial activity against bacterial biofilms using a new device and clinical-used catheters. J. Microbiol. Methods 83, 307–311. https://doi.org/10.1016/j.mimet.2010.09.017

Gaytán, I., Sánchez-Reyes, A., Burelo, M., Vargas-Suárez, M., Liachko, I., Press, M., Sullivan, S., Cruz-Gómez, M.J., Loza-Tavera, H., 2020. Degradation of recalcitrant polyurethane and xenobiotic additives by a selected landfill microbial community and its biodegradative potential revealed by proximity ligation-based metagenomic analysis. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02986

Goveas, L.C., Nayak, S., Kumar, P.S., Rangasamy, G., Vidya, S.M., Vinayagam, R., Selvaraj, R., Vo, D.V.N., 2023. Microplastics occurrence, detection and removal with emphasis on insect larvae gut microbiota. Mar. Pollut. Bull. 188, 114580. https://doi.org/10.1016/j.marpolbul.2023.114580

Haft, D.H., DiCuccio, M., Badretdin, A., Brover, V., Chetvernin, V., O'Neill, K., Li, W., Chitsaz, F., Derbyshire, M.K., Gonzales, N.R., Gwadz, M., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Yamashita, R.A., Zheng, C., Thibaud-Nissen, F., Geer, L.Y., Marchler-Bauer, A., Pruitt, K.D., 2018. RefSeq: An update on prokaryotic genome annotation and curation. Nucleic Acids Res. 46, D851–D860. https://doi.org/10.1093/nar/gkx1068

Jansen, B., Schumacher-Perdreau, F., Peters, G., Pulverer, G., 1991. Evidence for degradation of synthetic polyurethanes by *Staphylococcus epidermidis*. Zentralblatt fur Bakteriol. 276, 36–45. https://doi.org/10.1016/S0934-8840(11)80216-1

Jiménez, D.J., Wei, R., Bugg, T.D., Amaya, V., Castro-mayorga, J.L., Saldarriaga, F., Tarazona, A., 2021. Merging plastics, microbes, and enzymes: highlights from an international workshop. Appl. Environ. Microbiol. 88, e00721-22.

Karam, B.R.S., Ribeiro, P.M.A.P., 2022. *Staphylococcus warneri*: brief literature

review / Brazilian J. Heal. Rev. 5, 4358–4364. https://doi.org/10.34119/bjhrv5n2-043

Kaur, R., Singh, P., Tanwar, S., Varshney, G., Yadav, S., 2022. Assessment of bio-based polyurethanes: Perspective on applications and bio-degradation. Macromol 2, 284–314. https://doi.org/10.3390/macromol2030019

Kemona, A., Piotrowska, M., 2020. Polyurethane recycling and disposal: Methods and prospects. Polymers (Basel). 12. https://doi.org/10.3390/POLYM12081752

Khruengsai, S., Sripahco, T., Pripdeevech, P., 2022. Biodegradation of polyester polyurethane by *Embarria clematidis*. Front. Microbiol. 13, 1–12. https://doi.org/10.3389/fmicb.2022.874842

Kim, J.H., Choi, S.H., Park, M.G., Park, D.H., Son, K.H., Park, H.Y., 2022. Biodegradation of polyurethane by *Japanese carpenter* bee gut-associated symbionts *Xanthomonas* sp. HY-71, and its potential application on bioconversion. Environ. Technol. Innov. 28, 102822. https://doi.org/10.1016/j.eti.2022.102822

Lau, W.W.Y., Shiran, Y., Bailey, R.M., Cook, E., Stuchtey, M.R., Koskella, J., Velis, C.A., Godfrey, L., Boucher, J., Murphy, M.B., Thompson, R.C., Jankowska, E., Castillo, A.C., Pilditch, T.D., Dixon, B., Koerselman, L., Kosior, E., Favoino, E., Gutberlet, J., Baulch, S., Atreya, M.E., Fischer, D., He, K.K., Petit, M.M., Sumaila, U.R., Neil, E., Bernhofen, M. V., Lawrence, K., Palardy, J.E., 2020. Evaluating scenarios toward zero plastic pollution. Science (80-.). 369, 1–6. https://doi.org/10.1126/SCIENCE.ABA9475

