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Laccase immobilization on polyacrylonitrile particles

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“No me es posible saber si ya la infinita selva ha iniciado en mí el proceso que ha llevado a tantos otros a la locura total e irremediable.

Si es el caso, sólo me queda disculparme y pedir tu comprensión, ya que el despliegue que presencié durante esas encantadas horas fue tal que me parece imposible describirlo en un lenguaje que haga entender a otros su belleza y esplendor; sólo sé que cuando regresé, ya me había convertido en otro hombre.”

Theodor von Martius, Amazonas 1909.

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RESUMO

Este trabalho possui como objetivo principal imobilizar a lacase (Lac) de *Trametes versicolor* em partículas de poliacrilonitrila (PAN) sintetizadas via polimerização em lama. Na primeira etapa do trabalho, foram produzidas partículas de PAN e suas propriedades morfológicas, texturais e estruturais foram analisadas por microscopia ótica, Espectroscopia de Infravermelho com Transformada de Fourier (FTIR) e fisissorção de nitrogênio, com o intuito de identificar as técnicas mais apropriadas de imobilização enzimática em consonância com as características do suporte. As partículas foram quimicamente modificadas em quatro etapas: hidrólise alcalina, hidrólise ácida, aminação e aplicação de Glutaraldeído (Glu) como agente reticulante. Nestas etapas, foram testados o tempo de reação com o NaOH (na hidrólise alcalina) – 1; 2 e 3 horas, e a concentração de Glu (2; 4; 6; 8 e 10 % v/v). O tempo de reação em solução alcalina na primeira etapa da funcionalização que melhor representou alterações químicas no suporte foi o de 2 horas, enquanto a concentração de glutaraldeído não exerceu influência no rendimento de imobilização. A concentração de enzima, testada em 0,1; 0,25 e 0,5 mg/mL, também não apresentou influência significativa na imobilização. Ademais, o rendimento de imobilização foi de 99,48 e 14,29 % com o uso das partículas funcionalizadas e não funcionalizadas, respectivamente. A última etapa do trabalho consistiu na determinação de propriedades bioquímicas, químicas e físicas da lacase na sua forma livre e imobilizada por ligação covalente. Em geral, o derivado PAN+Lac exibiu bons resultados de atividade relativa e estabilidade na reação de oxidação do 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfonato) (ABTS) em diferentes pHs e temperaturas testados, exceto quando as condições impostas atingiram pH 8 e 70 °C. Nestas condições, ambas as enzimas (livre e imobilizada) perderam sua atividade vertiginosamente. Além disso, a lacase imobilizada foi capaz de reter aproximadamente 89 % da atividade inicial após 30 dias de armazenamento sob refrigeração a 5 °C. Também foi possível reutilizar o biocatalisador heterogêneo mantendo sua atividade inicial em até 50 % por 5 ciclos. Portanto, é possível apontar que o método de síntese foi eficiente para produção de partículas de PAN que, por sua vez, foram aplicadas com sucesso como suporte para imobilização da lacase de *Trametes versicolor* por ligação covalente. Estes resultados mostraram a potencialidade deste biocatalisador heterogêneo frente a aplicações de interesse industrial.

Palavras-chave: Lacase; *Trametes versicolor*; partículas de poliacrilonitrila funcionalizadas; imobilização enzimática.

RESUMO EXPANDIDO

Introdução

No que se refere ao uso de biocatalisadores na indústria, as lacases se destacam como enzimas de baixa especificidade, ou seja, seu sítio catalítico pode converter compostos altamente recalcitrantes em moléculas possivelmente menores e menos tóxicas, o que a torna uma enzima voltada principalmente para aplicações ambientais, como o tratamento de efluentes. Apesar disso, a estabilidade desta enzima precisa ser melhorada para uma aplicação consonante com os requisitos industriais. A estratégia que mais se encaixa nestes requisitos é a imobilização enzimática, a qual costuma conferir uma maior estabilidade térmica e química à enzima. A combinação de enzimas e propriedades do suporte de imobilização garante a eficiência do biocatalisador final. Sob esse ponto de vista, os polímeros sintéticos têm recebido significativa atenção como suportes enzimáticos por apresentarem fácil produção e baixo custo de síntese. Além disso, a presença de grupos funcionais ou funcionalizáveis na estrutura dos polímeros utilizados na imobilização permite a formação de interações relativamente fortes entre o suporte e a enzima (DARONCH *et al*., 2020). A poliacrilonitrila (PAN) é um polímero sintético que possui resistência a solventes orgânicos e a corrosão microbiológica, além de boas propriedades de estabilidade mecânica e térmica (ADEGBOLA; AGBOOLA; FAYOMI, 2020). A versatilidade deste polímero permite seu uso em aplicações como a imobilização enzimática, uma vez que possui grupos funcionalizáveis capazes de permitir uma ligação covalente com a lacase (VIEIRA *et al*., 2021). Nesse sentido, no presente estudo, partículas milimétricas de poliacrilonitrila foram preparadas por polimerização em solução com precipitação (*slurry*), com posterior funcionalização dos grupos nitrila para uso na imobilização da lacase de *Trametes versicolor* por ligação covalente e adsorção (sem funcionalização). O derivado enzimático foi submetido a testes de estabilidade térmica e de pH e testes de estabilidade operacional e de armazenamento, utilizando 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfonato) (ABTS) como substrato, apontando, assim, uma perspectiva positiva para aplicação futura

Objetivos

O cerne deste trabalho é a imobilização de lacase (Lac) de *Trametes versicolor* em partículas de poliacrilonitrila (PAN) sintetizadas via polimerização em lama. Os objetivos específicos compreendem a síntese das partículas de PAN, a caracterização destas em termos de morfologia e composição química de superfície, a funcionalização das partículas através de quatro etapas: hidrólise alcalina, hidrólise ácida, aminação e reticulação, a imobilização da lacase nas

partículas funcionalizadas e sem funcionalização, bem como a avaliação das propriedades físicas e químicas da enzima livre e imobilizada (estabilidade em temperatura e pH, operação/reuso e armazenamento).

Metodologia

As partículas foram preparadas por polimerização em solução com precipitação em reator de vidro borossilicato encamisado com capacidade de 1 L. A temperatura foi controlada a 85 °C por banho termostático anexado ao reator e agitação sub-regulada a 460 rpm por agitador mecânico. Para o resfriamento do condensador de vidro acoplado ao reator, foi utilizado outro banho termostático com temperatura controlada a 5 °C. Nesta ordem, 0,75 L de água destilada, 80 g (~10 % m/m) de monômero de acrilonitrila e 3,8 g de iniciador Persulfato de potássio (KPS) foram adicionados ao reator. Após 4 horas de polimerização, o reator foi resfriado e as partículas foram coletadas, filtradas a vácuo e lavadas com água destilada. Após a secagem em incubadora com temperatura controlada a 50 °C por 24 h, as partículas foram separadas por tamanho em peneiras (com abertura da malha de 1,20, 1,40 e 1,70 mm). As propriedades morfológicas, texturais e estruturais foram analisadas por microscopia ótica, Espectroscopia de Infravermelho com Transformada de Fourier (FTIR) e fisissorção de nitrogênio. Após a preparação, 0,1 g de partículas de PAN foram pesadas e funcionalizadas em quatro etapas. Inicialmente, elas foram imersas em 50 mL de NaOH 1 mol/L em uma incubadora mantida a 40 ºC por diferentes tempos (1, 2 e 3 h). Após este tempo, as amostras foram lavadas com água destilada e direcionadas para a segunda etapa, onde foram imersas em solução 50 mL de HNO_3/H_2SO_4 (50:50 v/v) 10% v/v (50:50 v/v) por 2 h a 25 °C. As partículas de PAN ativadas foram lavadas novamente com água destilada e imersas em 50 mL de solução de 1 mol/L de etilenodiamina pH 4,7/0,1 mol/L de carbodiimida à 25 °C por 1,5 h. Após a nova lavagem em água destilada, a quarta etapa consistiu na imersão das partículas em uma solução contendo diferentes alíquotas de glutaraldeído (2, 4, 6, 8 e 10 % v/v) por 1,5 h a 25 ºC. Além disso, para determinar as modificações na cadeia polimérica alcançadas pela ativação do suporte em NaOH, amostras foram incrustadas em pellets de Brometo de potássio (KBr) e analisadas por FTIR. Por fim, a PAN ativada foi tratada com 8 mL de lacase em solução tampão de acetato (0,1 mol/L, pH 4,5) (0,5 mg/mL) por até 6 horas sob agitação de 120 rpm e temperatura de 25°C. Este mesmo tratamento com solução de lacase (0,5 mg/mL) foi testado com PAN sem nenhuma etapa de funcionalização prévia para avaliar a capacidade do suporte em imobilizar a enzima sem qualquer indução de ligação covalente. Para avaliar a influência da concentração de enzima na imobilização, foram feitos testes com a partícula funcionalizada imersa em

