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PRODUÇÃO CONCOMITANTE DE LIPASES E BIOSSURFACTANTES EM FERMENTAÇÃO EM ESTADO SÓLIDO PARA USO EM BIORREMEDIAÇÃO

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Tese de Doutorado apresentada ao Programa de Pós Graduação em Engenharia Civil e Ambiental da Universidade de Passo Fundo, como parte dos requisitos para a obtenção do título de Doutor em Engenharia.

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“Seria uma atitude ingênua esperar que as classes dominantes desenvolvessem uma forma de educação que proporcionasse às classes dominadas perceber as injustiças sociais de maneira crítica.”
Paulo Freire

“Eu acredito sinceramente que a única maneira que nós podemos criar uma paz mundial é através não só da educação das nossas mentes, mas também dos nossos corações e nossas almas.”
Malala Yousafzai

Dedico esse trabalho a minha família, a prof. Lu e a todas as mulheres que não tiveram a oportunidade do estudo no Brasil.

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RESUMO

Os biossurfactantes e lipases são biocompostos que podem ser obtidos através de fermentação em estado sólido (FES) com potencial aplicação ambiental, como no uso em biorremediação de contaminantes oleosos em solos, tais como o biodiesel, o qual embora seja menos nocivo ao ambiente que o diesel, pode ocasionar a contaminação de solos e águas. Dentre os microrganismos utilizados em FES, os fungos são os mais recorrentes, havendo uma lacuna de dados em relação ao uso de bactérias em FES. Objetivou-se produzir simultaneamente lipases e biossurfactantes via FES a partir do fungo filamentoso *Aspergillus niger* e da bactéria *Bacillus methylotroficus* e avaliar o uso do meio fermentado na biorremediação de solo contaminado com 20% de biodiesel. Também foram realizados estudos da influência da adição dos biocompostos sobre a adsorção do contaminante ao solo. Diferentes resíduos agroindustriais foram avaliados para composição do meio de cultivo (melaço de cana de açúcar, farelo de trigo, sabugo de milho, casca e farelo de soja), e diferentes percetuais de nitrogênio, umidade e indutores foram avaliados. A otimização das condições de produção de biocompostos para o fungo *Aspergillus niger* ocorreu com a utilização de 80% de farelo de trigo, 20% de sabugo de milho, 0,5% de melaço de cana de açúcar, 60% de nitrogênio e 5% de indutor (10,74 ± 0,54 Unidades de atividade lipolítica e 6,67 ± 0,06 Unidades de Emulsificação). Ao longo de 90 dias de ensaio de biorremediação, a maior biodegradação (74,40±1,76%) foi verificada quando utilizada a aplicação do farelo fermentando contendo os biocompostos produzidos. Para a bactéria *Bacillus methylotrophicus* o meio de cultivo composto por 80% de farelo de trigo e 20% de sabugo de milho, 1% de melaço de cana de açúcar, 2% de fonte de nitrogênio, 75% umidade e 1% indutor apresentou a melhor produção concomitante de biossurfactantes e lipases (24,61% de Redução de Tensão Superficial e 43,54 ± 1,20 U, respectivamente). No ensaio de biorremediação avaliado ao longo de 90 dias, a maior biodegradação do contaminante foi observada em 60 d de experimento (72,08 ± 0,36%), quando aplicado o farelo fermentando contendo os biocompostos produzidos. Atingiu-se o objetivo de produção simultânea dos biocompostos via FES tanto para o fungo filamentoso quanto para a bactéria e concluiu-se que os processos de FES possuem potencial de contribuir com a aceleração dos processos de biorremediação de resíduos oleosos em solos, podendo reduzir os custos relacionados ao uso de biossurfactantes e lipases purificados.

Palavras-chave: Biodegradação, biodiesel, atividade lipásica, tensão superficial, solo contaminado

ABSTRACT

Biosurfactants and lipases are biocompounds that can be obtained through solid-state fermentation (FES) with the potential for environmental application, as in the use in bioremediation of oily contaminants in soils, such as biodiesel, which although is less harmful to the environment than diesel, can cause contamination of soil and water. Among the microorganisms used in FES, fungi are the most recurrent, with a data gap to the use of bacteria in FES. The aim was to simultaneously produce lipases and biosurfactants via FES from the filamentous fungus *Aspergillus niger* and the bacterium *Bacillus methylotroficus* and to evaluate the use of the fermented medium in the bioremediation of soil contaminated with 20% biodiesel. Studies were also carried out on the influence of the addition of biocompounds on the adsorption of the contaminant to the soil. Different agro-industrial residues were evaluated for the composition of the culture medium (sugar cane molasses, wheat bran, corn cob, soybean meal and soybean waste), and different nitrogen, moisture, and inducers percentages were evaluated. The optimization of biocompound production conditions for the *Aspergillus niger* fungus occurred with the use of 80% wheat bran, 20% corn cob, 0.5% sugarcane molasses, 60% nitrogen, and 5% of inducer (10.74 ± 0.54 Units of lipolytic activity and 6.67 ± 0.06 Emulsification Units). Over 90 days of bioremediation assay, the greatest biodegradation (74.40±1.76%) was verified when using the application of fermenting bran containing the produced biocompounds. For the bacterium *Bacillus methylotrophicus*, the culture medium composed of 80% wheat bran and 20% corn cob, 1% sugar cane molasses, 2% nitrogen source, 75% moisture, and 1% inducer presented the better concomitant production of biosurfactants and lipases (24.61% Surface Tension Reduction and 43.54 ± 1.20 Units of lipolytic activity in soil, respectively). In the bioremediation assay evaluated over 90 days, the greatest biodegradation of the contaminant was observed in 60 d of the experiment (72.08 ± 0.36%), when the fermenting bran containing the produced biocompounds was applied. The aim of simultaneous production of biocompounds via FES was achieved for both the filamentous fungus and the bacteria and it was concluded that the FES processes have the potential to contribute to the acceleration of the bioremediation processes of oily residues in soils, and may reduce the costs related to the use of biosurfactants and purified lipases.

Keywords: Biodegradation, biodiesel, lipase activity, surface tension, contaminated soil

SUMÁRIO

1. CAPÍTULO 1: INTRODUÇÃO GERAL

O cultivo de microrganismos para obtenção de lipases e biossurfactantes através da fermentação em estado sólido (FES) é considerado uma forma sustentável e de custo reduzido, uma vez que o meio de cultivo utilizado pode ser proveniente de subprodutos e resíduos agroindustriais, como cascas, grãos, bagaços e sementes (MAHMOUD et al., 2015). Ainda, por ser uma técnica na qual é requerida umidade somente para manter o crescimento microbiano possível, o consumo de água para o bioprocesso é reduzido. Como alguns cultivos em estado sólido não requerem agitação, também há redução de gastos com energia elétrica, razões pelas quais este tipo de fermentação pode ser considerado de menor impacto ambiental (LIZARDI-JIMÉNEZ and HERNÁNDEZ-MARTÍNEZ, 2017; PANDEY, 2003).

Dentre os microrganismos capazes de atuarem no cultivo em estado sólido, o fungo filamentoso *Aspergillus niger* destaca-se como um potencial produtor de biocompostos devido a sua adaptação em diversas condições ambientais (CAVKA et al., 2014, SCHUSTER et al., 2002). As bactérias também podem desenvolverem-se em FES (DAS, MUKHERJEE, 2007; SLIVINSK et al., 2012), entretanto a espécie *Bacillus methylotrophicus* é recentemente datada, e seu desenvolvimento neste tipo de cultivo é desconhecido. Entretando, já ocorrem registros da sua capacidade de produção quando utilizada a fermentação submersa, (DECESARO et al., 2016).

Dentre os biocompostos passíveis de produção, destacam-se as enzimas lipolíticas e biossurfactantes, que possuem aplicabilidade bem estabelecida na indústria de alimentos, cosmética e de tingimento (FAI et al.,2015; GHARAEI-FATHABAD, 2011). Entretanto, a capacidade destes compostos atuarem em aplicações ambientais é ainda pouco explorada, e sua obtenção de forma concomitante nos cultivos em estado sólido é pouco descrita na literatura (ZARINVIARSAGH et al., 2017; MARTINS et al., 2008). Isto pode ser justificado pelo alto custo observado nas etapas posteriores ao cultivo, como processos de purificação e recuperação, denominados de *downstream*, que podem representar até 60% do custo do cultivo (DECESARO et al., 2015; DESAI; BANAT, 2007).

Dentre as aplicações ambientais, as lipases e os biossurfactantes apresentam possibilidade de serem utilizados no tratamento de solos contaminados por resíduos oleosos. A literatura geralmente reporta o uso destes biocompostos de forma isolada e sendo produzidos em bioprocessos individuais. Ainda, a maior parte dos relatos de produção de biossurfactantes utiliza a fermentação submersa como método de produção, em função das características dos microrganismos utilizados, geralmente bactérias ou leveduras (SOBRINHO et al., 2008;

SILVA et al., 2010). Outro aspecto diz respeito ao uso destes compostos nas aplicações ambientais, em geral somente após as etapas de purificação.

A contaminação dos solos com resíduos oleosos é gerada em virtude de falhas no sistema de armazenagem e transporte de combustíveis, como vazamento em tanques e acidentes envolvendo derramamentos durante o transporte (CHIARANDA, 2011). O aumento do uso do biodiesel como fonte energética, devido a sua produção ser considerada sustentável e viável em grande escala, fomenta a demanda por este tipo de combustível, e consequentemente a preocupação com potenciais acidentes envolvendo seus sistemas de mobilidade e estocagem (LIN et al., 2011). Quando ocorre, a contaminação superficial e subterrânea de solos compromete o meio ambiente e a saúde humana, requerendo tratamento adequado.

As técnicas de remediação tradicionais, como bombeamento e tratamento e lavagem de solos, utilizam grande quantidade de produtos químicos, água e energia para a remoção de contaminantes. Priorizando o baixo custo do processo do tratamento do contaminante, a preocupação com o impacto ambiental gerado na execução do projeto de remediação dificilmente é considerada um parâmetro de importância na escolha da técnica de remediação ideal (KIM et al., 2013).

As técnicas de biorremediação, como a bioestimulação e a bioaumentação, podem ser consideradas sustentáveis e de baixo impacto ambiental. Para otimizar o processo e reduzir custos, busca-se por alternativas que atuem, paralelamente, como fonte nutricional no processo de biodegradação, auxiliares na biodisponibilidade do contaminante para o processo de biodegradação e microrganismos capazes de atuar na biodegradação de compostos oleosos. Assim, pode-se citar o uso de biossurfactantes e as enzimas lipolíticas (JAIN et al., 2013), biocompostos produzidos por microrganismos em condições de cultivo pré-estabelecidas (PIMMATA et al., 2013).

O uso de lipases e biossurfactantes coproduzidos para tratamento de contaminantes oleosos poderá contribuir para a biodegradação dos resíduos oleosos em solos, pois sua adição é positiva quando comparada ao uso de técnicas como a de atenuação natural monitorada. A adição de enzimas no tratamento de solos contaminados promove maior interação com o contaminante, acelerando o processo de biorremediação (KARIGAR; RAO, 2011). As lipases possuem a capacidade de catalisar reações envolvendo ácidos graxos (JÚNIOR et al., 2016), possuindo por isso potencial aplicação em biorremediação. Os biossurfactantes possuem o potencial de, quando adicionados no solo, interagirem com a fração aquosa da partícula do solo, emulsionando compostos oleosos e facilitando a biodegradação (DECESARO et al., 2017; LAMICHHANE et al., 2017; GUPTA et al., 2016).

Desta forma, propõe-se neste trabalho o uso associado de técnicas de bioestimulação, bioaumentação e adição de lipases e biossurfactantes na biorremediação de solos contaminados com biodiesel. Ainda, por tratar-se de compostos orgânicos, é importante analisar como o comportamento da adição dos biocompostos produzidos impacta na biodisponibilidade e na adsorção do contaminante no solo (CECCHIN et al., 2016). Para isso, é preciso identificar quais efeitos da biorremediação são causados pelo solo e quais são resultado da influência dos microrganismos e biocompostos adicionados ao meio, visto que as propriedades específicas dos diferentes tipos de solo, como capacidade de troca catiônica, fração argilomineral e área de superfície, podem influenciar no comportamento da adsorção e da degradação de contaminantes no solo (YARON et al., 2012).

A temática do comportamento de contaminantes no solo, aliado a adição de bioestimulantes tem sido muito bem explorada pela linha de pesquisa em Infraestrutura Sustentável do Programa de Pós-Graduação em Engenharia Civil e Ambiental (PPGENG), buscando reduzir os danos ao meio ambiente. Dentro da linha de pesquisa, trabalhos envolvendo produção de biocompostos e sua aplicação em solos e águas contaminadas são desenvolvidos através de parcerias entre o Laboratório de Bioquímica e Bioprocessos e o Grupo de Pesquisa em Geotecnia Ambiental da Universidade de Passo Fundo.

Esta tese foi dividida em 4 capítulos, correspondentes a cada artigo elaborado e enviado para publicação. O Capítulo 1 consiste na Introdução Geral do estudo, o Capítulo 2 é a revisão bibliográfica, e engloba os conceitos e artigos pesquisados que possibilitaram a fundamentação teórica desta tese, indicando a necessidade de artigos aprofundados na temática de aplicação de farelo fermentado em processos de biorremediação, bem como o uso de metodologias para determinação dos biocompostos in situ no solo. O Capítulo 3 envolve o estudo de otimização de condições de cultivo com o uso de farelo de trigo e sabugo de milho na produção de lipases e biossurfactantes por *Aspergillus niger* e sua aplicação em um processo de biorremediação de solo contaminado com biodiesel.

O Capítulo 4 apresenta a aplicação dos biocompostos produzidos em fermentação em estado sólido e sua direta aplicação em um processo de biorremediação de solo contaminado com óleo diesel, sem a necessidade de purificação dos biocompostos. Demonstra também a mensuração da atividade lipásica e de biossurfactantes produzidos in situ no solo ao longo do processo de biorremediação. O Capítulo 5 apresenta a produção de lipases e biossurfactantes em fermentação em estado sólido utilizando a bactéria *Bacillus methylotroficus* e sua aplicação na biorremediação de um solo contaminado com biodiesel, incluindo a mensuração dos biocompostos produzidos in situ no solo. Dentre os artigos produzidos, o apresentado no

Capítulo 2 foi publicado na revista Journal of Environmental Engineering. Os demais capítulosencontram-se atualmente enviados a revistas para análise de publicação. O Capítulo 6 apresentaa Conclusão Geral.

O objetivo geral deste trabalho foi utilizar resíduos agroindustriais para produção de biocompostos via fermentação em estado sólido, e aplicação direta dos meios fermentados obtidos em processo de biorremediação de solo contaminado com biodiesel.

Os objetivos específicos foram:

a) Utilizar o fungo *Aspergillus niger* e a bactéria *Bacillus methylotroficus* para a produçãode biossurfactantes e lipases de forma simultânea em fermentação em estado sólido;

b) Otimizar a produção dos biocompostos;

c) Aplicar os meios fermentados na biorremediação de solo contaminado com 20 % debiodiesel;

d) Avaliar a produção in situ de lipases e biossurfactantes no solo contaminado;

e) Verificar o efeito dos tratamentos propostos sobre os processos de adsorção do contaminante no solo.

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2. CAPÍTULO 2: USE OF BIOCOMPOUNDS IN BIOREMEDIATION PROCESSES: PRODUCTION, APPLICATION, AND METHODS OF DETERMINATION IN SOILS[1]

Abstract: Biosurfactants and lipases are studied in many ways for application in several industrial sectors. However, although well explored, its environmental relevance is flawed in terms of application in bioremediation processes. Bioremediation is a sustainable technology, increasingly used in cases of accidents, to mitigate the impacts generated on the environment and public health. Thus, the aim was to carry out a bibliometric and systematic review involving the production of these biocompounds, their application in bioremediation processes contaminated with oily compounds, and the existing methods for their analytical determination in situ in situ the soil. With the evaluated articles' survey, we verified that lipases and biosurfactants had been mentioned as auxiliary compounds in the bioremediation process of oily contaminated soils. However, most of the strategies used involve using these compounds isolated, without evaluating the possible increase in the production of these compounds in soil due to their effect on microorganisms' biostimulation. We verified as a need the study of the simultaneous production of lipases and biosurfactants to reduce the costs of production and the evaluation of the effects of the addition in the soil beyond the simple impact on the degradation of contaminant, being necessary the use of analytical methods that allows the determination of production in situ.

Keywords: biostimulation, bioaugmentation, natural attenuation, contamination, bibliometric analysis

1. INTRODUCTION

Contamination of soils with oily residues is due to flaws in the production system, storage, and transportation of fuels, mainly through leaking tanking systems and accidents involving spills during transport (Baniasadi and Mousavi, 2018). Consequently, surface and underground soil contamination compromises the environment and human health and requires adequate treatment. Traditional remediation techniques, such as heat treatment, advanced oxidative processes, and soil washing, use large amounts of chemicals, water, and energy to remove contaminants, increasing the process's cost (Liu et al. 2020; Ren et al. 2020).

Bioremediation techniques, such as biostimulation and bioaugmentation, can be considered sustainable and have a reduced environmental impact. To optimize the process and reduce costs, the use of compounds that can assist the biodegradation processes is sought, increasing the contaminants' bioavailability to be used by the microorganisms. Examples of these compounds are biosurfactants and lipolytic enzymes (Deng et al. 2020; Ganapathy et al. 2019), which microorganisms can produce during *in situ* bioremediation, or can be produced

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previously under conditions optimized by bioprocesses, to obtain sufficient quantities and at acceptable costs to be added in the bioremediation processes (Kreling et al. 2020; Kreling et al. 2019; Pimmata et al., 2013).

The use of lipases and biosurfactants results in the highest biodegradation of oily compounds, increasing their bioavailability, as it allows higher interaction of the hydrophobic contaminant with microbial cells (Safdari et al. 2018). The addition of lipolytic enzymes in the treatment of contaminated soils accelerates the bioremediation process due to catalyzed reactions involving fatty acids (Marchut-Mikolajczyk et al. 2020; Júnior et al. 2016). Biosurfactants interact with the aqueous fraction of soil particles, emulsifying oily compounds and facilitating biodegradation (Machado et al. 2020; Decesaro et al. 2017; Lamichhane et al. 2017).

The cultivation of microorganisms to obtain biocompounds is an alternative that has been widely investigated (Janek et al. 2020; Radha et al. 2020; Grüninger et al., 2019), with benefits for several areas of science and, more recently, in the environmental area. With the advancement of environmental policies, the aim is to minimize biotechnological processes' impacts, using sustainable raw materials to produce biocompounds (Prado et al. 2019; Salim et al. 2017). Thus, bioprocesses are considered sustainable and low cost when the culture medium used to produce microorganisms or biocompounds contains by-products and agro-industrial residues, such as whey, husks, grains, bagasse, and seeds (Sadh et al. 2018; Mahmoud et al. 2015; Pandey, 2003). One of the advantages of using bioprocesses is the possibility of concomitant production of several bioproducts during a single process, and these reports are still scarce in the literature (Kreling et al. 2020; Zarinviarsagh et al. 2017).

When lipases and biosurfactants are added to bioremediation processes, it is essential to assess whether the oily compounds' biodegradation affected the addition of the biocompounds. With biostimulation, microorganisms can produce lipases and biosurfactants *in situ*, increasing the rates of biodegradation. This work aimed to carry out a bibliometric analysis on the bioremediation of oily compounds in soils, emphasizing the use of lipases and biosurfactants and demonstrating the potential of these biocompounds in the bioremediation of contaminated soils. Also, we sought to review the existing methods for the analytical determination of these biocompounds in soil.

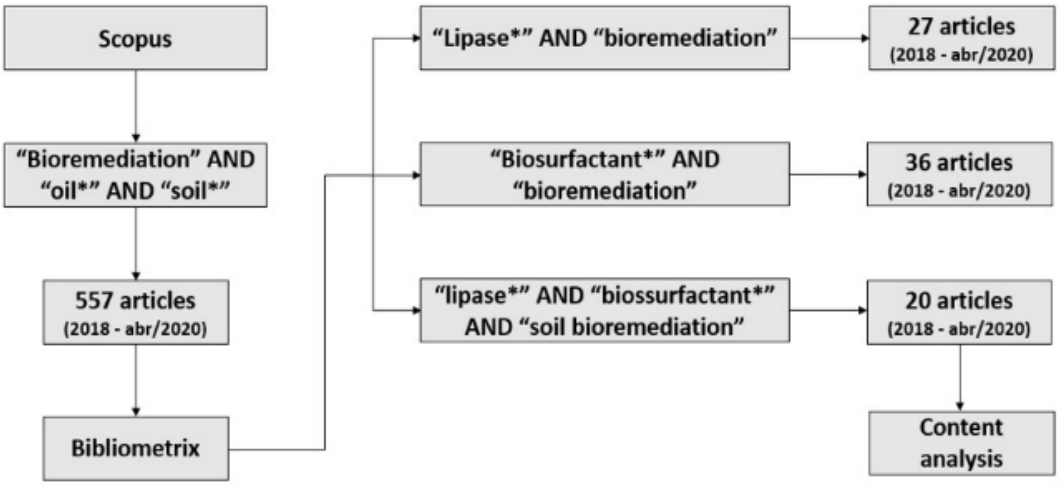
2. METHODOLOGY

The methodology of bibliometric research is shown in Figure 1. The search for articles was based on keywords inserted in the Scopus database, one of the largest and most used repositories of peer-reviewed literature, containing more than 70 million scientific articles (Duque-Acevedo et al. 2020).

The articles' survey was limited between the years 2018 and 2020, to present the technological advances in the area. It was carried out by inserting the search terms "Bioremediation" AND "Oil *" AND "Soil *" in the Scopus database. The articles found in this item were exported to the Bibliometrix tool of the RStudio system version 1.2.5033 for Desktop, in BibTex format (Scopus, 2020), allowing an overview of scientific production in that period, totaling 557 articles. For each of these items, thematic maps were created based on the analysis and grouping of word networks, subdivided by different colors, based on the interaction between authors, countries, abstracts, keywords, and relevance of the terms covered, identifying trends between the most cited words in the articles (Nazari et al. 2020; Aria and Cuccurullo, 2017).

For each of the items researched, articles that best describe the terms were approached separately, and for that purpose, all 24 scientific productions were selected for content analysis. In the items "Lipase *" AND "Bioremediation"; "Biosurfactant *" AND "Bioremediation" were mainly concerned with the production of biocompounds for the bioremediation of oily compounds, and in the item "Lipase *" AND "biosurfactant *" AND "soil bioremediation" the use of biocompounds in the bioremediation of oily compounds in soil, and the lipases mainly were used as an indicator of the production of biosurfactants. Scientific productions from periods before to bibliometric research were used to support better the subjects covered in this bibliographic review.

Figure 1 - Methodology used in the elaboration of bibliometric research

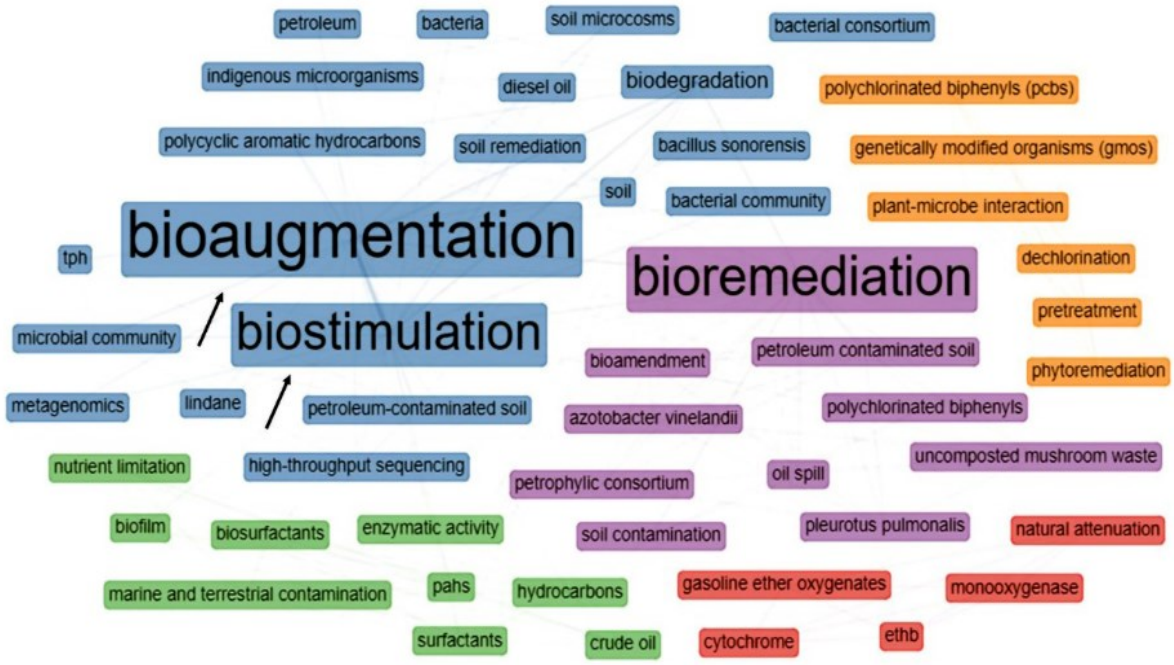


3. RESULTS AND DISCUSSION

3.1 Bioremediation for the treatment of oily contaminants in soils

Figure 2 shows the word cloud obtained in the Scopus database, using the terms "Bioremediation" AND "Oil*" AND "Soil*". The words that stood out the most in the word cloud when related to the bioremediation processes of oily compounds were "biostimulation" and "bioaugmentation" (Figure 2). The treatment of oily contaminants using approaches for adding microorganisms and nutrients to soils is widely known (Safdari et al. 2018; Sarkar et al. 2017; Abdulsalam and Omale, 2009; Singh et al. 2007).

Figure 2 - Thematic map of the articles found with the terms "Bioremediation" AND "Oil*" AND "Soil*"



Bioaugmentation is defined as the technique in which microorganisms are added to the contaminated environment to increase the biodegradation rate. It is used in cases where endogenous microorganisms cannot effectively biodegrade the contaminant, requiring the insertion of specific microorganisms to assist in its dissipation and accelerate the bioremediation process (Lawniczak et al. 2020; Pimmata et al. 2013).

Biostimulation is defined as the induction of the growth of microorganisms present in the contaminated environment through supplementation of the nutritional source, with the supply of nutrients such as nitrogen, phosphorus, potassium, and oxygen biodegradation of the contaminant. Inorganic (urea, sawdust, and sludge) and organic nutrients (such as inactive biomass and bioprocess residues) can be added to the medium in sufficient quantity to increase the degradation of the contaminant (Baniasadi and Mousavi, 2018; Herrero and Stuckey, 2015).

Among the vast bioremediation compounds, petroleum and its derivatives stand out. Zhang et al. (2019) evaluated the petroleum degradation capacity; when inserted into the soil, bacteria immobilized on biochar, free bacteria, and biochar isolated. After 60 days of testing, the technique of immobilizing bacteria on biochar was more effective in removing the contaminant. It was proven through gas chromatography where the highest carbon chain degradation and reduction of total petroleum hydrocarbons were observed. Also, changes in the

soil's physical-chemical properties were observed in the study, with increased fertility due to the greater diversity of microorganism's present.

Vargas et al. (2017) evaluated the use of two nitrogen-fixing bacteria (BFN), capable of eliminating hydrocarbons and producing biosurfactants. In a site contaminated with oil, 80% of the hydrocarbon was removed after 16 months of bioaugmentation, there was an increase in the BFN from 13.10^4 UFC to 2.10^9 UFC. Nwankwegu and Onwosi (2017) evaluated the degradation of total petroleum hydrocarbon (TPH) in soil contaminated with gasoline through bioaugmentation using the bacterium *Micrococcus luteus* and the fungus *Rhizopus arrhizus* added separately and in a consortium for eight weeks. The best biodegradation result was obtained using *M. luteus* (75.70%), followed using *R. arrhizuz* (71.10%) in bioremediation, and the consortium between the microorganisms acquired removal of 66.40%.

Combining bioaugmentation and biostimulation techniques, Al-Kaabi et al. (2018) evaluated the biodegradation of contaminated soil in an oil company using a combination of microorganisms and nutrients. The study showed degradations between 83% and 88% when using *Bacillus sonorensis* and nutritional supplementation with nitrogen, phosphorus, and Tween 80. The research suggests that the combination of techniques is an advance to be achieved, especially with microorganisms adapted to the contaminant, since they provide degradation.

Due to the biofuel production chain's growth, such as biodiesel, studies have been carried out to evaluate these fuels' bioremediation processes. Biostimulation was evaluated by Kreling et al. (2018), where inactive biomass and mannoproteins extracted from yeast *Saccharomyces cerevisiae* were added in bioremediation soil contaminated with biodiesel. After 60 days of testing, the biodegradation rate of the contaminant reached 81%. Decesaro et al. (2017) used phycocyanin, inactive biomass of the microalgae *Spirulina platensis*, as a biostimulant in the bioremediation of soil contaminated with two different contaminants, diesel, and biodiesel, obtaining after 60 days of testing the removal of 88.75% of the biodiesel present in the contaminated soil and 63.89% of biodegradation of diesel contaminated soil as well, after 60 days of testing.

The strategies of biostimulation and bioaugmentation are considered valid for environmental treatment. However, it is necessary to investigate alternative biocompounds that can assist in the biodegradation of oily contaminants (Lamichhane et al. 2017). Among these biocompounds, lipases and biosurfactants stand out, microbial metabolic agents obtained from different culture conditions and microorganisms, in culture media containing specific inducers for their synthesis. The culture media can be prepared using the most diverse types of macro

and micronutrient sources. In this phase, agro-industrial residues can be used to reduce the production costs of biocompounds and the valuation of these residues (Sperb et al. 2018; Colla et al. 2010). The use of these biocompounds represents an alternative to conventional bioremediation treatments. It may be positive due to the ability to increase the contaminant's bioavailability for microbial degradation, even when low concentrations are used, speeding up the environmental treatment process (Martins et al. 2008; Kumar et al. 2012).

When analyzing the word cloud subdivisions obtained in the bibliometric analysis of Figure 2, it appears that indigenous bacteria or microbial consortia most commonly treat pHAs. An attempt has been made to identify the microbial community involved (words metagenomics and sequencing). Contamination treatments with PCBs usually use GMOs (blue). Soils contaminated with petroleum compounds, in general, including the use of consortia. Still, words come up that include the use of fungi (mushroom, Pleurotus) and nitrogen-fixing bacteria (acetobacter) (purple), as well as a good part of the articles, involve the use of biocompounds, such as lipases and biosurfactants (green), however, the production of lipases and biosurfactants, mainly simultaneously, and their application in soils contaminated with oily compounds is not widely explored, being the object of this study. Also, together with the highlighted keywords, the term "nutrient limitation" is presented. Oily compounds have a hydrophobic characteristic, hindering their degradation through microbial activity, as microorganisms have difficulty using these compounds as a nutritional source for their development. According to Falony et al. (2006) and Burkert et al. (2004), the presence of biocompounds in the soil increases the bioavailability of oily compounds, increasing degradation rates, as it allows the highest assimilation of the contaminant as a nutritional source for microbial growth. In addition, the presence of biosurfactants in contaminated soils has the function of a biostimulant, since they are composed of organic molecules, they provide macronutrients and micronutrients for the development of soil microbiota. This occurs under favorable nutritional conditions, otherwise, it is necessary to supplement the medium by inserting essential nutrients, such as nitrogen and phosphorus. Macro and micronutrient limitations are considered limiting factors to produce biocompounds both *in situ* and *ex situ* (Varjani and Upasani, 2017; Fontes et al. 2008).

3.2 Lipolytic enzymes and their use in bioremediation

Lipases are enzymes responsible for the hydrolysis of oils and greases in fatty acids and glycerol. In addition, they present considerable levels of activity, stability in non-aqueous

environments and are able to catalyze reactions such as esterification, transesterification, acidolysis, alcoholism and ammonolysis. They are part of the family of α/β hydrolases and are widely used due to their versatility and ease of production (Sharma et al. 2018). The mechanism of action of lipases is based on their high specificity to the substrates and on the active sites present, formed by the amino acids serine-histidine-aspartate/glutamate (Barbosa, 2017).

Lipases represent the enzymes with the greatest biotechnological application in industrial processes, with emphasis on the bioremediation of oily compounds, due to their ability to hydrolyze oils and greases (Sandi et al. 2020). Many bacteria, filamentous fungi and yeasts are currently tested to produce lipases (Aguieiras et al. 2018).

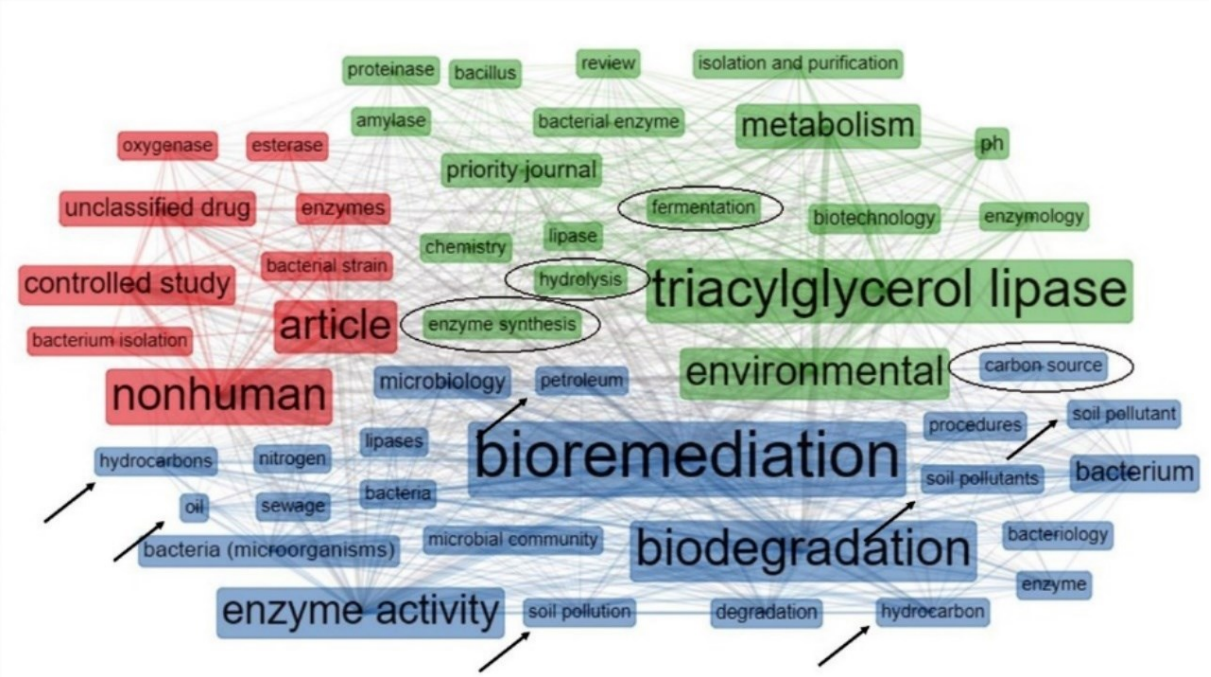
In this context, a content analysis was carried out, among the 24 articles selected from the bibliometric analysis, of scientific articles related to the production of lipase enzymes with the potential for degradation of oily compounds. The scientific articles to be addressed are shown in Table 1.