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows – Wheeler transform 25, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Li, J., Kim, H.R., Lee, H.M., Yu, H.C., Jeon, E., Lee, S., Kim, D.H., 2020. Rapid biodegradation of polyphenylene sulfide plastic beads by *Pseudomonas* sp. Sci. Total Environ. 720, 137616. https://doi.org/10.1016/j.scitotenv.2020.137616

Li, W., O'Neill, K.R., Haft, D.H., Dicuccio, M., Chetvernin, V., Badretdin, A., Coulouris, G., Chitsaz, F., Derbyshire, M.K., Durkin, A.S., Gonzales, N.R., Gwadz, M., Lanczycki, C.J., Song, J.S., Thanki, N., Wang, J., Yamashita, R.A., Yang, M., Zheng, C., Marchler-Bauer, A., Thibaud-Nissen, F., 2021. RefSeq: Expanding the prokaryotic genome annotation pipeline reach with protein family model curation. Nucleic Acids Res. 49, D1020–D1028. https://doi.org/10.1093/nar/gkaa1105

Liu, J., He, J., Xue, R., Xu, B., Qian, X., Xin, F., Blank, L.M., Zhou, J., Wei, R., Dong, W., Jiang, M., 2021. Biodegradation and up-cycling of polyurethanes: Progress, challenges, and prospects. Biotechnol. Adv. 48, 107730. https://doi.org/10.1016/j.biotechadv.2021.107730

Liu, J., Zeng, Q., Lei, H., Xin, K., Xu, A., Wei, R., Li, D., Zhou, J., Dong, W., Jiang, M., 2023. Biodegradation of polyester polyurethane by *Cladosporium* sp. P7: Evaluating its degradation capacity and metabolic pathways. J. Hazard. Mater. 448, 130776. https://doi.org/10.1016/j.jhazmat.2023.130776

Liu, Jiawei, Liu, Jingyuan, Xu, B., Xu, A., Cao, S., Wei, R., Zhou, J., Jiang, M., Dong, W., 2022. Biodegradation of polyether-polyurethane foam in yellow mealworms (*Tenebrio molitor*) and effects on the gut microbiome. Chemosphere 304, 135263. https://doi.org/10.1016/j.chemosphere.2022.135263

Lobelle, D., Cunliffe, M., 2011. Early microbial biofilm formation on marine plastic debris. Mar. Pollut. Bull. 62, 197–200. https://doi.org/10.1016/j.marpolbul.2010.10.013

Louail, R., Florin, F., Bernard, S., Michaud, J.B., Breton, J., Achamrah, N., Tavolacci,

M.P., Coëffier, M., Ribet, D., 2023. Invasion of intestinal cells by *Staphylococcus warneri*, a member of the human gut microbiota. Gut Pathog. 15, 1–9. https://doi.org/10.1186/s13099-022-00528-7

Luo, L., Wang, Y., Guo, H., Yang, Y., Qi, N., Zhao, X., Gao, S., Zhou, A., 2021. Biodegradation of foam plastics by *Zophobas atratus* larvae (Coleoptera: Tenebrionidae) associated with changes of gut digestive enzymes activities and microbiome. Chemosphere 282, 131006. https://doi.org/10.1016/j.chemosphere.2021.131006

Magnin, A., Pollet, E., Phalip, V., Avérous, L., 2020. Evaluation of biological degradation of polyurethanes. Biotechnol. Adv. 39, 107457. https://doi.org/10.1016/j.biotechadv.2019.107457

Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M., 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14. https://doi.org/10.1186/1471-2105-14-60

Meier-Kolthoff, J.P., Carbasse, J.S., Peinado-Olarte, R.L., Göker, M., 2022. TYGS and LPSN: A database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. Nucleic Acids Res. 50, D801–D807. https://doi.org/10.1093/nar/gkab902

Mohanan, N., Montazer, Z., Sharma, P.K., Levin, D.B., 2020. Microbial and enzymatic degradation of synthetic plastics. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.580709

Morton, R.D., 2001. Aerobic Plate Count, in: Compendium of methods for the microbiological examination of foods, (4th ed.), American Public Health Association-APHA, Washington, 63-67 (Chapter 7).