solução contendo lacase em tampão de acetato (0,1 mol/L, pH 4,5) nas concentrações de 0,1; 0,25 e 0,5 mg/mL. O rendimento de imobilização foi determinado a partir do decaimento da atividade enzimática no sobrenadante após 6 horas de imobilização. A determinação da atividade da lacase foi monitorada pela taxa de oxidação do ABTS. A estabilidade térmica do catalisador foi avaliada adicionando 0,1 g do derivado enzimático a 2,4 mL de solução tampão fosfato-citrato 0,1 mol/L (pH 6,0) e colocado em diferentes temperaturas (30, 50, 60 e 70 °C) em banho-maria, por diferentes períodos (0,08; 0,25; 0,5; 1; 2; 4; 6 e 8 horas). A cada intervalo foram coletadas amostras de suspensão contendo o derivado enzimático, acompanhando sua atividade de oxidação do ABTS. Da mesma forma, para comparação, 0,3 mL de solução de lacase (0,5 mg/mL) foram adicionados a 2,4 mL de solução tampão de fosfato-citrato 0,1 mol/L (pH 6,0) e colocados sob agitação nas mesmas temperaturas e tempo que a enzima imobilizada. A estabilidade do catalisador em diferentes valores de pH foi realizada adicionando 0,1 g de derivado enzimático em soluções tampão fosfato-citrato 0,1 mol/L com diferentes valores de pH (3, 5 e 8) mantidos à temperatura de 25 °C por diferentes períodos (0,08; 0,25; 0,5; 1; 2; 4; 6 e 8 horas). Também foi avaliada a atividade de oxidação do ABTS nas amostras de suspensão coletadas a cada intervalo. Adicionalmente, o mesmo procedimento foi realizado para a lacase em sua forma livre, que foi adicionada em soluções 0,1 mol/L de tampão fosfato-citrato com os mesmos valores de pH, pelo mesmo tempo na concentração de 0,5 mg/mL. A estabilidade de armazenamento foi estabelecida ao longo de 30 dias. O derivado imobilizado e a enzima livre foram armazenados a 5 °C. A cada 5 dias foram coletadas amostras e monitoradas as atividades enzimáticas com oxidação do ABTS. A estabilidade operacional da lacase imobilizada foi determinada testando ciclos de oxidação ABTS consecutivos. O ensaio de atividade residual foi realizado a 50 °C em banho-maria e 0,1 mol/L de tampão fosfato-citrato (pH 3).

Resultados e Discussões

De acordo com os dados obtidos por FTIR, as amostras de PAN apresentaram um pico de absorção de vibração de alongamento na faixa de 2243 cm^{-1}, indicando a presença clara do grupo nitrila (C≡N), e outro pico em 2939 cm^{-1}, representando uma ligação C–H de $–CH_2$ em acrilonitrila, características deste polímero (BAJAJ; SREEKUMAR; SEN, 2001; WANG *et al*., 2022). A vibração de estiramento na faixa de 1456 cm^{-1} também indica uma ligação C–H do grupo $–CH_2$ (ASHRAFI; FIROUZZARE, 2021; KARBOWNIK *et al*., 2019). Também é possível apontar um pico na faixa de 1629 cm^{-1}, que denota uma ligação dupla carbono-carbono (C=C) de baixa intensidade (XUE; LIU; LIANG, 2013) ou bandas atribuídas a vibrações de deformação em o plano N-H (SHEN *et al*., 2011). Pela análise é possível apontar que o método

de polimerização aplicado correspondeu à formação bem sucedida de partículas de poliacrilonitrila. A triagem das partículas produzidas foi realizada e o tamanho médio das partículas selecionadas para imobilização de lacase foi de $1{,}2 \leq D_p \leq 1{,}7$ mm. A partir dos modelos de BET e BJH, os resultados indicam que o valor médio do diâmetro dos poros na amostra de partículas de PAN foi de 28,05 Å, o que implica a classificação do material como mesoporoso, segundo a IUPAC – 20 Å a 500 Å (HWANG; BARRON, 2011). Isso é reforçado pelas isotermas de adsorção e dessorção de N_2 para partículas de PAN classificadas como tipo II (SING *et al*., 1985). Nos resultados obtidos pelo modelo BJH fica clara a predominância de mesoporos na ordem de 20 a 200 Å na amostra. Além disso, o volume máximo de poros de dessorção medido é de 0,4236 cm^3/g. Quanto à imobilização da lacase, os resultados apontam discrepância entre a taxa de imobilização em partículas funcionalizadas e sem funcionalização, 99,48 e 14,29 %, respectivamente. Ao se testar a concentração de lacase no rendimento de imobilização em partículas funcionalizadas, não foi percebida nenhuma influência relevante, uma vez que em ambas as concentrações testadas os resultados foram bem próximos, acima de 98 %. Quanto à funcionalização da PAN, a primeira etapa consistiu na imersão das partículas em solução contendo NaOH para hidrólise alcalina. Foi notado que houve uma diminuição gradativa, ao longo do tempo de reação, da intensidade relativa dos picos relacionados aos grupos –CN e a ligação CH de –CH2, manifestada nos picos em torno de 2243 cm^{-1} e 1456 cm^{-1}, respectivamente. Esta diminuição torna-se mais evidente após 2 horas de reação. Além disso, a intensificação do pico em torno de 3450 cm^{-1} aponta para evidências concretas da formação do radical –OH na amostra. No entanto, a formação do grupo carboxílico não foi clara, pois não foi observado pico de estiramento C=O na faixa de 1710 - 1780 cm^{-1} (OH; JEGAL; LEE, 2001). Essa mesma ligação formada pode ser observada na faixa de 1680 cm^{-1} a 1630 cm^{-1}. C=O das amidas primárias (AMIR *et al*., 2013), estas podem ser convertidas em grupos carboxílicos na etapa de hidrólise ácida. Bem como a quantidade de lacase, a concentração de glutaraldeído também exerceu pouca ou nenhuma influência no rendimento de imobilização, ambas as concentrações testadas apresentaram rendimentos de, aproximadamente, 99 %. Quanto aos testes referentes às propriedades da lacase livre e imobilizada, em geral, o derivado enzimático exibiu bons resultados de atividade relativa e estabilidade na reação de oxidação do ABTS em diferentes pHs e temperaturas testados. À 50 °C, para lacase livre e imobilizada, houve um aumento de aproximadamente duas vezes em relação às suas atividades iniciais, que foram medidas à 25 °C. No entanto, a 60 °C, a lacase imobilizada em PAN foi mais estável que sua contraparte livre, mantendo sua atividade relativa em 50 % até seis horas de incubação. A 70 °C, é possível observar o perfil de desativação térmica de ambas as enzimas após duas horas

de reação. Quanto ao pH, os melhores resultados para a lacase imobilizada em PAN foram obtidos em pH 3, onde se observa uma hiperativação nos primeiros cinco minutos de imersão em solução tampão. Sua atividade relativa aumentou cerca de duas vezes. Após quatro horas, essa atividade atinge seu pico, aumentando aproximadamente 4 vezes. Em pH 5, a enzima imobilizada ainda apresenta melhores resultados que a enzima livre, atingindo seu ponto máximo em 2,25 vezes o valor da atividade inicial após seis horas de incubação. A hiperativação sob condições de pH favoráveis é relatada na literatura (DAÂSSI *et al*., 2014; CHONG-CERDA *et al*., 2020). No entanto, não é possível afirmar que a imobilização tornou a lacase mais estável em termos de variação de pH, pois ambas as enzimas apresentaram estabilidade em pH 3 e 5. Os resultados trazem uma discussão interessante sobre a atividade de enzimas livres e imobilizadas em condições de pH semelhantes, o que seria interessante do ponto de vista de aplicações industriais por longos períodos, como pode ser visualizado durante as 8 h de incubação. Em pH 8, tanto as enzimas livres quanto as imobilizadas perderam suas atividades após os primeiros cinco minutos de incubação. Os resultados encontrados para o armazenamento mostram que a atividade relativa da lacase livre após 30 dias foi de aproximadamente 46 %, enquanto a enzima imobilizada reteve aproximadamente 89 % de sua atividade inicial. Enquanto para os testes de estabilidade operacional, os resultados apontam que a lacase imobilizada em PAN por ligação covalente retém sua atividade catalítica usando ABTS como substrato em aproximadamente 50 % da atividade inicial após 5 ciclos.

Conclusão

É possível destacar que o método de síntese foi eficiente para produção de partículas de PAN que, por sua vez, foram aplicadas com sucesso como suporte para imobilização da lacase de *Trametes versicolor* por ligação covalente. A imobilização enzimática pelo método de ligação covalente apresentou um rendimento de imobilização muito superior ao método de adsorção. Isso foi possibilitado pela funcionalização do suporte em quatro etapas (hidrólise alcalina, hidrólise ácida, aminação e reticulação). De maneira geral, o derivado enzimático apresentou boa estabilidade em diferentes pHs e temperaturas, além de reter atividade após 30 dias de armazenamento e possibilitar o reuso por pelo menos 5 ciclos. Neste sentido, ficou clara a potencialidade deste biocatalisador heterogêneo frente a aplicações de interesse industrial, sobretudo no que diz respeito à oxidação de compostos recalcitrantes.