Table 1 - Production of microbial lipases for use in bioremediation of oily compounds.

Enzyme Production	Producing microorganism	Contaminant	Inductor	Best results	Reference
Submerged fermentation	*Alcanivorax borkumensis*	Petroleum hydrocarbons	Mixture of hexadecane, engine oil and BTEX	Enzymatic production of 3628,57 U/mg; Removal of petroleum hydrocarbons of 73.75% for hexadecane, 82.80% for motor oil, 64.70% for BTEX and 88.52% for contaminated soil.	Kadri et al (2018)
Submerged fermentation	*Aspergillus fumigatus*	-	Tween 80	The highest production of lipases was 2,41 U/mL after 72 hours of incubation at 45 ºC and pH 10.	Mehta et al. (2018)
Tributyrin agar and rhodamine oil agar	*Pseudomonas palleroniana*	-	Olive oil	The highest production of lipases was observed in 7 days of incubation at 25 ºC (28,81 U/mL)	Jain et al. (2019)
Production *ex situ* (soil)	*B. cereus* *B. toyonensis* *L. macroides* *P. illinoisensis* *P. xylanilyticus* *S. hominis* *S. pasteuri*	-	-	High lipase activity with clearance zone diameter produced from 1.6 to 2.2 ± 0.16 mm. 48% of microorganisms were able to degrade Tween 80.	Haldar and Nazareth (2018)
Production *in situ* (oilfield)	*Pseudomonas synxantha* LSH-7	Petroleum hydrocarbons	Crude oil	Lipase activity of 12 μmol.min^{-1}. 50% degradation of the oily contaminant.	Meng et al. (2018)
Production *ex situ* (soil)	-	Petroleum hydrocarbons	Petroleum compounds	Lipase activity decreased between 15d and 22d, with a significant increase in production at 43d ($p<0,05$).	Mora et al (2020)
Production *in situ* (soil)	-	Diesel oil	-	There was an increase in enzyme production in 56 days and a reduction in TPH concentration from 14,221 mg / kg to 270 mg / kg.	Onwosi et al. (2019)

Figure 3 allows assessing the frequency of the words cited when lipases and bioremediation are common terms in database research. Initially, it is possible to observe the relevance of the word's "fermentation", "carbon source", "hydrolysis" and "enzyme synthesis". This indicates that for the period evaluated, there are articles involving the use of enzymes in the bioremediation process that address production processes or the conditions necessary for the synthesis of the biocompound and the carbon sources necessary for enzymatic synthesis. The carbon source is usually an oily inducer for the conversion of the oily compound into fatty acid by microbial metabolism, a hydrolysis process carried out by the lipolytic enzyme, which is produced at this stage of fermentation (Sperb et al. 2015; Salihu et al. 2012; Contesini et al. 2010).

Figure 3 - Thematic map of the 50 most relevant words when searched for "lipase *" AND "bioremediation"



Solid or submerged fermentation are methods used to produce microbial lipases and have advantages, as they allow the use of agro-industrial residues, reducing environmental impacts of destination and reducing production costs. Several residues have the potential for biotechnological use due to accessibility and nutritional composition, containing sources of macro and micronutrients. These residues can have the function of providing nutritional support for microbial growth and as an inducer for the synthesis of lipases, participating in two stages of the bioprocess (Sharma et al. 2001). Many studies have addressed the use of waste and

effluents to produce lipases using wheat bran, rice, soybeans, olive and soybean oils, sugarcane bagasse, dairy effluents, effluents from slaughterhouse and vegetable oil refineries. These residues contain a combination of easily and not easily assimilable substrates that act as inducers to produce lipases by microorganisms (El-katony et al. 2020; Salihu et al. 2012).

Kadri et al. (2018) evaluated the production of several hydrolytic enzymes, including lipases, from the bacterium *Alcanivorax borkumensis* in the submerged culture medium, for application in the biodegradation of petroleum hydrocarbons (motor oil, BTEX, hexadecane and soil contaminated with compounds of Petroleum). The enzymatic production was carried out in reactors containing synthetic seawater in order to simulate oil spills in the environment (high carbon concentrations and nitrogen source restrictions). As inducers for the production of lipases, a primary source of carbon and energy was used, a mixture composed of 3% hexadecane, 3% motor oil and 3% BTEX. The enzymes produced were extracted and applied in batch tests containing different concentrations of the contaminants evaluated. Enzymatic production reached values of 3628.57 U/mg, leading to high biodegradation of petroleum hydrocarbons, which reached 73.75% removal for hexadecane, 82.80% for motor oil, 64.70% for BTEX and 88.52% for contaminated soil. The difference in the percentage of degradation is due to the chemical structure and physical properties of the compounds used in the study.

Mehta et al. (2018) performed the optimization of lipase production, using the fungus *Aspergillus fumigatus* isolated from an oil-contaminated soil, to evaluate possible future applications. The highest production of lipases was observed in the culture medium supplemented with 1% galactose as a carbon source and 0.1% peptone as a nitrogen source (2.41 U/mL) after 72 hours of incubation at 45 ºC and pH 10. Among all the variables tested, there was an increase in the production of lipases, when evaluated one at a time, all of which favored the production, as it presented the fractions of carbon, nitrogen and lipids needed in the culture medium for the extracellular production of lipases. The enzymes produced showed good activity with alkaline pH, with potential for application in bioremediation of soils with oily compounds.

Tanyol et al. (2015) verified the production of lipases from the bacterium *Pseudomonas fluorescens* (NRLL B-2641), using a culture medium composed of residue from the production of sunflower oil as a carbon source, and peptone and ammonium sulfate as nitrogen sources. The study found that the optimum values for the production of lipases are based on a culture medium composed of 11.1% sunflower oil, 1.18% peptone and 0.83% ammonium sulfate, with maximum activity lipolytic of 10.8 U/mL, reached after 48 h of fermentation.

Selvakumar and Sivashanmugam (2017) studied the use of pomegranate, pineapple and orange peel for the production of lipases, aiming at the application in the production of biodiesel. The ideal cultivation condition was verified with a culture medium composed of 35 grams of pomegranate peel, 20 grams of orange peel and 35 grams of pineapple peel, obtaining a lipolytic activity of 9.7 U/mL, in pH 6.0 and cultivation temperature of 30ºC. The enzyme used in this bioprocess was extracted directly from the organic waste from the production of biodiesel.

The carbon sources generally used in the production of lipolytic enzymes using microorganisms come from olive, palm, sunflower, cotton, soy and corn oils, Tween 20 and 40 and fructose. For the enzymatic synthesis, lipid sources of carbon are needed, essential for high performance in the production of lipases (Jimoh and Lin, 2019).

Jain et al. (2019) performed the optimization of lipase production from the bacterium *Pseudomonas palleroniana* isolated from Himalayan soil. The enzymatic production was evaluated at different temperatures and pH, being carried out in a medium enriched with 1% olive oil. In addition, the effects of the oil source (1%), organic and inorganic nitrogen (0.5% and 0.3%, respectively) and non-ionic detergent source (1%) were evaluated. The production of lipases was evaluated daily for 7 days of incubation and expressed in units of lipase activity. The highest production of lipases was observed in 7 days of incubation at 25 ºC (28.81 U / mL). Still, when at low temperatures (4 - 14 ºC), there was slow enzymatic production, however, it continues. The pH significantly affected the production of lipases, with the highest values observed at pH 9 (35.95 U/mL) in 7 days of incubation. The oil was used as a primary carbon source in this study, is necessary as an inducer in the production of lipases, as well as the source of organic nitrogen, and in its absence, there was no enzymatic production. When using detergents, there was an increase in the availability of substrates for bacteria through the formation of emulsion, increasing enzyme production. In addition, the detergents acted as destabilizers of the bacterial membrane, thus releasing lipase enzymes. The lipases produced showed potential for use in bioremediation of oily compounds in regions with low temperatures, since the microorganism is resistant to extreme environments.

The yeast *Candida cylindracea* ATCC 14830 was used by Salihu et al. (2011) to verify the production of lipases using residues from the production of palm oil as a lipid carbon source, and peptone and Tween-80 as nitrogen sources. The highest lipolytic activity, of 20.26 U/mL, was verified using a culture medium composed of 0.45% peptone, 0.65% Tween-80 and 2.2% residue.

Xiaoyan et al. (2017) used cooking oil residue as the only carbon source to produce lipases by *Yarrowia lipolytica*. The inducer was added at a concentration of 30 g/L. In an expansion of the production scale for a 5 liter bioreactor, the production of lipases reached 12.7 U/mL in 24 h of culture.

Oliveira et al. (2017) aimed to produce lipases from *Aspergillus ibericus* by testing different oily residues. The highest production of the enzyme was achieved with 8.5% of palm oil residue, with a lipolytic activity of 127.00 U/g (in relation to the substrate dry mass).

Almeida et al. (2013) verified the production of lipases from the yeast *Candida viswanathii*, testing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5%) of olive oil. As a source of nitrogen, peptone, tryptone, yeast extract and urea were tested. The highest lipolytic activity, of 12.79 U/mg, was obtained using yeast extract and a 1.5% concentration of olive oil as a lipid carbon source.

The use of soybean oil as an inducer was confirmed by Reinehr et al. (2016), who evaluated the production of lipases for different strains of fungi, in 6 d of solid-state fermentation. The highest enzymatic activity (26.04 U/g) was verified for the *Aspergillus niger* strain (O-4), cultivated with the addition of 2% soybean oil inducer and 65% moisture. The humidity variation was also evaluated, with the influence of different percentages (60%, 65% and 70%) on the production of lipolytic enzymes. The statistical analysis carried out by the author found that lower moisture favors the production of lipolytic enzymes.

Numerous microorganisms, mainly bacteria, fungi and yeasts have in their nature a chemical composition that allows the production of biocompounds. Most of them are microorganisms isolated from contaminated soil, effluents, or wastewater and could develop in environments hostile to other microorganisms (López et al. 2018; Ojewumi et al. 2018).

Currently, fungi are the microorganisms most used in the production of lipases, mainly for their ability to produce extracellular enzymes, however, bacteria have received attention for their fast cell growth when related to fungi (Jaeger et al. 1999). Still, lipases produced by bacteria, have the ability to synthesize in mediums with high temperatures and may present activity in mediums with temperatures varying between 40° C and 70 °C, in addition to the wide range of pH in which the production of lipases by bacteria is verified, which may vary from 2.0 to 12.0, showing higher stability in alkaline pH (Cho et al. 2000; Ruiz et al. 2003). Among the bacteria used in the production of lipases, *Pseudomonas* sp., *Bacillus subtilis*, *B. cereus* and others can be mentioned.

Mahanta et al. (2008) evaluated the production of lipolytic enzymes using solid-state fermentation from the bacterium *Pseudomonas aeroginosa*, using Jatropha curcas seeds as a

substrate with a production of 1,084 U/g of lipase activity in 4 days of culture with a medium containing maltose as carbon source, sodium nitrate as a nitrogen source and a moisture of 50%. In addition, Ananthi et al. (2014) seeking to optimize the production of lipases by *Bacillus cereus* obtained a lipolytic production of 407.85 U/g, where onion peel was used as a substrate, 10% moisture, 0.5% maltose (carbon source), 0.5% ammonium bicarbonate (nitrogen source), 0.4% sesame oil as an inducer of microbial growth.

Solid-state fermentation was used to produce lipolytic enzymes by Mazhar et al. (2016), using the bacterium *Bacillus subtilis,* with the best result obtained of 34.93 g/mL in a culture medium containing wheat bran and sunflower residues as a substrate, ammonium chloride as a nitrogen source and banana residue as a source of carbon.

Meng et al. (2018) evaluated the production of several enzymes, including lipases, during the biodegradation process of petroleum hydrocarbons, using bacteria from an oilfield identified as *Pseudomonas synxantha* LSH-7. Lipases were evaluated due to their ability to degrade triacyl glycerides during the degradation of alkanes, therefore, they are used as indicators of oil degradation. Maximum lipase activity was observed at the end of the incubation with a production of 12 $\mu mol.min^{-1}$. Despite presenting moderate activity, the lipase enzyme was essential for the hydrolysis of triglycerides in glycerol and free fatty acids, resulting in the biodegradation of 50% of the oily contaminant.

Haldar and Nazareth (2018) taxonomically evaluated microorganisms from mangroves in Zuari and their ability to produce enzymes to be used in biodegradation processes. Twelve bacterial phyla were evaluated for enzymatic production, with emphasis on lipase activity, carried out in different media and expressed in the diameter of the clearance zone. The microorganisms showed potential for use in bioremediation, due to the diameter of the clearance zone produced from 1.6 to 2.2 ± 0.16 mm. Among the isolated microorganisms, 48% showed the ability to degrade Tween 80, which is a surfactant compound (has a hydrophilic and hydrophobic fraction) and has the characteristic of increasing the solubility of oily substrates in water and, consequently, the bioavailability for microbial action.

Regarding the ideal cultivation conditions for enzymatic production, several studies are based on the search for optimal production conditions, in addition to investigating nutritional requirements and alternative substrates (Sharma et al. 2001).

Jia et al. (2015) evaluated different organic sources (yeast extract, peptone and soybeans) and inorganic sources (sodium nitrate) in the production of the same enzyme using the fungus *Aspergillus niger*, in a culture carried out for 2 days. The inorganic source used at a concentration of 10 g/L produced approximately 1800 U/mL of lipase activity.

Sooch and Kauldhar (2013) analyzed the influence of different cultivation parameters on Pseudomonas sp's lipase production process. BWS-5. Different conditions for the bioprocess were evaluated, such as pH of the medium (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and temperature (25, 30, 35, 37, 40 and 45ºC). It was observed that the highest enzyme activity of 298.0 IU/mL was achieved in 30 h of fermentation, with a culture medium at pH 6.5. For the temperature, the enzyme activity increased linearly with the temperature rise, stabilizing at 37ºC.

Garlapati and Banerjee (2010) sought to optimize extracellular lipases produced by *Rhizopus oryzae* NRRL 3562 in solid-state fermentation. Cultivations were carried out by varying the pH between 4.5, 5.0, 5.5, 6.0, and 6.5 and the temperature between 25, 30, 35, 40, and 45ºC. The highest lipolytic activity of 96.52 U/gds was verified using a pH of 5.5 and a temperature of 35ºC for the bioprocess.

Still, it is possible to observe in the word cloud the presence of the words "petroleum", “hydrocarbons”, “oil,” and “soil pollution”. This indicates the presence of articles related to the use of lipases in the bioremediation of soils contaminated by oily compounds (Mora et al. 2020; Onwosi et al. 2019). Lipases catalyze the total or partial hydrolysis of triacylglycerol, providing diacylglycerol, monoacylglycerol, glycerol, and free fatty acids esterification, transesterification, and interesterification reactions of lipids. These enzymes have a unique ability to act at the oil/water interface, with potential for environmental application, in the bioremediation of soils contaminated with oily compounds, such as petroleum-derived fuels. (Sperb et al. 2015).

Mora et al. (2020) carried out the bioremediation of oily sludge from the bottom of a petroleum company storage tank, using old soil contaminated with hydrocarbons from a petrochemical plant. The study aimed to evaluate the efficiency of hydrocarbon biodegradation, using enzyme activity as an indicator. At the end of the bioremediation, the total removal of hydrocarbons was observed in all tests performed. Lipase activity showed a decrease between 15d and 22d, with a significant increase in production at 43d ($p < 0.05$). The contaminant's high toxicity can explain the initial decline, and the microorganisms go through an adaptation phase for development. The increased production of lipases in time 43d is associated with a decrease in petroleum hydrocarbons and an increase in biodegradable bacteria in the medium. The microorganisms produce lipases that help in the hydrolysis of the most complex structure of the contaminants in more superficial structures, used as a carbon source of easy assimilation. In the same way, intermediate metabolites have been produced that increase the solubility and bioavailability of the contaminants, allowing the microbiota to use the oily compound as a

nutritional source, reducing the concentrations in the environment. (Pacwa-plociniczak et al. 2011; Harms and Bosma, 1997).

Onwosi et al. (2019) used natural attenuation and biostimulation (sawdust) in the bioremediation of soil contaminated with diesel. Several parameters were analyzed to evaluate the bioremediation processes, emphasizing lipase activity and the concentration of total petroleum hydrocarbons (TPH). After 56 days of testing, an increase in enzyme production reduced TPH concentration from 14,221 mg/kg to 270 mg/kg when using sawdust as a biostimulant. At the end of the experiments, there was a decrease in lipase production, probably due to toxic intermediates' formation. Also, exhaustion of nutrients presents in the soil for microbial development.

Sharma et al. (2014) evaluated the bacterium *Pseudomonas aeruginosa* to bioremediate a site contaminated with diesel oil, using the enzymatic activity of enzymes present in the soil dehydrogenase, catalases, and lipases for investigation during the period of bioremediation. The study concluded that the soil's enzymatic activity increased as the hydrocarbon concentration decreased, reaching biodegradation of 66% of diesel oil in 30 days of bioremediation.

The bioremediation process of cooking oil residue, using lipases produced by the fungus *Penicillium chrysogenum* was studied by Kumar et al. (2012), who used an enzyme purified with ammonium sulfate. The highest value of released fatty acid (26.92 mg/g) was verified for the mustard oil residue, demonstrating that the enzyme can be applied in used cooking oil bioremediation processes.

Tsuji et al. (2013) tested the application of lipases from the yeast *Mrakia blollopis* to treat effluent with milk fat. The values of removal of Biochemical Oxygen Demand (BOD) reached 63.8% without the enzyme's addition. In comparison, the effluent treatment added with the enzyme obtained a BOD removal rate of 83.1%, demonstrating that the lipases are viable in the treatment of effluents.

Azhdarpoor et al. (2014) studied surface water treatment contaminated by oily residues using the lipolytic enzyme produced by the bacterium *Pseudomonas* sp. For a residual oil concentration below 8.4 g/L, the percentage of removal of the contaminant was 95%. With the increase of the residual oil concentration in the effluent to 22 g/L, the removal efficiency reached 85%.

Salgado et al. (2016) evaluated the production of enzymes from three filamentous fungi (*Aspergillus ibericus, Aspergillus uvarum,* and *Aspergillus niger*) and the bioremediation of wastewater from olive mills and wineries. Higher reductions in chemical oxygen demand (COD), color, and phenolic compounds (65.1%, 56.1%, and 43.3%, respectively) were seen

when both effluents were treated together, in a 1:1 ratio, by the fungus *Aspergillus uvarum*. However, the highest value of lipase produced was obtained with *Aspergillus ibericus* (1,253.7 U/L) during the wastewater treatment of the olive mill.

3.2.1 Measurement of lipolytic enzymes in soils

The need to evaluate the treatment of oily contaminants in soils has led to the emergence of new methodologies that make it possible to monitor the bioremediation process when lipolytic enzymes are inserted in the medium. Enzymes are indicators of the progress of the biodegradation process of oily compounds, as they catalyze a series of metabolic reactions that lead to the decomposition of organic contaminants. However, they have little explored potential (Margesin et al. 1999).

Evaluating the word cloud, mention is made of determination methodologies with the term “procedures”. However, when making a detailed analysis of the selected articles, few report the determination of the biocompound in soil within the specified period (Mora et al. 2020; Onwosi et al. 2019).

Margesin et al. (1999) evaluated lipase activity as a tool to monitor the biodegradation process. Using a soil contaminated with diesel oil, the author compared two bioremediation techniques, using hydrocarbon biodegradation as a response variable, the lipase activity of the soil and the number of oil-degrading microorganisms in sterile and non-sterile soil, during 116 days, in contamination of 5 mg of diesel oil for each 8 kg of soil. The soil's lipase activity was measured using the modified method described by Pokorna (1964), where the released butanoic acid was extracted with ethyl acetate and quantified by titration with 5 mM NaOH. The authors demonstrated that lipase activity increased with time while hydrocarbon concentration decreased for sterile and non-sterile soil. After reaching maximum lipase activity, production levels remain constant. The maximum lipase activity in the soil with natural attenuation was reached between 90-100 days of bioremediation and 30 days for the biostimulated soil. It was concluded that the enzymatic systems involved in lipid degradation are similar to those involved in the biodegradation of oily compounds. That nutrient supplementation considerably increases microbial biomass and its metabolic activity, capable of degrading hydrocarbons and lipids.

Another study carried out by Margesin et al. (2002) used a colorimetric method to determine lipase activity in soils. The authors evaluated two soil types: simulated contamination of 20 g of diesel oil per kg of soil and soil contaminated by anthropic action. The study demonstrated that the lipases present in recent contamination hydrolyze the substrate twice as

fast as the soil enzymes with old contamination. In the ideal conditions proposed by the methodology (0.1 g of soil, 10 min at 30ºC, pH 7.25 and 1 mM pNPB) there was a lipase activity of 516 and 194 µg pNPB/g soil.min, for contaminated soil recently and to the soil with old contamination. The study also showed that the enzyme activity of the soil increases significantly after contamination.

Cooper and Morgan (1981) defined a methodology for determining lipases in soil. The determination was based on the analysis of four soil types collected in the superficial layer (0-15 cm) of the soil. The method consists of preparing 1 g of soil with the addition of 10 mL of a pH 7.5 buffer solution, incubation at 30ºC for 3 h at 120 rpm, and later centrifugation at 4000 rpm for 15 minutes. A 0.1 ml aliquot of the centrifuged supernatant was pipetted together with 3 ml of 0.1 M buffer solution at pH 7.5 and 0.1 ml (10 moles/L) of 4-methylumbelliferone. The emulsion was placed in a spectrofluorometer at 30ºC for 10 minutes to assess the increase in fluorescence. Through a standard curve, it was possible to correlate the fluorescence response with the 4-methylumbelliferone used. It is necessary to use a standard curve since the response variable can be changed depending on the reaction of 4-methylbuelliferil at different pHs or a substrate's presence.

The use of Tween 20 as a water-soluble substrate was evaluated for the determination of lipase activity in soils by Sakai and collaborators (2002). The test was carried out for the upper fraction of the soil layer (0-10 cm) and did not include contaminants' addition. The methodology was performed with 1.0 g of soil added with 0.2 ml of toluene, 0.6 ml of Tween 20, 1.15 ml of distilled water, and 0.2 mol/L of sodium acetate solution, incubated for 18 hours, at a temperature of 30ºC and agitation of 240 rpm. After this period, 8 ml of ethanol was added, agitated for 10 seconds, and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and titrated with 0.02 mol/L sodium hydroxide. The authors also proved a relationship between the reaction incubation time and the total measured enzymes and that the optimal pH for the reaction of the proposed methodology is 7.5. The results show that a hydrophilic substrate, such as Tween 20, demonstrated a high potential for enzyme determination in soils since hydrophilic substrates can easily interact with the enzyme molecule through diffusion.

Regarding the application of lipase enzymes in soil bioremediation processes contaminating with oily compounds, there is difficulty finding standardized and easily accessible methods for the determination of this biocompound in soils. Enzymes significantly influence microbial biodegradation. However, methods consolidated in the literature are needed so that the enzymatic concentration in contaminated soils is precisely determined to assess their influence concerning other biocompounds added to the soil, such as biosurfactants. In this way,

it may be possible to analyze which biocompound is most likely to biodegrade in a contaminated environment.

3.3 Biosurfactants and their use in bioremediation

In the bioremediation of oily compounds, the biosurfactant's role is to increase the bioavailability of the contaminant through its distribution in the aqueous phase. The biocompound also assists in microbial activity during the biodegradation process. It alters the hydrophobicity and permeability of the cell membrane, increasing the contaminant's absorption by the microorganism (Patowary et al., 2018).

Biosurfactants have production advantages, such as greater tolerances to the variation of pH and temperature ranges, and can be produced in extreme environmental conditions. They are products of microbial origin and present biodegradability in waters and soils, making them ideal for environmental application to treat contaminants. Due to their low toxicity and do not present a risk to human health, they have applications in the food, cosmetic and pharmaceutical industries (Machado et al. 2020; Araujo et al. 2013).

Biosurfactants are classified according to their microbial origin and chemical composition. They are divided into classes such as glycolipids (rhamnolipid, sophorolipid subgroups), lipopeptides and lipoproteins (viscosin, surfactin, polymyxin subgroups, among others), fatty acids, neutral lipids, and phospholipids (fatty acids, neutral lipids, phospholipids) (polymers) mannan-lipid-protein, emulsan, liposan, among others), mannoproteins and sophorolipids (Desai and Banat, 1997).

The type of biosurfactant produced also varies according to the property of the biosurfactant and the molecular weight. Low molecular weight biosurfactants are mainly composed of carbohydrates and aliphatic fatty acids and belong to the class of glycolipids (subclass rhamnolipids and surfactin), being associated with the property of reducing the surface tension of the medium. High molecular weight biosurfactants are mainly formed by lipoproteins, polysaccharides, and proteins related to emulsion formation properties (Rosenberg and Ron, 1999).

According to bibliometric analysis, among the 24 articles evaluated, scientific productions related to biosurfactants in the bioremediation of oily compounds were selected. The articles to be addressed are shown in Table 2.

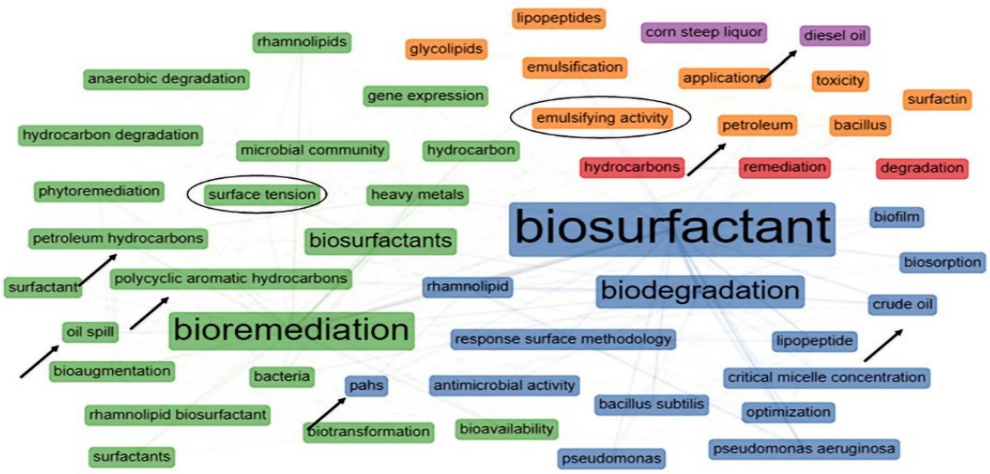
Table 2 - Biosurfactants for use in bioremediation of oily compounds.

Biosurfactant Production	Producing microorganism	Inductor	Contaminant	Treatment technique	Best results	Reference
Production *ex situ* (soil)	*Burkholderia xenovorans* LB400 DSM-17367 *Paenibacillus* sp. *Lysinibacillus* sp	-	TPH e PAH	Bioestimulation/bio augmentation	There was no reduction of the contaminant attributed to the addition of a low concentration of biosurfactant.	Anza et al. (2019)
Submerged fermentation	*Serratia marcescens*	Corn oil	Burnt motor oil	Addition of biosurfactant	Surface tension in the production of biocompound (25.92 mN / m) 94% removal of contaminant from the soil	Araújo et al. (2019)
Production *ex situ* (soil)	*Pseudomonas aeruginosa*	-	Hexadecane	Bioaugmentation/bi osurfactant	97,5% de removal of the contaminant.	Hajieghrari e Hejazi (2020)
-	*Paenibacillus* sp. D9	Frying oil	Motor oil and diesel oil	Addition of biocompounds	Removal of the contaminant (73,2% e 77,60% for motor oil, 71,8% and 74,30% for diesel oil)	Jimoh e Lin (2020)
Submerged fermentation	*Saccharomyces cerevisiae*	Soybean oil	Biodiesel	Addition of biosurfactant	48,75% removal of the contaminant from the soil.	Kreling et al. (2018)
Submerged fermentation	*Saccharomyces cerevisiae*	Soybean oil, glycerol e biodiesel	Biodiesel	Addition of biosurfactant	Higher emulsifying activity (6.95 EU/d) using glycerol 5.71% removal of contaminant from the soil	Kreling et al. (2020)
-	-	-	Petroleum hydrocarbons	Addition of biosurfactant	92,30% removal of contaminant from the soil.	Li et al. (2018)
Production *ex situ* (soil)	*Pseudomonas aeruginosa* PF2	-	PAH	Bioaugmentation/de sorption	The biosurfactant had a positive effect on the desorption of organic compounds	Pourfadakari et al. (2019)
Production *ex situ* (soil)	*Shewanella seohaensis* BS18	-	Hydrocarbons	Fitoremediation/bio augmentation/biosur factant	Best efficiency combining the three techniques	Ram et al. (2019)
Production *ex situ* (soil)	*Sphingomonas changbaiensis* e *Pseudomonas stutzeri*	-	Petroleum hydrocarbons	Bioaugmentation/bi osurfactant	Best removal efficiency combining the two techniques (59%)	Li et al. (2020)

The most frequently cited words when using the terms biosurfactant and bioremediation include mainly “emulsification activity”, “surface tension” (Figure 4). The reduction of surface tension and the formation of emulsions are the main properties of biosurfactants. Emulsification is defined as the dispersion of one liquid into another, leading to a mixture of two immiscible phases. The reduction in surface tension occurs when the concentration of biosurfactant cells in the medium increases, forming micelles (aggregates of biosurfactant molecules). With the formation of micelles in the medium, it is possible to define the Critical Micellar Concentration (CMC), which establishes the minimum concentration of biosurfactants in the medium necessary to reduce the surface tension the maximum (Previdello et al. 2006). Biosurfactants are formed by a hydrophilic fraction (soluble in water and polar) and a hydrophobic fraction (soluble in lipids and nonpolar). Their main properties are the ability to form oil/water and water/oil emulsions and to reduce the surface and interfacial tension of media, justifying the emphasis on words such as "surface tension" and "emulsifying activity". (Satpute et al. 2010).

Likewise, words such as “oil spill”, “crude oil”, “diesel oil”, “petroleum hydrocarbons”, “petroleum”, “PAHs” are observed, which indicate the presence of articles where biosurfactants are used in the remediation of oily compounds Biosurfactants have emulsification properties, phase separation, reduction of surface activity and viscosity of crude oil, with potential for environmental application, mainly in the bioremediation of soils and waters contaminated with oily compounds, accelerating the process and improving the performance of microorganisms (Durval et al. 2020; Vanjani and Upasani et al., 2017).

Figure 4 - Thematic map of the 50 most relevant words when searched for “biosurfactant *” AND “bioremediation”.



Most of the biosurfactants' production from microorganisms is reported by bacteria and yeasts, such as *Bacillus* sp. and *Candida* sp. (Decesaro et al. 2013; Rufino et al. 2014). Fungal biosurfactants also show the viability of cultivation and production and can present good yields, mainly when animal fat, glycerol, and oleic acids are used as substrates (Bhardwaj et al. 2013). The study of varied cultivation conditions is a relevant aspect when the aim is to expand the production scale, as changes in the composition of the culture medium, such as pH, temperature, type, and concentration of soluble and insoluble carbon sources, lead to changes in the yields of the biosurfactant, also modifying the structure and properties of the biocompound produced. These factors are crucial when making biosurfactants for specific applications (Luna et al. 2015).

Almeida et al. (2015) evaluated different concentrations of residual carbon sources for biosurfactant production by Candida tropicalis, such as molasses, millhocin (residue from corn production), canola oil under other conditions of agitation, and percentage of inoculum added to the medium. The production was more effective (yield of 30g/L of biosurfactant and reduced surface tension to 29.52 mN/m) when 2.5% molasses, millhocin, and canola oil were used in the medium, with 250 rpm agitation and inoculum 2%.

A strain of the fungus *Cunninghamella echinulata* was isolated from the Brazilian Caatinga and evaluated for biosurfactants' production by Silva et al. (2014). The experiments were carried out using soybean oil residue (0.62%, 3.0%, 6.50%, 10.0% and 12.38%) and corn liquor (2.64%, 4.0 %, 6.0%, 8.0% and 9.36%) during 96 h of submerged fermentation. The culture medium's surface tension was reduced to 36 mN/m when 3.0% soybean oil residue and 4% corn liquor were used and reached an emulsification rate of 80% with motor oil as a forming agent emulsion.

Liu et al. (2011) evaluated the production of glycolipids by *Ustilago maydis* using different concentrations of glycerol as a carbon source (10, 20, 50, 80, 100, 120, 150, and 180 g/L) and several sources of inorganic nitrogen (ammonium sulfate, ammonium nitrate, and ammonium citrate) and organic (urea, tryptone, peptone, yeast extract). When 50 g/L of glycerol was used, the best yield of glycolipid production (6.7 g/L) was observed. Among the nitrogen sources tested, a higher yield in the production of glycolipids (4.8 g/L) was obtained when ammonium citrate was used as a nutritional source.

Rodrigues et al. (2014) studied the production of biosurfactants by the fungus *Aspergillus flavus* through solid-state fermentation for eight days, varying the carbon sources (olive oil and soybean oil) and the nitrogen sources (urea and sodium nitrate), all at a fixed

concentration of 3%. The highest water-in-oil emulsifying activity was obtained for the experiment using 3% urea and 3% soybean oil (79.75 EU).

Kiran et al. (2009) optimized biosurfactants' production using the fungus *Aspergillus ustus* MSF3, isolated from a marine sponge. The cultures were carried out in SDA medium (peptone, dextrose, agar, and distilled water), varying the pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), temperature (10ºC, 20ºC, 30ºC, 40ºC, and 50ºC), 1% carbon source (glucose, olive oil, kerosene, and vegetable oil) and nitrogen (peptone, yeast extract, and urea). The highest emulsification index obtained in the study was 30% when glucose was used as a carbon source and 25% when yeast extract was used as a nitrogen source, for an optimum pH of 7.0 and a temperature of 20ºC, for a 120 h period.