Mukherjee, K., Tribedi, P., Chowdhury, A., Ray, T., Joardar, A., Giri, S., Sil, A.K., 2011. Isolation of a *Pseudomonas aeruginosa* strain from soil that can degrade polyurethane diol. Biodegradation 22, 377–388. https://doi.org/10.1007/s10532-010-9409-1

Musharrafieh, R., Tacchi, L., Trujeque, J., LaPatra, S., Salinas, I., 2014. *Staphylococcus warneri*, a resident skin commensal of rainbow trout (*Oncorhynchus mykiss*) with pathobiont characteristics. Vet. Microbiol. 169, 80–88. https://doi.org/10.1016/j.vetmic.2013.12.012

Nadeau, L.J., Barlow, D.E., Hung, C.S., Biffinger, J.C., Crouch, A.L., Hollomon, J.M., Ecker, C.D., Russell, J.N., Crookes-Goodson, W.J., 2021. Colonization and degradation of polyurethane coatings by *Pseudomonas protegens* biofilms is promoted by PueA and PueB hydrolases. Int. Biodeterior. Biodegrad. 156. https://doi.org/10.1016/j.ibiod.2020.105121

Orts, J.M., Parrado, J., Pascual, J.A., Orts, A., Cuartero, J., Tejada, M., Ros, M., 2023. Polyurethane soam residue biodegradation through the *Tenebrio molitor* digestive tract: Microbial communities and enzymatic activity. Polymers (Basel). 15. https://doi.org/10.3390/polym15010204

Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J. V., Chuang, H.Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E.D., Gerdes, S., Glass, E.M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A.C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., PUch, G.D., Rodionov, D.A., Rückert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O., Vonstein, V., 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33, 5691–5702. https://doi.org/10.1093/nar/gki866

Peng, Y.H., Shih, Y. hsin, Lai, Y.C., Liu, Y.Z., Liu, Y.T., Lin, N.C., 2014. Degradation of polyurethane by bacterium isolated from soil and assessment of polyurethanolytic activity of a *Pseudomonas putida* strain. Environ. Sci. Pollut. Res. 21, 9529–9537. https://doi.org/10.1007/s11356-014-2647-8

Ren, L., Men, L., Zhang, Z., Guan, F., Tian, J., Wang, B., Wang, J., Zhang, Y., Zhang, W., 2019. Biodegradation of polyethylene by *Enterobacter* sp. D1 from the guts ofwax moth *Galleria mellonella*. Int. J. Environ. Res. Public Health 16, 1–3. https://doi.org/10.3390/ijerph16111941

Richter, M., Rosselló-Móra, R., 2009. Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U. S. A. 106, 19126–19131. https://doi.org/10.1073/pnas.0906412106

Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: A comprehensive review. Biotechnol. Adv. 26, 246–265. https://doi.org/10.1016/j.biotechadv.2007.12.005

Shah, Z., Gulzar, M., Hasan, F., Shah, A.A., 2016. Degradation of polyester polyurethane by an indigenously developed consortium of *Pseudomonas* and *Bacillus* species isolated from soil. Polym. Degrad. Stab. 134, 349–356. https://doi.org/10.1016/j.polymdegradstab.2016.11.003

Sivan, A., 2011. New perspectives in plastic biodegradation. Curr. Opin. Biotechnol. https://doi.org/10.1016/j.copbio.2011.01.013

Tatusova, T., Dicuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L., Lomsadze, A., Pruitt, K.D., Borodovsky, M., Ostell, J., 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 44, 6614–6624. https://doi.org/10.1093/nar/gkw569

Van Heel, A.J., De Jong, A., Song, C., Viel, J.H., Kok, J., Kuipers, O.P., 2018. BAGEL4: A user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Res. 46, W278–W281. https://doi.org/10.1093/nar/gky383

Verdon, J., Berjeaud, J.M., Lacombe, C., Héchard, Y., 2008. Characterization of anti-*Legionella* activity of warnericin RK and delta-lysin I from *Staphylococcus warneri.* Peptides 29, 978–984. https://doi.org/10.1016/j.peptides.2008.01.017

Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C.A., Zeng, Q., Wortman, J., Young, S.K., Earl, A.M., 2014. Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 9. https://doi.org/10.1371/journal.pone.0112963

Wang, Z., Xin, X., Shi, X., Zhang, Y., 2020. A polystyrene-degrading *Acinetobacter* bacterium isolated from the larvae of *Tribolium castaneum*. Sci. Total Environ. 726, 138564. https://doi.org/10.1016/j.scitotenv.2020.138564

Werneburg, G.T., Hettel, D., Adler, A., Mukherjee, S.D., Lundy, S.D., Angermeier, K.W., Wood, H.M., Gill, B.C., Vasavada, S.P., Goldman, H.B., Rackley, R.R., Shoskes, D.A., Miller, A.W., 2023. Biofilms on indwelling artificial urinary sphincter devices harbor complex microbe–metabolite interaction networks and reconstitute differentially in vitro by material type. Biomedicines 11. https://doi.org/10.3390/biomedicines11010215

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13, 1–22. https://doi.org/10.1371/journal.pcbi.1005595

Wierckx, N., Prieto, M.A., Pomposiello, P., de Lorenzo, V., O'Connor, K., Blank, L.M., 2015. Plastic waste as a novel substrate for industrial biotechnology. Microb. Biotechnol. 8, 900–903. https://doi.org/10.1111/1751-7915.12312

Yang, Y., Yang, J., Wu, W.M., Zhao, J., Song, Y., Gao, L., Yang, R., Jiang, L., 2015.

Biodegradation and mineralization of polystyrene by plastic-eating mealworms: Part 2. Role of gut microorganisms. Environ. Sci. Technol. 49, 12087–12093. https://doi.org/10.1021/acs.est.5b02663

Zeenat, Elahi, A., Bukhari, D.A., Shamim, S., Rehman, A., 2021. Plastics degradation by microbes: A sustainable approach. J. King Saud Univ. - Sci. 33, 101538. https://doi.org/10.1016/j.jksus.2021.101538

Zhang, J., Gao, D., Li, Q., Zhao, Yixuan, Li, L., Lin, H., Bi, Q., Zhao, Yucheng, 2020. Biodegradation of polyethylene microplastic particles by the fungus *Aspergillus flavus* from the guts of wax moth *Galleria mellonella*. Sci. Total Environ. 704, 1–8. https://doi.org/10.1016/j.scitotenv.2019.135931

Zhang, K., Hu, J., Yang, S., Xu, W., Wang, Z., Zhuang, P., Grossart, H.P., Luo, Z., 2022. Biodegradation of polyester polyurethane by the marine fungus *Cladosporium halotolerans* 6UPA1. J. Hazard. Mater. 437, 129406. https://doi.org/10.1016/j.jhazmat.2022.129406

CONCLUSÕES E PERSPECTIVAS

A capacidade da lipase secretada por *S. liquefaciens* L135 em se ligar *in silico* a diferentes PUs e a confirmação *in vitro* da biodegradação do Impranil® e do PCLMDI indica seu potencial de biorremediação, e ensaios de biodegradação de outros PU devem ser explorados. A busca de outros microrganismos e enzimas constituem em alternativas para a redução de polímeros plásticos na naureza e, neste estudo, confirmou-se que o intestino de larvas de *G. mellonella* é fonte de microrganismos biodegradadores de plásticos pelo isolamento de *S. warneri* UFV_01.21. Essa espécie foi relatada pela primeira vez como capaz de biodegradação de Impranil® e PCLMDI e de usar esses PUs como única fonte de carbono. Embora o consórcio formado pelos isolados de *S. liquefaciens* L135 e *S. warneri* UFV_01.21 não tenha mostrado efeito sinérgico na degradação de PUs, é possível explorar o co-cultivo dessas promissoras bactérias com outros microrganismos e otimizar condições para a produção de enzimas hidrolíticas de interesse industrial. Identificar os produtos de biodegradação, bem como as enzimas e vias metabólicas envolvidas no processo de biodegradação, tanto em cultura pura quanto em consórcio microbiano é importante para elucidar o mecanismo de biodegradação e para contribuir para a mitigação da contaminação ambiental por polímeros plásticos.