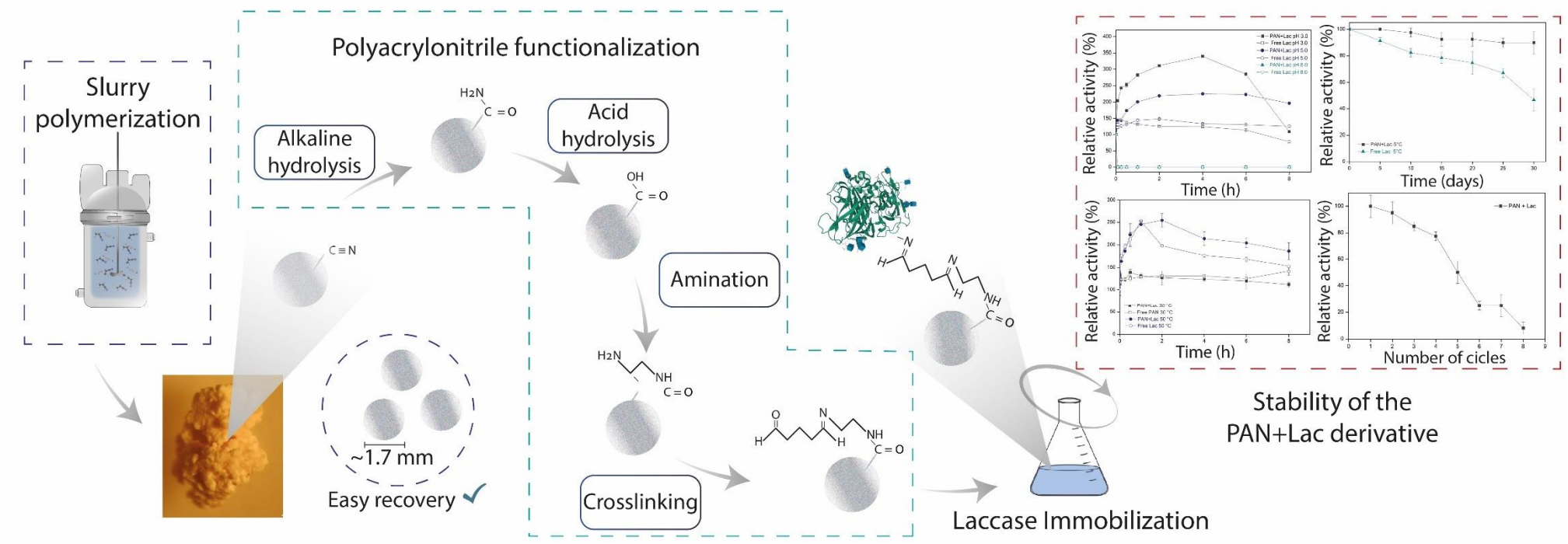
Palavras-chave: Lacase; *Trametes versicolor*; partículas de poliacrilonitrila funcionalizadas; imobilização enzimática.

ABSTRACT

The main objective of this work was to immobilize laccase (Lac) from *Trametes versicolor* in polyacrylonitrile (PAN) particles synthesized *via* slurry polymerization. In the first stage of the study, PAN particles were produced and their morphological, textural, and structural properties were analyzed by optical microscopy, Fourier Transform Infrared Spectroscopy (FTIR), and nitrogen physisorption, to identify the most appropriate techniques for enzymatic immobilization in line with the characteristics of the support. The particles were chemically modified in four steps: alkaline hydrolysis, acid hydrolysis, amination, and application of Glutaraldehyde (Glu) as a crosslinking agent. In these steps, the reaction time with NaOH (in alkaline hydrolysis) – 1; 2, and 3 h, and the Glu concentration (2; 4; 6; 8 and 10 % v/v) were tested. The reaction time in alkaline solution in the first part of the functionalization that best-represented chemical changes in the support were 2 h, while the glutaraldehyde concentration did not influence the immobilization yield. The enzyme concentration, tested at 0.1, 0.25, and 0.5 mg/mL, also showed no significant influence on immobilization. In addition, the immobilization yield was 99.48 and 14.29 % with the use of functionalized and non-functionalized particles, respectively. The last stage of the work consisted of the determination of biochemical, chemical, and physical properties of the laccase in its free form and immobilized by a covalent bond. In general, the PAN+Lac derivative exhibited good relative activity and stability results in 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation reaction at different pHs and temperatures tested, except when the imposed conditions reached pH 8 and 70 °C. In these conditions, both enzymes (free and immobilized) dramatically lost their activity. Furthermore, the immobilized laccase was able to retain approximately 89 % of the initial activity after 30 days of refrigerated storage at 5 °C. In operational stability tests, it was also possible to reuse the enzymatic derivative maintaining its initial activity at up to 50 % for 5 cycles. Therefore, it is possible to point out that the polymer synthesis method was efficient for the production of PAN particles, which, in turn, were successfully applied for the immobilization of *Trametes versicolor* laccase by covalent bonding. These results showed the potential of this heterogeneous biocatalyst for applications of industrial interest.

Keywords: Laccase; *Trametes versicolor*; functionalized polyacrylonitrile particles; enzyme immobilization**.**

GRAPHICAL ABSTRACT



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LIST OF ABBREVIATIONS AND ACRONYMS

ABS	Acrylonitrile-butadiene-styrene
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AN	Acrylonitrile
AOPAN	Amidoxime polyacrylonitrile
APs	Alkylphenols
BET	Brunauer, Emmett, and Taller method
BJH	Barrett, Joyner, and Halenda method
BPA	Bisphenol
BSA	Bovine serum albumin
CLEA	Cross-linked enzyme aggregate
CPs	2- and 4-chlorophenol
CTC	Chlortetracycline
DMF	Dimethylformamide
EDA	Ethylenediamine
EDC	Carbodiimide
EFMs	Electrospun fibrous membranes
EPR	Electronic Paramagnetic Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
Glu	Glutaraldehyde
GO	Graphene oxide
HAS	Hydroxylammonium sulfate
HZS	Hydrazinium sulfate
IGPAN	Granado Institute of Polyacrylonitrile Technology
IUPAC	International Union of Pure and Applied Chemistry
KBr	Potassium bromide
KPS	Potassium persulfate
Lac	Laccase
MMT	Montmorillonite

MOFs	Metal-organic structures
MPS	3-methacryloxypropyltrimethoxysilane
O-MMT	Organically modified montmorillonite
PAN	Polyacrylonitrile
PDA	Polydopamine
PEI	Polyethyleneimine
PET-g-MAH	Poly(ethylene) Terephthalate Grafted with Maleic Anhydride Nanofiber Mat
PPy	Polypyrrole
PU	Polyurethane
SAN	Styrene-acrylonitrile
TCP	2,4,6-trichlorophenol
β-CD	β-cyclodextrin

SUMMARY

CHAPTER 1

Here we have a general introduction to the research and its general and specific objectives.

 [1]

[1] Bring the reader to QR code to see how it all started.

1 INTRODUCTION

Biocatalysts are increasingly gaining evidence as a sustainable alternative for industrial processes and presenting high performance. In this scenario, laccases stand out as enzymes with low specificity, i.e., they can be used in several applications since their catalytic site can convert highly recalcitrant compounds into possibly smaller and less toxic molecules. As a result, laccases can be used for a wide range of applications, such as wine and fruit juice stabilization, biosensor for detection of molecules and compounds, decolorizing synthetic dyes, removing drugs and emerging contaminants, treating aromatic compounds, and detoxifying wastewater (KELBERT *et al.*, 2021; KHATAMI *et al.*, 2022; PERVEEN *et al.*, 2022; ZHANG, C. *et al.*, 2018). More recently, there has been an increase in studies that refer to laccase as an enzyme primarily aimed at toxicological and environmental applications (SHARMA *et al.*, 2021).

Laccases can be obtained from different organisms (plants, fungi, bacteria, insects, and lichens). Each laccase species can present peculiar catalytic characteristics, which further expands the range of applications of this type of protein (ARREGUI *et al.*, 2019). Lyophilized laccase has been commercially available since 1999 (RODRIGUEZ-COUTO, 2012). Since then, the market for this enzyme has soared. According to Global Laccase Market Research Report (2022), this industry sector is valued at USD 2,836.19 million in 2021, with an estimated growth of around 0.61 % until 2027. Currently, the main global players of laccase are industries such as Novozymes (Denmark), Amano Enzyme (Japan), DowDuPont (USA), Yiduoli (China), Denykem (UK), and Sunson (China). Brazilian production is still not so expressive considering the international scenario.

Despite the high enzymatic activities and the positive results in the biodegradation of toxic compounds, it is necessary to improve the stability and applicability of the enzyme according to industrial requirements. Strategies to recover and reuse the enzyme can reduce the operational costs of biocatalytic processes, not applied to the free enzyme. In this context, the application of immobilization techniques can overcome these drawbacks in using the enzyme in the free form (SHAKERIAN; ZHAO; LI, 2020). These techniques allow the laccase to have greater thermal and chemical stability. It enables reuse in several cycles and long storage periods, as long as they are kept under favorable conditions. In addition, heterogeneous enzyme systems allow for multiple reuses of enzymes, easy recovery and separation of enzyme and substrate, continuous flow operation, and diversity in bioreactor design (GU *et al.*, 2021).

The combination of enzyme and support properties ensures the stability of the supported biocatalyst. In this sense, the choice of support is an important step, as the reaction medium, the enzyme-support interaction, and the main objectives of the application must be considered (VIEIRA *et al.*, 2021). From that point of view, synthetic polymers have received significant attention lately as enzymatic supports because they present easy production and low synthesis costs. Furthermore, the presence of functional or functionalizable groups in the structure of the polymers used in immobilization allows the formation of relatively strong interactions between the support and the enzyme (DARONCH *et al.*, 2020).