Santos et al. (2014) evaluated the optimization of biosurfactants' production from *Candida lipolytica* in a culture medium containing 5% animal fat and 2.5% millhocin. The conditions of agitation (200, 300, and 400 rpm), aeration (0.1 and 0.2 volume of air per volume of medium), and time (48, 96, and 144 hours) were varied through experimental design 2^3) of the culture medium. The ideal condition was verified with agitation at 200 rpm, 144 hours of cultivation, and without aeration, favoring the reduction of surface tension (21.20 mN/m) and biomass production (7.32 g/L).

Castiglioni et al. (2009) used solid-state fermentation to produce biosurfactants from the fungus *Aspergillus fumigatus*. Rice husk and bran were used as a carbon source (15:85) and 1% soybean oil and diesel oil as inducers for biosurfactants' production. However, higher yields were observed when there was no supplementation of the culture medium with the presence of inductors (11.17 EU) compared to the addition of soybean oil (8.47 EU) and diesel oil (9.99 EU).

Biosurfactants help treat contaminants due to their mobilization properties, solubilization, and emulsification, which result in increased biodegradation. Through emulsification, biosurfactants increase pollutants' bioavailability, mainly hydrocarbons, for biodegradation (Lin, 2011).

The high molecular weight biosurfactants promote an increase in the emulsification of oily compounds. They can disperse in immiscible liquids, and the hydrophobic fraction of the molecule will bind with the oil adsorbed in the soil matrix. The hydrophilic fraction will be bound to the aqueous parts. soil particles (Nievas et al. 2008; Costa et al. 2010). Biosurfactants characterized as having high molecular weight can form emulsions with high efficiency and are therefore applied as additives to stimulate bioremediation.

Biosurfactants with the characteristic of reducing the medium's surface tension are classified as low molecular weight, forming micelles (aggregates of biosurfactant molecules) that destabilize the forces of fluid cohesion, reducing the surface tension among them. In bioremediation processes, the reduction in surface tension between fluids such as air, water, and contaminants (especially oily compounds) occurs in soil particles, increasing bioavailability and increasing biodegradation (Uzoigwe et al. 2015).

Anza et al. (2019) carried out the bioremediation of soil contaminated with total petroleum hydrocarbons and polycyclic aromatic hydrocarbons, using biostimulation and bioaugmentation techniques associated with rhizoremediation. The contaminated soil was supplemented with rhamnolipid biosurfactant and sodium dodecyl benzenesulfonate to increase the contaminant's bioavailability and biodegradability. At the end of the trials, there was no significant reduction in any treatments, including the control trial, where there was no application of bioremediation techniques, only monitoring. The results were attributed to the use of low concentrations of the biosurfactant and sodium dodecyl benzenesulfonate. The proper concentration of biosurfactants is related to different factors such as the nature, concentration, and recalcitrance of the contaminant and the soil's physical and chemical properties to be treated.

Araújo et al. (2019) evaluated biosurfactants' production by *Serratia marcescens* UCP 1549 in a fermentative medium containing cassava flour wastewater. A complete factorial design was carried out to select the best culture conditions. Subsequently, tests were carried out to remove burnt engine oil from sandy soil by applying the produced biosurfactant. *Serratia marcescens* showed a higher reduction in surface tension (25.92 mN/m) in the medium containing 0.2% lactose, 6% residual water from cassava flour, and 5% residual corn oil, after 72 h of fermentation at 28°C and 150 rpm. The microorganism used lactose and residual corn oil as carbon sources (carbohydrates and fatty acids) to induce the production of secondary metabolites. Likewise, wastewater was used mainly due to the presence of nitrogen. Biopolymers' high production can be attributed to the relationship between carbon and nitrogen present in the medium. For the production of secondary metabolites, a ratio of 50: 1 of C/N is recommended. The biosurfactant produced showed 94% removal of the contaminant from the sandy soil due to its ability to emulsify and solubilize the oily compound in water, reducing its surface tension and, consequently, biodegradation.

Hajieghrari and Hejazi (2020) investigated the biodegradation of hexadecane in soil using the bacterium *Pseudomonas aeruginosa*, free and isolated, known to be a producer of biosurfactants. The highest biodegradation of the contaminant was observed when using

immobilized bacteria (95.70%) and the highest degradation rate (0.06 day-1). Although the biosurfactant measurement was not performed, the study highlighted the ability of *P. aeruginosa* to produce rhamnolipid biosurfactants in previous studies. The high biodegradation of the organic contaminant is due to the biosurfactant structure, which has a complex and diversified effect on the performance of microorganisms, including changes in functional groups and enzymatic activities.

Jimoh and Lin (2020) evaluated the potential for using frying oils as a low-cost raw material in the production of biosurfactants by *Paenibacillus* sp. D9. The Biosurfactant produced was used in the bioremediation of soils and waters contaminated with engine oil and diesel. The biosurfactant was evaluated using the response surface methodology and presented the highest tension and yield results of 31.2 mN/m and 5.31 g/L, respectively. For bioremediation, a comparison was made between the biosurfactant and the chemical surfactant, with the best results obtained with the biosurfactant (73.2% and 77.60% for engine oil, 71.8% and 74.30% for diesel), respectively, on contaminated soil and water. The contaminant's amphiphilic nature was an essential factor in the solubilization of the oily contaminant, reducing surface tension and increasing the bioavailability of hydrophobic compounds, acting as a facilitator for local microbiota to use the contaminant as a nutritional source. Although the chemical surfactant has shown significant degradation values, it can cause toxic effects to the environment, favoring the biological surfactant's use.

Kreling et al. (2018) evaluated the bioremediation process for soil contaminated with biodiesel, and the soil treatment carried out by adding 0.5% of mannoprotein, an intracellular biosurfactant obtained from the yeast *Saccharomyces cerevisiae*, resulting in the removal of 48.75% of the contaminant, over 60 days of bioremediation.

The production and application of extracellular biosurfactants obtained by Kreling et al. (2020) were evaluated on biodiesel's biodegradation as a soil contaminant. The maximum production of the emulsifying agent (6.95 UE/d) was verified for the addition of 5g/L of glycerol as an inducer of biosurfactants' production in a culture medium of pH 5.5 and temperature of 30 ºC. The addition of 0.5% of this biosurfactant allowed removing 56.71% of the contaminant in the soil after 90 days of bioremediation.

Li et al. (2018) performed the bioremediation of soil contaminated with petroleum hydrocarbons for 36 days, using the nutrient solution and two biosurfactants (rhamnolipids and Tween 80). A positive effect was observed in the addition of nutrients. The removal of 92.30% of the contaminant was observed with the addition of 150 mg/kg of the rhamnolipid

biosurfactant. Rhamnolipids promoted the higher metabolic activity of microorganisms in the assimilation of hydrocarbons when compared to Tween 80.

Pourfadakari et al. (2019) investigated the performance of a biosurfactant produced by *Pseudomonas aeruginosa* PF2 in the desorption of polycyclic aromatic hydrocarbons from contaminated soil. The biosurfactant produced was characterized as rhamnolipid, with a critical micellar concentration of 60 mg/L and an emulsification index of 60.2% for n-hexadecane, 58.40% for h-heptane and 55.60% for h-hexane, 45.7% for diesel oil, and 33.40% for crude oil. It also had a positive effect on the desorption of organic compounds from contaminated soil.

Ram et al. (2019) evaluated the combined use of a rhamnolipid biosurfactant with phytoremediation techniques and bioaugmentation of soil contaminated with hydrocarbons. Bioremediation was performed with the microorganism *Shewanella seohaensis* BS18. When using diesel oil as a carbon source, production of 2.2 mg/g of rhamnolipids in dry weight was observed, with a reduction in surface tension of 28.6 mN/m and an emulsification potential 65.6%. The lowest hydrocarbons (2.1 mg/g of non-sterile soil) were observed when combined, bioaugmentation, phytoremediation, and the addition of the biocompound. A larger microbial population was produced in the same treatment, degrading the contaminant and higher plant biomass production. The treatments' efficiency is associated with a reduction in surface tension, increasing the contact area between bacteria and the contaminant. Also, there is a decrease in the interfacial tensions between water and oil, increasing hydrocarbons' solubility in the soil's aqueous phase.

Li et al. (2020) investigated the effects of polyglycosidic alkyl biosurfactant (APG) on improved biodegradation of soils contaminated with petroleum hydrocarbons using *Sphingomonas changbaiensis* and *Pseudomonas stutzeri*. Among the tests carried out, the results showed 39.2 ± 1.9% and 47.2 ± 1.2% of hydrocarbon degradation for bioaugmentation treatments with *Sphingomonas changbaiensis* and *Pseudomonas stutzeri*, respectively. The addition of the biosurfactant improved the bioremediation processes and the rates of biodegradation. The rates of biodegradation using *Sphingomonas changbaiensis* and *Pseudomonas stutzeri* associated with APG were 52.1 ± 2.0% and 59.0 ± 1.8%, respectively. The biosurfactant's addition increased the solubility and sorption of petroleum compounds in microbial cells, stimulating their activity and metabolism.

Morais and Abud (2012) evaluated the bioremediation potential of sandy soil contaminated with oil using a 1.5% concentration of biosurfactant produced by the yeast *Yarrowia lipolytica*, reaching a percentage of contaminant bioremediation of 78%.

The application potential of biosurfactants produced by *Candida sphaerica* and *Bacillus* sp. was evaluated by Chaprão et al. (2015). The authors confirmed that both biosurfactants were able to remove engine oil from sandy soil by 90% and 40% for the biosurfactants of *C. sphaerica* and *Bacillus* sp., respectively. It was observed that when molasses was added to the medium, the biodegradation of the oil contaminant reached approximately 100% for the biosurfactant of *Bacillus* sp. in 90 days of testing.

3.3.1 Measurement of biosurfactants in soils

The analytical determination of biosurfactants in soils still requires further studies to consider the type of biosurfactant produced, whether of high or low molecular weight and its classification, since both factors directly influence the chemical composition of the biosurfactant and, in the method of soil determination.

For the assessment of biosurfactants produced *in situ*, Ángeles and Refúgio (2013) adopted a method of the previous extraction of the biocompound in soil contaminated with oily compounds for the subsequent determination biosurfactants by reading the surface tension. The biosurfactants present in the soil were extracted using an electrolytic solution composed of 0.01 mol/L of potassium nitrate, 0.01 mol/L of tris hydrochloride, and 0.003 mol/L of sodium azide at pH 7.00 in a proportion of 1:2 of soil: solution (v/m). For the extraction, 10 mL of the solution was added in a tube containing 5 g of sample, with constant agitation at 240 rpm for three days at room temperature and kept for two days without agitation. Subsequently, the solution was filtered through a 0.22 μm filter, and the obtained solution was used to measure the surface tension. By evaluating the production of biosurfactants *in situ* through the analysis of the reduction in surface tension over 10 d of the bioremediation process, for soil contaminated with hydrocarbons, at the end of the test, they obtained a decrease in surface tension from 75.20 mN/m to 55.60 mN/m (26.06%) and 57.20 mN/m (23.94%), when using the techniques of biostimulation and bioaugmentation and only biostimulation, respectively.

Machado (2020) evaluated the surface tension as a response variable for the production of biosurfactants in situ in the soil contaminated with 20% of diesel oil, showing a reduction in the surface tension in the 30 to 60 days for the treatment of the contaminant, in tests where biostimulation and bioaugmentation were used together, and separately biostimulation and bioaugmentation, obtaining reductions from approximately 60.00 mN/m to 53.00 mN/m, equivalent to 11.67%.

Li et al. (2018) performed the bioremediation of soil contaminated with petroleum hydrocarbons and used the methodology for determining fatty acid phospholipids (PLFA). Lipid extraction is achieved with a mixture of chloroform, methanol, and phosphate buffer (ratio 1:2:0.8). The lipid extracts are then separated into neutrals, glycol, and phospholipids in silicic acid columns. After extraction, the phospholipids are separated into saturated, polyunsaturated, and monounsaturated fatty acids, quantified by gas chromatography (Helfrich et al. 2015).

Analytical determination from PLFA is used as an indicator of microbial development during bioremediation (Frostegard et al. 2011) and when biosurfactants are present in the bioremediation of organic contaminants (Mair et al. 2013). The use of this technique is promising for the evaluation of the biodegradation of pollutants in the soil, very limited in its description in the literature (LI et al., 2018).

Youssef et al. (2007) demonstrated the application of *in situ* biosurfactants produced by two different Bacillus strain*s* through microbial petroleum recovery (MEOR). The activity of biosurfactants was determined through the concentration of lipopeptides. The quantification of lipopeptides was performed using high-performance liquid chromatography (HPLC), using a C18 column with a reverse phase and 60% acetonitrile in water with a mobile phase. A 20 mL aliquot of 1:4 and 1:2 dilutions of the sample were injected into the column, with biosurfactant retention times 2, 2, and a half and 3.1 minutes, which correspond to three different fatty acid fractions of the lipopeptides. The areas of the three peaks were added, and the concentration was calculated from a standard curve. For 30 hours after the test, an average production of 90 mg/L of lipopeptide-type biosurfactant was observed, demonstrating the feasibility of using this technique.

The same author demonstrated (Youssef et al. 2013) the production of lipopeptides *in situ* by *Bacillus* strains aiming to use the microorganism petroleum recovery technique (MEOR). Biosurfactants' production was measured by high-performance liquid chromatography (HPLC), reaching the maximum concentration of 28 mg/L and 20 mg/L for each of the two walls evaluated, a concentration higher than the minimum required for the success of the application of the technique (11 mg/L).

For the determination of biosurfactants *in situ*, there is difficulty in adopting methods that evaluate biosurfactants' production with accessible value since most current methods have high costs, such as chromatography. The extraction of biocompound in the soil is not widely reported in the literature. It is not possible to say that the method is viable for all types of biosurfactants produced and added to the soil in the treatment of the contaminant, being

necessary to consider the specificity of the properties of the biosurfactant, such as high or low molecular weight.

Thus, the best bet is the techniques that only evaluate the contaminant and microbial growth degradation. These methodologies are indirect indications of the performance of the biocompounds in the environment, proving, in the same way, its efficiency about techniques for monitoring the contaminant, such as natural attenuation. Another determining factor for measuring biosurfactants in soils is the amount of biosurfactant needed to be applied to the soil. As these concentrations are generally low, below 1%, it is challenging to detect biosurfactants in the medium.

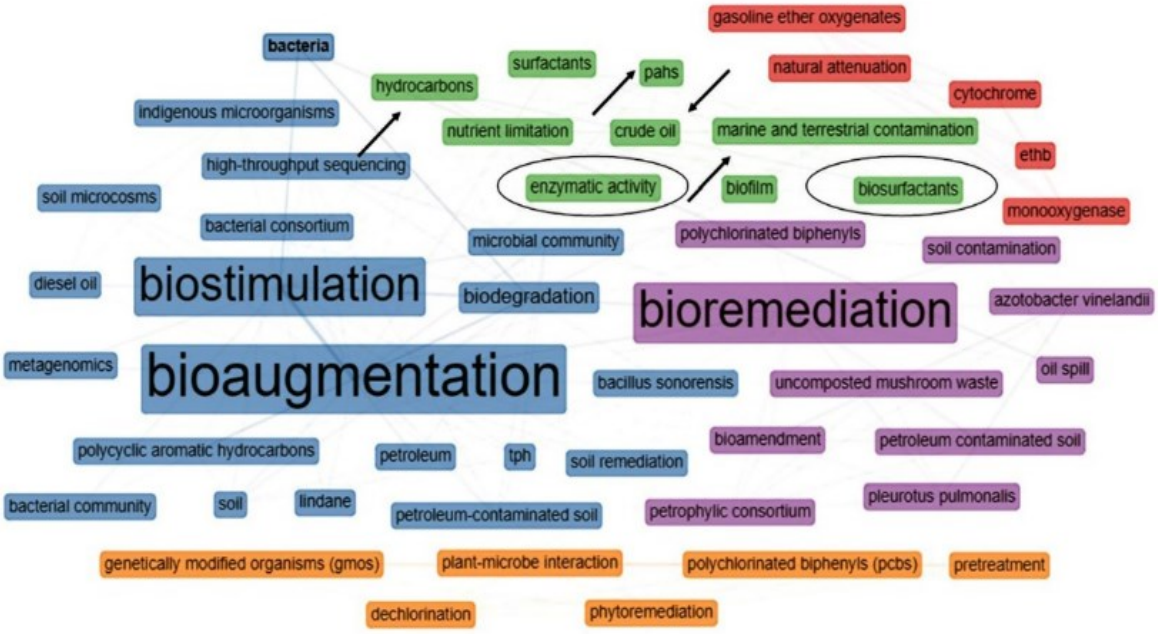
3.4 Relationship between biosurfactants and lipases in bioremediation process

Figure 5 allows evaluating the most cited words when lipases, biosurfactants, and bioremediation are standard terms in database research. First, it is possible to observe that the words "enzymatic activity" and "biosurfactant" are mentioned together with the words "marine and terrestrial contamination", "crude oil", "hydrocarbons," and "pahs". This indicates that relevant articles involve enzymes and biosurfactants in the bioremediation process, used mainly in the biodegradation of oily compounds.

Among the enzymes used in bioremediation, lipases stand out, which assist in the hydrolysis of triglycerides present in the oily contaminant of glycerol and free fatty acids, facilitating the use of the oily compound as a microbial nutritional source (Karigar and Rao, 2011). Thus, the higher the number of enzymes available for microbial use, the higher the interaction between the substrate (contaminant) and the microorganism through the formation of the enzyme-substrate complex, allowing higher pollutant degradation.

Another factor associated with the biodegradation of oily compounds is the availability of the contaminant. Many contaminants have an apolar character, which in most cases is not compatible with cell membrane entry and transporter sites, making them unavailable for intracellular metabolism. Some microorganisms circumvent this obstacle by producing biosurfactants and allowing nonpolar molecules' access into the cell (Gaylarde et al. 2005).

Figure 5 - Thematic map of the 50 most relevant words when searched for “lipase *” AND “biosurfactant *” AND “soil bioremediation”



Different authors report the production of biocompounds through microbial metabolism, using waste and oily compounds as the primary carbon source, used as essential nutrients to obtain significant amounts of lipases and biosurfactants (Saharan et al. 2011). There is a relationship between the production of lipases and biosurfactants, which can be justified by the need for microorganisms to metabolize compounds that are not soluble in water. Biosurfactants can be produced from reactions catalyzed by lipases (Desai and Banat, 1997; Paula et al. 2005).

The relationship in the production of both biocompounds can be justified by the lipolytic enzyme's action, responsible for the hydrolysis of triacylglycerol in fatty acids and glycerol (Kiran et al. 2016), from oily inducers. Fatty acids and glycerol can be metabolized according to the metabolic route explained by Fontes et al. (2008), producing biosurfactants. This metabolism can occur to form the nonpolar (lipid) fraction of the biosurfactant molecule, as easily assimilated carbon sources are used to synthesize the polar fraction of the biosurfactant molecule. Perfumo et al. (2018) also indicate that the synthesis of biosurfactants is associated with the production of extracellular enzymes, such as lipases, promoting the solubilization and mobilization of hydrophobic inducers thus increasing their bioavailability.

Few articles report the use of both biocompounds in the same bioremediation process for the period established in the bibliometric analysis (2018-2020). Mainly, the production of lipolytic enzymes is used to evaluate the production of biosurfactants to be inserted in

bioremediation processes. Studies show that lipolytic enzymes are closely related to the organic pollutants present in the soil. This enzyme activity is responsible for the reduction of a large part of hydrocarbons from contaminated soils and spills of oily compounds. Lipolytic activity is a very close indicator for assessing hydrocarbon degradation in soil, and it can serve as a parameter for the assessment of bioremediation (Karigar and Rao, 2011).

In this context, among the 24 articles evaluated in bibliometric analysis, seven papers were selected for content analysis involving lipases and biosurfactants in bioremediation processes of contaminated soils.

Kreling et al. (2020) evaluated the coproduction of lipases and biosurfactants using the fungal strain of *Aspergillus niger* and applying these biocompounds in a bioremediation process. In a culture medium composed of 80% wheat bran and 20% corn cob, 5% soybean oil and 60% moisture, biosurfactants (6.67 ± 0.06 EU) and lipases (10.74 ± 0.54) were produced U). When 10% of the culture medium containing the biocompounds was applied in bioremediation of soil contaminated with biodiesel, degradation of 64.09% of biodegradation of the contaminant was obtained after 90 days of testing. The study proved the possibility of simultaneous production of biocompounds and their application in environmental treatment.

Ra et al. (2019) evaluated the production of lipases and biosurfactants by different isolated microorganisms and their application in the bioremediation of soil contaminated with petroleum. 13 bacterial strains and four fungal strains were tested in the production of biocompounds and biodegradation of the oily contaminant. Biosurfactants' production was evaluated through an emulsification test, showing the best emulsification rates with four bacterial strains, A5, A14, G1, and G6 (60.87; 56.5; 58.33 and 41.67%, respectively). The plate method evaluated the lipase activity, which assesses the increase in diameter and the color change around the agar cavities. The best results were obtained by bacterial strains A1, G5, and G1 with diameters of 10, 8.50, and 7.75 mm. The G1 bacterial strain identified as *Acinetobacter calcoaceticus* showed the production capacity of both biocompounds and the petroleum compound's biodegradation (61.11 ± 2.30%).

Louhasakul et al. (2020) studied the production of lipases and biosurfactants by the microorganisms *Yarrowia lipolytica* and *Bacillus subtilis*, respectively, using industrial residues of palm oil for their valorization with the production of biocompounds and, later use as a raw material in the production of biodiesel. *B. subtilis* induced by crude glycerol showed concentrations of 1180 ± 60 mg/L, evaluated through surface tension, which showed a reduction from 70.25 to 34 mN/m. The lipases produced by the yeast obtained better results when induced by glycerol in the presence of biosurfactants (586.8 ± 41 U in total). There was no lipase activity

without the presence of the biocompound. The biocompounds produced showed synergy in the residue's degradation, reducing the interfacial lust between oil and water and increasing the affinity of microorganisms to hydrophobic surfaces. Besides, there was higher absorption of nutrients and lipid production, making the process promising and low cost to produce biodiesel.

Ojha et al. (2019) evaluated the bioremediation of indeno (1,2,3-cd) pyrene (InP) and also the production of biosurfactants by seven isolated yeasts using biosynthesized iron nanoparticles. The production of the biosurfactant was evaluated by different methodologies, among them lipase activity. The strain that showed the highest potential for biodegradation of InP (90.68 ± 0.7%) and biosurfactants' production (emulsification index of 69.9%) was identified as *Candida tropicalis* NN4. With the production of biosurfactants, the assimilation of hydrophobic and hydrophilic substrates was facilitated, allowing higher cell growth and higher production of lipases.

Likewise et al. (2018) studied biosurfactants' production to evaluate the potential in the bioremediation of petroleum compounds, using one of the analysis methodologies, the lipase activity directly involved in the emulsification of the biosurfactant. Bacterial species from brackish waters were isolated and tested for their ability to produce biosurfactants. Among the six isolated microorganisms, BS-6, identified as *Bacillus tequilensis*, presented the best results of the emulsification index (70%), and lipase activity, by the plate method, showed free zones, indicating high emulsification.

Larik et al. (2018) evaluated the production of lipases and biosurfactants during biodegradation tests of diesel and engine oil. The bacterium *Stenotrophomonas maltophilia* strain 5DMD isolated from oil drilling mud was used. The microorganism showed high potential for the production of lipases (lipolytic zone=1.8±0.1 cm and 2.2±0.3 cm) and biosurfactants (oil displacement test => 6 mm; surface tension = 33.9 and 34.4 mN/m) for diesel oil and engine oil, respectively. Biodegradation rates were assessed by UV-Vis spectrophotometric analysis, resulting in 80.5% for diesel oil and 70.8% for engine oil.

Suganthi et al. (2018) used microbial consortia to produce biocatalyst enzymes (lipases, oxyreductases, and catalases) and biosurfactants to maintain the constant level of bottom sludge in oil hydrocarbon tanks. The microorganisms showed oil degradation capacity and production of lipases (80 U/mL) and biosurfactants (152 mg/g of oil sludge) constantly, with a 96% reduction in total petroleum hydrocarbons. Complementary analyzes identified the complete degradation of hydrocarbons, making the process sustainable and promising for oil refineries.

Still, mention is made of authors who carried out the coproduction of lipases and biosurfactants in solid-state fermentation, using agro-industrial residues for bioremediation.

The scientific production to be addressed is related to a period before bibliometric analysis (2008-2015).

Colla et al. (2010) investigated the existence of a relationship between lipases and biosurfactants' production. The author obtained a linear relationship for the coproduction of lipases and biosurfactants when using submerged fermentation, up to 91%.

Velioglu and Urek (2015) evaluated the production of the fungus *Pleurotus ostreatus* in solid-state fermentation with a culture medium composed of sunflower husk and sunflower seed oil. In 5 d of cultivation, it was possible to verify that the surface tension was reduced to 30.6 mN/m.

Martins et al. (2008) evaluated the coproduction of biosurfactants and lipases by two different microorganisms *Phialemonium* sp. and *Aspergillus fumigatus,* for the use of compounds in bioremediation of soils contaminated with vegetable oils and hydrocarbons, using solid-state fermentation. The maximum lipolytic activity found for the fungus *Phialemonium* sp. was 129.50 U g-1 and 5.07 EU g^{-1} of water/oil emulsifying activity. For the *Aspergillus fumigatus* fungus, the maximum lipolytic activity was 119.46 U $g^{-1,}$ and emulsifying activity 7.36 EU g^{-1} for water/oil, where the amount of lipase generated in the fermentation medium did not interfere with the production of biosurfactants.

4. FINAL CONSIDERATIONS

Contamination of soils with oily compounds is a problem that requires proper treatment. Bioremediation is a sustainable technology, increasingly used in cases of accidents, to mitigate the impacts generated on the environment and public health. The application of biocompounds is viable, in this context, individually or through joint processes.

Lipases are enzymes with great potential for use in the bioremediation of soils contaminated with oily compounds due to their ability to hydrolyze complex fatty acids into simple structures and easily assimilated by microorganisms. They can be produced through microbial metabolism in different culture media, have high activity even in adverse conditions, and increased stability and specificity to the substrates.

Furthermore, the biodegradation of the contaminant is directly associated with its availability in the soil's aqueous phase. Because it has no polar character, it is not compatible with the cell membrane entry and transport sites, making it impossible to enter the cell. To enable the degradation of the contaminant, some microorganisms produce biosurfactants that, through emulsification and reduction of surface and interfacial tension, increase the

bioavailability of hydrophobic compounds in the aqueous phase of the soil, allowing their degradation through microbial metabolism, being used as a source nutritional.

For the synthesis of both biocompounds, the literature points out the importance of using oily inducers as a source of lipid carbon, used in vegetable oils or residues from the petroleum industries. Oily compounds, which when used in the culture medium as inducers, are used by microorganisms as a nutritional source for the formation of the biocompounds of interest. Initially, the lipases produced make the hydrolysis of triacylglycerols into fatty acids, subsequently metabolized to form the polar and nonpolar fraction of the biosurfactants.

The research pointed out that the study of enzyme production and biosurfactants is well established in the literature. However, most of them treat the production of biocompounds in isolation or use enzyme production to indicate the production of biosurfactants. Few scientific articles have been found that address the simultaneous production of lipases and biosurfactants. Still, they are relevant studies, which report the production of two biocompounds with high added value in the same biotechnological process. Besides, the direct application in bioremediation processes is little explored, requiring advances in publications involving the environmental application of these biocompounds.

Producing lipases and biosurfactants are also viable when using agro-industrial waste, one of the areas of significant development in industrial biotechnology. Low-cost substrates' choice generates a tremendous economic impact on the production processes, significantly reducing the total cost at the end of the bioremediation. In general, biocompounds are produced and undergo downstream processes for subsequent application, increasing bioremediation costs. When using agro-industrial residues in fermentative production processes, for example, biocompounds have the potential for direct application in the bioremediation of contaminated soils since several microorganisms present in soils can use lignocellulosic, pectinolytic, or amylolytic compounds as a nutritional source, culture medium used as a source of bio-stimulus, through the insertion of macro and essential micronutrients and, bio-increase, with the addition of active microorganisms present in the fermentation.

Still, the difficulty arises in measuring, through methodology already established in the literature, the specific behavior of these biocompounds and their behavior in the soil over the time of bioremediation, since the currently available chromatographic techniques involve high cost, and the analytical determinations, financially viable, take a long time.

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3. CAPÍTULO 3: SIMULTANEOUS PRODUCTION OF LIPASES AND BIOSURFACTANTS IN SOLID STATE FERMENTATION AND USE IN BIOREMEDIATION[2,3]

Abstract: The researchers aimed the simultaneous production of lipases and biosurfactants and the use of the culture media containing the biocompounds (without the process of precipitation and purification) in bioremediation of contaminated soil. Different concentrations of agroindustrial raw materials, moisture, oil inducers and nitrogen were studied. The bioremediation assay was evaluated for 90 days, using biodiesel as a contaminant, adding the fermented solid culture medium in soil and comparing it to natural attenuation. The culture media was composed of wheat bran:corncob (proportion 80:20), 5% of inducer and 60% of moisture, inoculated with *Aspergillus niger*, allowing the simultaneous production of biosurfactants (6.67 ± 0.06 UE) and lipases (10.74 ± 0.54 U). In the bioremediation assay, the highest degradation of contaminant (64.09%) was found after 60 d using 10% of the fermented solid culture medium containing lipases and biosurfactants. We allowed the coproduction of both biocompounds in the fermentation process, representing cost reduction and viability potential of the bioprocess when considered an industrial production scale, as well as its environmental application in the treatment of oily contaminants.

Keywords: *Aspergillus niger,* biodegradation, biodiesel, contaminated soil, emulsifying activity, natural attenuation.

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4. CAPÍTULO 4: IMPROVING THE BIOREMEDIATION AND IN SITU PRODUCTION OF BIOCOMPOUNDS OF A BIODIESEL-CONTAMINATED SOIL[4]

Abstract: The reduction of the impact of contaminations using not purified biocompounds produced by solid state fermentation (SSF) can contribute to the valuation of agro industrial residuals and reduction of contamination caused by oily compounds. We aimed to produce simultaneously biosurfactants and lipases in SSF using *Aspergillus niger*, followed by the use of the fermented media obtained on the bioremediation of oily contaminated soil. The biocompounds were produced using wheat bran and corncob (80:20), 5% of soybean oil and 0.5% of sugar cane molasses in SSF for 4 d, producing 4.58±0.69 UE of emulsifying activity and 7.77±1.52 U of lipolytic activity. This fermented media was used in the bioremediation of a 20% biodiesel contaminated soil, evaluating for 90 d microbial growth, contaminant degradation and production of lipases and biosurfactants in soils. Six experimental strategies (Natural attenuation; Biostimulation + Bioaugmentation + Biocompounds; Biostimulation + Biosurfactant; Biocompounds extract; Biostimulation; Adsorption of contaminant) were realized. The highest degradation of contaminant was verified in 90 d, of 74.40±1.76%, and the production of biosurfactants and lipases in situ in the soil was found in 30 d (6.02±0.24% of reduction in surface tension and 6.62±0.17 UL of lipid activity in soil) for the same experiment (Biostimulation + Bioaugmentation + Biocompounds). The use of solid fermented culture medium containing both biocompounds was feasible for the treatment of contaminants, demonstrating the potential for the environmental application without the need for purification processes.

Keywords: biosurfactant, surface tension, lipase, *Aspergillus niger*, soil adsorption, natural attenuation.

1. INTRODUCTION

Remediation techniques have been widely applied for the treatment of soils contaminated with oily compounds (Ossai et al. 2019), being based on physical, chemical and biological treatments, such as pumping and treatment, soil washing, chemical oxidation and bioremediation (Maletić et al. 2019; Olajuyigbe et al. 2020). Physical and chemical techniques can present disadvantages such as the use of large amounts of water, energy and chemicals, which increases costs and generates a negative environmental impact (Kim et al. 2013). Bioremediation techniques are considered more sustainable and with low environmental impact, aiding in the solubility of oily compounds and their consequent biodegradation (Bento et al. 2005).

Among the bioremediation technologies, stand out the (Herrero and Stuckey 2015), biostimulation, bioaugmentation and the addition of biocompounds, such as biosurfactants and

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enzymes (Sharma et al. 2014; Salgado et al. 2016; Huang et al. 2020). However, the use of microorganisms and biocompounds with high added value has its application compromised due to the high cost in the obtaining, being necessary the search for economically viable and sustainable means of production from the environmental point of view (Mahmoud et al. 2015; Kumar and Ray 2014).

Solid state fermentation (SSF) presents itself an alternative for the production of biocompounds for use in bioremediation (Martins et al. 2008), being a very well-known form of cultivation in the food industry (Pandey 2003; Soccol et al. 2017), but still with few environmental applications. Its process is based on microbial growth in solid substrates such as husks, bran and bagasse, which are constituted as agro-industrial residues, which can be valued for obtaining biocompounds useful in bioremediation processes, such as lipases and biosurfactants (Colla et al. 2010; Nayarisseri et al. 2019)

The biocompounds produced can be added directly to the contaminated soil, with the culture media, without the need for precipitation and purification processes after cultivation, a step that increases the cost of bioprocesses by up to 60% (Decesaro et al. 2015; Desai and Banat 1997). The materials used as culture media in the production of lipases and biosurfactants can be applied directly to the contaminated soils, resembling sawdust and straw, materials already used as auxiliaries in environmental treatment in landfarming techniques (Chikere et al. 2019).

Among the microorganisms that can be grown in a solid medium, the fungus *Aspergillus niger* has a good development since the solid matrices simulate their natural habitat (Cavka et al. 2014), is the production of lipolytic enzymes from this microorganism widely studied (Fleuri et al. 2014; Reinehr et al. 2017; Utami et al. 2017). However, the production of biosurfactants and lipases simultaneously was little explored, as well as the application of both to treat soils contaminated by oily compounds, such as biodiesel (SENA et al., 2018; QUINTELLA et al., 2019), being this one of the goals of the present study.

In Brazil, the production of biodiesel has been stimulated, as well as its commercialization increased in the world and Brazilian, where the compulsory addition to diesel reaches 12% (Brasil 2018). Increased production and transport of biofuel also increases the risks of accidents, spills and failures and leaks in storage systems (Singh et al. 2020). In 2019, automotive fuels were responsible for 4.724 contamination cases only for the state of São Paulo/Brazil (Cetesb 2019). These cases can cause surface and underground contamination of soils, compromising the environment and human health (Reddy and Adams 2015; Shahaeen et al. 2020).

The aim of this work was to evaluate if the addition of a fermented bran containing lipases and biosurfactants, produced by solid state fermentation using the fungus *Aspergillus niger*, could stimulate the biodegradation of a diesel contaminated soil and the in situ production of biosurfactants and lipases in soil during the bioremediation process.