Polyacrylonitrile (PAN) is a synthetic polymer that, in addition to presenting the characteristics mentioned above, has resistance against organic solvent, alkalis, acids, hydrolysis, oxidation, and microbiological corrosion, as well as good mechanical and thermal stability properties, when compared to natural polymers such as alginate and agar (ADEGBOLA; AGBOOLA; FAYOMI, 2020; WANG *et al.*, 2014a; WU *et al.*, 2012; ZHANG *et al.*, 2014). The versatility of this polymer allows its use in various applications, such as fiber cement (LI *et al.*, 2021), carbon concrete polyacrylonitrile composites (BÖHM *et al.*, 2018), nanocomposite membranes for the removal of heavy metals in effluents (AZAD *et al.*, 2022; HERATH; SALEHI; JANSONE-POPOVA, 2022), fabric with a flame retardant coating (LIU *et al.*, 2022) or even oxidized fibers for thermal and acoustic insulation (SUN, X. *et al.*, 2021). In other words, it is a polymer widely known and used around the world. According to the Granado Institute of Polyacrylonitrile Technology – IGTPAN (2022), in 2019, the consumption of acrylic fibers worldwide reached 1.9 million tons, with a tendency to remain stable for the next 10 years. The IGPAN also highlights that China has the largest polymer production, with around 40 % of world production, followed by regions covering Africa and the Middle East, with 20 %.

The current production of acrylonitrile (AN), a monomer that originates PAN, follows the method developed in the ‘50s, called “Sohio Acrylonitrile Process”, where there is only one step. The ease of production has made acrylonitrile a plentiful, pure, and inexpensive chemical. It was of unique importance for developing the acrylic fiber and plastic industries (SOHIO, 1996). In this sense, AN is often used as a precursor for resin, elastomers, thermoplastics and plays an important role in producing acrylic acid and acrylamide (HSU; ROBINSON, 2019). Furthermore, among the copolymers derived from acrylonitrile, the most important in the production of commodities is acrylonitrile-butadiene-styrene (ABS) and styrene-acrylonitrile (SAN) (FIROUZI *et al.*, 2019).

The physicochemical properties of PAN allow the formation of fibers, composite fibers, beads, or core-shell structures as based materials for immobilization (CATAPANE *et al.*, 2013; KAUSAR, 2019b; LI; CHEN; WU, 2007; VIEIRA *et al.*, 2021). Despite this, its low hydrophobicity, inert chemical groups, low chain symmetry, and strong molecular interaction restrict its applications (LI, Y. *et al.*, 2019; PAITAID; H-KITTIKUN, 2021). This disadvantage can be overcome through chemical reactions, with the modification and replacement of nitrile groups by functional groups capable of increasing the interaction between the support and the groups available in the three-dimensional structure of the enzyme (AGRAWAL; CHATURVEDI; VERMA, 2018).

Currently, there is a relatively high number of articles in the scientific literature about enzyme immobilization on polyacrylonitrile-based supports. However, this is restricted to the use of PAN in the form of fibers and composite fibers, to the detriment of granules and other possible morphologies. To the best of our knowledge, just a few articles discuss about the immobilization of laccase in this type of support. In this sense, in the present study, millimeter-sized particles of polyacrylonitrile were prepared by solution polymerization with precipitation (slurry), with subsequent functionalization of the nitrile groups for use in the immobilization of laccase from *Trametes versicolor* by covalent bonding. Laccase immobilization was also tested on particles without functionalization to compare the immobilization yields. The enzyme derivative was subjected to thermal and pH stability tests and operational and storage stability tests, using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate, thus pointing to a positive perspective for future application. The characterization of PAN particles was performed using nitrogen physisorption isotherms. Data such as surface area, diameter, and pore volume were obtained from the treatment by the method of Brunauer, Emmett, and Taller (BET) and Barrett, Joyner, and Halenda (BJH). The chemical composition of the particles was analyzed by Fourier Transform Infrared Spectroscopy - FTIR. Also, the morphology was observed by optical microscopy. Tests of enzymatic activity were performed, monitoring the increase in the absorbance of the ABTS oxidation reaction in a spectrophotometer to understand the properties of free and immobilized laccase.

Furthermore, the textual organization of this work is as follows: in addition to Chapter I, which contains this general introduction and the objectives (general and specific) of this study, Chapter II presents a literature review recently published in Chemical Record, presenting the use of PAN-based materials for laccase immobilization, adding more in-depth topics on laccase, enzymatic immobilization, and PAN polymerization technique in solution with precipitation. The details of the experimental methodology, as well as the equipment and

materials that made it possible to carry out the enzymatic activity tests and the proposed polymerization technique, are provided in Chapter III. In Chapter IV, in turn, the results obtained in the theoretical-experimental study of laccase immobilization are pointed out and discussed. Finally, Chapter V addresses the main conclusions and suggestions obtained with the development of this work. This work was funded by the National Council for Scientific and Technological Development (CNPq), and the entire experimental part was supported by the Biological Engineering Laboratory (LiEB), Carlos Henrique Neves Polymerization Process Control Laboratory (LCP), Analysis Center of the Chemical and Food Engineering Department of the Federal University of Santa Catarina (UFSC), and at the Analysis Center of the Department of Chemistry at UFSC.

1.1 OBJECTIVES

1.1.1 General objective

The core of this work is the immobilization of laccase (Lac) from *Trametes versicolor* in functionalized (*via* covalent bonding) and non-functionalized polyacrylonitrile (PAN) particles, synthesized via slurry polymerization.

1.1.2 Specific objectives

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

- Produce polyacrylonitrile particles by solution polymerization with precipitation;
- Characterize the particles produced in terms of morphology and surface chemical composition;
- Functionalize the particles produced through four steps: alkaline hydrolysis, acid hydrolysis, amination, and crosslinking;
- Immobilize the laccase enzyme in functionalized and non-functionalized particles;
- Evaluate the enzymatic activity of free and immobilized laccase through different physical and chemical parameters;
- Evaluate the reuse and storage of the biocatalyst after immobilization on PAN particles.

CHAPTER 2

This chapter presents a literature review on the use of polyacrylonitrile as a support for the immobilization of the oxidoreductase enzyme laccase. This review chapter was published in The Chemical Record in October 2021.

 [2]

[2] Bring the reader closer to the QR code to access the article "A Perspective Review on the Application of Polyacrylonitrile-Based Supports for Laccase Immobilization" published in The Chemical Record journal.

2 A PERSPECTIVE REVIEW ON THE APPLICATION OF POLYACRYLONITRILE-BASED SUPPORTS FOR LACCASE IMMOBILIZATION

ABSTRACT

The use of laccases applied in bioremediation processes has been increasingly studied, given the urgent need to overcome the environmental problems caused by emerging contaminants. It is known that immobilized enzymes have better operational stability under reaction conditions, allowing for greater applicability. However, given the lack of commercially available immobilized laccases, the search for immobilization materials and methods continues to gain effort. The use of polyacrylonitrile (PAN) to immobilize enzymes has been investigated since it is a low-cost material and can be modified and functionalized to interact with the enzyme well. This polymer can be used with different morphologies such as fibers, beads, and core-shell presented as an easily applicable alternative. This review presents the missing link between polymer and enzyme through an overview of PAN's current use as support for laccase immobilization and polymer functionalization methods, considering the importance of immobilized laccases in several industrial sectors.

Keywords: enzymes, immobilization, polymers.

2.1 INTRODUCTION

Biocatalysis reactions have been exploited for decades in the face of developing a greener route for chemical transformations. The growing interest in enzymes contributes to the development of modern biocatalysts with optimizing operational characteristics such as stability, high activity, resistance to inhibitors, selectivity, or specificity obtained through different immobilization techniques (CIPOLATTI *et al.*, 2019). In many cases, one of the major drawbacks in enzymatic catalysis is the high price of some enzymes compared to chemical catalysts, which can be solved by the possibility of recovery and reusing the enzyme after immobilization (BILAL *et al.*, 2018; CIPOLATTI *et al.*, 2016; SHELDON; VAN PELT, 2013). Currently, many industrial sectors use enzymatic biocatalysts in consolidated processes, while new applications continue to be heavily investigated (DI COSIMO *et al.*, 2013). Among the several technological and industrial applications, processes catalyzed by enzymes have also emerged as complementary alternatives for wastewater treatment concerning the degradation

of a range of chemical compounds, including emerging pollutants (ALCALDE *et al.*, 2006; GRANDCLÉMENT *et al.*, 2017).

Laccase is a copper oxidoreductase, so it directly uses this redox capacity to catalyze the oxidation of various aromatic compounds and ultimately form water and oxygen molecules (CAMPOS; LEVIN; WIRTH, 2016). This enzyme has four copper atoms in its catalytic site that confer both the protein's bluish color and its catalytic mechanism (AGRAWAL; CHATURVEDI; VERMA, 2018). Thus, the focus currently given by industries on cleaner production, supported by the evident need for maintaining human longevity on the planet, has made laccase an excellent alternative for bioremediation since this enzyme can act over a complete range substrate because it has a structure with low specificity. The use of laccase in biotechnology mainly considers the biodegradation potential of this enzyme. Therefore, several researchers worldwide have studied its use in decolorizing synthetic dyes, treating agro-industrial effluents, and removing emerging contaminants and drugs. Although many research studies demonstrate methods of immobilizing laccases in the most various supports, this enzyme is not yet commercialized in this form (DARONCH *et al.*, 2020). It reinforces the need to continue developing new materials that present the best requirements for developing immobilized laccases on a large scale, with good operational properties and low production cost.

Several organic, inorganic, and hybrid materials have been extensively investigated to support enzyme immobilization long the years (CIPOLATTI *et al.*, 2016; DATTA; CHRISTENA; RAJARAM, 2013). The success of the biocatalyst is reached by the combination of the properties of enzyme and support. The choice of support for enzymatic immobilization must consider the objectives of applying the biocatalyst and the reaction medium (SHELDON; VAN PELT, 2013), considering that characteristics such as particle size, composition, porosity, and surface functionality can be modified. In this way, the method needs to consider that the interaction between the support and the enzyme can affect the catalytic activity (BESTETI *et al.*, 2014).