2. METHODOLOGY

2.1 Production of biosurfactants and lipases by *Aspergillus niger* in SSF

The *Aspergillus niger* strain O-4, GenBank number KC545858.1 (Reinehr et al. 2017) used in this study was isolated by Colla et al. (2010), from a soil contaminated with diesel oil, kept in potato-dextrose-agar (PDA) medium, and stored under refrigeration at 4ºC until the use in this work.

The inoculum was prepared by adding 10 mL of a 0.01% (v/v) solution of Tween 80 in a test tube containing the isolated strain. The spore suspension (5 mL) was added to a 1 L Erlenmeyer containing 100 mL of PDA medium previously sterilized for 20 min at 121ºC. The microorganism was incubated in a greenhouse for 5 d at 30ºC for growth and hyphae formation. After this period, 50 mL of 0.01% Tween 80 solution and 3 sterile glass beads were added to the conical flask Erlenmeyer to obtain a new spore solution. The solid fermentation media were inoculated with 2 mL of this suspension, giving an initial spore concentration of 2.10^6 spores.g^{-1}.

The culture medium was prepared from 40 g of wheat bran and corn cob in an 80/20 ratio, 0.5% (w/w) of sugar cane molasses, 5% (w/w) of inducer (soybean oil) and 30 mL of a saline solution composed of 2 $g.L^{-1}$ of potassium phosphate (KH_2PO4), 1 $g.L^{-1}$ magnesium sulfate ($MgSO_4$) and 10 $mL.L^{-1}$ of trace solution, consisting of 0.63 $mg.L^{-1}$ iron sulfate ($FeSO_4.7H_2O$), 0.01 $mg.L^{-1}$ manganese sulfate ($MnSO_4$) and 0.62 zinc sulfate ($ZnSO_4$) as a source of micronutrients. These experimental conditions were previously optimized by Kreling et al. (2020). The moisture of the culture medium was adjusted to 60% with distilled water and the medium was sterilized for 20 min at 121ºC. Inoculation was carried out with the spore suspension and cultivation was carried out at 30ºC for 4 d. Samples were collected at the end of cultivation to determine the production of biosurfactants and lipases, according to the analytical determinations of item 2.3.

2.2 Bioremediation assay

The soil used in the study was classified pedologically by Streck et al. (2008) and geotechnically by Thomé et al. (2014), as dystrophic humic oxisol (Passo Fundo unit) and as high-plasticity clay, presenting acid pH, low CTC and high clay content, characteristics typical of soils with a predominance of kaolinite clay. It was collected in an open trench at a depth of 1.2 m, sieved (mesh 10, with 2 mm between the sieve grids), according to the American Society Testing and Materials (ASTM 2013).

The used soil presented initial moisture of 5.43%. This moisture was adjusted to 15% with distilled water. Subsequently, the samples were contaminated with 20% biodiesel (BSBIOS/Brazil), totaling initial moisture of 35% based on the dry weight of the soil sample. The percentage of contaminant added to the soil was adopted based on previous studies (Decesaro et al. 2016), to simulate ex situ contamination. The bioremediation assay was designed according to Table 1 and performed in duplicate for each test, using 500 g of dry soil and the other constituents, according to the experimental strategy.

Table 1 Experimental conditions for the bioremediation process

Experiment	Description of the technique
E1	*Natural attenuation*: soil + 20% biodiesel
E2	*Biostimulation* + *Bioaugmentation* + *Biocompounds*: soil + 20% biodiesel + 10% fermented bran containing nutrients from the culture medium, active *A. niger* fungus, lipases and biosurfactants
E3	*Biostimulation* + *Biosurfactant*: Soil + 20% biodiesel + 10% fermented bran containing nutrients from the sterilized culture medium (121 ºC/20 min), isolating the effect of biosurfactants and inactivating the fungus and enzymes
E4	*Biocompounds extract*: Soil + 20% biodiesel + 10% (v/m) of liquid extract obtained from fermented bran containing lipases and biosurfactants
E5	*Biostimulation:* Soil + 20% biodiesel + 10% bran containing nutrients from the sterilized culture medium (121 ºC/20 min), without the addition of the fungus and production of biocompounds
E6	*Adsorption of contaminant in the soil*: Sterile soil + 20% biodiesel + 10% fermented bran under the same conditions as in experiment 2 sterilized (121 ºC/20 min) to inactivate microbial activity

The bioremediation tests were carried out for 90 d, with sampling in the initial times, 15, 30, 60 and 90 d, evaluating the microbial growth using the microbial counting method, the degradation of the contaminant (quantitatively by the determination of oils and greases and qualitatively by the degradation of carbon chains from gas chromatography), determination of

lipase activity in situ and the production of biosurfactants in situ (reduction of surface tension and emulsifying activity), according to the analytical determinations of item 2.4.

2.3 Production of biosurfactants and lipases by *Aspergillus niger* in SSF

The extraction of biocompounds from SSF fermented bran was performed according to Colla et al. (2010) with modifications. To obtain the extract for the determination of lipolytic activity, 1 g of fermented bran was added with 10 mL of 2 M phosphate buffer, pH 7.0 at 160 rpm for 30 min at 37ºC, and the solids were filtered on cotton. For the determination of emulsifying activity, the extracts were obtained from 5 g of fermented bran, added with 30 mL of distilled water at 90ºC followed by extraction in a water bath for 30 min, with subsequent filtration of solids and collection of supernatant in cotton.

2.3.1 Water in oil emulsifying activity (EA w/o)

The analysis was performed in duplicate by adapting the emulsification index method (Cooper and Goldenbeg, 1987). An aliquot of 3.5 mL of cell free extract and 2 mL of biodiesel (used as the oil phase) were mixed and agitated on a Vortex agitator at 700 rpm for 1 min. After 24 h, the height of the water/oil emulsion formed and the total height of the emulsion (height of the emulsion plus height of the remaining oil layer) were measured. The water in oil emulsifying activity was determined according to Equations 1 and 2. Blank samples were made using extract in place of the sample.

$$E = \left(\frac{h_{emulsion}}{h_{total}}\right).100 \quad (1)$$

$$EA_{W/O} = (E_{sample} - E_{blank}) \quad (2)$$

Where $EA_{W/O}$= emulsifying activity of water in oil (UE); $H_{emulsion}$= percentage of the height of emulsified layer (mm); H_{total}=total height of the liquid column (mm); E_{Sample}=centesimal relation between the height of the water in oil emulsion and total height.

2.3.2 Lipase activity (LA)

To determine the lipase activity, the methodology described by Burkert et al. (2004) was used. To 75 mL of 7% (w/v) gum arabic solution was added 25 mL of olive oil. This mixture was stirred at 500 rpm in vortex shaker for 5 min for emulsion formation. For the enzymatic reaction, 5 mL of the prepared emulsion, 1 mL of the enzyme extract and 2 mL of 2 M phosphate buffer solution pH 7.0 were added in 250 mL Erlenmeyer. The reaction occurred for 30 min at 160 rpm on a shaker at 37 °C and was then stopped with 15 mL of 1:1 alcohol:acetone solution. The obtained solution was titrated with 0.01 $mol.L^{-1}$ NaOH. One unit of lipase activity was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute per gram of wet fermented bran (1 U = 1 $\mu mol\ min^{-1}g^{-1}$) according to Equation 3.

$$LA = \frac{v*M*f*11000}{t*m} \quad (3)$$

Where: LA=a unit pf lipase activity (U); v=volume of NaOH spent on titration (mL); M=molar mass of NaOH used for titration ($mmol.mL^{-1}$); f=correction factor of NaOH; t=time spent reaction 1 mL of enzyme extract (min); m=mass of wet fermented bran (g).

2.4 Bioremediation assay

2.4.1 In situ production of biosurfactants and lipases in soil

The extraction of biosurfactants from the soil was carried out according to the methodology proposed by Ángeles and Refúgio (2013), performed in the initial time, 15 d, 30 d, 60 d and 90 d. An electrolyte solution was prepared with 0.01 M potassium nitrate (KNO_3), 0.01 M hydrochloric tris (Tris-HCl) and 0.003 M sodium azide (NaN_3), with pH adjusted to 7.0. The extraction was carried out with the proportion 1: 2 (w/v) and maintained for 72 hours under agitation at 240 rpm and temperature between 25ºC to 27ºC. The sample rested for 48 hours, being filtered in 0.22 μm Merck millipore (Cotia/SP/Brazil). The filtered solution obtained was used to determine the water-in-oil emulsifying activity ($AE_{A/O}$) as described in item 2.3.1.1, according to the methodology proposed by Cooper and Goldenberg (1987) and the surface tension by the ring method (Du-Nuoy's ring method), where a volume of 30 mL of the extract was added to a Biolin Scientific tensiometer (São Paulo/SP/Brazil), model Sigma

702, with the surface tension expressed in millinewton per meter ($mN.m^{-1}$). The reduction of the surface tension of the media in relation to the time of the beginning of the cultivation was calculated according to Equation 4.

$$STR\ (\%) = \frac{ST_{initial} - ST_{final}}{ST_{initial}} * 100 \quad (4)$$

Where: STR=surface tension reduction (%); $ST_{initial}$=surface tension obtained at the initial time of cultivation ($mN.m^{-1}$) and ST_{final}=surface tension obtained at the final time of cultivation ($mN.m^{-1}$).

The determination of lipase concentration in soils was carried out according to the methodology proposed by Margesin et al. (1999), performed in the initial time, 15 d, 30 d, 60 d and 90 d. 1 g of soil was weighed in 100 ml Erlenmeyer, adding 1.5 ml of toluene, shaking the flasks for 15 min. After stirring, 9 ml of distilled water and 1 ml of tributyrin were added. The samples were incubated for 72 hours at 37º C in a water bath with shaking. Subsequently, 40 mL of ethyl acetate was added to the sample, 10 mL of which was collected for titration with 5 drops of thymolphthalein and 0.05 M sodium hydroxide. A control was performed by adding tributyrin only after the samples were incubated. Lipase activity is expressed in lipase units (LU), according to Equation 5.

$$AL\ (LU) = \frac{40 * 100 * v}{10 * 10 * (\frac{100}{g})} \quad (5)$$

Where: AL = one unit of lipase activity per gram of soil (LU), v = NaOH volume spent on titration (mL); 40 = volume of the extract used (mL); 10 = conversion factor from 0.005 M to 0.05 M; 10 = sample aliquot (mL); g = conversion factor for dry soil mass (100.dm-1).

2.4.2 Microbial growth

Microbial growth was evaluated through microbial counting, performed in the initial time, 15 d, 30 d, 60 d, and 90 d (except for the experiment where the soil was sterilized), using the count of total heterotrophic microorganisms methodology (Brazil, 2003). In Erlenmeyer, 10 g of soil was added in 90 mL of 0.1% peptone saline solution and then stirred at 25 °C for 1 hour at 150 rpm. Subsequent dilutions were made and plated in PCA Petri dishes by pour-plate technique, adding 1 mL of the dilutions. The plates were incubated at 36 °C for 48 h, and then

the number of colonies forming units was counted and the result was expressed in log UFC.g soil^{-1}.

2.4.3 Degradation of the contaminant

Contaminant degradation was assessed quantitatively through the residual content of the contaminant, with the removal of the contaminant being evaluated in the initial time, 15 d, 30 d, 60 d and 90 d. 10 g of soil were used to extract oils and greases according to the methodology of USEPA 3550B (1996), using an ultrasonic probe (Unique brand – Indaiatuba/SP/Brazil), allowing the quantification of volatile and semi-volatile substances in the soil. The residual content was evaluated by Equation 6, and the result expressed as degradation of the oily contaminant, according to Equation 7.

$$Residual\ content\ (\%) = \left(\frac{P_2 - P_1}{P_0}\right) * 100 \quad (6)$$

$$Contaminant\ degradation\ (\%) = \frac{\%OG_{initial} - \%OG_{final}}{\%OG_{initial}} * 100 \quad (7)$$

Where: P_0=amount of the soil sample in dry weight used in the analysis (g); P_1=weight of the beaker (g); P_2=weight of the beaker plus contaminant extracted from the contaminated soil (g); $OG_{initial}$=initial content of oils and greases (%); OG_{final}=final content of oils and greases (%).

Contaminant degradation was evaluated qualitatively through the analysis of the degradation of carbon chains, performed by gas chromatography in the initial time, 30 d, 60 and 90 d, using a gas chromatograph with a mass spectrometer in the series type Triple Quadrupolo, Software GCSolutions version 4,11, column Rtx-5MS with 30 mx 0.25 um x 0.25 mm, through an oven-heating program with an initial temperature of 90 °C (3.0 min). The temperature was then increased from 6 ºC.min^{-1} to 185 ºC (0 min), and again from 2 ºC.min^{-1} to 300 ºC (1 min), with an injector temperature of 250 °C, injection volume of 1.0 µL (splitless), a drag gas flow (helium) constant at 0.96 mL.min^{-1}, a transfer line temperature of 300 °C, ionization source temperature of 200 °C, full scan acquisition mode and a total running time of 77 min.

The identification of carbon chains was performed by external standardization from a commercial standard mixture containing a carbon range from C15 to C30. From the

concentration results of the compounds at the different sampling times, the removal efficiency of each compound was evaluated through Equation 8:

$$Carbon\ chains\ degradation\ (\%) = \frac{C_{initial} - C_{final}}{C_{initial}} .100 \quad (8)$$

Where: $C_{initial}$ = initial value of the carbon area analyzed; C_{final} = final value of the carbon area at the end of each analysis time.

2.5 Treatment of the data

The data obtained for the bioremediation assay were analyzed using the Tukey test for a 95% confidence level.

3. RESULTS AND DISCUSSION

3.1 Production of biosurfactants and lipases by *Aspergillus niger* in SSF

The extracts obtained in solid-state fermentation with the fungus *Aspergillus niger* presented water-in-oil emulsifying activity of 4.58±0.69 UE (Units of Emulsifying activity) and lipolytic activity of 7.77 ± 1.52 U (Units of lipase activity), under the culture conditions of: wheat bran and corn cob (proportion 80:20), 0.5% sugar cane molasses, 5% soybean oil as inducer and 60% of moisture.

The use of soybean oil as an inducer in the culture medium, combined with soluble carbon sources from the culture medium composed of wheat bran and sugar cane molasses is the combination for the synthesis of the hydrophilic and hydrophobic portions of the molecule that produce the biosurfactants (Satpute et al. 2010). Also, as soybean oil is a compound of vegetable origin, its assimilation by the microorganism can be facilitated, favoring the formation of the desired biocompounds (Makkar and Rockne 2003). The biosurfactant from *Aspergillus niger* has been described by Rodrigues et al. (2014) as high molecular weight, characterized by the ability to form emulsions. The emulsification process occurs from the interaction between the hydrophobic portion of the molecule attached to the oil inside the molecule, and the hydrophilic fraction attached to the aqueous portion of the exterior of the molecule (Nievas et al. 2008).

For the synthesis of the lipase enzyme by microorganisms, it is necessary to use inducers that act as a lipid source of carbon, which when present in the culture medium can provide the necessary nutrients for microbial growth and to induce the production of lipase enzymes (Contesini et al., 2010). Lipases have an active site formed by a hydrophobic peptide chair, composed of polar amino acids on the outside and nonpolar on the inside, the latter being in contact with the active site. The contact between the peptide chain and the lipid-water interface from the substrate and the culture medium promotes the interaction between both, making the chain move, changing the structural form of the enzyme, exposing the active site and allowing the catalysis of reactions (Sperb et al. 2015).

Colla et al. (2010) evaluated the existence of a correlation between the production of lipases and biosurfactants, observing a second-order polynomial relationship (R^2 of 0.91) for the production of both biocompounds grown in submerged fermentation. Kreling et al. (2020) found that the correlation of production also occurs in solid-state fermentation, obtaining a second-order polynomial ratio (R^2 of 0.98) for the same cultivation conditions proposed in this study. The co-production of these biocompounds is justified by the action of the lipolytic enzyme, responsible for the hydrolysis of triacylglycerol in fatty acids and glycerol (Kiran et al. 2016), from the soybean oil inducer. The fatty acids are then metabolized and could be converted in the nonpolar (lipid) fraction of the biosurfactant molecule, being the polar (hydrophilic) fraction formed through soluble and easily assimilated carbon sources (Fontes et al. 2008). Perfumo et al. (2018) indicated that the synthesis of biosurfactants is associated with the production of extracellular enzymes, such as lipases, promoting the solubilization and mobilization of hydrophobic inducers, thus increasing their bioavailability.

3.2 Bioremediation assay

3.2.1 In situ production of biosurfactants and lipases in soil

Fig. 1 present the results of the determination of biosurfactants and lipases obtained in the soil for the bioremediation process carried out over 90 d. The reduction in surface tension (RTS) is considered an indicative of the production of biosurfactants. The experiment E6 (sterilized soil without microbial activity), demonstrating the absence of production of these biocompounds in the absence of microorganisms, due to the soil having been sterilized and also added with sterile biostimulants. In this experiment (E6) low reduction in RST (3.39% in 30 d

- 43.04±0.03 $mN.m^{-1}$) or no reduction (60 and 90 d - 46.74±0.17 $mN.m^{-1}$ and 46.17±0.02 $mN.m^{-1}$, respectively) is observed.

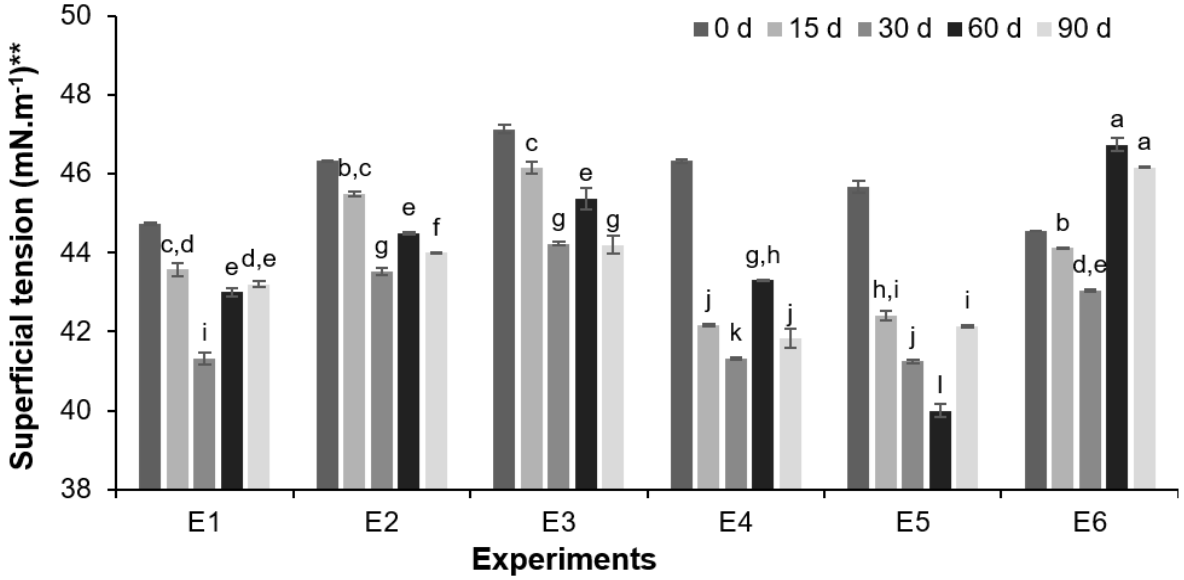
The other experiments performed showed an increase in the RTS in 30 d of cultivation, which can be justified by the increase in the biodegradation of the contaminant in the medium. This can be observed due to the time required for the adaptation of microorganisms to the contaminated environment (Decesaro et al. 2016), close to 15 d, starting the effective biodegradation of the oily compound after 30 d of bioremediation. Also, for tests E2 to E5, the production of compounds with properties to reduce surface tension may occur due to the addition of biostimulants and biocompounds to the soil to be bioremediated, promoting the growth of microorganisms, producing biocompounds in situ, positively influencing the bioremediation process.

The highest RTS in the soil was verified for the E5 experiment - biostimulation, presenting 9.68±0.40% in 30 d (41.25±0.05 $mN.m^{-1}$) and 12.43±0.09% (39.99±0.17 $mN.m^{-1}$) in 60 d for the experiment E4 (biocompounds extract), from 10.80±0.16% in 30 d. A statistical difference was observed comparing E4 in 30 d and E5 in 30 d and 60 d ($p_{E4\ 30d\ -\ E5\ 30d}$=0.0037; $p_{E4\ 30d\ -\ E5\ 60d}$ =0.0001) and statistical difference between the E5 experiment, in 30 d and 60 d ($p_{E5\ 15d\ -\ p_{E5\ 30d}}$=0.0001; $p_{E5\ 15d\ -\ p_{E5\ 60d}}$=0.0001). As these experiments presented the highest RTS averages, they are considered the best results of the entire experiment.

When biocompounds are not added to the soil where bioremediation occurs, native microorganisms need to perform the synthesis of the biosurfactant to use the oily contaminant as a carbon source. For the experiment where the biocompounds extract was added directly to the soil (E4), greater diffusion of the extract may have occurred (enzyme, biosurfactant and water) in the soil matrix when compared to the addition of the whole fermented bran (experiments E2, E3 and E5), facilitating the interaction between biosurfactants and contaminants and promoting greater access to microorganisms (Pacwa-Plociniczak et al. 2011).

The microorganisms present in the soil are majority species of *Bacillus* sp. and *Pseudomonas* sp. (Mulligan 2009), which produce low molecular weight biosurfactants. Low molecular weight biosurfactants are known to have the property of reducing surface tension (Satpute et al. 2010), justifying the higher percentages of RTS in experiments where there are no fungal biosurfactants previously added to the soil. Fungal biosurfactants, because they have polymeric characteristics, act preferentially in increasing emulsifying activities to reducing the surface tension of the media (Rodrigues et al. 2014).

Figure 1 - Production of biosurfactants in situ in the soil over the 90 d of experiment*



*Equal letters between all experiments and times indicate that there was no statistical difference ($p<0.05$) by the Tukey test for means ± standard deviation; **percentage of reduction in surface tension in relation to the initial cultivation time; E1: Natural attenuation; E2: Biostimulation + Bioaugmentation + Biocompounds; E3: Biostimulation + Biosurfactant; E4: Biocompounds extract; E5: Biostimulation; E6: Adsorption of contaminant in the soil.

In 30 d, the autochthonous microorganisms from the soil may have released compounds with surfactant activity, which reduce as a function of time. This behavior is verified for the E1 experiment (natural attenuation), where there was 7.64±0.44% of RTS in 30 d (41.32±0.16 $mN.m^{-1}$), 3.87±0.31% in 60 d (43.01 ± 0.11 $mN.m^{-1}$) and 3.42±0.10% in 90 d (43.21±0.08 $mN.m^{-1}$) to the initial time (44.74±0.04 $mN.m^{-1}$). In this experiment, the action of low molecular weight biosurfactants occurs, which reduces surface tension be greater in this experiment when compared to experiment E2, where both biocompounds were added for the same period ($p_{E1\text{-}E2\ 30d}$=0.0001).

The experiments where there was the addition of fungal biosurfactants to the soil showed lower percentages of RTS (6.02±0.24% for E2 and 6.16±0.12% for E3, both at 30 d, with no statistical difference between these experiments $p_{E2\text{-}E3\ 30d}$=1.0000). This can be justified by the addition of biosurfactants produced by the fungus *Aspergillus niger* at the beginning of the bioremediation process. In this way, as biosurfactants are already present in the medium, the production of in situ biosurfactants by indigenous microorganisms is lower, obtaining a low percentage of RTS.

Machado et al. (2020) evaluated the surface tension as a response variable for the production of biosurfactants in situ in the soil contaminated with 20% diesel oil, presenting RTS in the 30 to 60 d of bioremediation, obtaining reductions of approximately 11.67%. In comparison to our study, the possibility of using the measurement of soil RTS as an indicative

technique for the production of biosurfactants by indigenous microorganisms is corroborated, with significant reductions being observed in periods between 30 and 60 d in both studies, this being the period of greater reduction in surface tension of the soil.

Ángeles and Refugio (2013) evaluated the production of biosurfactants in situ through the analysis of the reduction in surface tension over 10 d of the bioremediation process, for soil contaminated with hydrocarbons. At the end of the test, they obtained a reduction in surface tension from 75.20 $mN.m^{-1}$ to 55.60 $mN.m^{-1}$ (26.06%) and 57.20 $mN.m^{-1}$ (23.94%), when using the techniques of bioaugmentation+biostimulation and biostimulation, respectively.

Regarding the determinations of water in oil (EA $_{W/O}$) emulsifying activities in bioremediation soils, little emulsion formation was observed, and the values obtained over time remained close to those obtained in the initial time, around between 3.00 and 4.00 UE overall 90 d of testing. There was no statistical difference in the production of EA $_{W/O}$ over time ($p>0.05$) for all experiments. As justified previously, the biosurfactants produced in the soil are of low molecular weight, therefore, the measurement of EA $_{W/O}$ as an indicator of the production of biosurfactants in soils was not adequate, since this analytical determination is carried out for high molecular weight biosurfactants (Uzoigwe et al. 2015).

Table 2 present the production of lipases in situ in the soil. For the E6 (sterilized soil without microbial activity) and E1 (natural attenuation) experiments was constant and minimal over the bioremediation time, with values of the order of 0.30±0.06 UL in 30 d for the experiment E6 and 1.03±0.03 UL in 90 d for experiment E1. It was found that the production of lipases in situ in soils was low when the extract of biocompounds was added (E4), and also when added only the sterilized culture medium as a biostimulant in the soil (E5), with lipase activities in situ 1.05±0.15 UL in 30 d for E4 and 1.17±0.02 UL in 30 d for E5.

In E2 experiment (Biostimulation + Bioaugmentation + Biocompounds) and in E3 (Biostimulation + Biosurfactant), higher lipase activities were observed in situ on soil (2.50±0.51 UL in 30 d for E3; 6.62±0.17 UL in 30 d for E2, $p_{E3\text{-}E2\ 30d}$=0.0001), these being the highest lipase activities obtained. The higher lipase activities of the E2 and E3 experiments compared to the E4 ($p_{E2\text{-}E4\ 30d}$= 0.0001; $p_{E3\text{-}E4\ 30d}$=0.0008) and E5 ($p_{E2\text{-}E5\ 30d}$= 0.0001; $p_{E3\text{-}E5\ 30d}$=0.0024) experiments can be explained by the influence of the production of biosurfactants in soils. These, when acting on soils, interact with the aqueous fraction of the soil, leading to an increase in the bioavailability of the contaminant for biodegradation, increasing the production of contaminant-degrading enzymes in the soil, as is the case of lipase enzymes (Urum and Pekdemir 2004).

Lin et al. (2009) evaluated a soil contaminated with diesel oil at a concentration of 50 $g.kg^{-1}$ of soil, using as treatment the addition of 0.5% of fungal cultures and 0.5% of bacteria culture, monitoring lipase activity over 2 years of bioremediation. It was observed that the lipase activity was higher for the first year of bioremediation (15.41 $g.772\ h^{-1}$), decreasing in the second year of testing (9.44 $g.772\ h^{-1}$), indicating that the enzymatic activity is able to promote the increase in growth and the consequent metabolism of the contaminant, which is also observed in our study.

Table 2 Production of lipases in situ in the soil over the 90 days of experiment*

Experiment	Lipase activity in soil (UL)				
	0 d	15 d	30 d	60 d	90 d
E1	$0.94\pm0.21^{a,b,c,d,e}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$0.19\pm0.03^{a,b}$	$1.03\pm0.03^{b,c,d,e}$
E2	3.55 ± 0.39^{h}	$0.73\pm0.28^{a,b,c,d,e}$	6.62 ± 0.17^{i}	$2.08\pm0.24^{f,g}$	$1.33\pm0.17^{c,d,e,f}$
E3	0.0 ± 0.0^{a}	$2.66\pm0.88^{g,h}$	2.50 ± 0.51^{g}	$0.82\pm0.15^{a,b,c,d,e}$	$1.68\pm0.13^{e,f,g}$
E4	$1.45\pm0.57^{d,e,f}$	$0.40\pm0.06^{a,b,c}$	$1.05\pm0.15^{b,c,d,e}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
E5	$0.25\pm0.06^{a,b}$	$0.22\pm0.06^{a,b}$	$1.17\pm0.02^{b,c,d,e,f}$	$0.80\pm0.09^{a,b,c,d,e}$	$0.49\pm0.00^{a,b,c,d}$
E6	0.0 ± 0.0^{a}	$0.16\pm0.04^{a,b}$	$0.30\pm0.06^{a,b,c}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

*Equal letters between all experiments and times indicate that there was no statistical difference ($p<0.05$) by the Tukey test for means ± standard deviation; E1: Natural attenuation; E2: Biostimulation + Bioaugmentation + Biocompounds; E3: Biostimulation + Biosurfactant; E4: Biocompounds extract; E5: Biostimulation; E6: Adsorption of contaminant in the soil.

Analyzing the production of biosurfactants and the production of lipases in situ, it is possible to verify a relationship between the RTS and the increase in the production of enzymes in the soil, since the highest values occurred in 30 d of study (experiments E5 and E4). The increase in the bioavailability of the contaminant through the production of biosurfactants and the increase in the use of the contaminant by microorganisms present in the soil as a nutritional source leads to the need for the production of lipases for the use of biodiesel as a carbon source. This may also be related to the adaptation of microorganisms to the environment, since greater enzyme and biosurfactant production occurs between 15 d and 30 d, indicating the beginning of the production of biocompounds that act in the degradation of the contaminant by the native microorganisms in this period.

3.2.2 Microbial growth

Table 3 shows the count of microorganisms in the soil over the 90 d of bioremediation. Among experiments E1 to E5, the E2 (Biostimulation + Bioaugmentation + Biocompounds) showed the highest microbial count in the soil. For this experiment, the high count in the initial

period since the active fungus was added together with the biocompounds to the medium, causing the microbial count to remain high until 60 d of bioremediation. The microbial count remains high over time (7.08±0.06 log UFC.g soil^{-1} in 30 d and 6.62±0.70 log UFC.g soil^{-1} in 60 d) in relation to the other experiments where there was no addition of the active fungus over time, such as E5 (5.26±0.07 log UFC.g soil^{-1} in 15 d and 4.62±0.17 log UFC.g soil^{-1} in 30d, with no microbial count in 60 and 90 d). For having the highest averages and statistical equality among all tests performed (p=0.9874), adaptation of native microorganisms to the contaminant is observed when lipases and biosurfactants are added to the soil (experiment E2), indicating the use of the contaminant as a nutritional source and greater degradation of the contaminant (Machado et al. 2020).

The E3 (Biostimulation + Biosurfactant) and E4 (Biocompounds Extract) assays showed equal p>0.05 microbial counts (5.71±0.17 log UFC.g soil^{-1} and 6.37±0.53 log UFC.g soil^{-1}) for the E4 experiment at times 0 and 15 d, and 5.28±0.25 log UFC.g soil^{-1} and 5.57±0.10 log UFC.g soil^{-1} for experiment E3, at 0 and 15 d). In the subsequent times of bioremediation, a reduction in the microbial count was observed, which may indicate that the adaptation of the native microorganisms to the contaminant also occurs when only one biocompound or the liquid extract of both is added, but that the use of the contaminant as a nutritional source is less, and therefore there is less degradation of the contaminant.

The E1 (natural attenuation) and E5 (Biostimulation) experiments showed low microbial count in relation to the initial time of both tests (3.45±0.21 log UFC.g soil^{-1} and 4.62±0.17 log UFC.g soil^{-1} at 0 and 30 d for the experiments E5 and 3.87±0.03 log UFC.g soil^{-1} and 4.38±0.39 log UFC.g soil^{-1} at 0 and 30 d for experiment E1, respectively), and statistical equality between these tests at initial time ($p_{E1\text{-}E5\ 0d}$=0.9963) and 30 d ($p_{E1\text{-}E5\ 30d}$ =0.9999). This suggests that when biocompounds such as lipases and biosurfactants are not added to the soil, the ability of indigenous microorganisms to use the contaminant as a nutritional carbon source is less, as can be seen in the biodegradation results presented below.

The possibility of using biodiesel as a nutritional source of carbon is due to its chemical structure, which is less complex when compared to other oily contaminants, such as diesel and gasoline, is a compound of plant origin. Still, the use of biosurfactants in the environment promoted a greater interaction between biodiesel and the autochthonous microbial cells, and subsequently the biodegradation of the contaminant by the action of the lipolytic enzymes made available in the medium and other enzymes that participate in the central microbial metabolic pathways (Teixeira 2007). This allows the fatty acids in the chemical structure of biodiesel to

be converted into acetyl-CoA through beta-oxidation, and into CO_2 by the Krebs cycle (Berg et al. 2006).

Lin et al. (2009) observed bacterial growth in the soil in a two-year period of bioremediation, from $1.49x10^8$ UFC.g of dry $soil^{-1}$ in the initial period, $8.91x10^7$ UFC.g of dry $soil^{-1}$ in the first year of testing and $3.87x10^8$ UFC.g of dry $soil^{-1}$ at the end of the experiment, for contamination of 50 g of diesel oil.kg^{-1} of soil, indicating the microbial growth capacity and consequent use of the contaminant for cellular nutrition, which was also indicated in our study.

Table 3 Count of microorganisms present in the soil over the 90 d of bioremediation*

Experiment	Microbial growth (log UFC.g $soil^{-1}$)				
	T0	T15	T30	T60	T90
E1	$3.87\pm0.03^{b,c}$	ND**	$4.38\pm0.39^{b,c,d}$	ND**	ND**
E2	8.30 ± 0.08^{j}	$8.16\pm0.20^{i,j}$	$7.08\pm0.06^{h,i}$	$6.62\pm0.70^{g,h}$	ND**
E3	$5.28\pm0.25^{d,e,f}$	$5.57\pm0.10^{e,f,g}$	3.36 ± 0.07^{b}	ND**	ND**
E4	$5.71\pm0.17^{e,f,g}$	$6.37\pm0.53^{f,g,h}$	$6.15\pm0.21^{f,g,h}$	$5.62\pm0.87^{e,f,g}$	ND**
E5	3.45 ± 0.21^{b}	$5.26\pm0.07^{d,e,f}$	$4.62\pm0.17^{c,d,e}$	ND**	ND**

*Equal letters between all experiments and times indicate that there was no statistical difference ($p<0.05$) by the Tukey test for means ± standard deviation; **ND: Not Detected by the method; E1: Natural attenuation; E2: Biostimulation + Bioaugmentation + Biocompounds; E3: Biostimulation + Biosurfactant; E4: Biocompounds extract; E5: Biostimulation; E6: Adsorption of contaminant in the soil.

3.2.3. Degradation of the contaminant

Fig. 2 shows the biodegradation and the retention of the contaminant, for the 90 d of experiments. There was an increase in the degradation of the contaminant in the soil for all experiments analyzed over time, except for the experiment E6 (adsorption of the contaminant in the soil), where there was no microbial intervention. In this experiment, the removal of the contaminant (between 42.12±0.17% and 44.45±0.34%) was stable over the 90 d of bioremediation, and statistical equality ($p>0.05$) was observed between the initial time and all other tests evaluated over time, indicating no biodegradation.

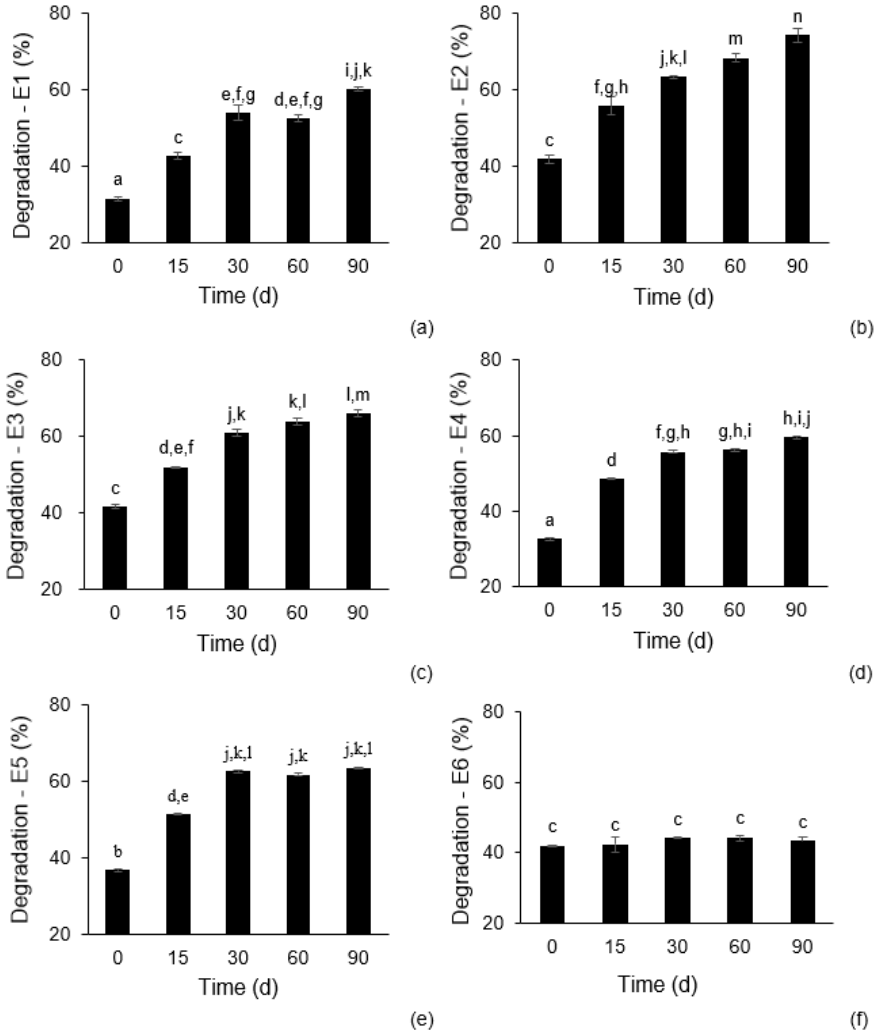
Cecchin et al. (2016) demonstrated that for sterile soil, the contaminant that is not removed by the solvent in the extraction process is adsorbed by the soil particles. The authors indicate that clay soils, similar to that used in the present study, retain a lower percentage of contaminants in their matrix the higher the percentage of compounds of organic origin added to the soil, such as lipases, biosurfactants and the solid culture medium. For the addition of 10% fermented bran (E6), the oil removal observed in our study is up to 44.45±0.34%. Kreling et al. (2019) indicate values of up to 25% removal of the contaminant in the microbial absence using 0.5% of biosurfactant in the contaminated soil. As the value observed in our study is higher

(44.45±0.34%), it can be said that the greater the addition of organic compounds added to the soil, the lesser retention of the contaminant is observed. This phenomenon can be explained by the preferential adsorption of the mineral particles of the soil by nitrogen, phosphorus and potassium ions, present in the biocompounds, reducing the interaction of the contaminant with the soil, since the reactivity of the clay minerals depends on the adsorption surfaces if saturated with ions from organic compounds (Cecchin 2016).

The experiment E2 (Biostimulation+Bioaugmentation+Biocompounds) showed greater degradation of the contaminant (68.32±1.11% in 60 d and 74.40±1.76% in 90 d), and statistical difference ($p<0.05$) in relation to most results of degradation of the other experiments in 60 and 90 d. (. The E1 experiment (natural attenuation) showed biodegradation of the contaminant of 52.47±0.95% in 60 d and 60.09±0.52% in 90 d. Compared to the percentage of contaminant adsorbed in the soil (about 44%, obtained in the E6 experiment), there is a 30% degradation of the contaminant for the E2 experiment, which can be attributed to the addition of biocompounds in the soil. The removal of contaminant disregarding the adsorption was 16% for experiment E1, demonstrating that the use of biodiesel as a nutritional source by indigenous microorganisms also occurs in the absence of biocompounds added to the soil. However, the addition of biosurfactants and lipolytic enzymes reduces the time required for bioremediation (52.47±0.95% for E1 and 68.32±1.11% for E2, in 60 d of bioremediation), positively influencing the treatment of the contaminant. The biodegradation presented for the experiment E2 in 60 d is statistically equal ($p=0.8112$) to the E3 test (Biostimulation + Biosurfactant), at the end of the 90 d test, proving the importance of adding biosurfactants for the treatment of oily compounds in soils.

Regarding the mechanism of action of biosurfactants in the treatment of contaminated soils, Nievas et al. (2008) indicates that the addition of biosurfactants to the soil increases the biodegradation of hydrocarbons through mobilization and solubilization processes, carried out by low molecular weight biosurfactants, such as *Bacillus* sp. and *Pseudomonas* sp. and emulsification process, carried out by high molecular weight biosurfactants, such as *Aspergillus niger* added to the soil.

Figure 2 - Biodegradation and retention of the contaminant over the 90 d of bioremediation (%)*



*Contaminant removal (biodegradation+soil retention) for experiments E1-E5 and contaminant retention in experiment E6; Equal letters between all experiments and times indicate that there was no statistical difference ($p<0.05$) by the Tukey test for means ± standard deviation; E1: Natural attenuation; E2: Biostimulation + Bioaugmentation + Biocompounds; E3: Biostimulation + Biosurfactant; E4: Biocompounds extract; E5: Biostimulation; E6: Adsorption of contaminant in the soil.

The mobilization process occurs in concentrations lower than the critical micellar (CMC) of the biosurfactant, where the biocompound reduces the surface tension of the soil/water fraction, increasing the angle of contact of the biosurfactant with the soil/oil fraction, bringing soil and contaminant closer. In the solubilization mechanism, which occurs above CMC, biosurfactants increase the solubility of the contaminant from the connection between the hydrophobic fraction of the molecule with the oil, and the hydrophilic fraction with the aqueous part of the exterior of the molecule. Solubilization also facilitates the transport of contaminants adsorbed in the solid phase to the aqueous phase (Chu and Chan 2003). In the emulsification process, through the stability of emulsions, high molecular weight biosurfactants

make hydrocarbons bioavailable for biodegradation, acting as emulsifying agents (Pacwa-Plociniczak et al. 2011).

It was observed that the addition of the liquid extract containing the lipases and biosurfactants (E4) was not sufficient to improve the biodegradation of the contaminant, presenting results similar to the test E1 in 90 d of bioremediation (60.09±0.52% for E1 and 59.58±0.34% for the E4 test; p=1.00). However, as verified in experiment E2, the association of lipase enzymes with the biosurfactant and fermented bran added to the soil promoted the greatest biodegradation of this study, indicating that the association of both is positive for the treatment of oily compounds in soils.

Margesin et al. (1999) evaluated the influence of lipases on the biodegradation of soil contaminated with 5 mg of diesel oil for 8 kg of soil, for 116 d. The authors found that in biostimulated soils, biodegradation and lipase activity are higher and that supplementation with nutrients increases microbial biomass and metabolic activity. This is verified in our study when the addition of the E5 (biostimulation) experiment, without lipases and biosurfactants, promoted greater degradation of the contaminant to the E4 experiment - extract containing the lipases and biosurfactants (63.54±0.14% for E5 and 59.58±0.34% for E4, in 90 d), since the culture medium is composed of agro-industrial residues, including carbon, nitrogen and phosphorus sources in its composition, acting as a biostimulant in the soil, favoring the increase of biomass and promoting biodegradation of the contaminant.

The application potential of biosurfactants produced by *C. sphaerica* and *Bacillus* sp. was evaluated by Chaprão et al. (2015). The authors confirmed that both biosurfactants were able to remove engine oil from a sandy soil by 90% and 40%, for *C. sphaerica* and *Bacillus* sp. biosurfactants, respectively. When molasses was added to the medium, biodegradation reached almost 100% for the biosurfactant from *Bacillus* sp., in 90 d of the experiment. The bioremediation of cooking oil residue using lipases produced by the fungus *Penicillium chrysogenum* was studied by Kumar et al. (2012), with the highest fatty acid released at 026.92 $mg.g^{-1}$, demonstrating that the enzyme can be applied in bioremediation processes. Both studies corroborate the research carried out, which also highlights the potential for the use of biosurfactants and lipases in bioremediation processes, obtaining a high level of contaminant degradation when both biocompounds are inserted concomitantly in the medium (74.40±1.76% in 90 d).

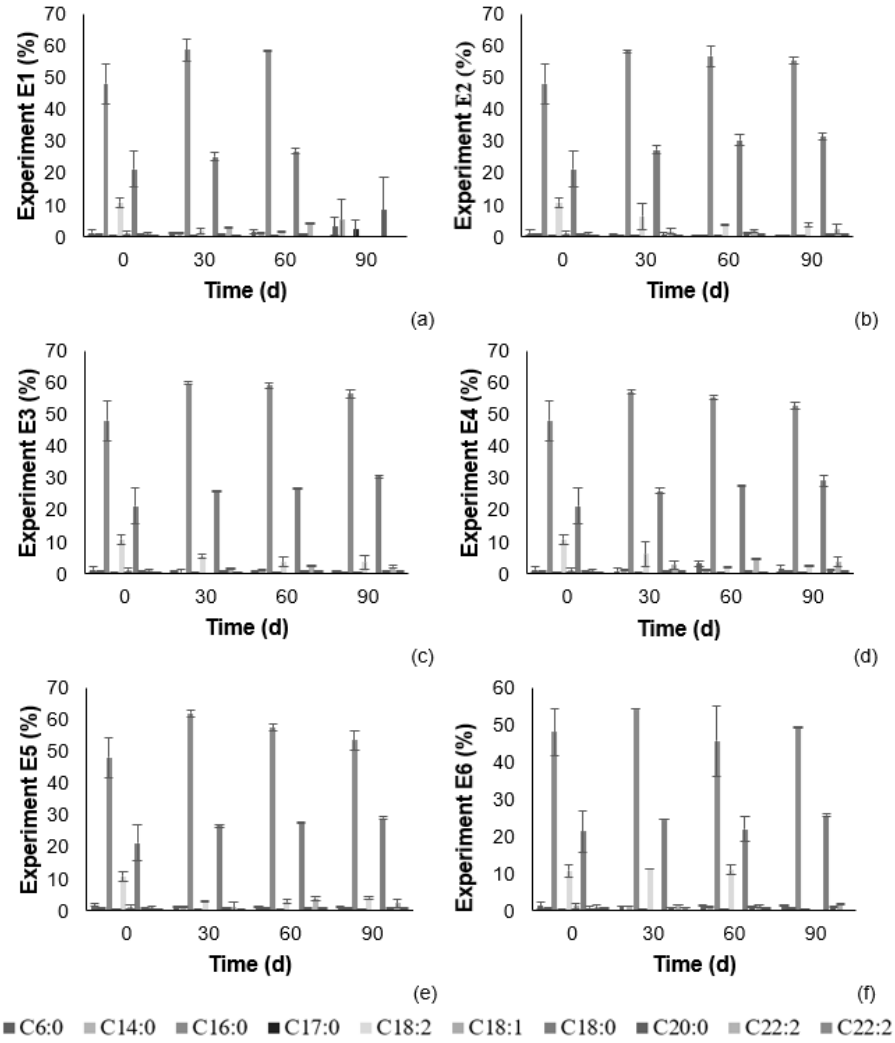
The analysis of the contaminant degradation by quantifying the carbon chains of the contaminant shown in Fig. 3 allows verifying an increase or reduction in the percentage of

carbonic bonds that make up the contaminant fatty acid, were these, mostly present in biodiesel, and their degradation, are analyzed throughout the bioremediation period.

The efficiency of adding biocompounds for the experiment E2 - Biostimulation + Bioaugmentation + Biocompounds (Fig. 3b) can be confirmed by verifying a reduction in the percentage of carbons over time, as for C18:2 carbons (10.87%, 6.58%, 3.85% and 8.83% at times 0, 30, 60 and 90 d), C14:0 (1.03%, 0.82%, 0.67% and 0.25% at 0, 30, 60 and 90 d) and C6:0 (1.45%, 0.72%, 0.45% and 0.52% at 0, 30, 60 and 90 d), indicating the use of the contaminant as a microbial nutritional source. There is also an increase in the percentage of C16:0 (48.21%, 58.39%, 56.92% and 55.41% at 0, 30, 60 and 90 d), indicating the occurrence of contaminant degradation, with the conversion of carbons C18:2, C18:1 and C18:0 in smaller chains, proven by reducing the percentage of carbons over time. This reduction occurs slowly for the experiment E1 - natural attenuation (Fig. 3a), where higher percentages of degradation are observed only at the end of the 90 d, for carbons C14:0 (1.03%, 1.32%, 1.32% and 5.51% at 0, 30, 60 and 90 d) and C6:0 (1.45%, 1.51%, 1.55% and 3.33% at 0, 30, 60 and 90 d). In the experiment E6 (Fig. 3f), the percentages of carbon chains stabilized over time, indicating no degradation, as seen for C14:0 (1.03%, 1.09%, 1.08% and 0.88% at 0, 30, 60 and 90 d), and C 22:2 (1.16%, 1.50%, 1.34% and 1.85% at 0, 30, 60 and 90 d).

It is confirmed that experiment E3 - Biostimulation + Biosurfactant (Fig. 3c) promoted better results compared to E4 – Biocompounds extract (Fig. 3d), where the degradation of carbon chains was greater for the biosurfactant along the bioremediation test period (C14:0 with percentages of 1.03%, 0.59%, 1.25% and 0.82% for times 0, 30, 60 and 90 d) compared to adding the extract for the same C14:0 (1.03% , 1.25%, 1.27% and 1.05% for times 0, 30, 60 and 90 d), where the degradation of C14:0 is similar only to experiment E5 (Fig. 3e), without the addition of biocompounds (1.03% , 1.21%, 1.16% and 1.03% at 0, 30, 60 and 90 d).

Figure 3 - Degradation of the carbon chains of the contaminant over the 90 d of bioremediation (%)



E1: Natural attenuation; E2: Biostimulation + Bioaugmentation + Biocompounds; E3: Biostimulation + Biosurfactant; E4: Biocompounds extract; E5: Biostimulation; E6: Adsorption of contaminant in the soil.

Kreling et al. (2019) also describe that in environments where the microbial influence is null, the degradation of carbon chains is not observed during the period of bioremediation, but that the degradation occurs when biocompounds are added to the medium, confirming that the microbial action promotes increased biodegradation. The authors also indicate the existence of the contaminant adsorption process in the soil matrix and that this adsorbed contaminant is not degraded, which is proven by maintaining the percentages of C18:0 unsaturated carbons over time over time, with percentages (21.39%, 15.93%, 8.43% and 28.77%) similar to those obtained in our study (21.40%, 24.67%, 22.07% and 25.72%), reinforcing the need to use biocompounds that assist in the process of biodegradation of the contaminant, such as biosurfactants and lipases.

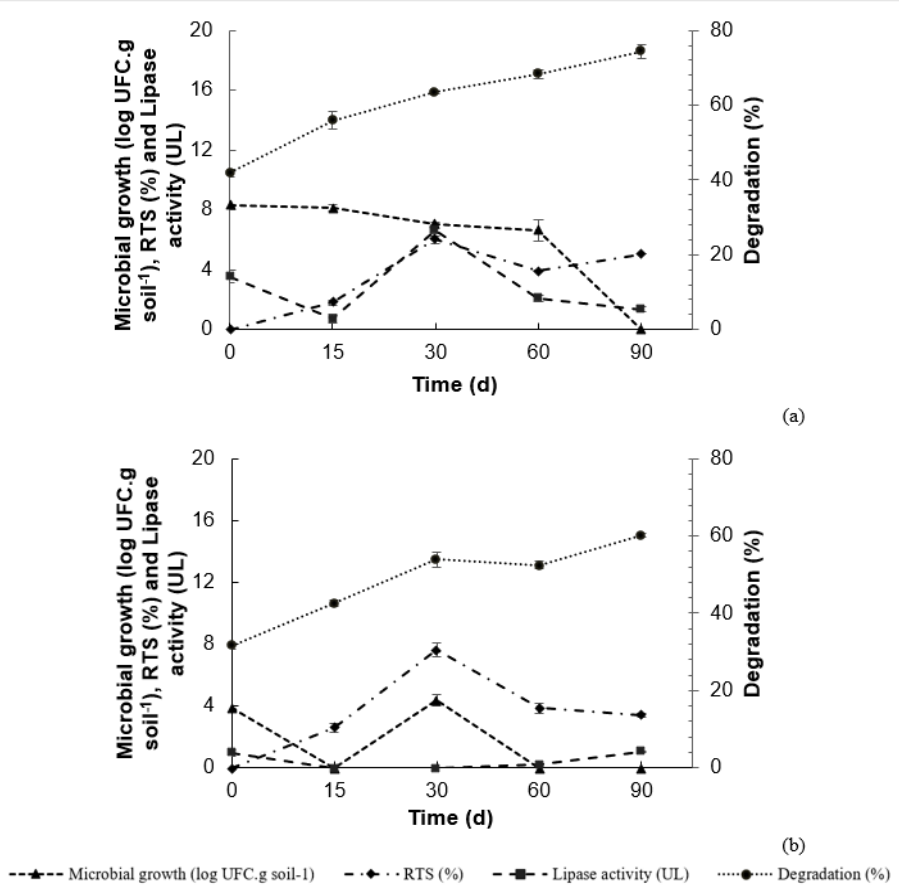
Meneghetti (2007) also observed the occurrence of reduction of biodiesel carbon chains when using the bioaugmentation technique to natural attenuation (C14:0 with an area of 1,484 for natural attenuation and 350 for bioaugmentation, respectively), for a period 120 d of bioremediation. Meyer et al. (2014) also proved the efficiency of contaminant biodegradation (97%) when compared to the monitored natural attenuation technique (50%) for a bioremediation process using biodiesel as a contaminant.

Fig. 4 shows the comparison for all parameters evaluated during bioremediation, for the test with the highest degradation observed (E2 - Biostimulation + Bioaugmentation + Biocompounds) and natural attenuation (experiment E1). The degradation increases over the bioremediation time, reaching its maximum (74.40±1.76%) at 90 d in the experiment added with biocompounds (Fig. 4a). The production of both biocompounds in the soil goes through an adaptation period in the first 15 d, increasing by 30 d, where there is also an increase in biodegradation. The degradation of the contaminant in the soil effectively occurs in 30 d of bioremediation, where microbial growth is also observed up to this period. Subsequently, the production of both biocompounds in situ and the increase in soil biomass are reduced, reaching the stationary growth phase and using the contaminant as a nutritional source, justifying the increase in the percentage of biodegradation between 60 and 90 d.

For experiment E1, an increase in the percentage of biodegradation of the contaminant is observed over time, occurring, however, in a reduced percentage (60.09±0.52% in 90 d). Microbial growth is not kept constant over time, having the same behavior as the production of lipases and biosurfactants in situ in the soil (adaptation in 15 d, with an increase in the production of biocompounds, microorganisms and biodegradation in 30 d, indicating in this period production of biocompounds in situ in soils). In 60 and 90 d, the degradation of the contaminant continues to occur, with a reduction in the production of biocompounds and microbial biomass.

The comparison between both experiments shows that when there is a previous addition of biocompounds, their production in the medium is reduced, as they are already available in the medium, accelerating the biodegradation process of the contaminant. When they are not previously added to the soil, their production by the native microorganisms is higher, but not in enough quantity to promote a high biodegradation percentage at the end of the 90d of bioremediation test.

Figure 4 - Comparison between experiments E2 (addition of lipases and biosurfactants – a) and E1 (natural attenuation – b) for the study variables over 90 d



The results obtained in our study prove the possibility of co-producing two biocompounds in the same culture medium, with the ability to be applied in bioremediation. The environmental application allows the absence of processes after the conventional bioprocess, such as extraction and purification, making the process economically viable, which uses agro-industrial waste as a means of cultivation, also making its raw material cheaper.

The concomitant application of both produced biocompounds, when applied in a bioremediation process of oily contaminants, promotes greater biodegradation than to the natural attenuation technique, or even when only one biocompounds is used in the process of treating contaminants in the soil. The study also demonstrated that the higher the percentage of biocompounds added to the soil, the smaller the contaminant adsorption process in the soil matrix. Few studies indicate the possibility of applying biosurfactants and lipases produced by *Aspergillus niger* in solid culture media and the direct application of this medium in environmental treatments, which highlights the importance of this work, expanding the economic viability of the production of biocompounds and the reduction of the environmental

impact by the use of residual raw materials, as well as the feasibility of its use in the removal of contaminants of oily origin.

4. CONCLUSIONS

The potential for application of solid culture medium containing lipases and biosurfactants in bioremediation processes of oily compounds has been confirmed, excluding the need for precipitation and purification methods. The bioremediation experiment carried out over 90 d demonstrated greater biodegradation of the contaminant (74.40 ± 1.76%) when using both biocompounds, biostimulation and bioaugmentation (experiment E2). In the synthesis of biocompounds in situ in the soil, maximum production is verified in 30 d of bioremediation, for all evaluated tests. For the same experiment (E2), the production of biosurfactants in situ in the soil was lower, as this biocompound was previously added to the soil (reduction of surface tension by 6.02 ± 0.24% in 30 d), and the enzymatic production in situ in the soil remained high (6.62 ± 0.17 UL) even when the enzyme was previously added.

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5. CAPÍTULO 5: BIORREMEDIAÇÃO DE UM SOLO CONTAMINADO COM BIODIESEL UTILIZANDO BIOCOMPOSTOS PRODUZIDOS POR *Bacillus methylotrophicus*[5]

Resumo: A produção de biossurfactantes e lipases via processos de fermentação sólida (FES) é pouco explorada quando leva-se em consideração o uso de bactérias. O potencial de aplicação das matrizes sólidas contendo biocompostos produzidos, sem a necessidade de processos de precipitação e recuperação, ajudam a viabilizar processos de biorremediação, reduzindo a contaminação dos solos por compostos oleosos. Objetivou-se a produção simultânea de biossurfactantes e lipases via FES utilizando a bactéria *Bacillus methylotrophicus* e o uso do farelo proveniente deste cultivo, com os biocompostos produzidos, na biorremediação de um solo contaminado com biodiesel. Para isso, realizou-se o screening de meios de cultivo compostos por resíduos agroindústrias, como o sabugo de milho (SM) e farelo de trigo (FT) em diferentes proporções, adicionados de melaço, indutor oleoso e concentrações de nitrogênio e indutor de acordo com delineamento experimental fracionário e completo. Os meios foram inoculados com a bactéria *Bacillus methylotrophicus*, e durante 8 d avaliaram-se a atividade lipásica e a redução da tensão superficial (RTS). O meio FT+SM 80/20 possibilitou a produção simultânea de lipases e biossurfactantes nas condições de 75% umidade, 1% de indutor, 2% nitrogênio e 1% de melaço de cana de açúcar (24,61% de RTS e 43,54 ± 1,20 U, respectivamente. Para o ensaio de biorremediação, 20% de biodiesel foi adicionado a um solo argiloso, sendo a biodegradação avaliada por um período de 90 d, mensurando-se a atividade lipásica e produção de biossurfactantes in situ no solo, além do percentual de biodegradação e crescimento microbiano. O ensaio que apresentou maior biodegradação em relação aos experimentos de atenuação natural e bioestimulação foi o experimento contendo, mutuamente, os biocompostos produzidos, o meio de cultivo íntegro e a bactéria em sua forma ativa (72,08 ± 0,36% de biodegradação em 60 d). Para a síntese dos biocompostos in situ no solo, máxima produção de biossurfactantes e lipases foram verificadas em 30 d de biorremediação (RTS de 23,97% e produção enzimática no solo de 1,52 ± 0,19 UL). Confirma-se a possibilidade da coprodução de dois biocompostos em um mesmo meio de cultivo, representando redução de custo e potencial de viabilização do bioprocesso quando considerada uma escala de produção industrial, além de sua aplicação ambiental no tratamento de contaminantes oleosos.

Palavras chave: bactéria, tensão superficial, bioprocesso, aplicação ambiental, atenuação natural, biodegradação.

1. INTRODUÇÃO

Priorizando o desenvolvimento econômico, faz parte da cultura de muitos países a exploração agrária e o cultivo de grãos. Consequentemente, elevado volume de resíduos provenientes desta atividade é gerado, sendo que estes resíduos possuem pouco ou nenhum valor econômico (GUERRERO; MUÑOZ, 2018), gerando impacto ambiental negativo (SCHERHAUFER et al., 2018). Para a valoração de resíduos agroindustriais, como farelos,

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sabugos e cascas, uma alternativa de aplicação é possível através da conversão destes em matérias primas de cultivo microbiano para a produção de biocompostos. Este uso é viável pela potencial fonte nutricional fornecida por estes resíduos (LIZARDI-JIMÉNEZ; HERNÁNDEZ-MARTÍNEZ, 2017).

Dentre os tipos de cultivos utilizados para a produção de biocompostos, a fermentação em estado sólido (FES) é considerada um método sustentável e de baixo custo, principalmente pelo baixo consumo de água e energia, devido principalmente a ausência de água livre no sistema e de necessidade de agitação do meio e o uso de resíduos como substrato para o crescimento microbiano (PANDEY et al., 2003; SHARMA et al., 2014). Este tipo de cultivo vai ao encontro do avanço das políticas ambientais que visam a redução do uso de novos insumos e a sustentabilidade, sendo uma alternativa para a redução de impacto ambiental (SALIM et al., 2017).

As enzimas lipolíticas e os biossurfactantes são biocompostos cuja produção já tem sido realizada (TANYOL et al. 2015, OLIVEIRA et al. 2017, RUFINO et al., 2014, ALMEIDA et al., 2013). Entretanto, a produção simultânea destes bioprodutos é pouco descrita na literatura (COLLA et al., 2010; ZARINVIARSAGH et al., 2017; MARTINS et al., 2008). Ainda, quando considerada a produção de biossurfactantes e lipases via FES destacam-se fungos e leveduras, sendo as bactérias pouco avaliadas quanto a sua capacidade de desenvolvimento em matrizes sólidas (THOMAS et al., 2013).

Especificamente, o uso de bactérias do gênero *Bacillus* na produção de lipases e biossurfactantes é conhecido (SLIVINSK et al., 2012; MACHADO et al., 2020), entretanto não são encontrados estudos que avaliam seu potencial para a produção concomitante de biocompostos quando em matrizes sólidas, meio onde apresenta vantagem de ser cultivado em virtude de sua capacidade de crescimento em baixa humidade (MAZHAR et al., 2016; ZOUARI et al., 2015). A espécie *Bacillus methylotrophicus* é recentemente datada, sendo por isso pouco avaliada para o cultivo e produção de biocompostos, mas já há registros da sua capacidade de produção de biossurfactantes (DECESARO, 2016), restando a avaliação de sua capacidade de produção enzimática e de coprodução de bioprodutos de forma simultânea.

Biossurfactantes e lipases são amplamente utilizados em indústrias farmacêuticas, químicas e de alimentos (FAI et al., 2015; GHARAEI-FATHABAD, 2011; NITSCHKE, 2002), sendo a capacidade de atuação destes compostos em aplicações ambientais ainda pouco explorada (TSUJI et al., 2013, AZHDARPOOR et al., 2014). Dentre os potenciais usos ambientais destes biocompostos, destaca-se a biorremediação, técnica onde o tratamento de solos contaminados baseia-se na conversão de contaminantes de origem oleosa (como

biodiesel, gasolina e óleo diesel) em compostos simples e atóxicos (SALGADO et al., 2016; LARIK et al., 2019). O uso de lipases e biossurfactantes produzidos via FES pode ser vantajoso no tratamento de contaminantes (QUINTELLA et al., 2019) pois possibilita a aplicação de toda a matriz sólida, incluindo o microrganismo cultivado, os biocompostos produzidos e o próprio meio de cultivo diretamente no solo, anulando a etapa de precipitação e recuperação destes biocompostos, reduzindo significativamente os custos de ambos os processos de produção e de aplicação dos bioprodutos (BANAT et al., 2014).

Objetivou-se a produção simultânea de biossurfactantes e lipases em fermentação em estado sólido utilizando a bactéria *Bacillus methylotrophicus* e o uso do farelo proveniente deste cultivo incluindo os biocompostos produzidos na biorremediação de um solo contaminado, avaliando a remoção do contaminante e a produção dos biocompostos in situ no solo durante o processo de biorremediação.

2. METODOLOGIA

2.1 Microrganismo e preparo do inóculo

O microrganismo utilizado foi a bactéria *Bacillus methylotrophicus*, isolada de um solo contaminado com óleo diesel, identificada segundo gênero e espécie através do sequenciamento e análise filogenética do gene RNA ribossomal 16S (DECESARO, 2016), armazenada no banco de cepas do Laboratório de Bioquímica e Bioprocessos da Universidade de Passo Fundo, mantida sob refrigeração a 4ºC.

O inóculo foi preparado em meio Plate Count (PC) contendo triptona (5g/L), extrato de levedura (2,5g/L) e glicose (1g/L). Posteriormente, em Erlenmeyer de 250mL foi adicionado 50mL do meio PC, esterilizado a 120°C por 20 minutos. Após, a inoculação foi realizada com duas alçadas de *B. methylotrophicus* previamente preparadas em meio PCA (Plate Count Agar). O meio PC já inoculado em erlenmeyer foi incubado em agitador orbital por 48h a 30°C, até apresentar densidade óptica maior ou igual a 1,0 a 660 nm (padrão de turvação baseado na escala nefelométrica de McFarland, onde a densidade maior ou igual a 1,0 a 660nm significa que há aproximadamente $3x10^8$ Unidades Formadoras de Colônias (UFC)/mL).

2.1.1 Preparo dos meios de cultivo e delineamentos experimentais

Os substratos utilizados no preparo dos meios de cultivo foram o farelo de trigo (FT), proveniente do Laboratório de Cereais da Universidade de Passo Fundo, sabugo de milho (SM) obtido de cultivo experimental realizado no Centro de Extensão e Pesquisa Agropecuária (CEPAGRO) da Universidade de Passo Fundo, melaço de cana-de-açúcar (MCA) obtido junto a empresa Alisul Alimentos S.A., na forma de borra de tanque de armazenamento para processo produtivo de ração animal, em unidade localizada na cidade de Carazinho/RS. Os substratos foram caracterizados quanto aos teores de proteínas, lipídeos, resíduo mineral fixo (cinzas), umidade, carboidratos totais, tamanho de partícula e concentração de glicose presente no resíduo de melaço (KRELING et al., 2020).

A fim de realizar o screening de meios e condições de cultivo apropriadas para a obtenção simultânea de lipases e biossurfactantes, realizou-se um Delineamento Experimental Fracionado 2^{5-1} resolução IV, com adição de 4 pontos centrais, totalizando 20 experimentos (Delineamento I, Tabela 2). Para o Delineamento I, os meios de cultivo foram preparados a partir de 8 g de material seco contento proporções variadas de FT/SM (70/30, 90/10, 80/20). O percentual de indutor, umidade, MCA e nitrogênio foram adicionados ao meio de acordo com as variações da Tabela 2. Acrescentou-se 75% de solução salina composta por 2 g/L de fosfato de potássio (KH_2PO_4), 1 g/L de sulfato de magnésio ($MgSO_4$) e 10 mL/L de solução traço, composta por 0,63 mg/L de sulfato de ferro ($FeSO_4.7H_2O$), 0,01 mg/L de sulfato de manganês ($MnSO_4$) e 0,62 mg/L de sulfato de zinco ($ZnSO_4$) como fonte de micronutrientes.

Os experimentos foram realizados em Erlenmeyers de 125 mL, sendo os meios de cultivo esterilizados a 121ºC durante 20 minutos. Após arrefecimento, foi realizada a inoculação a partir da adição de 0,4 mL de inóculo, preparado conforme descrito no item 2.1. Os ensaios foram incubados em estufa durante 8 dias a 30°C, sendo a cada 2 dias retiradas amostras para determinação da atividade emulsificante água em óleo ($AE_{A/O}$) e redução da tensão superficial (RTS) como indicativo da produção de biossurfactantes e para a determinação da atividade lipásica (AL), como indicativo da produção da enzima lipase.

A partir das variáveis que apresentaram efeitos significativos ($p<0,10$) no primeiro delineamento experimental, um novo Delineamento Experimental Fatorial Completo 3^2 com adição de três pontos centrais, totalizando 12 experimentos (Delineamento II) foi proposto. Para o Delineamento II, mantiveram-se como variáveis fixas a proporção de FT/SM em 80/20, 1,0% de adição de MCA e 2% de sulfato de amônio como fonte de nitrogênio. O indutor glicerol foi variado em 0,5% (-1), 1,5%(+1) e 1,0% (0), e a umidade em 65% (-1), 75%(+1) e 70% (0). Os

procedimentos de preparo dos meios, inoculação, incubação, retirada de amostras e determinações analíticas foram os mesmos utilizados para ambos os Delineamentos I e II.

2.2 Ensaio de biorremediação

A condição de cultivo que apresentou a melhor correlação entre a produção de lipases e biossurfactantes foi utilizada para um ensaio de biorremediação. O ensaio foi preparado diretamente com o farelo fermentando proveniente da fermentação em estado sólido contendo o microrganismo, biossurfactante e enzima produzida, sem a precipitação ou recuperação dos biocompostos.