Among the supports available for immobilization, polyacrylonitrile (PAN) has interesting characteristics for the immobilization of laccases, such as low cost, ease of synthesis, and microbiological resistance corrosion compared to biopolymers like chitosan and alginate (VILLEGAS *et al.*, 2016). The nitrile group's presence on the PAN's surface can be modified and replaced for new functional groups of interest through chemical reactions to facilitate the interaction between the support and the functional groups from the enzyme (AGRAWAL; CHATURVEDI; VERMA, 2018).

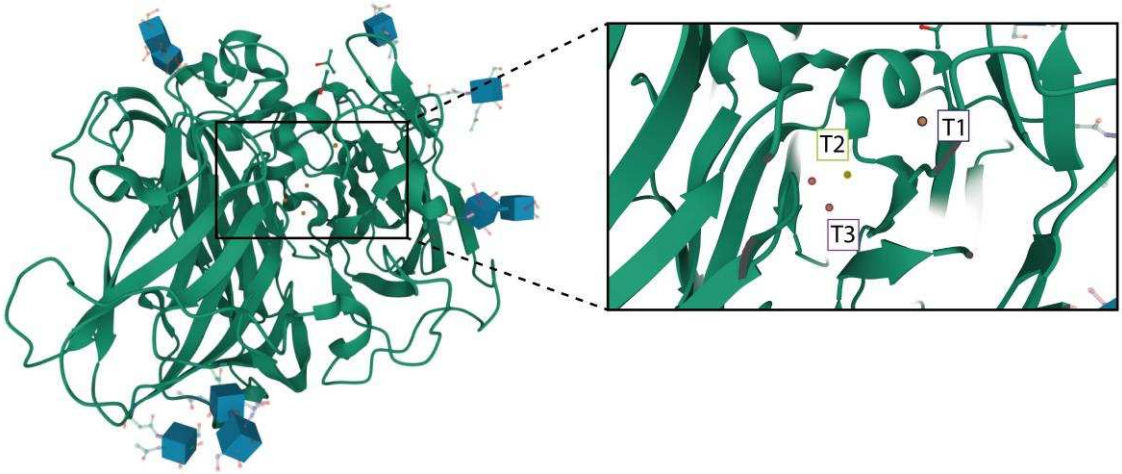
From this perspective, this review aims to provide a comprehensive explanation of PAN and its application as support for laccase immobilization, focused on the importance of this biocatalyst in bioremediation. Furthermore, this chapter is an adaptation of the review article published in The Chemical Record in October 2021. Sections 2.2, 2.2.1, and 2.5 were added to complement the understanding of the topics discussed in this master's thesis.

2.2 LACCASE

Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) is a member protein of blue copper oxidoreductases, also called multicopper oxidases. It directly uses the redox capacity of cupric ions, catalyzing the oxidation of several phenolic compounds with the simultaneous reduction of four electrons from O_2 to H_2O (CAMPOS; LEVIN; WIRTH, 2016). Most of the laccases characterized in the literature come from white-rot fungi, such as the protein produced by *Pleurotus sajor-caju* (JUNIOR *et al.*, 2020). In addition, it is possible to find current works whose focus of the study is the production of this enzyme by bacteria, such as *Escherichia coli* (ZHANG *et al.*, 2019), from the intestine of insects such as the termite *Reticulitermes flavipes* (COY *et al.*, 2010) or by the crustacean *Litopenaeus vannamei* (CHEN *et al.*, 2020), and also by plants such as *Prunus avium L.* (BERNI *et al.*, 2019). Fungal laccases, in turn, are usually extracellular and have molecular masses in the range of 60 to 80 kDa. The ideal pH of the medium for there to be no denaturation is acid, in the range between 3.0 and 6.0 (PERALTA *et al.*, 2017).

Each enzyme has a specific structure, so their catalyzed reactions define their classification. Generically, enzymes are formed by a specific chain of amino acids that constitutes their primary structure. The spatial arrangement of this chain forms regions with regular structure through hydrogen bonds or others, thus composing its secondary structure. A third structure is formed from the tangle of secondary structures. The quaternary structure is formed when more than one peptide chain and its interactions with the spatial arrangement formed by the tertiary structures (TORRES, 2001). In laccases, the backbone comprises approximately 500 amino acid residues organized into three domains: domain I with the first 150 amino acids; domain II encompasses from 150 to 300 amino acids and domain III with the last 200 (AGRAWAL; CHATURVEDI; VERMA, 2018).

Figure 1 – Three-dimensional structure of a laccase from *Trametes versicolor* with emphasis on the active site composed of T1, T2, and T3 coppers



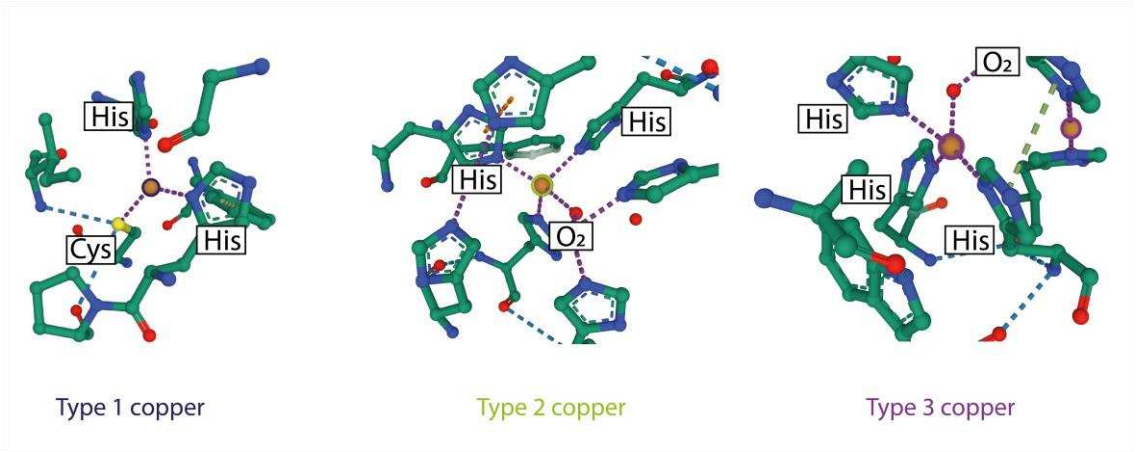
Source: Adapted from Protein Data Bank

The three-dimensional structure of the laccase shown in Figure 1 has four copper atoms at its catalytic site that can be classified into three domains using EPR (Electronic Paramagnetic Spectroscopy) and UV/visible resonance. Type 1 copper (T1) imparts a bluish color to multicopper proteins due to the intense electronic absorption promoted by the copper-cysteine covalent bond. This type of copper has trigonal coordination, with one cysteine and two histidine residues as conserved equatorial ligands, in generally variable positions. On the other hand, type 2 copper (T2), although detectable by EPR, is colorless due to the absorption deficiency in the visible region. In addition, two histidine residues confer its coordination. Type 2 and Type 3 (T3) coppers form a tri-nuclear center strategically located close together. This center is involved in the enzyme's catalytic mechanism, as it is where the reduction of molecular oxygen occurs and, consequently, the release of water occurs. T3 copper, on the other hand, has no coloring or even EPR signal due to the antiferromagnetic coupling between the two copper atoms, which is associated with the presence of hydrogen bonds (AGRAWAL; CHATURVEDI; VERMA, 2018).

These copper atoms present in the laccase active site coordinate the free electrons of the sulfur and nitrogen atoms belonging to the amino acid residues of the enzyme's three-dimensional structure. The amino acid sequence is then coordinated by these cupric ions so that T1 copper coordinates the sulfur atom of cysteine and the nitrogen atom of two histidine

residues. The T1 copper ligands are positioned equatorially and therefore assume a trigonal geometry (BRUGNARI *et al.*, 2021). This pattern is shown in Figure 2.

Figure 2 – Four copper ion centers with coordinating amino acid residues. Cysteine (Cys) and Histidine (His)



Source: Adapted from Protein Data Bank

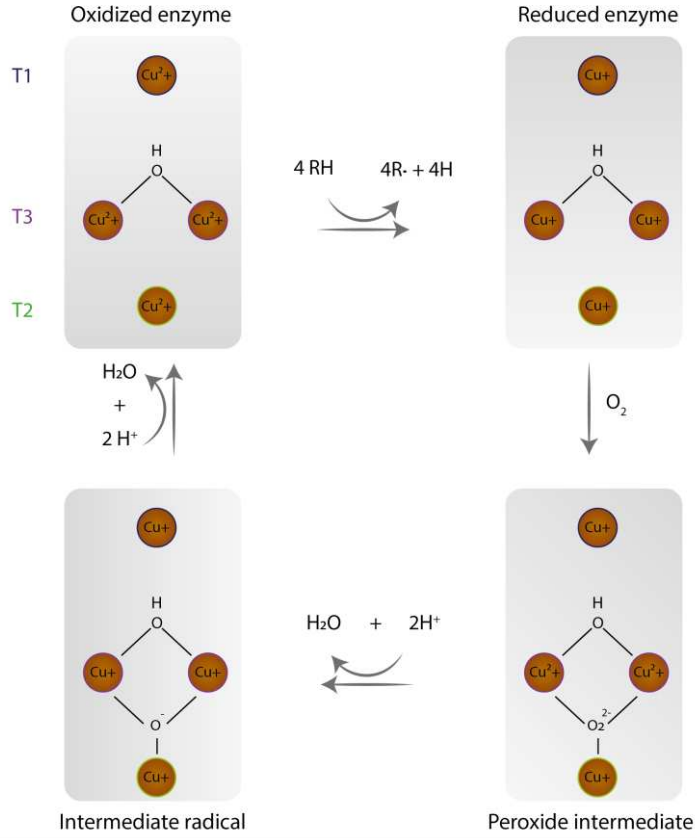
In addition to these bonds, copper T1 establishes another bond in an axial position with phenylalanine. T2 copper is also responsible for coordinating an oxygen atom of water and two nitrogen atoms of two different histidine residues in trigonal geometry. Each atom of the T3 copper pair, in turn, coordinates three nitrogen atoms from three histidine residues, plus one oxygen atom from a hydroxyl group.

Laccases can act on a wide range of substrates due to their low specificity. Amoncanible applications can biodegrade lignin and detoxify the products derived from it (DU *et al.*, 2018). These characteristics give the enzyme considerable value for the development of clean technologies ranging from the biodegradation of agro-industrial effluents such as sugarcane vinasse (VILAR *et al.*, 2018) and Pulp-wash (CRUZ *et al.*, 2020), including recent studies in the biodegradation of bisphenol A (TAGHIZADEH *et al.*, 2020), to the discoloration of several synthetic dyes, such as Congo red (DAS *et al.*, 2016). In general, the mechanism of the catalytic action of laccase consists of three moments: (i) copper T1 oxidizes four molecules of the substrate, then the reduction of copper T1 occurs by the reduction of the substrate; (ii) then, an intramolecular transport of electrons from T1 copper to T2 and T3 coppers; (iii) and finally, the reduction of O_2 to H_2O in the trinuclear center formed by T2 and T3 copper (SINGH; GUPTA, 2020).

In more detail, the second moment is that an oxygen molecule is attached to the trinuclear center, where it receives two electrons that are transferred from the reduced T3

coppers. This movement results in the disposition of a peroxide intermediate (O_2^{-2}). From there, the third moment occurs, where this intermediate peroxide undergoes a scission in the O–O bond releases a molecule of H_2O and decays into an oxygen radical. Concomitantly, copper T2 reduces this O* radical, releases one more molecule of H_2O, and returns to the initial state of the enzyme (DWIVEDI *et al.*, 2011; WONG, 2009). The laccase catalysis mechanism is shown in Figure 3.

Figure 3 – Mechanism of the catalysis reaction in the copper centers of the laccase



Source: adapted from Wong (2009)

2.2.1 Laccase immobilization

Although they generally have low energy costs and low environmental impact due to mild operating conditions (such as temperature and pH), enzymatic processes can become expensive due to the high cost of enzymes available on the market. The use of supports for immobilization leads to the reuse of these enzymes, making the process cheaper. The immobilization processes consist of the confinement of the enzyme in supports by mechanisms

that can be divided into three main categories: binding to a support (covalent bonds, ionic bonds, physical adsorption), entrapment (encapsulation or microencapsulation), and cross-linking. (crossing) (SHELDON; VAN PELT, 2013).

Here, physical adsorption and covalent bonding will be focused on, which are the methods that involve binding to support most discussed in the scientific literature. Immobilization by adsorption consists of binding the enzyme to a solid support through weak chemical interactions, such as van der Waals force and hydrogen bonds (physical adsorption), in addition to ionic bonds (ionic adsorption) (REN *et al.*, 2020). The set of these interactions allows little stability so that the enzymes remain adhered to the support for only a few cycles and leach with the reuse and washing of the support (JESIONOWSKI *et al.* 2014). Among the main advantages of the method, adsorption generally requires a simple operation, has low cost, easy reproducibility, and does not cause changes in the enzyme's three-dimensional structure (AGGARWAL *et al.* 2021). Enzymatic desorption can be circumvented by combining the adsorption technique with another, allowing a more effective binding between the support and the enzyme. In the study developed by Fathali *et al.* (2019), laccase was immobilized by adsorption on mesoporous silica materials and aggregated by crosslinking. The researchers investigated the efficiency of this immobilization methodology in removing phenolic compounds and maintaining stability over treatment cycles. After 20 cycles of successive operations, the immobilized biocatalyst continued to present 79% of its initial activity.

When it comes to immobilization by covalent bonding, it is worth mentioning that it involves the covalent bond between the functional groups of the amino acid residues belonging to the enzymatic surface with the functional groups of the support (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). In this method, the enzyme generally does not detach easily from the support, and, in this sense, chemical interactions allow for more extreme operating conditions. The main disadvantage of covalent bonding as a method of immobilization is the possibility of losing enzymatic activity. The structural conformation of the enzyme may undergo some modifications with the chemical interaction between the enzyme and the support (DAMIN *et al.*, 2021). For example, Lin *et al.* (2017) covalently immobilized laccase on magnetic particles prepared by the hydrolytic polycondensation reaction of tetraethoxysilane and 3-methacryloxypropyltrimethoxysilane (MPS). Comparing the enzymatic activities in the free and immobilized forms, they concluded that the immobilized laccase conferred greater thermal stability and storage durability; moreover, it decolorized methyl red more efficiently than free laccase.

Enzyme immobilization solves the main setbacks encountered by the use of free enzymes, such as short enzyme shelf life, low reuse, and low stability under adverse environmental conditions such as pH, temperature, and salinity (BERTRAND; MARTÍNEZ-MORALES; TREJO-HERNÁNDEZ, 2017). Considering the nature of the support and the immobilization method, Table 1 compares the kinetic and stability parameters found in the literature, showing the values for free and immobilized enzymes. It is worth mentioning that the values presented are those whose relative activity is greater than 60%. Another observation concerns pH and temperature, whose free and immobilized enzyme ranges can be close; however, the relative activities are higher in immobilized enzymes.

Table 1 – Comparison of kinetic and stability parameters between free and immobilized laccase

Parameter	Free	Immobilized	Nature of the support	Immobilization method	Reference
pH	4 to 6	3 to 9	Chitosan microspheres (2 mm) using glutaraldehyde as a crosslinking agent PAN membranes with fiber diameters from 200 nm to 300 nm	Crosslinking	(ASGHER; NOREEN; BILAL, 2017)
Temperature (ºC)	30 to 55	40 to 75			
Storage (days)	< 15	30			
Cycles	1	10			
Km (mM)	0.07	0.032			
pH	4 to 5.5	3 to 6	PAN electrospun fibrous membrane with a diameter ranging from 200 nm to 500 nm. Activation by amidination reaction	Covalent bonding	(XU *et al.*, 2013)
Temperature (ºC)	20 to 50	20 to 60			
Storage (days)	5	18			
Cycles	1	10			
Km (mM)	0.9	1.5			
pH	3	2 to 4	Poly(glycidyl methacrylate) microspheres with an average size of 2.85 μm	Covalent bonding	(VERA *et al.*, 2020)
Temperature (ºC)	40 to 65	30 to 65			
Storage (days)	15	25			
Cycles	1	8			
Km (mM)	2.3	2.5			
pH	4.5 to 6.5	4.5 to 8.5	Sodium zeolite Y and its modified desilicated and dealuminated forms with sizes between 500–1000 nm	Hydrogen bonding	(TAGHIZADEH *et al.*, 2020)
Temperature (ºC)	20 to 30	20 to 55			
Storage (days)	< 5	20			
Cycles	1	10			
Km (mM)	1.01	0.26			
pH	3	3 to 5	TiO_2 sol-gel coated polyacrylonitrile/organically modified montmorillonite fibers (138.27 ± 23.22 nm)	Covalent bonding	(WANG *et al.*, 2020)
Temperature (ºC)	40 to 50	30 to 60			
Storage (days)	-	-			
Cycles	2	8			
Km (mM)	0.12	0.35			

pH	5 to 6	4 to 7	Polypropylene chloride film and poly(glycidyl methacrylate)-grafted film with a specific surface area of 3.79 m^2/g	Covalent bonding	(ARICA *et al.*, 2017)
Temperature (ºC)	25 to 55	25 to 65			
Storage (days)	< 15	30			
Cycles	1	8			
Km (mM)	0.38	0.53			
pH	3 to 6	3 to 9	Poly(methyl methacrylate)/polyaniline electrospun fiber (695 nm ± 352 nm)	Adsorption/ Covalent bonding	(JANKOWSKA *et al.*, 2020)
Temperature (ºC)	30 to 50	20 to 70			
Storage (days)	15	30			
Cycles	1	10			
Km (mM)	0.059	0.119			
pH	3 to 4	4 to 5	Poly(diallyldimethylammonium chloride) modified halloysite nanotubes with a specific surface area of 24.54 m^2/g	Covalent bonding	(CHAO *et al.*, 2017)
Temperature (ºC)	15 to 45	35 to 65			
Storage (days)	-	-			
Cycles	1	6			
Km (mM)	2.09	3.11			
pH	2 to 4	2 to 4	Gelatin beads (diameter of 2 mm) using glutaraldehyde as a cross-linking agent.	Crosslinking/ Covalent bonding	(HARGUINDEGUY *et al.*, 2020)
Temperature (ºC)	25 to 45	25 to 55			
Storage (days)	-	-			
Cycles	1	10			
Km (mM)	0.0556	0.038			
pH	4 to 5.5	3.5 to 6	Chitosan-coated Fe_3O_4 nanoparticles (10.81 nm ± 1.32 nm)	Covalent bonding	(ZHANG, K. *et al.*, 2020)
Temperature (ºC)	20 to 40	20 to 40			
Storage (days)	10	30			
Cycles	1	10			
Km (mM)	0.0263	0.0301			