O solo utilizado no estudo foi classificado geotecnicamente por Thomé et al. (2014), como Latossolo Vermelho Distrófico Húmico e como argila de alta plasticidade, apresentando pH ácido, baixa CTC e alto teor de argila, sendo estas características típicas de solos com predominância do argilomineral caulinita. Foi coletado em uma trincheira aberta a 1,2 m de profundidade, no Centro Tecnológico de Engenharia Civil (CETEC) da Universidade de Passo Fundo/RS, peneirado (peneira mesh 10, com 2 mm entre as grades das peneiras), de acordo com a American Society Testing and Materials (ASTM, 2013).

O solo utilizado apresentou uma umidade inicial de 4,45%. Esta umidade foi ajustada para 15% com água destilada. Posteriormente, as amostras foram contaminadas com 20% de biodiesel (BSBIOS/Passo Fundo/Brasil), totalizando uma umidade inicial de 35% baseado no peso seco da amostra de solo. O percentual de contaminante adicionado no solo foi adotado com base em estudos anteriores (DECESARO et al., 2016), a fim de simular uma contaminação ex situ. O ensaio de biorremediação delineado conforme a Tabela 1 foi realizado em duplicata para cada ensaio, utilizando em cada um 500 gramas de solo seco e os demais constituintes, conforme estratégia experimental. Os ensaios de biorremediação foram realizados durante 90 dias, com revolvimento do solo a cada 2 dias, e amostras foram coletadas nos tempos inicial, 30, 60 e 90 dias, avaliando-se o crescimento microbiano a partir do método da contagem microbiana, a degradação do contaminante (quantitativamente pela determinação de óleos e graxas), determinação da atividade lipásica in situ e da produção de biossurfactantes (redução da tensão superficial), de acordo com determinações analíticas do item 2.3.

Tabela 1 – Condições experimentais para o processo de biorremediação

Experimento	Técnica	Descrição*
E1	Atenuação natural	Solo + biodiesel
E2	Bioestimulação + Bioaumentação + Biocompostos	Solo + biodiesel + 10% de farelo fermentado íntegro
E3	Bioestimulação	Solo + biodiesel + 10% de farelo fermentado sem biocompostos
E4	Adsorção do contaminante no solo	Solo estéril + biodiesel + 10% de farelo fermentado íntegro estéril

* Experimento 2: Farelo fermentado com nutrientes do meio de cultivo, fungo *A. niger* ativo e lipases e biossurfactantes; Experimento 3: Farelo fermentado com nutrientes do meio de cultivo esterilizado em autoclave (121 ºC/20 min) sem adição do fungo e produção de biocompostos; Experimento 4: Solo estéril e farelo fermentado nas mesmas condições do Experimento 2 esterilizado em autoclave (121 ºC/20 min) para inativação da atividade microbiana.

2.3 Determinações Analíticas

2.3.1 Produção simultânea de biossurfactantes e lipases

2.3.1.1 Obtenção dos extratos do meio de cultivo

Após o cultivo em estado sólido, os meios foram submetidos à extração dos biocompostos para determinação das atividades lipolíticas e emulsificantes (COLLA et al., 2010). Para a obtenção do extrato para a determinação da atividade lipolítica, 1 g de meio de cultivo foi adicionado de 10 mL de solução tampão fosfato 2 M, pH 7,0 a 160 rpm durante 30 mim a 37ºC, sendo os sólidos filtrados posteriormente em algodão. Para a determinação da atividade emulsificante, os extratos foram obtidos a partir de 5 g de meio de cultivo, adicionados de 30 mL de água destilada a 90ºC seguido de extração em banho maria durante 30 min, com posterior filtração dos sólidos e coleta do sobrenadante em algodão.

2.3.1.2 Atividade emulsificante e tensão superficial

A produção de biossurfactantes foi analisada através da determinação da atividade emulsificante água em óleo, de acordo com método adaptado do índice de emulsificação, proposto por Cooper e Goldenberg (1987), no qual 3,5 mL do extrato obtido foi adicionado em tubo de ensaio juntamente com 2 mL de biodiesel. A mistura foi agitada em agitador Vórtex a 700 rpm por 1 min. Após 24 h de repouso foi realizada a leitura da altura da emulsão água/óleo

formada e da altura total da emulsão (altura da emulsão mais altura da camada remanescente de óleo), com paquímetro eletrônico digital, gerando a atividade emulsificante água/óleo (Equação 1 e 2). Brancos foram realizados utilizando água destilada no lugar da amostra.

$$E = (\frac{h_{emulsão}}{h_{total}}) * 100 \quad (1)$$

$$AE_{A/O} = (E_{amostra} - E_{branco}) \quad (2)$$

Sendo: $AE_{A/O}$= atividade emulsificante água em óleo (UE), $H_{emulsão}$= altura da camada de emulsão, H_{total}= altura da camada total, E= relação centesimal entre a altura da emulsão água/óleo e a altura total.

A tensão superficial foi avaliada através do método do anel (Du-Nuoy's ring method). Neste método, foi utilizado um volume de 30 mL do extrato utilizado para a determinação da atividade emulsificante água em óleo foi adicionado em tensiômetro da marca Biolin Scientific, modelo Sigma 702. A redução da tensão superficial dos meios em relação ao tempo de início do cultivo foi calculada conforme a Equação 3.

$$RTS\ (\%) = \frac{TS_{inicial} - TS_{final}}{TS_{inicial}} * 100 \quad (3)$$

Sendo: $TS_{inicial}$= Tensão superficial obtida no tempo inicial do cultivo (mN/m) e TS_{final}= Tensão superficial obtida no tempo final do cultivo (mN/m).

2.3.1.3 Atividade lipásica

Para a determinação da atividade lipásica foi utilizada metodologia descrita por Burkert et al. (2004). A 75 mL de solução 7% (m/v) de goma arábica adicionou-se 25 mL de azeite de oliva. Esta mistura foi agitada a 500 rpm em agitador vórtex durante 5 min para formação de emulsão. Para a reação enzimática, 5 mL da emulsão preparada, 1 mL do extrato enzimático e 2 mL de solução tampão fosfato 2 M pH 7,0 foram adicionados em erlenmeyer de 250 mL. A reação ocorreu durante 30 min a 160 rpm em mesa agitadora a 37 ºC, sendo posteriormente paralisada com 15 mL de solução álcool:acetona 1:1. Em seguida a solução obtida foi titulada com NaOH 0,01 mol/L. Uma unidade de atividade lipásica foi definida como a quantidade de enzima que libera 1 μmol de ácido graxo por minuto por grama de farelo fermentado úmido (1 U = 1 μmol $min^{-1}g^{-1}$), de acordo com a Equação 4.

$$AL\ (U) = \frac{v*M*f*11000}{t*m} \quad (4)$$

Sendo: AL= Uma unidade de atividade lipásica (U), v= Volume de NaOH gasto na titulação (mL); M = massa molar do NaOH utilizado para a titulação (mmol/mL), f= Fator de correção do NaOH, t = Tempo gasto na reação de 1 mL de extrato enzimático (min), m = Massa de farelo fermentado úmido (g).

2.3.2 Ensaio de biorremediação

2.3.2.1. Biossurfactantes e lipases em solos

A extração dos biossurfactantes do solo foi realizada de acordo com metodologia proposta por Ángeles e Refúgio (2013), realizada nos tempos inicial, 30 d, 60 d e 90 d. Preparou-se uma solução eletrolítica com 0.01 M de nitrato de potássio (KNO_3), 0.01 M de tris hidroclorídrico (Tris-HCl) e 0.003 M de azida de sódio (NaN_3), com pH ajustado para 7.0. A extração foi realizada com a proporção 1:2 (p/v) e mantida por 72 horas sob agitação em 240 rpm e temperatura entre 25ºC à 27ºC. Posteriormente, a amostra repousou por 48 horas, sendo filtrada em milipore 0.22 µm. A solução filtrada obtida foi utilizada para a determinação da tensão superficial através do método do anel (Du-Nuoy's ring method), descrita no item 2.3.1.2.

A determinação da concentração de lipases em solos foi realizada de acordo com metodologia proposta por Margesin et al. (1999), realizada nos tempos inicial, 30 d, 60 d e 90 d. Pesou-se 1 g de solo em erlenmeyer de 100 mL, adicionando-se 1.5 mL de tolueno, agitando-se os frascos por 15 min. Após agitação, adicionou-se 9 mL de água destilada e 1 mL de tributirina. As amostras foram incubadas por 72 horas a 37º C em banho maria com agitação. Posteriormente, 40 mL de acetato de etilo foi adicionado a amostra, sendo deste coletado 10 mL para titulação com 5 gotas de timolftaleína e hidróxido de sódio 0.05 M. Um controle foi realizado adicionando-se tributirina somente após a incubação das amostras. A atividade lipásica é expressa em unidades lipásicas (U), de acordo com a Equação 5.

$$AL\ (UL) = \frac{40*100*v}{10*10*(\frac{100}{g})} \quad (5)$$

Sendo: AL= Uma unidade de atividade lipásica por grama de solo (UL), v = Volume de NaOH gasto na titulação (mL); 40 = volume do extrato utilizado (mL); 10 = fator de

conversão de 0.005 M para 0.05 M; 10 = alíquota da amostra (mL); g = fator de conversão para massa de solo seco ($100.dm^{-1}$).

2.3.2.2 Crescimento microbiano

O crescimento microbiano foi avaliado através da contagem microbiana, realizada nos tempos inicial, 30 d, 60 d, e 90 d (com exceção do ensaio onde o solo estava esterilizado), sendo utilizada metodologia proposta por Trindade (2002) através da contagem de microrganismos heterotróficos totais. Em Erlenmeyers foram acrescentados 10 g de solo juntamente com 90 mL de solução peptonada e posteriormente, inseridos em shaker para agitação da suspensão com temperatura de 25ºC e 150 rpm por 1 hora. Os extratos obtidos a partir da agitação foram diluídos sucessivamente e em seguida plaqueados através da técnica pour-plate em meio Plate Count Agar (PCA), utilizando 1 mL das diluições em cada placa de Petri. O plaqueamento foi realizado em duplicata e as placas foram incubadas em estufa a 36 ºC por um período de 48 horas. Após foi realizada a contagem do número de unidades formadoras de colônias (UFC), sendo o resultado expresso em UFC/g de solo.

2.3.2.3 Degradação do contaminante

A degradação do contaminante foi avaliada quantitativamente através do teor residual do contaminante, sendo a remoção do contaminante avaliada nos tempos inicial, 30 d, 60 d e 90 d. 10 g de solo foram utilizados para extração de óleos e graxas segundo metodologia da USEPA 3550B (1996), com uso de sonda ultrassônica (Marca UNIQUE), permitindo a quantificação de substâncias voláteis e semi-voláteis do solo. O cálculo do teor residual foi realizado através da Equação 6, e o resultado expresso em degradação do contaminante oleoso, de acordo com a Equação 7.

$$Teor\ Residual\ de\ Óleo\ (\%) = \left(\frac{P_2 - P_1}{P_0}\right) * 100 \quad (6)$$

$$Degradação\ do\ contaminante\ (\%) = \frac{\%OG_{inicial} - \%OG_{final}}{\%OG_{inicial}} * 100 \quad (7)$$

Onde: P_0 = Quantidade de amostra de solo em peso seco utilizada na análise (g); P_1 = Peso do balão de fundo chato (g); P_2 = Peso do balão mais a mistura extraída do solo

contaminado (g); $OG_{inicial}$ = teor inicial de óleos e graxas (%); OG_{final} = teor final de óleos e graxas (%).

2.4 Tratamento dos dados

O delineamento experimental fracionado foi analisado através de análise de variância (ANOVA) para um nível de confiança de 90%, e o delineamento experimental completo para um nível de confiança de 95%. O ensaio de biorremediação foi analisado através de teste de Tukey para um nível de confiança de 95%.

3 RESULTADOS E DISCUSSÃO

3.1. Screening de variáveis com uso de delineamentos fatoriais para definição de meios de cultivo

3.1.1 Delineamento I: Delineamento Experimental Fracionado 2^{5-1} IV

Na Tabela 2 estão apresentados os valores máximos de atividade emulsificante água em óleo ($AE_{A/O}$), atividade lipásica (AL) e os percentuais de redução de tensão superficial obtidos, bem como os tempos em que estes eventos ocorreram, respectivamente.

As maiores $AE_{A/O}$ foram verificadas no tempo de 8 d de cultivo, nos ensaios 14 (90/10 FT/SM, 0,5% MCA, 80% umidade, 3% indutor e 1% nitrogênio) e 19 (80/20 FT/SM, 1% MCA, 70% umidade, 2% indutor e 2% nitrogênio), com valores de 5,37 ± 0,07 UE e de 5,04 ± 0,48 UE, no 8 d de cultivo, respectivamente. Observou-se também que 16 dos 20 experimentos realizados apresentaram maior formação de emulsão no tempo de 8 d de cultivo, indicando estabilidade de síntese desses compostos nesse tempo de cultivo, optando-se pela realização da análise estatística dos dados nesse período.

Em relação aos resultados de tensão superficial dos extratos obtidos a partir da fermentação em estado sólido (Tabela 2), verificou-se que 11 ensaios apresentaram redução na tensão superficial em relação ao tempo inicial de cultivo. Dentre estes, 8 ensaios apresentaram a maior redução da tensão superficial em relação ao valor do tempo inicial ocorre no tempo de 6 d de cultivo. Os experimentos que apresentaram maior redução na tensão superficial foram os experimentos 4 (90/10 FT/SM, 1,5% MCA, 60% umidade, 1% indutor e 3% nitrogênio), com redução de 35,08 mN/m ± 0,10 para 31,29 ± 0,35 mN/m, 3 (70/10 FT/SM, 1,5% MCA,

60% umidade, 1% indutor e 1% nitrogênio), de 33,77 mN/m ± 0.15 para 30,67 ± 0,04 mN/m e 1 (70/10 FT/SM, 0,5% MCA, 60% umidade, 1% indutor e 3% nitrogênio), de 34,61 mN/m ± 0,13 para 31,45 ± 0,08 mN/m), representando percentuais de 10,80%, 9,17% e 9,13%, respectivamente. Desta forma, definiu-se o tempo de 6 d como o melhor tempo de cultivo para redução de tensão superficial deste delineamento experimental, sendo a análise estatística desses resultados realizadas nesse período.

Para a atividade enzimática, verificou-se produção em 15 dentre os 20 experimentos realizados. No tempo de 2 d de cultivo foram observadas as maiores produções, para 13 experimentos. Os melhores resultados de atividade lipásica foram obtidos nos ensaios 6 (90/10 FT/SM, 0,5% MCA, 80% umidade, 1% indutor e 3% nitrogênio), 9 (70/30 FT/SM, 0,5% MCA, 60% umidade, 3% indutor e 1% nitrogênio), e 1 (70/30 FT/SM, 0,5% MCA, 60% umidade, 1% indutor e 3% nitrogênio), de 9,50 ± 0,00, 8,50 ± 0,99 e 8,48 ± 0,25 U, respectivamente. Por esta razão, este foi considerado o melhor tempo de cultivo para análise estatística desta variável.

Tabela 2 – Matriz do Delineamento Experimental I, resultados de produção de biossurfactantes e lipases e tempos de maiores produções

Experimento	Variáveis dependentes					Variáveis de resposta					
	Proporção FT/SM (%)	Melaço (%)	Umidade (%)	Indutor* (%)	Nitrogênio** (%)	$AE_{A/O}$ (UE)	Tempo (d)	RTS (%)***	Tempo (d)	Atividade lipásica (U)	Tempo (d)
1	70/30 (-1)	0.5 (-1)	60 (-1)	1 (-1)	3 (+1)	1,64 ± 0,22	8	9,13	6	8,48 ± 0,25	2
2	90/10 (+1)	0.5 (-1)	60 (-1)	1 (-1)	1 (-1)	2,04 ± 0,12	8	8,57	6	ND	-
3	70/30 (-1)	1.5 (+1)	60 (-1)	1 (-1)	1 (-1)	1,72 ± 0,25	8	9,17	6	5,85 ± 0,71	2
4	90/10 (+1)	1.5 (+1)	60 (-1)	1 (-1)	3 (+1)	2,16 ± 0,47	2	10,80	6	6,74 ± 0,50	2
5	70/30 (-1)	0.5 (-1)	80 (+1)	1 (-1)	1 (-1)	3,85 ± 0,30	8	ND	-	3,17 ± 0,95	2
6	90/10 (+1)	0.5 (-1)	80 (+1)	1 (-1)	3 (+1)	4,47 ± 0,99	8	ND	-	9,50 ± 0,00	2
7	70/30 (-1)	1.5 (+1)	80 (+1)	1 (-1)	3 (+1)	3,16 ± 0,20	8	ND	-	6,21 ± 0,49	2
8	90/10 (+1)	1.5 (+1)	80 (+1)	1 (-1)	1 (-1)	1,34 ± 0,03	6	ND	-	7,00 ± 0,25	2
9	70/30 (-1)	0.5 (-1)	60 (-1)	3 (+1)	1 (-1)	1,11 ± 0,07	2	2,34	6	8,50 ± 0,99	2
10	90/10 (+1)	0.5 (-1)	60 (-1)	3 (+1)	3 (+1)	2,16 ± 0,16	8	5,40	6	ND	-
11	70/30 (-1)	1.5 (+1)	60 (-1)	3 (+1)	3 (+1)	1,92 ± 0,53	8	2,42	2	ND	-
12	90/10 (+1)	1.5 (+1)	60 (-1)	3 (+1)	1 (-1)	1,99 ± 0,59	8	1,64	6	3,86 ± 0,98	2
13	70/30 (-1)	0.5 (-1)	80 (+1)	3 (+1)	3 (+1)	2,41 ± 0,45	4	ND	-	8,33 ± 1,27	2
14	90/10 (+1)	0.5 (-1)	80 (+1)	3 (+1)	1 (-1)	5,37 ± 0,07	8	ND	-	7,59 ± 0,24	6
15	70/30 (-1)	1.5 (+1)	80 (+1)	3 (+1)	1 (-1)	3,79 ± 1,19	8	2,04	6	2,03 ± 0,48	2
16	90/10 (+1)	1.5 (+1)	80 (+1)	3 (+1)	3 (+1)	3,54 ± 0,03	8	ND	-	0,17 ± 0,00	2
17	80/20 (0)	1.0 (0)	70 (0)	2 (0)	2 (0)	3,53 ± 0,31	8	1,89	2	7,14 ± 0,49	2
18	80/20 (0)	1.0 (0)	70 (0)	2 (0)	2 (0)	3,70 ± 0,36	8	0,89	2	3,98 ± 0,32	4
19	80/20 (0)	1.0 (0)	70 (0)	2 (0)	2 (0)	5,04 ± 0,48	8	ND	-	ND	-
20	80/20 (0)	1.0 (0)	70 (0)	2 (0)	2 (0)	4,05 ± 1,08	8	ND	-	ND	-

*Indutor: Glicerol; **Fonte de nitrogênio: Sulfato de Amônio; ***RTS: Redução da Tensão Superficial; ND: Não detectado pelo método

Para a atividade emulsificante, somente a umidade apresentou efeito significativo (efeito positivo +1,80345 e $p_{umidade}$= 0.0128). A redução da tensão superficial em relação ao tempo inicial de cultivo foi influenciada pela umidade (efeito negativo -5,6262 e $p_{umidade}$=0.0148) e pela concentração do indutor glicerol (efeito negativo -3.2823 e $p_{indutor}$=0.0749). Para a atividade lipásica, a adição do indutor glicerol também apresentou efeito significativo (efeito negativo -4,2549 e $p_{indutor}$=0.0879), sendo que as demais variáveis não apresentaram significância dentro do intervalo avaliado.

O efeito positivo da umidade sobre a atividade emulsificante indica que maior umidade no meio de cultivo eleva a produção de biossurfactantes. O teor elevado de umidade no meio de cultivo é uma condição significativa do processo de fermentação, principalmente para bactérias, pois esses microrganismos são considerados os mais adequados para o crescimento em teores de umidade mais altos, visto que a disponibilidade de água no meio favorece a oxigenação do mesmo e contribui para o crescimento bacteriano (SELLA et al., 2009; KARANTH et al., 1999).

O efeito negativo das variáveis umidade e concentração de indutor sobre a redução da tensão superficial e sobre a produção enzimática indicam que menores percentuais de ambos no meio de cultivo favorece a redução da tensão superficial. Utami et al (2017) avaliou que maiores concentrações de indutor podem apresentar toxicidade para o meio, influenciando negativamente na absorção de nutrientes pelo microrganismo e na transferência de oxigênio no meio, inibindo e/ou reduzindo o crescimento microbiano e consequentemente, a produção de biossurfactantes. Fator semelhante pode estar associado à necessidade de menor umidade no meio de cultivo, visto que a saturação do meio implica em redução da porosidade, colaborando para a redução da transferência de oxigênio no meio e na redução do crescimento microbiano (PANDEY et al, 2003).

Como a concentração de indutor apresentou efeito negativo sobre a RTS e a atividade lipásica, esta concentração foi reduzida na sequência dos ensaios propostos. O percentual de umidade, que apresentou efeito negativo sobre a RTS, também foi reduzido a fim de analisar as propriedades dos biossurfactantes bacterianos em meio de cultivo sólido. É importante que a umidade ideal do meio de cultivo seja verificada cuidadosamente, em razão de que baixa umidade também pode representar queda no crescimento microbiano e na consequente produção dos biocompostos de interesse, pois dificulta a solubilidade de nutrientes no meio de cultivo (MAHADIK et al., 2002).

A fim de selecionar e estudar as variáveis que efetivamente influenciaram no processo de produção de biossurfactantes e lipases, um novo Delineamento Experimental Fatorial

Completo 3^2 com adição de três pontos centrais foi executado (Delineamento II). As variáveis selecionadas foram:

- Umidade (variada em 65, 75 e 70%) por apresentar efeito significativo na redução da tensão superficial, optando-se pela redução dos níveis da variável em relação ao ensaio anterior;
- Indutor glicerol (variação em 0,5, 1,0 e 1,5%) por apresentar efeito significativo na redução da tensão superficial e atividade lipásica, também optando-se pela redução da concentração desta variável em relação ao ensaio anterior.

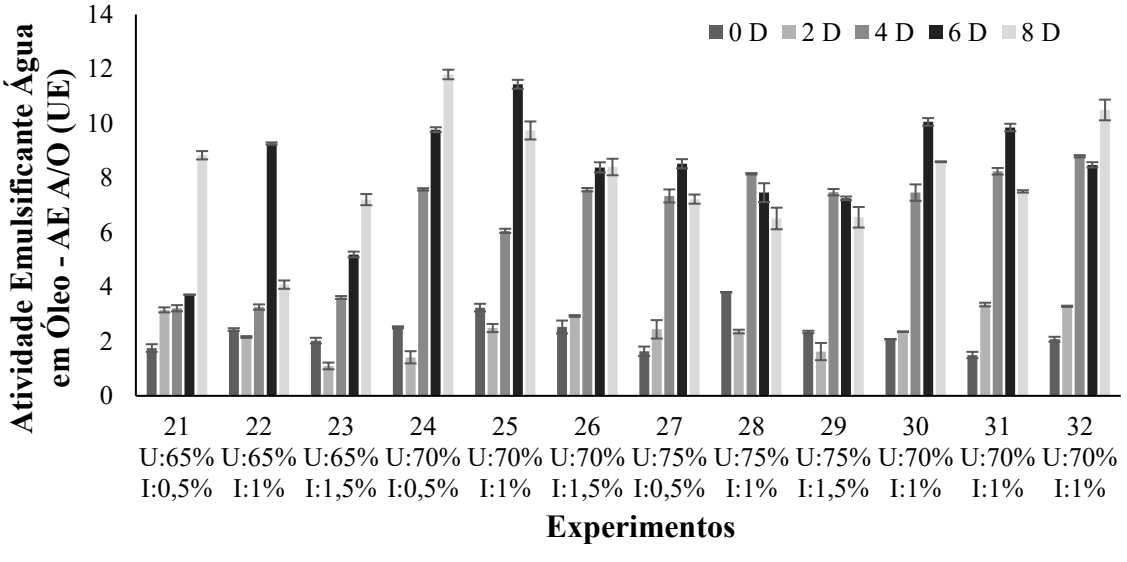
Foram fixadas as variáveis proporção de FT/SM, MCA e nitrogênio nos níveis centrais do primeiro delineamento (80/20 FT/SM, 1% de MCA e 2% de nitrogênio), visto que nenhuma destas variáveis apresentou significância para as três variáveis de resposta analisadas.

3.1.2 Delineamento II: Delineamento Experimental Fatorial Completo 3^2

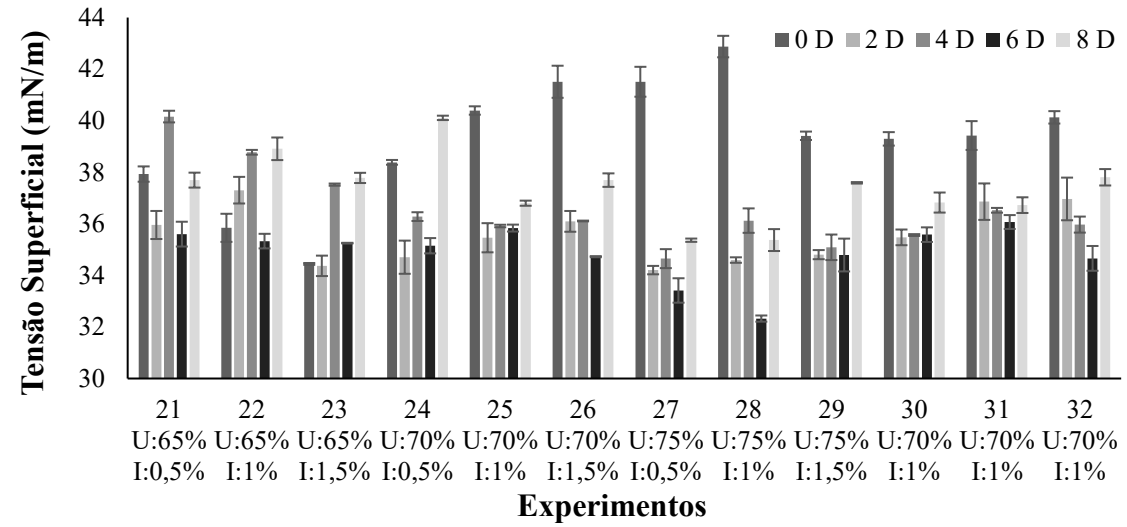
As Figuras 1a e 1 b mostram a produção de biossurfactantes ao longo de 8d de cultivo. Para a $AE_{A/O}$, percebe-se, de maneira geral, aumento na formação de emulsões entre o 6d e o 8d de cultivo. As maiores emulsões foram observadas para os ensaios 24 (70% umidade e 0,5% indutor) e 25 (70% umidade e 1% indutor), com 11,80 ± 0,18 e 11,44 ± 0,16 UE, em 8d e 6d de cultivo, respectivamente. É possível perceber que, para a maioria dos ensaios realizados, o tempo de maior formação de emulsões é o sexto dia de cultivo, sendo esse selecionado para a análise de variância realizada, representando de melhor forma o comportamento verificado nos experimentos.

Para a redução da tensão superficial, observa-se o mesmo comportamento verificado no Delineamento I, onde os maiores percentuais de redução da tensão superficial foram observados no 6d de cultivo. As maiores reduções foram verificadas para os experimentos 28 (75% umidade e 1% indutor) e 27 (75% umidade e 0,5% indutor) com percentuais de redução de tensão superficial de 24,61% e 19,50%, respectivamente. Com exceção do ensaio 23 (65% umidade e 1,5% indutor), todos os experimentos apresentaram reduções em relação ao tempo inicial de cultivo também no 6d, selecionando-se esse período para a análise de variância realizada.

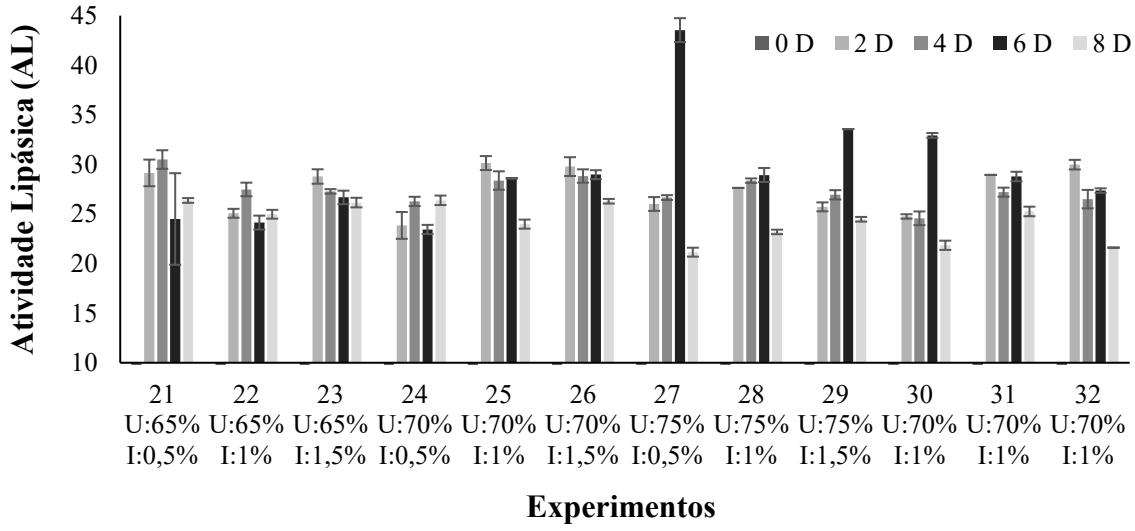
Figura 1 - Produção de biossurfactantes (a, b) e enzimas lipolíticas (c) do Delineamento II*



(a)



(b)



(c)

*U: umidade (%); I: indutor (%)

Para a produção enzimática (Figura 1c), observou-se aumento na atividade lipásica em todos os ensaios realizados. As maiores produções enzimáticas foram verificadas também no 6 de cultivo, para os experimentos 27 (75% umidade e 0,5% indutor), 29 (75% umidade e 1,5% indutor) e 28 (75% umidade e 1% indutor), com 43,54 ± 1,20, 33,56 ± 0,00 e 28,93 ± 0,71 U, respectivamente. Portanto, sendo este o tempo selecionado para a análise de variância posteriormente realizado.

A análise de variância realizada para um nível de confiança de 95% não apresentou variáveis significativas para a atividade emulsificante água em óleo ($p_{umidade}$= 0.7177 e $p_{indutor}$= 0.1860). Para a redução de tensão superficial, foi significativo e positivo o efeito da adição de indutor no meio de cultivo (efeito positivo +16,0874 e $p_{indutor}$=0.0031), indicando que maior concentração do indutor glicerol induz a maiores percentuais de redução de meio de cultivo. Quanto a produção enzimática, o efeito da concentração de indutor também foi significativo e positivo (efeito positivo +10,2424 e $p_{indutor}$=0.0331), indicando que elevadas concentrações deste favorecem a produção enzimática. O percentual de umidade adicionado no meio de cultivo não foi significativo para nenhuma das variáveis de resposta analisadas.

Cooper e Paddock (1984) indicam que a efetiva produção de biossurfactantes com propriedades tensoativas demonstram tensões superficiais inferiores a 35 mN/m, o que foi verificado neste estudo, para o experimento 28 no 6d de cultivo (32,33 mN/m). Isto indica que no meio de cultivo utilizado para a produção de ambos os biocompostos, a bactéria *Bacillus methylotrophicus* é capaz de produzir biossurfactantes com capacidade de redução de tensão superficial (UZOIGWE et al., 2015).

A elevada produção enzimática pode ser justificada pelo uso do indutor glicerol, necessitando de elevada produção de lipases para o uso desta fonte como nutriente. A síntese da enzima lipásica ocorre a partir da necessidade da degradação de lipídeos, sendo que à medida que o processo de fermentação ocorre, a disponibilidade do substrato diminui com o tempo, sendo necessária a produção de enzimas extracelulares para promover a degradação do substrato, garantindo a sobrevivência celular. A liberação das enzimas para o meio de cultivo aumenta o contato do complexo enzima-substrato, e consequentemente aumenta a assimilação de nutrientes, obtendo-se a máxima atividade das lipases extracelulares (CONTESINI et al., 2010, SPERB et al., 2015).

Desta forma, dentre os experimentos avaliados, os experimentos 28 (75% umidade e 1% indutor) e 27 (75% umidade e 0,5% indutor) são considerados como o meio de cultivo ideal para a produção de biossurfactantes e enzimas, por apresentar a maior redução de tensão superficial observada em relação ao tempo de cultivo inicial (24,61% e 19,50%,

respectivamente) e elevada produção enzimática (43,54 ± 1,20 e 28,93 ± 0,71 U, respectivamente). Para a aplicação dos biocompostos de interesse utilizados no ensaio de biorremediação posterior, o meio de cultivo do experimento 28 foi selecionado, em virtude de sua maior capacidade de redução de tensão superficial, desta forma, maior indício de produção de biossurfactantes.

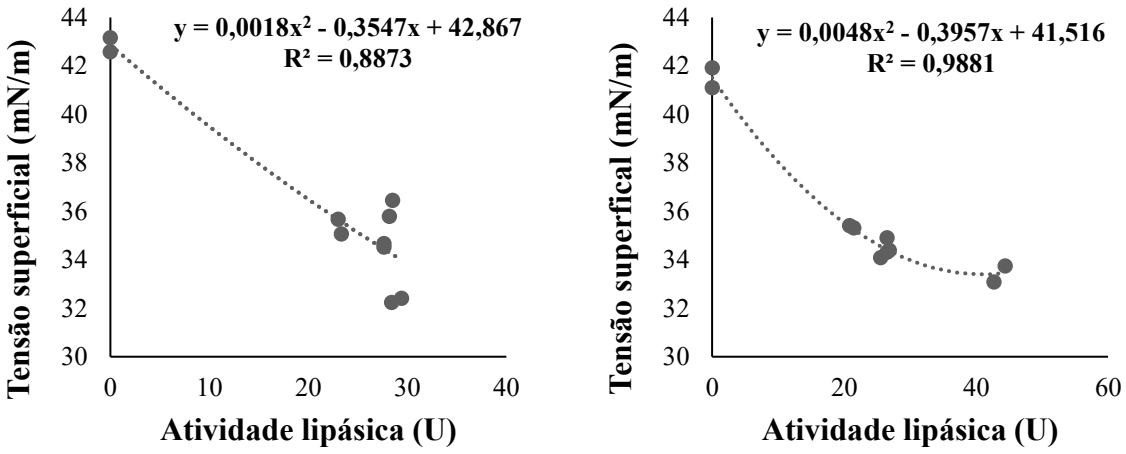
A produção de lipases em meios de cultivo sólidos utilizando bactérias também foi explorada por Ananthi et al. (2014), onde o uso da bactéria *B. cereus* foi capaz de produzir 407,58 U/g de meio de cultivo, quando utilizado 10% de óleo de gergelim como indutor. O uso do glicerol como indutor foi confirmado por Das e Mukherjee (2007), que avaliaram a produção de biossurfactantes por *B. subtilis* utilizando 0,5% do indutor, utilizando casca de batata como substrato. Os autores obtiveram uma produção máxima de biossurfactantes de 92 mg/g de meio de cultivo. A capacidade de produção enzimática em cultivos sólidos e uso de indutor que promove a redução superficial de meio de cultivos, indicando a produção de biossurfactantes, também foi comprovada em nosso estudo.