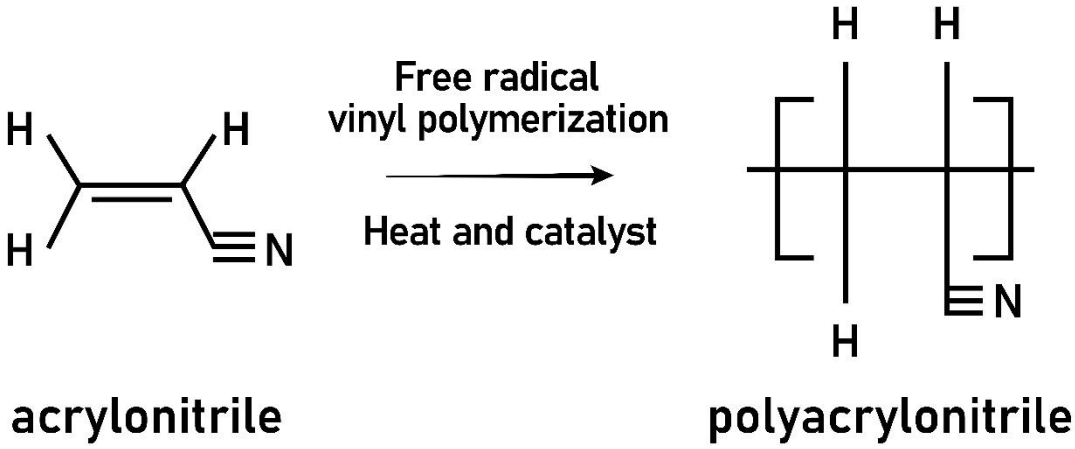
Source: from author

Many researchers have studied the use of polymeric supports, and the balance of advantages and disadvantages allows us to observe increasingly favorable results. Natural polymers such as chitosan (QIU *et al.*, 2021) and alginate (CAMPAÑA *et al.*, 2020) are presented in the literature as supports with advantageous characteristics due to their good biodegradability, non-toxicity, and biocompatibility. At the same time, synthetic polymers have received a lot of attention lately because they are, in general, easy to produce and have a low cost of synthesis. Another advantage concerns that they can be manipulated to maintain a better enzyme-support interaction. Characteristics such as particle size, composition, porosity, and surface functionality can be modified considering the immobilization methodologies and the structural needs of the enzyme because, no matter how light it is, the bond between the support and the enzyme causes some kind of catalytic effect activity (BESTETI *et al.*, 2014). Next, the application of polyacrylonitrile-based materials for enzyme immobilization will be presented.

2.3 APPLICATION OF POLYACRYLONITRILE-BASED MATERIAL AS SUPPORT FOR ENZYME IMMOBILIZATION

PAN is a synthetic polymer with the linear formula $(C_3H_3N)_n$ obtained in granulated or powder form from the polymerization of acrylonitrile monomer (Figure 4) (ADEGBOLA; AGBOOLA; FAYOMI, 2020) that can be polymerized using appropriate methods and conditions.

Figure 4 – Polymerization of acrylonitrile monomer



Source: from author

Different techniques have been used to form PAN, such as bulk polymerization, free radical, and anionic. There is a formation of a ladder structure by the polymerization of nitrile (KAUSAR, 2019a). Although acrylonitrile is a highly toxic monomer, the polymer formed is a non-toxic compound (NATARAJ; YANG; AMINABHAVI, 2012). Moreover, acrylonitrile polymerization is known by high reaction rates and can be completely converted into the reaction medium.

The PAN molecule's dominant characteristic is the presence of strongly polar nitrile groups due to the nitrogen atom's greater electronegativity than the carbon atom (LIU *et al.*, 2019). The highly dipolar nitrile groups repel within a chain and form dipolar bonds between the chains, which boosts the crystallinity of PAN, which is a semicrystalline polymer despite being atactic. In addition, these interactions force the PAN to adopt an extended, fully transformed conformation, which results in a hexagonal chain arrangement (KOPEĆ *et al.*, 2019).

Polyacrylonitrile can maintain its structure and composition when subjected to acidic conditions (hydrolysis exists but is quite slow), alkaline, oxidation, and organic solvents. All this shows the versatile application of the PAN product. (WANG; WAN; XU, 2007). This polymer has good thermal, chemical, and mechanical stability; besides, it has good efficiency as sorbent and biocompatibility (MINET *et al.*, 2010; NATARAJ; YANG; AMINABHAVI, 2012). It has attracted a lot of attention and popularity due to its unique structure and potential to transform it under high temperatures. These characteristics are attributed to its carbon-carbon structure. Its versatility is due to its high carbon content (PAN yields up to 65% carbon upon carbonization) (ADEGBOLA; AGBOOLA; FAYOMI, 2020; QIN, 2016).

Almost all PAN resins are copolymers made from mixtures of monomers, with acrylonitrile as the main component. This polymer appears in several types of applications in literature used to produce a large variety of products, including fibers for textiles, ultra-filtration membranes, hollow fibers for reverse osmosis, etc. (QIN, 2016).

Polyacrylonitrile can offer high stability and resistance to microbiological corrosion than carriers of common natural polymers such as chitosan and alginate. For example, the structure of wet chitosan is easy to collapse after drying, while alginate is unstable in electrolyte solutions with high concentrations (WU *et al.*, 2017). In addition, PAN is reported in the literature as the main precursor in forming carbon nanofibers for different applications, achieving porous structured materials with a high surface area, which can be interesting when discussing biocatalysis (NATARAJ; YANG; AMINABHAVI, 2012). Some methods for immobilizing enzymes in PAN have already been reported.

Li, Chen and Wu (2007) evaluated the efficiency of the covalent immobilization of lipase from *Candida rugosa* in PAN fibers after activation by amidination reaction. Due to the huge specific surface area provided by the fiber, the protein load can reach 2.1% (w/w) in the support, and the immobilized enzyme retained high activity (> 80%). Also, storage stability has been substantially improved over the free enzyme (LI; CHEN; WU, 2007). Pataid and H-Kittikun (2020) investigated the immobilization of lipase from *Aspergillus oryzae ST11* by crosslinking glutaraldehyde and bovine serum albumin (BSA) in magnetic nanoparticles coated with PAN for later application as a biocatalyst for biodiesel production. In that study, immobilized lipase could be reused five times, with 65% relative activity in biodiesel production (PAITAID; H-KITTIKUN, 2020). Table 2 shows enzymes immobilized on different PAN-based supports, their source and applications, and the support type, among other information about the papers, illustrating the potential of polyacrylonitrile as proper support for enzyme immobilization. (LI *et al.*, 2019).

The versatility of polyacrylonitrile allows the extensive use of this polymer in different morphologies, added to another polymer or compound that makes it more functional. Most of the works found in the literature use PAN in nanofibrous membranes produced by electrospinning.

Although natural polymers are good candidates for enzyme immobilization because they have functional groups that allow covalent bonds between enzyme and support, polysaccharides or proteins need to be electrospinning in an aqueous solution is a more difficult method than the use of organic solvents (LI; CHEN; WU, 2007). Another disadvantage of natural polymers is that they are generally more chemically and mechanically unstable compared to synthetic polymers. In this sense, polyacrylonitrile has the stability and fundamental mechanical properties for its good use as promising support for enzyme immobilization.

Features such as interconnectivity, high porosity, a large volume-to-surface ratio, and microscale interstitial space (NATARAJ; YANG; AMINABHAVI, 2012) mean electrospinning PAN nanofibers can be successfully applied in biotechnology and biochemical engineering. Due to the small diameter of the fibers, this type of support has a large relationship between surface area and volume compared to other known morphologies. These characteristics are desirable when thinking about enzymatic immobilization since the density of the immobilized enzyme in a given catalyst volume can be vastly improved compared to traditional methods (SAKAI *et al.*, 2010). The ability to retain large amounts of enzyme on the support is another positive point for polyacrylonitrile-based supports; this can be seen in Tables 2 and 3.

Other characteristics that justify the use of nanofibers are the high adhesion capacity of enzymes with a homogeneous dispersion, less probability of denaturation and enzymatic dysfunctions, and less resistance to mass transfer (ZAHIRINEJAD *et al.*, 2021). Adsorption on electrospinning PAN membranes is the easiest and least expansive immobilization method. However, despite providing high enzymatic retention on the support, it is less efficient than covalent binding, as the adsorbed enzymes are more likely to be gradually released with the reuse of the biocatalyst. In this sense, PAN pearls are preferred as laccase support for uses in bioreactors (MENALE *et al.*, 2012).

From an economic point of view, natural polymers and inorganic materials used as support for enzymes immobilization are usually cheaper than synthetic polymers (ZHOU; ZHANG; CAI, 2021). However, inherent characteristics of synthetic polymers may be more advantageous, such as greater strength to corrosion. Additionally, polyacrylonitrile has been used as a low-cost core material in fibers compared to other polymers, which tend to be more expensive (ADEGBOLA; AGBOOLA; FAYOMI, 2020).

Inorganic materials used for laccase immobilization may present nonspecific interactions, with low laccase-support interaction and diffusional barriers (DARONCH *et al.*, 2020). On the other hand, it was shown that PAN could be easily functionalized to obtain the best possible interaction with laccase, which will be discussed further in the next section.