3.2 Análise da coprodução de biocompostos para os meios de cultivo avaliados

Para identificar em quais condições foi verificada maior coprodução dos biocompostos, a Figura 2 apresenta a correlação entre a tensão superficial e a atividade lipásica para os melhores ensaios selecionados no Delineamento Fatorial Fracionário II (substrato FT/SM 80/20, 1% de MCA e 2% de sulfato de amônio) – Ensaios 28 (75% umidade e 1% indutor) e 27 (75% umidade e 0,5% indutor).

Os coeficientes de determinação verificados foram de R^2=0,8873 e R^2=0,9881 para os ensaios 28 e 27, respectivamente. Resultados semelhantes de correlação entre a produção de biossurfactantes e a atividade lipolítica foi verificado por Colla et al. (2010), onde os autores obtiveram uma correlação polinomial de segunda ordem de 0.91 para a produção de ambos os biocompostos para a fermentação submersa, quando utilizado o fungo *Aspergillus niger*. Kreling et al. (2020) também obtiveram uma alta correlação (de R^2=0,98) para a produção de ambos os biocompostos também em fermentação em estado sólido para o fungo *Aspergillus niger.* Nosso trabalho indica que esta correlação também ocorre para a fermentação em estado sólido quando o cultivo é realizado para bactérias que normalmente desenvolvem-se em matrizes submersas, como é o caso da bactéria *Bacillus methylotrophicus*.

Figura 2 – Correlação entre a produção de biossurfactantes e lipases nos ensaios 28 (a) e 27 (b) do Delineamento II



(a) (b)

A relação na produção de ambos os biocompostos pode ser justificada pela ação da enzima lipolítica, responsável pela hidrólise de triacilglicerol em ácidos graxos e glicerol (KIRAN et al., 2016), provenientes do indutor. Os ácidos graxos podem ser metabolizados de acordo com a rota metabólica explanada por Fontes et al. (2008), produzindo assim biossurfactantes. Esta metabolização pode ocorrer para a formação da fração apolar (lipídica) da molécula de biossurfactante, ao passo que as fontes de carbono de fácil assimilação são utilizadas para a síntese da fração polar da molécula de biossurfactante. Perfumo et al. (2018) também indicam que a síntese de biossurfactantes está associada a produção de enzimas extracelulares, como é o caso da lipase, promovendo a solubilização e a mobilização dos indutores hidrofóbicos, aumentando assim sua biodisponibilidade.

Desta forma, verifica-se que a coprodução de biocompostos utilizando-se fermentação em estado sólido é possível para bactérias quando utilizado resíduos agroindustriais com baixo teor de material lignocelulósico, como o farelo de trigo e o sabugo de milho, reduzindo custos de produção e tornando o processo viável do ponto de vista ambiental e econômico (HASAN et al, 2006), trazendo vantagens em relação a fermentação submersa, onde o uso de insumos como água e energia são maiores (COLLA et al., 2016), influenciando negativamente na produção de biocompostos em larga escala quando este tipo de cultivo é levado em consideração.

3.3 Ensaio de biorremediação

A composição de meio de cultivo (Delineamento II – FT/SM 80:20, 1% MCA, 2% nitrogênio, 75% umidade e 1% de indutor) que apresentou correlação de R^2=0.8873 entre a produção de biossurfactantes (redução de tensão superficial de 24,61%) e lipases (28,93 ± 0,71 U) foi utilizada para o ensaio de biorremediação.

3.3.1 Produção in situ de biossurfactantes e lipases no solo e crescimento microbiano

A Figura 3 apresenta os resultados da determinação da produção de biossurfactantes em solo para o processo de biorremediação realizado ao longo dos 90 d. Em relação a redução da tensão superficial (RTS), observa-se, para o experimento E4 (solo sem atividade microbiana), baixa ou nenhuma redução em 90 d de cultivo (3,35% em 30 d, e 0,00% em 60 d e 90 d). Isto comprova a ausência de produção destes biocompostos na ausência microbiana.

Para os demais ensaios realizados, percebe-se aumento no percentual da RTS em 30 d de cultivo. De acordo com Decesaro et al. (2016), a redução da tensão superficial pode ser explicada pelo aumento da biodegradação do contaminante no meio, sendo nos primeiros 30 d o início efetivo da biodegradação do composto oleoso pelos microrganismos do meio, fenômeno que ocorre inclusive para o experimento E1 (atenuação natural). Para os ensaios E2 e E3, a produção de compostos com propriedades de redução da tensão superficial do meio pode ocorrer devido a adição do bioestimulante e biocompostos ao processo de tratamento do contaminante, incrementando a biomassa microbiana presente no solo, que iniciaram a produção in situ dos biocompostos.

A maior RTS no solo foi verificada para o experimento E2 (bioestimulação com a adição de biocompostos), apresentando uma redução de 23,97% em 30 d de ensaio. Para este experimento, houve diferença estatística quando comparado com os demais ensaios realizados ($p_{E1\ 30d}$=0,0193; $p_{E3\ 30d}$=0,0084; $p_{E4\ 30d}$=0,0001) e diferença estatística entre os próprios ensaios do experimento E2, em 60 d e 90 d ($p_{E2\ 60d}$=0,0001; $p_{E2\ 90d}$=0,0001). Este ensaio apresentou o maior percentual de RTS, sendo portando considerado a maior redução da tensão superficial de todo o experimento.

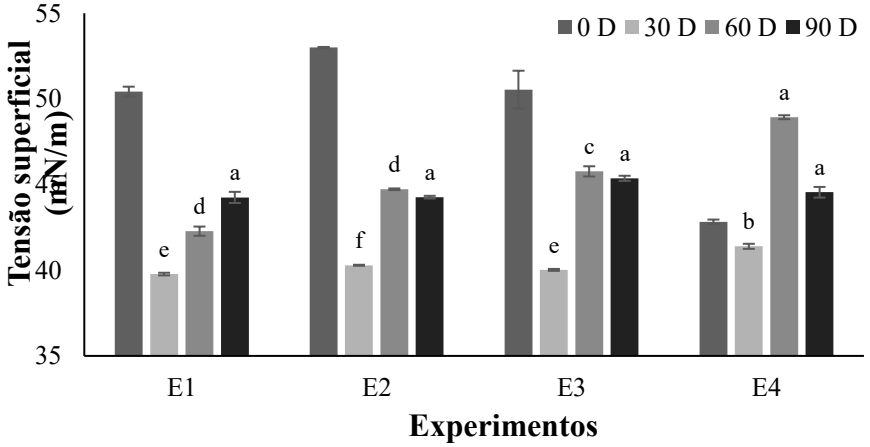
Os microrganismos presentes no solo são em grande maioria espécies de *Bacillus sp*. e *Pseudomonas sp*. (MULLIGAN, 2009). Estes possuem como característica principal a produção de biossurfactantes de baixo peso molecular. Os biossurfactantes de baixo peso molecular são conhecidos por apresentar a propriedade de redução da tensão superficial

(SATPUTE et al., 2010), justificando os maiores percentuais de RTS em ensaios onde há a adição da bactéria *Bacillus methylotrophicus*. Os ensaios onde não houve a adição de biocompostos e da bactéria apresentaram menores percentuais de RTS em 30 dias de ensaio (20,79% para o experimento E3 – bioestimulação e 21,12% para o experimento E1 – atenuação natural), não havendo diferença estatística entre estes ensaios (p=0,9999). Isto pode ser justificado pela adição dos biocompostos produzidos bactéria *Bacillus methylotrophicus* no início do processo de biorremediação.

Machado et al. (2020) analisou a produção de biossurfactantes in situ em solo contaminado com 20% de óleo diesel, apresentando redução da tensão superficial nos tempos de 30 d e 60 d de para o tratamento do contaminante, em ensaios onde utilizaram-se em conjunto bioestimulação e bioaumentação, e separadamente bioestimulação e bioaumentação, obtendo-se reduções de aproximadamente 60,00 mN/m para 53,00 mN/m (11,67%). Em comparação ao nosso estudo, corrobora-se a possibilidade do uso da mensuração da redução de tensão superficial do solo como técnica indicativa da produção de biossurfactantes por microrganismos autóctones, sendo que no período de 30 d observaram-se reduções significativas em ambos os estudos, sendo este o período de maior redução de tensão superficial do meio e, consequentemente, de maior presença de biossurfactantes no solo.

Ángeles e Refugio (2013) avaliaram a produção de biossurfactantes in situ ao longo de 10 d de biorremediação para um solo contaminado com hidrocarbonetos. Ao final do ensaio, obtiveram uma redução da tensão superficial de 75,20 mN/m para 55,60 mN/m (26,06%) e 57,20 mN/m (23,94%), quando utilizado as técnicas de bioaumentação e bioestimulação e apenas bioestimulação, respectivamente.

Figura 3 – Produção de biossurfactantes in situ no solo durante dos 90 d de experimento*



*letras iguais entre todos os ensaios e tempos indicam que não houve diferença estatística (p<0.05) pelo teste de Tukey para as médias ± desvio padrão; E1: Atenuação Natural; E2: Bioestimulação + Bioaumentação + Biocompostos; E3: Bioestimulação; E4: Adsorção do contaminante no solo.

Em relação a mensuração da atividade enzimática in situ no solos (Tabela 3), percebe-se, de uma forma geral, baixa atividade lipásica para todos os experimentos avaliados. Principalmente para os ensaios E4 (solo sem atividade microbiana) e E1 (atenuação natural), sendo os valores máximos verificados de 0,23±0,05 UL para o experimento E4 e 0,93±0,001 UL para o experimento E1, ambos em 60 d.

É possível verificar também que a maior produção de lipases in situ no solos foi verificada no experimento E2 (1,52±0,19 UL), quando o solo foi adicionado de biocompostos, a bactéria *Bacillus methylotrophicus* e os bioestimulantes presentes no meio de cultivo. Este portanto apresentou-se como o maior valor obtido entre todos os experimentos avaliados durante os 90 d de ensaio, com a maior média e diferença estatística dentre as demais médias apresentadas ($p_{E2\ 30\text{-}E1\ 30d} = 0{,}0001$; $p_{E2\ 30\text{-}E3\ 30d} = 0{,}0001$; $p_{E2\ 30\text{-}E4\ 30d} = 0{,}0001$). Os valores de atividade lipásica serem maiores em relação aos ensaios E1 e E3 pode ser explicado pela influência da produção de biossurfactantes em solos. Estes, quando atuam em solos, interagem com a fração aquosa do solo, levando ao aumento da biodisponibilidade do contaminante para biodegradação, aumentando a produção de enzimas degradadoras do contaminante no solo, como é o caso das enzimas lipásicas (URUM; PEKDEMIR, 2004).

Lin et al. (2009) avaliaram um solo contaminado com óleo diesel na concentração de 50000 mg/kg de solo, utilizando como tratamento a adição de 0.5% de culturas de fungos e 0.5% de cultura de bactérias, monitorando a atividade lipásica ao longo de 2 anos de biorremediação. Observou-se que a atividade lipásica foi maior para o primeiro ano de biorremediação (15.41 g/72 h), decaindo no segundo ano de ensaio (9.44 g/72 h), indicando que a atividade enzimática é capaz de promover o aumento no crescimento microbiano e na consequente metabolização do contaminante, o que também é indicado em nosso estudo.

Em relação ao crescimento microbiano (Tabela 3), para os ensaios realizados onde o solo não estava esterilizado (experimentos E1 a E3), é possível verificar que o ensaio E2 (bioestimulação + bioaumentação + biocompostos) apresentou o maior número de unidades formadoras de colônias (UFC). Para este ensaio, a elevada contagem no período inicial (10,28 ± 0,10 log UFC/g solo em 0 d) deve-se ao fato de que a bactéria em sua forma ativa foi adicionada juntamente com os biocompostos ao meio, fazendo com que a contagem microbiana permanecesse elevada até os 60 d de biorremediação, indicando boa adaptação dos microrganismos presentes no solo ao contaminante, e a ambos os biocompostos adicionados ao meio de cultivo, podendo-se indicar portanto maior biodegradação do contaminante e seu uso como fonte nutricional quando lipases e biossurfactantes são adicionados ao meio de cultivo (MACHADO et al., 2020). O número de unidades formadoras de colônias permanece elevado

nos tempos 30 d e 60 d (6,69 ± 0,24 log UFC/g solo em 30 d e 4,54 ± 0,39 log UFC/g solo em 60 d), possuindo as maiores médias dentre todos os ensaios realizados ($p_{E2\ 0d\ -\ 30d} = 0,0001$; $p_{E2\ 30-60d} = 0,0001$).

Para o ensaio E3 (Bioestimulação), percebe-se similaridade na contagem microbiana (4,71 ± 0,01 log UFC/g solo em 30 d e 2,94 ± 0,02 UFC/g solo em 90 d), com redução na formação de colônias no período final do ensaio de biorremediação. Isto pode indicar que a biodegradação do contaminante no solo também ocorre quando apenas bioestimulantes são adicionados aos solos contaminados, entretanto sua eficiência é menor quando comparada a adição das três estratégias de biorremediação (ensaio E1 - bioestimulação + bioaumentação + biocompostos). Para o ensaio E1 (atenuação natural), não houve a formação significativa de número de unidades formadoras de colônias, para todos os tempos avaliados.

Lin et al. (2009) observaram crescimento bacteriano no solo em um período de dois anos de biorremediação, de $1.49x10^8$ UFC/g de solo seco no período inicial, $8.91x10^7$ UFC/g de solo seco no primeiro ano de ensaio e $3.87x10^8$ UFC/g de solo seco ao final do experimento, para uma contaminação de 50000 mg de óleo diesel/kg de solo, indicando a capacidade de crescimento microbiano e consequente uso do contaminante para a nutrição celular, o que também foi indicado em nosso estudo.

A contagem microbiana é um dos métodos utilizados para indicar a potencial ação dos microrganismos no tratamento do contaminante, podendo ser melhor avaliada quando o percentual de degradação do contaminante é levado em consideração.

Tabela 3 – Produção de lipases in situ e contagem dos microrganismos presentes no solo ao longo dos 90 dias de biorremediação*

Experimento	**Atividade lipásica no solo (UL)**			
	0 d	**30 d**	**60 d**	**90 d**
E1	$0,00 \pm 0,00^{a}$	$0,00 \pm 0,00^{a}$	$0,93 \pm 0,01^{d,e}$	$0,51 \pm 0,17^{b,c}$
E2	$0,02 \pm 0,01^{a}$	$1,52 \pm 0,19^{f}$	$0,21 \pm 0,00^{a,b,c}$	$1,04 \pm 0,16^{e}$
E3	$0,00 \pm 0,00^{a}$	$0,58 \pm 0,14^{c,d}$	$0,02 \pm 0,01^{a}$	$1,12 \pm 0,21^{e}$
E4	$0,13 \pm 0,02^{a,b}$	$0,00 \pm 0,00^{a}$	$0,23 \pm 0,05^{a,b,c}$	$0,00 \pm 0,00^{a}$
Experimento	**Crescimento microbiano (log UFC/g solo)**			
	0 d	**30 d**	**60 d**	**90 d**
E1	ND	ND	ND	ND
E2	$10,28 \pm 0,10^{d}$	$6,69 \pm 0,24^{c}$	$4,54 \pm 0,39^{b}$	ND
E3	ND	$4,71 \pm 0,01^{b}$	$2,94 \pm 0,02^{a}$	ND

*letras iguais entre todos os ensaios e tempos indicam que não houve diferença estatística ($p<0.05$) pelo teste de Tukey para as médias ± desvio padrão; **ND: Não detectado pelo método; E1: Atenuação Natural; E2: Bioestimulação + Bioaumentação + Biocompostos; E3: Bioestimulação; E4: Adsorção do contaminante no solo.

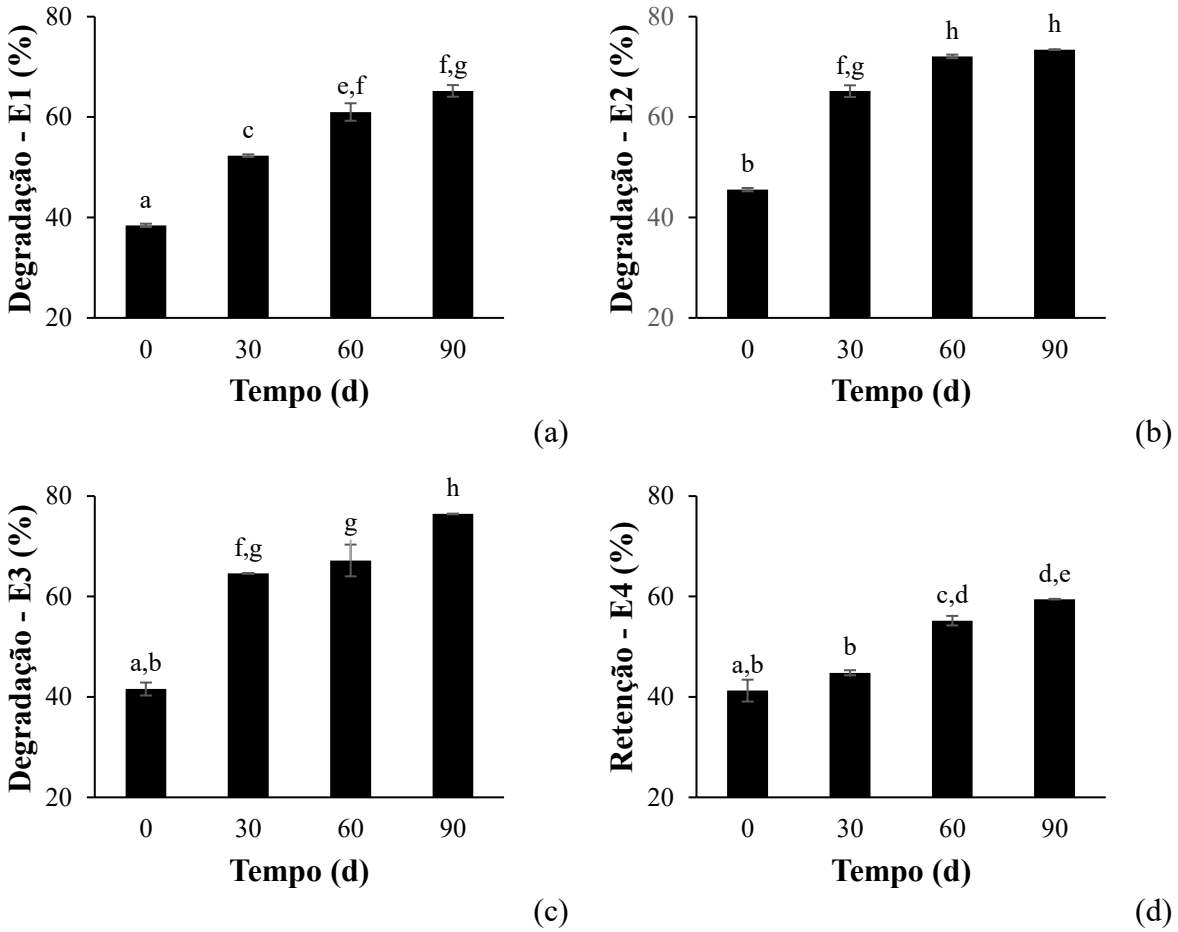
3.3.2 Degradação do contaminante

Em relação a biodegradação a Figura 4 indica os resultados da biodegradação e da retenção do contaminante, para os 90 dias de experimentos. Verifica-se aumento da degradação do contaminante no solo para todos os experimentos analisados ao longo do tempo. A menor degradação foi verificada para o experimento E4, em que o solo foi esterilizado (onde não houve intervenção microbiana), mantendo-se a remoção do contaminante (entre 41,26 ± 2,18% e 59,48 ± 0,06%) abaixo do observado nos demais ensaios, onde houve a ação da ação microbiana e da adição dos biocompostos, ao longo dos 90 dias de biorremediação.

Quando adicionado lipases e biossurfactantes ao meio de cultivo (ensaio E2 – bioestimulação + bioaumentação + biocompostos), verifica-se aumento na remoção do contaminante (de 73,44 ± 0,02 ao final de 90 d de estudo). Este aumento também ocorre quando bioestimulantes são adicionados ao solo contaminado (ensaio E3), onde uma biodegradação de 76,44 ± 0,07 ao final de 90 d de biorremediação foi observada. A atenuação natural (ensaio E1), ou seja, o monitoramento ao longo do tempo da biodegradação do contaminante mostra que o uso do biodiesel como fonte nutricional pelos microrganismos também ocorre na ausência dos biocompostos. Entretanto, o tempo apresenta-se como um fator de influência no processo de biodegradação, visto que quando biocompostos são adicionados ao solo, o tempo de biorremediação é menor, levando a biodegradação do contaminante a ocorrer em um período reduzido do que quando comparado a atenuação natural (61,01 ± 1,74% para o ensaio E1 e 72,08 ± 0,36% para o ensaio E2, em 60 d de biorremediação). Isto comprova que a adição de enzimas lipolíticas e biossurfactantes no solo influencia positivamente no tratamento do contaminante, aumentando a biodegradação ao longo do tempo.

Para um nível de significância de 95%, houve diferença significativa entre todos os ensaios avaliados (p=0.00). Para o ensaio E4 (solo sem atividade microbiana), observou-se igualdade estatística entre os tempos inicial e 30 dias (p_{0d}=0,3005; p_{30d}=0,3005) e entre os tempos de 60 e 90 dias (p_{60d}= 0,1154; p_{90d}=0,1154), observando-se nestes ensaios as menores médias de remoção de contaminante ao longo do tempo, em virtude da ausência de microrganismos biodegradadores do contaminante.

Figura 4 – Biodegradação e retenção do contaminante em 90 d de biorremediação (%)*



*Remoção de contaminante (biodegradação + retenção do solo) para os experimentos E1-E3 e retenção do contaminante para o experimento E4; Letras iguais entre todos os ensaios e tempos indicam que não houve diferença estatística ($p<0.05$) pelo teste de Tukey para as médias ± desvio padrão; E1: Atenuação Natural; E2: Bioestimulação + Bioaumentação + Biocompostos; E3: Bioestimulação; E4: Adsorção do contaminante no solo.

Cecchin et al. (2016) indicam que quando o solo não possui atividade microbiana, o contaminante que não é removido pelo solvente no processo de extração é adsorvido pelas partículas do solo, sendo o valor de remoção do contaminante observado ao longo do tempo atribuído aos processos de adsorção do solo. Os autores indicam que solos argilosos, como o utilizado no presente estudo, retém um percentual do contaminante em sua matriz, sendo este percentual menor quando utilizados compostos de origem orgânica, como lipases, biossurfactantes e o meio de cultivo sólido. Kreling et al. (2020) indicam valores de até 44% de adsorção. Os valores encontrados em nosso estudo assemelham-se ao verificado pela autora em virtude do elevado percentual de compostos orgânicos adicionados ao solo (10% em ambos os estudos).

Desta forma, conclui-se que quanto maior é o percentual de compostos de origem orgânica (lipases, biossurfactantes e o meio de cultivo sólido) no solo, menor é a retenção do

contaminante, pois reduz a capacidade de sorção dos contaminantes no solo. Este fenômeno pode ser explicado pela adsorção preferencial das partículas minerais do solo por íons de nitrogênio, fósforo e potássio, presentes nos biocompostos, reduzindo a interação do contaminante com o solo, visto que a reatividade dos argilominerais depende das superfícies de adsorção, que encontram-se saturadas com os íons provenientes dos compostos orgânicos (CECCHIN et al., 2016).

Para os demais tratamentos realizados, percebe-se aumento na biodegradação ao longo dos 90 d de cultivo. Os experimentos E2 (bioestimulação + bioaumentação + biocompostos) e E3 (bioestimulação) apresentaram os maiores valores de biodegradação do contaminante (72,08 ± 0,36% e 67,18 ± 3,16% em 60 d, respectivamente; 73,44 ± 0,02% e 76,44 ± 0,07% em 90 d respectivamente). Entretanto, a biodegradação apresentada para o ensaio E2 em 60 d é igual estatisticamente ($p=0.1047$) ao ensaio E3 (bioestimulação), ao final de 90 d de ensaio. Desta forma, comprova-se a importância da adição de biossurfactantes e lipases para o tratamento de compostos oleosos em solos, onde o tempo de tratamento da biorremediação ocorre de maneira mais acelerada quando estes são adicionados aos solos contaminados.

Em relação ao mecanismo de ação de biossurfactantes no tratamento de solos contaminados, Nievas et al. (2008) indica que a adição de biossurfactantes ao solo aumenta a biodegradação de hidrocarbonetos através de processos de mobilização e solubilização, realizados por biossurfactantes de baixo peso molecular, como as cepas de *Bacillus sp*. adicionadas ao processo de biorremediação. O processo de mobilização ocorre em concentrações menores do que a micelar crítica (CMC) do biossurfactante, onde o biocomposto reduz a tensão superficial da fração solo/água, aumentando o ângulo do contato do biossurfactante com a fração solo/óleo, aproximando solo e contaminante. No mecanismo de solubilização, que ocorre acima da CMC, biossurfactantes aumentam a solubilidade do contaminante a partir da ligação entre a fração hidrofóbica da molécula com o óleo, e a fração hidrofílica com a parte aquosa do exterior da molécula. A solubilização também facilita o transporte de contaminantes adsorvidos na fase sólida para a fase aquosa (CHU; CHAN, 2003).

A influência da adição na enzima lipásica na biodegradação de solo contaminado foi avaliada por Margesin et al. (1999). Para uma contaminação de 5 mg de óleo diesel para 8 kg de solo, os autores verificaram que em solos bioestimulados a biodegradação e a atividade lipásica são maiores, e que a suplementação com nutrientes incrementa a biomassa microbiana e a atividade metabólica. Isto é verificado em nosso estudo quando a adição do ensaio E3 (bioestimulação), sem lipases e biossurfactantes, promoveu elevada biodegradação ao final de 90 d de ensaio. Isto pode ocorrer pois o meio de cultivo adicionado ao solo para tratamento do

contaminante é composto por resíduos agroindustriais, incluindo em sua composição fontes de carbono, nitrogênio e fósforo, atuando como um bioestimulante no solo, favorecendo o incremento de biomassa e promovendo a biodegradação do contaminante.

O potencial de aplicação de biossurfactantes produzidos por *C. sphaerica* e *Bacillus sp.* foi avaliado por Chaprão et al. (2015). Os autores confirmaram que ambos os biossurfactantes foram capazes de remover óleo de motor de um solo arenoso em 90% e 40%, para os biossurfactantes de *C. sphaerica* e *Bacillus sp*., respectivamente. Quando molase foi adicionado ao meio, a biodegradação atingiu quase 100% para o biossurfactante de *Bacillus sp*., em 90 dias de ensaio. Ambos os estudos corroboram com a pesquisa realizada, que também evidencia o potencial de uso de biossurfactantes e lipases em processos de biorremediação, obtendo alto teor de degradação do contaminante quando ambos os biocompostos são inseridos de forma concomitante no meio.

Os resultados obtidos em nosso estudo comprovam a possibilidade de coprodução de dois biocompostos em um mesmo meio de cultivo, com a capacidade de aplicação em biorremediação. A aplicação ambiental possibilita ausência de processos posteriores ao bioprocesso convencional, como extração e purificação, viabilizando economicamente o processo, que usa como meio de cultivo resíduos agroindustriais, barateando também sua matéria prima.

A aplicação concomitante de ambos os biocompostos produzidos, quando aplicados em um processo de biorremediação de contaminantes oleosos, promove maior biodegradação do que em relação a técnica de atenuação natural, ou ainda quando apenas bioestimulantes são utilizados no processo de tratamento de contaminantes no solo. O estudo demonstrou ainda que, quanto maior o percentual de biocompostos adicionados ao solo, menor é o processo de adsorção do contaminante na matriz deste. Poucos são os estudos que indicam a possibilidade de produção de biocompostos bacterianos em fermentação em meios de cultivo sólido e a aplicação direta deste em tratamentos ambientais, o que destaca a importância deste trabalho, ampliando a viabilidade econômica da produção de biocompostos e a redução do impacto ambiental pelo uso de matérias primas residuais, bem como a viabilidade de seu uso na remoção de contaminantes de origem oleosa.

4. CONCLUSÕES

A bactéria *Bacillus methylotrophicus* possui viabilidade para produção simultânea de lipases e biossurfactantes quando cultivado em estado sólido. Dentre os meios de cultivo

avaliados, os ensaios 28 (75% umidade e 1% indutor) e 27 (75% umidade e 0,5% indutor) do Delineamento Fatorial Fracionário II (substrato FT/SM 80/20, 1% de MCA e 2% de sulfato de amônio) apresentaram as maiores produções de biossurfactantes (redução de tensão superficial observada em relação ao tempo de cultivo inicial de 24,61% e 19,50%, respectivamente) e lipases (43,54 ± 1,20 e 28,93 ± 0,71 U, respectivamente). A coprodução destes biocompostos também foi confirmada, havendo um coeficiente polinomial de segunda ordem de produção de 0,8873 para o ensaio 28 e de 0,9881 para o ensaio 27. O potencial de aplicação de todo o meio de cultivo contendo os biocompostos produzidos em processos de biorremediação de compostos oleosos foi confirmando, excluindo-se a necessidade de etapas de precipitação e purificação destes. O ensaio de biorremediação realizado ao longo de 90 dias demonstrou melhor biodegradação do contaminante em 60 d de experimento (72,08 ± 0,36%), quando utilizado ambos os biocompostos, bioestimulação e bioaumentação no meio (ensaio E2). Na síntese de biocompostos in situ no solo, máxima produção de biossurfactantes e lipases foram verificadas em 30 d de biorremediação, também para o ensaio E2 (redução da tensão superficial de 23,97% e produção enzimática in situ no solo de 1,52 ± 0,19 UL).

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6. CAPÍTULO 6: CONCLUSÕES GERAIS

Para a aplicação do fungo *Aspergillus niger*, cultivos foram realizados com sabugo de milho, farelo de trigo e resíduo e casca de soja. Quando utilizado sabugo de milho e farelo de trigo, a produção de lipases e biossurfactantes foi viável para um meio de cultivo composto de 0,5% de melaço de cana de açúcar, 60% de nitrogênio e 5% de indutor (10,74 ± 0,54 Unidades de Atividade Lipásica e 6,67 ± 0,06 Unidades de Emulsificação). A aplicação deste meio de cultivo contento os biocompostos e o fungo ativo em um ensaio de biorremediação de solo contaminado apontou que a maior biodegradação (74,40 ± 1,76%) foi verificada em 90 d de ensaio. Também se comprovou a viabilidade da produção simultânea de biocompostos a partir da bactéria *Bacillus methylotrophicus* quando cultivado em estado sólido. Dentre os meios de cultivo avaliados, o ensaio composto por farelo de trigo e sabugo de milho, 1% de melaço de cana de açúcar, 2% de fonte de nitrogênio, 75% umidade e 1% indutor apresentou a melhor produções de biossurfactantes e lipases (redução de tensão superficial de 24,61% e 43,54 ± 1,20 Unidades de Atividade Lipásica, respectivamente). Para o ensaio de biorremediação avaliado ao longo de 90 dias, a maior biodegradação do contaminante foi observada em 60 d de experimento (72,08 ± 0,36%), quando utilizado meio de cultivo contento os biocompostos e a bactéria ativa.

Desta forma, percebe-se que o uso da bactéria *Bacillus methylotrophicus* possuir maior potencial de biodegradação do contaminante biodiesel quando comparado ao uso do fungo *Aspergillus niger*, visto que em um período menor (60 dias) a bactéria foi capaz de biodegradar até 72,08% do contaminante em relação a aplicação do fungo *Aspergillus niger* (74,40% em 90 dias). Comprova-se o potencial de desenvolvimento da bactéria *Bacillus methylotrophicus* inclusive em matrizes sólidas com baixa umidade, onde esse microrganismo mostrou-se adaptável as condições de cultivo e com capacidade de produção de biossurfactantes e enzimas. Isso possibilita a escolha por matérias primas residuais, promovendo um impacto econômico positivo e reduzindo custos com a produção dos biocompostos e da biorremediação. A necessidade de processos de precipitação e recuperação dos biocompostos para sua posterior comercialização também é eliminado do bioprocesso, o que aumenta a viabilidade econômica do uso ambiental de lipases e biossurfactantes. Como sugestão para trabalhos futuros, pode ser analisado a ampliação de escala de produção concomitante dos biocompostos estudados, e a aplicação do ensaio de biorremediação em diferentes tipos de solos, tais como os arenosos, verificando-se inclusive a mudança na capacidade de adsorção do contaminante.

APÊNDICE A: Estudos preliminares

OBTAINING OF FUNGAL BIOCOMPOUNDS BY SOLID STATE FERMENTATION[6]

Abstract: The cultivation of microorganisms to obtain biosurfactants and lipases using solid-state fermentation (SSF) is considered a sustainable and low-cost form. The simultaneous production of these biocompounds is still little explored, especially when using solid matrices as a substrate aiming at reducing residues and environmental impacts and the potential environmental application of the produced biocompounds, in bioremediation processes and effluent treatment. The aim was the simultaneous production of lipases and biosurfactants via SSF from the fungus *Aspergillus niger.* Screening of cultivation media and conditions was carried out, with the studied variables being the proportions of soybean meal (SM) and soybean waste (SW), moisture, concentrations of carbon source (molasses), oily inducer and nitrogen source, through experimental designs. The media were fermented for 6 days, evaluating the lipase and emulsifying activities and the reduction in surface tension obtained every 2 days. In the culture medium composed of SM + SW (70/30), production of lipases (31.13 ± 0.55 U) and biosurfactants (11,92 % of reduction of surface tension and emulsifying activity below 2.00 UE) was observed under conditions of 50% of moisture, 1.5% of nitrogen source and 1% of oily inducer.

Keywords: lipases, biosurfactants, *A. niger*, surface tension, agroindustrial waste, emulsifying activity

1. INTRODUCTION

Solid-state fermentation (SSF) can be used as a cultivation method to obtain fungi biocompounds, being considered a sustainable and low-cost method, due to the low consumption of water and energy, due to the absence of free water in the system, absence of agitation of the medium and use of residues as a substrate for microbial growth [1, 2, 3]. Among the possible residues to be used in the SSF, agro-industrial residues, such as shells, bran, cob, bagasse, and seeds stand out for promoting microbial growth acting as a nutritional source [4, 5, 6].

Several microorganisms are studied for the production of biocompounds via SSF, among them fungi, yeasts and bacteria, however, filamentous fungi stand out for their ability to grow in solid or semi-solid matrices with low moisture [7, 8, 9]. The *Aspergillus niger* fungus is characterized by its potential producer of lipolytic enzymes and extracellular biosurfactants [10, 11, 12, 13].

Biosurfactants and lipases are widely used in the pharmaceutical, chemical, and food industries, with a still limited environmental application [14, 15, 16]. Among the potential

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environmental uses of these biocompounds, there are the degradation processes of oily contaminants in soils [17, 18] and water [19, 20], which promotes this study, given the potential they have to act in the degradation of compounds of oily origin [21-23].

The aim of this study was to evaluate the influence of cultivation conditions and carbon sources on the release of lipases and extracellular biosurfactants produced in SSF by the fungus *Aspergillus niger* using agro-industrial waste as a substrate.

2. METHODOLOGY

2.1 Characterization of waste used as substrate

The substrates used in the preparation of the culture media were soybean waste (SW) and soybean meal (SM) (BSBios, Passo Fundo/RS, Brazil). The SM is obtained from the process of extracting soybean oil with hexane, and the SW is the result of the soybean cleaning process, consisting of stalks, pods, soy husks, and a small percentage of broken grains and soy straw. The sugar cane molasses (SCM) (Alisul Alimentos S.A., Carazinho/RS, Brazil), was collected in a storage tank for the animal feed production process.

The substrates were characterized in terms of protein, lipid content, fixed mineral residue (ash), moisture, total carbohydrates, and particle size, according to standard methodology [24]. The concentration of glucose present in the molasses residue was also quantified by the determination of total reducing sugars (TRS) using 3,5 dinitrosalicylic acid (DNS), after pre hydrolysis of sucrose in an acid medium (HCl 2 N) in a water bath (67.5 ºC, 12 minutes) and the determination of TRS from a standard anhydrous glucose curve [25].

2.2 Microorganism, preparation of inoculum and culture media

The microorganism used was the fungus *Aspergillus niger* strain O-4, GenBank number KC545858.1 [26], belonging to the strain bank of the Biochemistry and Bioprocess Laboratory of the University of Passo Fundo, maintained in test tubes with Potato Dextrose Agar (PDA) under refrigeration at 4ºC.

The inoculum was prepared by adding 10 mL of a 0.01% (v/v) solution of Tween 80 in a test tube containing the isolated strain. The spore suspension (5 mL) was added to a 1 L conical flask containing 100 mL of PDA previously sterilized in an autoclave for 20 min at 121ºC, followed by incubation for 5 days at 30ºC for growth and hyphae formation. After this period,

50 mL of 0.01% Tween 80 solution and 3 sterile glass beads were added to the flask to obtain a spore solution, used later for inoculation of the media.

The culture media were prepared from 40 g of dry material containing varying proportions of SM/SW (70/30, 90/10, 80/20). The percentages of inductors, moisture, and SM were added to the medium according to the variations proposed in the Fractional Factor Design (item 2.3) and the Full Factorial Design (item 2.4). Then, 30 ml of a saline solution composed of 2 $g.L^{-1}$ of potassium phosphate (KH2PO4), $1g.L^{-1}$ of magnesium sulfate (MgSO4) and $10ml.L^{-1}$ of trace solution, composed of 0.63 $mg.L^{-1}$ were added of iron sulfate (FeSO4.7H2O), 0.01 $mg.L^{-1}$ of manganese sulfate (MnSO4) and 0.62 $mg.L^{-1}$ of zinc sulfate (ZnSO4) as a source of micronutrients. The experiments were carried out in 250 mL beakers, with the culture media sterilized at 121ºC for 20 minutes. Inoculation was performed using 2 ml of spore suspension for each 40 g of the prepared medium, making an initial spore concentration of 2,106 $spores.g^{-1}$. Erlenmeyer's were incubated in an oven at 30 ºC for 6 days, samples being taken every 2 days to evaluate the production of biosurfactants by determining the water-in-oil emulsifying activity and surface tension, and production of lipolytic enzymes, through lipase activity.

2.3 Experimental strategy

To screen the significant variables on the simultaneous production of lipases and biosurfactants, a Fractional Experimental Design 2^{5-1} IV presented in Table 2 was used, with the addition of central points, totaling 20 experiments. The procedures for media preparation, inoculation, and incubation were performed according to item 2.2.

From the variables that showed significant effects ($p < 0.10$) [27] in the first experimental design, a second Factorial Experimental Design with the addition of central points was proposed. For this Design, the proportion of SM/SW in 70/30 and the addition of 1% SCM were kept as fixed variables. The varied percentage of glycerol used as an inducer was 1% (-1), 5% (+1) and 3% (0), moisture at 50% (-1), 60% (+ 1) and 55% (0) and sodium nitrate by 1.5% (-1), 4.5% (+1) and 3% (0). The procedures for media preparation, inoculation, and incubation were performed according to item 2.2.

2.4 Analytical determinations

After the SSF process, the bran fermented in the crops was subjected to methods of extracting the biocompounds produced to identify the emulsifying and lipolytic activities and

to reduce surface tension [8]. To obtain the extract and to determine the emulsifying activity, in 250 ml Erlenmeyer, 5 g of fermented bran and 30 ml of distilled water at 90 ° C was added, is then placed in a water bath for 30 min for extraction and after the filtration of the solids was carried out. with cotton to obtain the supernatant for use in the analyzes. In the extraction to determine the lipolytic activity in 250 ml Erlenmeyer, 1 g of fermented bran was added with the addition of 10 ml of 2 M phosphate buffer solution, pH 7.0, which was stirred at 160 rpm for 30 minutes at 37ºC, and the solids were subsequently filtered in cotton.

2.4.1 Determination of emulsifying activity and surface tension

The determination of the water-in-oil ($EA_{W/O}$) emulsifying activity was carried out using the method adapted from the emulsification index [28], where 3.5 mL of the extract previously obtained and 2mL of biodiesel respectively, were added in test tubes and then stirred for 1 min on a Vortex shaker at 700 rpm. After 24 hours of rest, the total emulsion height (water/oil emulsion plus the remaining height of the oil layer) and the water/oil emulsion formed was measured with a digital caliper. From Equations 1 and 2, the water-in-oil emulsifying activity produced was calculated. Blanks will be made using distilled water instead of the extract.

$$E = (\frac{h_{emulsion}}{h_{total}}) * 100 \quad (1)$$

$$EA_{W/O} = (E_{sample} - E_{blank}) \quad (2)$$

Where: $EA_{w/o}$ = water-in-oil (EU) emulsifying activity, $H_{emulsion}$ = height of the emulsion layer, H_{total} = height of the total layer, E = centesimal relationship between the height of the water/oil emulsion and the total height.

The surface tension was assessed using the ring method (Du-Nuoy's ring method). In this method, a volume of 30 mL of the extract used for the determination of the water-in-oil emulsifying activity was added to a Biolin Scientific tensiometer, model Sigma 702. The reduction of the surface tension of the media concerning the time of the beginning of the culture was calculated according to Equation 3.

$$STR\ (\%) = \frac{TS_{initial} - TS_{final}}{TS_{initial}} * 100 \quad (3)$$

Where: $TS_{initial}$ = Surface tension obtained in the initial cultivation time ($mN.m^{-1}$) and TS_{final} = Surface tension obtained in the final cultivation time ($mN.m^{-1}$).

2.4.2 Determination of lipase activity

To determine lipase activity, 75 ml of 7% solution (w/v) of gum arabic was added 25 ml of olive oil. This mixture was stirred at 500 rpm on a vortex shaker for 5 min to form an emulsion. For the enzymatic reaction, 5 ml of the prepared emulsion, 1 ml of the enzyme extract, and 2 ml of 2 M phosphate buffer solution pH 7.0 were added in a 250 ml conical flask. The reaction occurred for 30 min at 160 rpm on a shaking table at 37 ºC and was subsequently paralyzed with 15 mL of 1:1 alcohol: acetone solution [29]. Then, the obtained solution was titrated with 0.01 $mol.L^{-1}$ NaOH. A unit of lipase activity was defined as the amount of enzyme that releases 1 µmol of fatty acid per minute per gram of moist fermented bran (1 U = 1 µmol $min^{-1}g^{-1}$), according to Equation 4.

$$LA = \frac{v*M*f*11000}{t*m} \quad (4)$$

Where: LA = A unit of lipase activity (U), v = NaOH volume spent on titration (mL); M = molar mass of NaOH used for the titration ($mmol.mL^{-1}$), f = NaOH correction factor, t = Time spent in the reaction of 1 mL of enzyme extract (min), m = Mass of moist fermented bran (g).

2.5 Treatment of the data obtained

The fractional experimental design was analyzed using analysis of variance (ANOVA) for a 90% confidence level, and the complete experimental design for a 95% confidence level.

3. RESULTS AND DISCUSSION

3.1 Characterization of waste used as a substrate

Table 1 and Fig. 1 show the results of the chemical characterization and particle size of the substrates used in the bioprocesses. The soybean waste presented results similar to those

demonstrated by Silva et al. [30] concerning the content of lipids (1.67%) and ash (5.20%). The protein and carbohydrate content of SW obtained in our study were high (18.31% and 57.21%, respectively), indicating the potential of this residue to be used as a macro nutrient source. Karr-Lilienthal et al. [31] evaluated the composition of the SM obtaining protein content between 45.1% and 52.6%, similar to that obtained in this study (38.21%). The lipid content obtained by the authors was low, varying between 4.1% and 9.0%, which was also verified in the present study (2.14%). García-Rebollar et al. [32] evaluated the influence of the origin of soybean meal on its chemical composition, observing ash content (between 7.18% and 7.57%) close to that obtained in our study (5.87%). Regarding the particle size of the residues, both have a particle diameter greater than 14 mm (82.65% for the SM and 83.36% for the SR), while the SM is still 16.93% composed of particles smaller than 20 mm.

The high content of carbohydrates (57.21%) present in SW is mainly due to the structure of this residue, formed by lignocellulosic materials already characteristic of vegetables, these materials being characterized by the presence of cellulose, hemicellulose and lignin, where cellulose is generally the structural polysaccharide dominant plant cell walls (35 - 50%), followed by hemicellulose (20 - 35%) and lignin (10 - 25%) [33, 34]. According to nee'Nigam et al. [35] residues from soy have a lignocellulosic composition of 34.5% cellulose, 24.8% hemicellulose and 19.8% lignin. Lignocellulosic materials, mainly because they have a complex structure, need to be subjected to the hydrolysis process to convert the polysaccharides into fermentable sugars, hydrolysis can be carried out using acid or enzymatic catalysts, where enzymatic hydrolysis stands out because it requires less energy input and greater conversion efficiency, however, enzymatic hydrolysis may be hampered by the high recalcitrant structure of lignocellulosic biomass that inhibits them from decomposing into favorable by-products for later use [36-38].

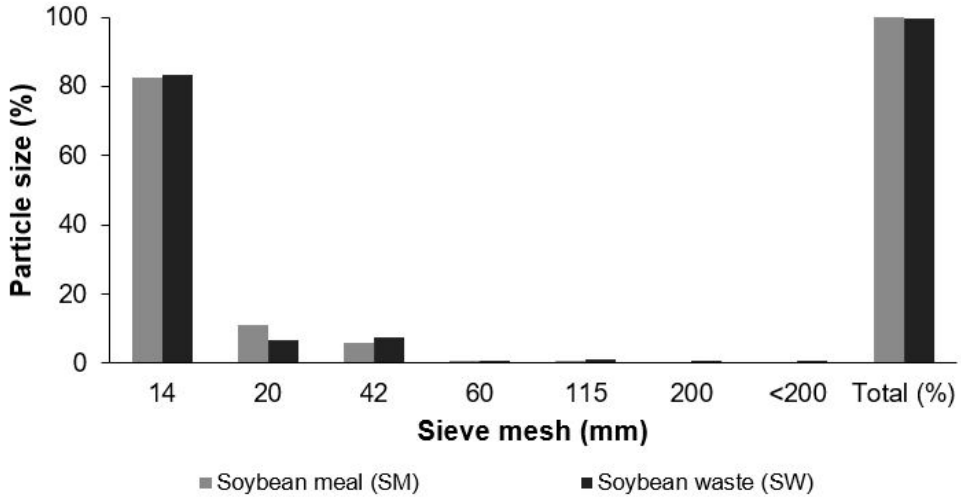
The molasses showed a high concentration of total reducing sugars (325.30 $g.L^{-1}$) as well as a high percentage of total carbohydrates (70.07%) according to the previous characterization performed by Kreling et al. [39], characterizing molasses as a simple and easily assimilated carbon source for microorganisms contributing to their growth and also helping to form the hydrophilic portion of biosurfactants [40, 41]. In their study Costa et al. [42] evaluated, for use in animal feed, the composition of sugar cane molasses, where 54.3% of the composition is total carbohydrates, 20.89% moisture, 8.5% ash, 2.6% protein and 0.70% lipids. In addition to being a carbohydrate source, sugarcane molasses has high concentrations of calcium (1.0 - 1.1%), magnesium (0.4 - 0.5%), potassium (3 - 4%), chlorine (2 - 3%) and sulfur (0.45 - 0.60%),

but it is low in phosphorus (0.1%) and also has trace metal concentrations such as aluminum, iron and potassium [43].

Table 1 Chemical characterization of residues used as a culture medium for solid state fermentation

Characteristics	Residues	
	Soybean meal (SM)	Soybean waste (SW)
Moisture (%)	13.54 ± 0.04	16.28 ± 0.13
Lipids (%)	2.14 ± 0.06	2.15 ± 0.15
Proteins (%)	38.21 ± 0.02	18.31 ± 0.04
Ashes (%)	5.78 ± 0.02	6.05 ± 0.08
Total carbohydrates (%)	40.25 ± 0.06	57.21 ± 0.02

Fig. 1 Particle size of residues used as a culture medium for solid state fermentation.



3.2 Results of the screening of variables from the Fractional Factorial Design

Table 2 shows the maximum productions of biosurfactants and lipases and the times when the maximum productions occurred for the 20 tests performed in the Fractional Experimental Design 2^{5-1} IV. Only 8 tests showed emulsion production in 6 days of cultivation. The greatest emulsifying activities were verified for experiments E1 (70/30 SM/SW, 0% SCM, 55% moisture, 1% inducer and 3% nitrogen) and E11 (70/30 SM/SW, 1% SCM, 55% moisture, 5% inducer and 3% nitrogen), of 6.36 ± 0.83 UE and 4.88 ± 0.13 UE, respectively, in 6d of cultivation, the results of this cultivation time being used to perform the statistical analysis of this response variable.

Only a considerable reduction in surface tension was observed in the final time of the bioprocess (6d), which was defined as the best cultivation time for the analysis of the effects performed. The greatest reductions were seen in the E9 tests (70/30 SM/SW, 0% SCM, 55% moisture, 5% inductor, 0% nitrogen), from 37.53 ± 0.45 to 34.10 ± 0.00 $mN.m^{-1}$ (9.13%), and in the E16 test (90/10 SM/SW, 1% SCM, 65% moisture, 5% inductor, 3% nitrogen), from 37.26 ± 0.15 to 34.15 ± 0.70 $mN.m^{-1}$ (8.35%), in the 6d periods and 4d of cultivation, respectively. Regarding the production of lipolytic enzymes, the highest yields of 39.28 ± 1.77 U and 35.15 ± 0.28 U were verified in the E1 tests (70/30 SM/SW, 0% SCM, 55% moisture, 1% inducer and 3% nitrogen) and E11 (70/30 SM/SW, 1% SCM, 55% moisture, 1% inducer and 0% nitrogen), both in 4 days of cultivation. For this reason and verifying that 9 of the 20 experiments evaluated showed high enzymatic production in the 4 d of cultivation, this is selected as the best production time for the analysis of the subsequent effects.

The analysis of effects performed at a 90% confidence level indicated, to produce $EA_{W/O}$, that the variables moisture (negative effect -2.3778 and $p_{moisture}$= 0.0285) and sodium nitrate as a source of nitrogen (positive effect) were significant. +1.9768 and p = 0.0495), thus, lower percentages of moisture and higher nitrogen concentration favored the increase in the production of water-in-oil emulsions. Regarding the reduction of the surface tension of the culture medium, the addition of the nitrogen source had a significant and negative effect (-2.1591 and p = 0.0934), indicating that lower concentrations of this promote a reduction in the surface tension of the culture medium. To produce lipases, moisture had a significant negative effect (-17.8701; p=0.0213), indicating that it should be reduced to increase enzyme production.

Moisture was an important variable to produce both biocompounds, with the best results obtained at lower levels of moisture. This variable directly affects the oxygenation of the culture medium, influencing microbial growth, and the consequent production of biocompounds of interest [44].

Table 2 - Results of production of biosurfactants and lipases and times of greatest production for the Fractional Factor Design

Experiments	Dependent variables					Response variables					
	Proportion SM/SR (%)	Molasses (%)	Moisture (%)	Inducer* (%)	Nitrogen** (%)	EA w/o (EU)	Time (d)	STR (%)***	Time (d)	Lipase activity (U)	Tempo (d)
E1	70/30 (-1)	0 (-1)	55 (-1)	1 (-1)	3 (+1)	6.36 ± 0.83	6	10.44	2	39.28 ± 1.77	4
E2	90/10 (+1)	0 (-1)	55 (-1)	1 (-1)	0 (-1)	3.02 ± 0.15	6	9.14	6	5.07 ± 0.29	2
E3	70/30 (-1)	1 (+1)	55 (-1)	1 (-1)	0 (-1)	ND	-	1.96	6	31.55 ± 1.83	4
E4	90/10 (+1)	1 (+1)	55 (-1)	1 (-1)	3 (+1)	2.83 ± 0.10	6	6.11	6	27.99 ± 0.26	4
E5	70/30 (-1)	0 (-1)	65 (+1)	1 (-1)	0 (-1)	ND	-	3.11	6	6.40 ± 0.89	2
E6	90/10 (+1)	0 (-1)	65 (+1)	1 (-1)	3 (+1)	ND	-	2.75	6	12.86 ± 0.28	4
E7	70/30 (-1)	1 (+1)	65 (+1)	1 (-1)	3 (+1)	3.19 ± 0.49	6	0.01	6	8.32 ± 0.25	4
E8	90/10 (+1)	1 (+1)	65 (+1)	1 (-1)	0 (-1)	ND	-	2.47	6	24.73 ± 1.03	4
E9	70/30 (-1)	0 (-1)	55 (-1)	5 (+1)	0 (-1)	0.25 ± 0.02	4	9.13	6	4.99 ± 0.22	2
E10	90/10 (+1)	0 (-1)	55 (-1)	5 (+1)	3 (+1)	3.36 ± 0.19	6	10.38	2	30.63 ± 0.81	4
E11	70/30 (-1)	1 (+1)	55 (-1)	5 (+1)	3 (+1)	4.88 ± 0.13	6	ND	-	35.15 ± 0.28	4
E12	90/10 (+1)	1 (+1)	55 (-1)	5 (+1)	0 (-1)	1.52 ± 0.45	6	4.01	-	20.84 ± 1.13	4
E13	70/30 (-1)	0 (-1)	65 (+1)	5 (+1)	3 (+1)	ND	-	ND	-	6.44 ± 0.00	2
E14	90/10 (+1)	0 (-1)	65 (+1)	5 (+1)	0 (-1)	ND	-	1.50	6	11.61 ± 1.13	2
E15	70/30 (-1)	1 (+1)	65 (+1)	5 (+1)	0 (-1)	ND	-	3.54	6	11.06 ± 0.56	2
E16	90/10 (+1)	1 (+1)	65 (+1)	5 (+1)	3 (+1)	ND	-	8.35	4	9.61 ± 0.54	2
E17	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	6.91	6	14.25 ± 0.57	2
E18	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	5.53	2	10.63 ± 1.38	2
E19	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	8.66	6	27.1 ± 2.85	2
E20	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	9.73	6	7.64 ± 0.00	2

*Inducer: Glycerol; **Nitrogen source: Sodium nitrate; ***STR: Surface tension reduction; ND: Not detected by method

Greater reductions in surface tension were obtained with lower concentrations of nitrogen in the culture medium. Cooper and Paddock [45] indicate that the effective production of biosurfactants with surfactant properties demonstrate surface tensions below 35 $mN.m^{-1}$, which was not verified in this study, since the tensions presented for the experiments reach values very close to 35 $mN.m^{-1}$, remaining between 34 and 33 $mN.m^{-1}$. This indicates that, regardless of the culture medium used for the production of both biocompounds, the fungus *Aspergillus niger* is not capable of producing biosurfactants with the capacity to reduce surface tension [46], making the use of this variable unviable response to the indicative of the production of biosurfactants.

The nitrogen source was significant for the formation of water-in-oil emulsions, indicating the need to increase its concentration for greater production of biosurfactants. This nutritional source is necessary for protein synthesis, leading to cell growth and the consequent production of biocompounds [47, 48]. The concentration of the glycerol inducer to produce biosurfactants and lipases was not significant ($p=0.3962$). It appears that the formation of emulsions was less and that they occurred in a longer cultivation time. The low productivity of water-in-oil emulsions and the consequent production of biosurfactants when using the glycerol inducer can be justified by the solubility of this inducer in aqueous media. Thus, it is used for the production of the hydrophilic portion of the biosurfactant molecule, the formation of the hydrophobic portion of the molecule being compromised due to the absence of an oil-insoluble inducer in water, making it difficult to produce biosurfactants, which does not occur when using soybean oil [49].

For the subsequent complete factorial design, the percentage of moisture was reduced (50, 55 and 60%), the percentage of the nitrogen source added in the form of sodium nitrate was increased (1.5, 3 and 4.5%) and the variation of glycerol concentration added to the culture medium was maintained at 1, 3 and 5%. The other variables, which did not show significance in the production of the biocompounds, were maintained in the following conditions: the proportion of SM/SW in 70/30 to guarantee the greater oxygenation of the culture medium since the soybean residue has greater granulometry, and consequent greater porosity to the culture medium; and adding 1% SCM. Aiming at increasing the production of biosurfactants, since this is considered an easily assimilated carbon source.

3.3 Complete Factorial Design

Fig. 2 shows the production of biosurfactants over 6 days of cultivation. Regarding $EA_{W/O}$, the low production also verified in the Fractional Factor Design is confirmed. The production of emulsions was not observed for any experiment in the 2d of culture. In the 4d and 6d of cultivation, low yields were verified for the tests, the highest observed for the E28 tests (60% moisture, 4.5% nitrogen and 5% inductor), from 2.55 ± 0.01 UE in the 6d culture, and 1.84 ± 0.32 UE in the E31 experiment (55% moisture, 3% nitrogen and 3% inductor), in the 4d of cultivation.

The difficulty in the production of biosurfactants may be related to the use of bran and soybean residue as a base medium for cultivation, since both are composed of lignocellulosic materials, consisting of highly complex carbonic bonds, as stated by Cassales et al. [50], who indicate that compounds from soybeans may have a high composition (74.3%) of cellulose, hemicellulose, and lignin, which may compromise its use as a carbon source for the microorganism used, due to the need for hydrolysis of this residue for conversion to glucose, hindering the metabolization of this nutritional source as a hydrophilic carbon source, necessary for the formation of the polar fraction of the biosurfactant molecule [51, 52], thus compromising the formation of this biocompounds. Still, Utami et al. [53] indicate that high concentrations of inducers can be toxic to the culture medium, negatively influencing the transfer of oxygen and absorption of nutrients, reducing microbial growth, and the production of biocompounds.

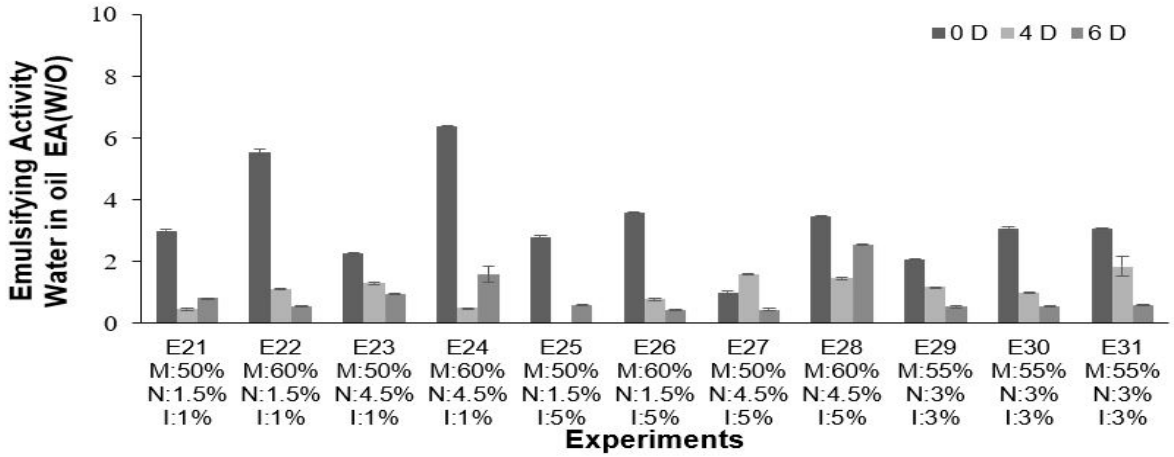
Fig. 3 shows the results of the reduction of surface tension evaluated over 6d of cultivation. The greatest reductions were seen in the E27 tests (50% moisture, 5% inductor, 4.5% nitrogen) with a reduction from 41.77 ± 0.25 to 36.37 ± 0.13 $mN.m^{-1}$ (12.92%) and E21 (50% moisture, 1% inductor, 1.5% nitrogen) showing a reduction from 41.31 ± 0.16 to 36.38 ± 0.04 $mN.m^{-1}$ (11.92%) both results were obtained in the 4d of culture. The reduction in surface tension is directly related to the production of biosurfactants observed in the present study, since the reduction in surface tension is proportional to the concentration of biosurfactants in the medium, as assessed by SILVA et al. [54] who in their study evaluated the production of biosurfactants from *Pseudomonas cepacia*, obtained a maximum production of biosurfactants of $8g.L^{-1}$ in submerged fermentation in 0.1 L medium at the same time obtained a reduction of surface tension of 70.00 to 27.00 $mN.m^{-1}$.

For the formation of lipolytic enzymes, high production was observed in all experiments performed (Fig. 4), with the highest yields observed in 6d of cultivation time, being in the E21 experiment (50% moisture, 1.5% nitrogen and 1% inductor) verified the highest enzymatic

production for all tests performed (31.13 ± 0.55 U). The high production in this context can be justified using the glycerol inducer, requiring high production of lipases for the use of this source as a nutrient. The synthesis of the lipase enzyme occurs from the need for lipid degradation, and as the fermentation process occurs, the availability of the substrate decreases over time, requiring the production of extracellular enzymes to promote the degradation of the substrate, ensuring cell survival. The release of enzymes into the culture medium increases the contact of the enzyme-substrate complex, and consequently increases the assimilation of nutrients, obtaining the maximum activity of extracellular lipases [55, 56].

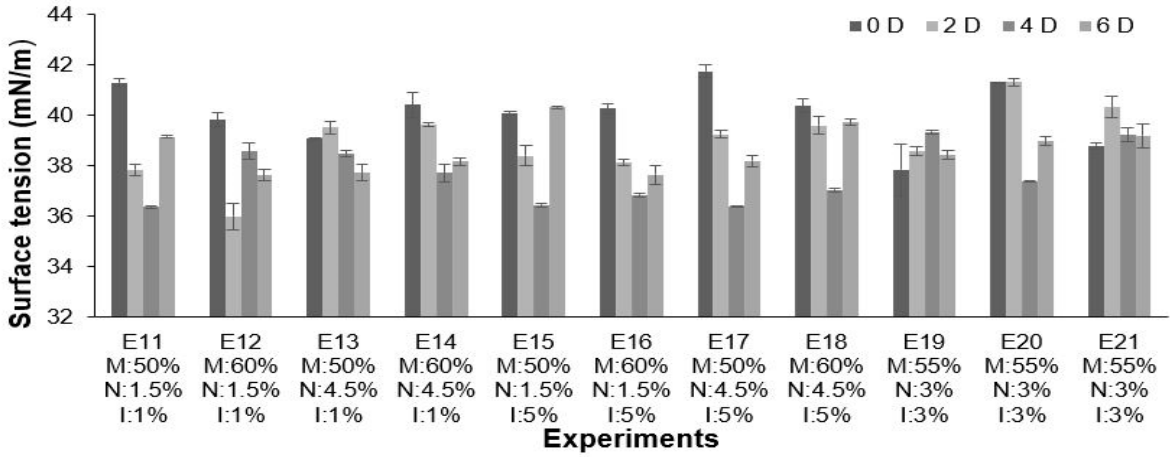
The effects analysis was performed at a 95% confidence level for the 4d of cultivation for $EA_{W/O}$ and reduction of surface tension and in the 6d of cultivation for LA, where the highest yields were observed, respectively. For the formation of emulsions, the addition of higher concentrations of nitrogen in the culture medium was significant (p=0.0003) and positive (0.6211). For the reduction of surface tension, there was no statistically significant difference between the variables analyzed (p> 0.05). For the lipase activity, significant ($p_{moisture}$ = 0.0016, $p_{nitrate}$=0.0024, $p_{inducer}$=0.0046) and negative (effect $_{moisture}$=-5.4433, $effect_{nitrate}$=-5.1571, inducing effect=-4.7019) were analyzed. This, considering the above, the E21 experiment (50% moisture, 1.5% nitrogen, and 1% inducer) is selected as the best composition of culture medium, as it has the lowest added percentages of moisture, nitrogen, and inductor. The choice is justified by the high enzyme production verified in this assay (31.13 ± 0.55 U), corroborating what was demonstrated in the analysis of effects to produce lipases.

Fig. 2 Emulsifying activity of biosurfactants produced by *Aspergillus niger* in the Complete Factorial Design *



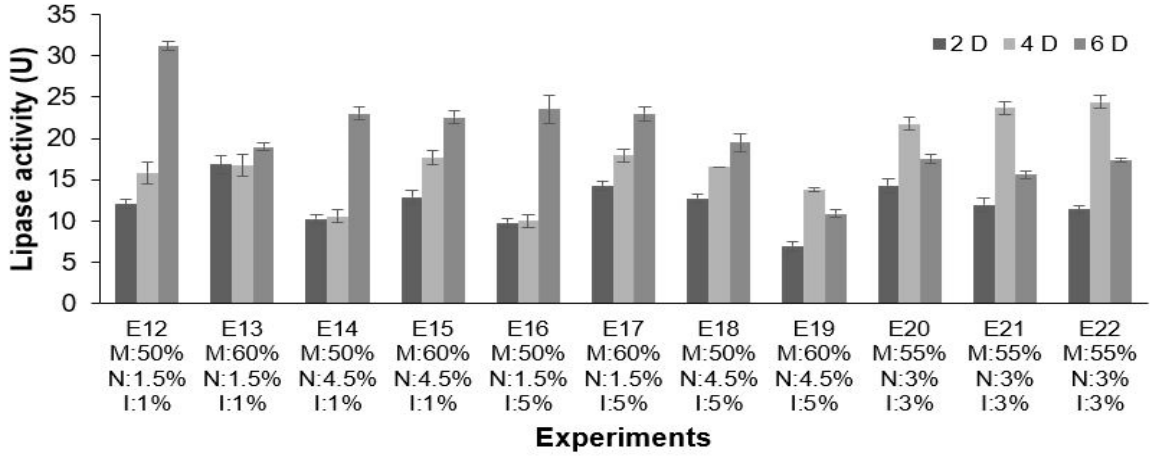
*M: moisture (%); N: nitrogen (%), I: inducer (%)

Fig. 3 Reduction of surface tension of biosurfactants produced by *Aspergillus niger* in the Complete Factorial Design *



*M: moisture (%); N: nitrogen (%), I: inducer (%)

Fig. 4 Lipase activity of lipolytic enzymes produced by *Aspergillus niger* in the Complete Factorial Design *



*M: moisture (%); N: nitrogen (%), I: inducer (%)

Rigo et al. [57] verified the production of lipases in a culture medium composed of 10 g of soybean meal, 55% moisture, 1% urea, and 0.3% soybean oil as an inducer. Using the microorganism *Penicillium* P58, the authors obtained an approximate production of 200 $U.g^{-1}$ of enzymatic activity, indicating that soybean meal associated with low moisture and inducer concentrations promote high enzymatic production.

The type of substrate used in the SSF was evaluated by Prabaningtyas et al. [58], who cultivated Aspergillus niger in different solid matrices (soybean meal, palm oil meal, and coconut flour) and 2% olive oil inducer. The authors verified the highest production of lipases for the 7 d of cultivation (110.83 $U.g^{-1}$) with soybean meal as a substrate, stating that high concentrations of inducer can interfere with enzyme production, an observation also verified in our study, where lower concentrations inducer promoted greater lipase activity.

Souza et al. [59] evaluated the use of 2% glycerol as an inducer in the production of biosurfactants for the microorganism *Bacillus subtilis* ATCC 6633. The verified emulsifying activities were below 2.2 UE, proving that even in low concentrations the use of the glycerol inducer can be inefficient to produce biosurfactants, which was also verified in our study. The study confirms the high potential not yet fully explored of the application of biosurfactants and lipases in several areas, mainly in the environmental area, in which it can be applied in processes of bioremediation of oily contaminants as a low-cost alternative compared to other treatment processes of contaminants [60].

4. CONCLUSIONS

Among the tests carried out for the Fractional Factor Design, the best results to produce biosurfactants were 6.36 ± 0.83 UE of emulsifying activity for the E1 experiment and the greatest reduction in surface tension was 9.13% reduction in E9, both in the 6d time. For lipolytic activity, the maximum production was observed in E1 as 39.28 ± 1.77 U in 4d time. For the Complete Factorial Design, the best production of lipases occurred for the E12 test (50% moisture, 1.5% nitrogen, and 1% inductor), of 31.13 ± 0.55 U. For the same experiment, the reduction in surface tension was with a reduction of 11.92% and the production of biosurfactants was less than 2.00 UE, indicating that when using lignocellulosic materials and water-soluble inducers in the composition of the culture medium, the production of biocompounds is compromised.

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