Table 2 – PAN-based materials used to immobilize different enzymes

Support	Support modification	Enzyme	Immobilization method	Protein loading	Substrate	Reuse/ Enzymatic activity	Application	Reference
Acrylonitrile copolymer membranes	Modified with hydroxylammonium sulfate (HAS) and hydrazinium sulfate (HZS)	Urease	Covalent bonding	~26 μg/cm²	-	68% (HAS) and 67% (HZS)	-	(GODJEVARGOVA; GABROVSKA, 2003)
Polyacrylonitrile fiber membrane	Activation by amidination reaction	Lipase (*Candida rugosa*)	Covalent bonding	21.2 ± 1.3 mg/g	P-nitrophenyl palmitate	70% of the initial activity after 10 cycles	-	(LI, S.; CHEN; WU, 2007)
Polyacrylonitrile copolymer membranes	Activation with glutaraldehyde	Pectinase (*Aspergillus niger*)	Adsorption/ Covalent bonding	-	Pectin solution in C-P buffer	50% of the initial activity after 20 min	-	(DELCHEVA; DOBREV, 2007)
Polyaniline-grafted PAN Films	Use of potassium dichromate as an oxidizing agent	Uricase (*Arthrobacter globiformis*)	Adsorption	216 μg/cm²	Substrate solution (uric acid)	96% of the initial activity after 6 cycles	-	(BAYRAMOGLU, Gülay; ALTINTAS; ARICA, 2011)
Amidoxime polyacrylonitrile (AOPAN) fibrous membrane	Modified by Fe(III) chelation	Catalase (Bovine liver)	Coordination bonds	48.3 mg/g	Hydrogen peroxide solution	70% of the initial activity after 15 days	-	(FENG *et al.*, 2012)
Polyacrylonitrile fiber membrane	Activation by amidination reaction	Chitosanase (*Aspergillus* sp.)	Covalent bonding	-	Chitosan substrates	60% of initial activity after 30 days of storage	Chitosan hydrolysis/ glucosamine production	(SINHA *et al.*, 2012)

Polyacrylonitrile beads	Use of ethylenediamine and glutaraldehyde	Tyrosinase	Covalent bonding	18.3 mg/g	Phenolic compounds	50% after 40 days of storage / 80% of the initial activity after 6 cycles	Degradation of phenolic compounds	(WU, Q. *et al.*, 2017)
Polyacrylonitrile membrane with polyethyleneimine	Use of Glutaraldehyde, epichlorohydrin or $CaCl_2$	Lipase (*Candida antarctica*)	Crosslink	36.9 mg/g	Soy oil	89% of the initial activity after 20 cycles	Catalysis in biodiesel production	(LI, Y. *et al.*, 2019)
Magnetic nanoparticles coated with polyacrylonitrile	Use of glutaraldehyde and BSA as crosslinking agents	Lipase (*Aspergillus oryzae ST11*)	Crosslink	-	Palm oil	80% of the initial activity after 13 days of storage	Catalysis in biodiesel production	(PAITAID; H-KITTIKUN, 2020)

Source: from author

2.4 FUNCTIONALIZED SURFACES AND IMMOBILIZATION METHODS

This work has already discussed the importance of the large surface area of polyacrylonitrile in laccase immobilization processes, whether, through adsorption or covalent bonding, the protein retention capacity in immobilization is essential from the point of view of application and reuse. However, several methods can be applied to modify the polymer surface to promote support with improved properties, such as chemical cross-linking and composite coating (LI *et al.*, 2019).

The most common methods of immobilizing laccase on PAN supports are crosslink, surface adsorption, and covalent bonding (Table 3). Some review articles summarize these methods strongly, addressing their advantages and disadvantages (DARONCH *et al.*, 2020; MORSHED *et al.*, 2021; NDLOVU; BA; MALINGA, 2020; ZHOU; ZHANG; CAI, 2021). Each enzyme and each immobilization method may require a different type of functionalization of the polyacrylonitrile surface, and in the scientific literature, several methodologies aim to optimize the interaction.

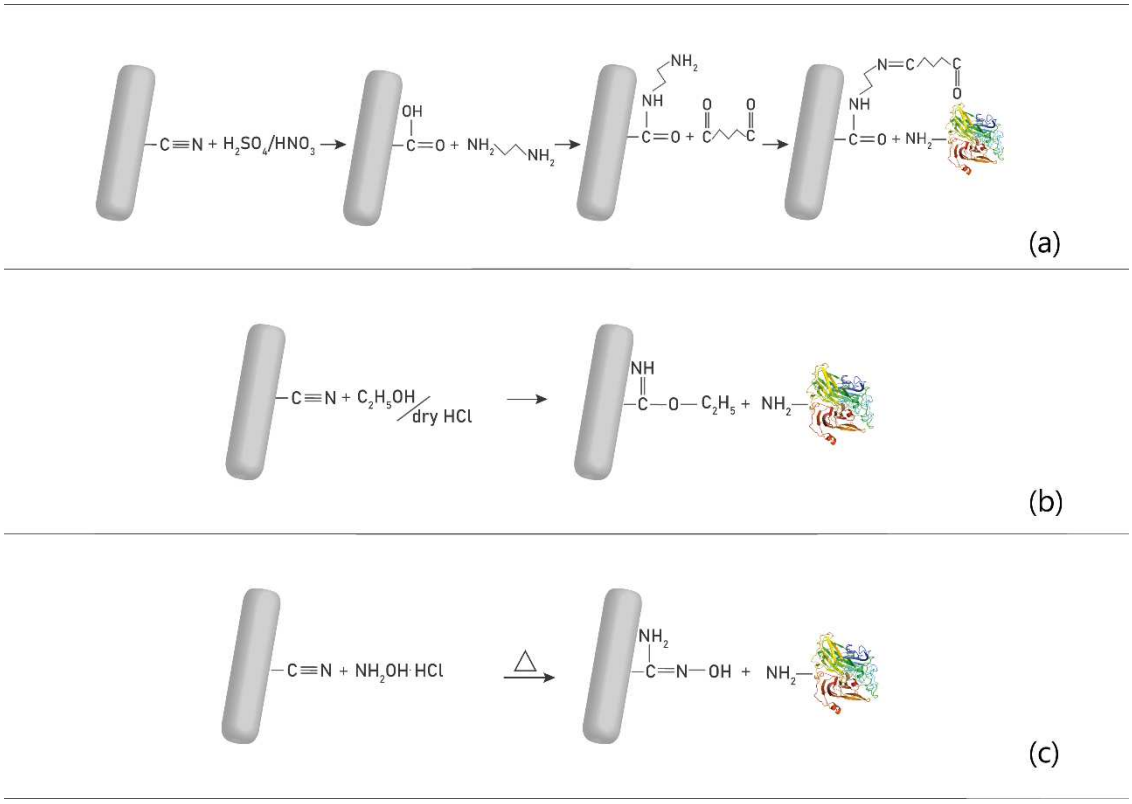
Ren, Luo, and Wan (2018) promoted a three-dimensional modification in polyacrylonitrile membranes using polydopamine (PDA), polyethyleneimine (PEI), and Metal-organic Frameworks (MOF) for laccase immobilization. In this process, the insertion of PEI in the selective separation layer resulted in charge reversal, and the laccase immobilization took place by electrostatic adsorption. The stability of the biocatalyst resulted in the removal of up to 92% bisphenol A in a continuous flow cycle (REN; LUO; WAN, 2018).

Immobilization processes involving laccase demand knowledge about the three-dimensional structure of this enzyme. Histidine residues are known to be present at the catalytic site of laccase. In this sense, when immobilizing laccase on PAN beads, Catapane *et al.* (2013) used a methodology involving diazotization of the phenolic groups of tyrosine residues, which are not in the active site (CATAPANE *et al.*, 2013). This process was chosen over the condensation process, as it would covalently bind the polyacrylonitrile to the histidine residues. In contrast, condensation was performed by Nicolucci *et al*. (2011) for tyrosinase immobilization and efficient application to remove bisphenols (NICOLUCCI *et al.*, 2011).

Glutaraldehyde is a bifunctional crosslinker widely used in immobilization processes involving polyacrylonitrile and laccase (Figure 5(a)). This crosslinking agent

is popular because it can form stable intra- and inter-subunit covalent bonds, in addition to being soluble in aqueous solvents (DATTA; CHRISTENA; RAJARAM, 2013). Among the greatest advantages of its use is its economical and easily obtainable crosslinking reagent (HASSAN *et al.*, 2019). In covalent bond immobilizations, the efficiency is increased due to the formation of the Schiff base that binds to the amino group present in the enzyme (ASLAM *et al.*, 2021).

Figure 5 – Detailed schematic illustration of polyacrylonitrile functionalizations for laccase immobilization. a) with the use of glutaraldehyde; b) amidination; c) amidoximation



Source: from author

Taheran *et al*. (2017) assertively describe the process of immobilization of laccase in PAN-Biochar nanofibrous membrane, using glutaraldehyde as a crosslinking agent. Throughout the process, the researchers say, the free aldehyde group reacts with the amino groups on the enzyme resulting in imino groups (–CH=N–)(TAHERAN *et al.*, 2017a). Thus, it is possible to see the influence of the concentration of glutaraldehyde as a parameter for laccase immobilization. It was reported by Feng *et al.* (2016) when they

covalently immobilized the laccase on amidoxime polyacrylonitrile/montmorillonite (AOPAN/MMT) nanofibrous membrane. The researchers noticed that the increase in the concentration of glutaraldehyde provided an increase in the amount of immobilized laccase until reaching a saturation point of 5%, where the amount of laccase reached 89.12 mg/g, maximum retention of enzyme in the support (FENG *et al.*, 2016).

Another method used is the activation of the PAN surface by amidination (Figure 5(b)), where the nitrile groups of polyacrylonitrile are activated in a medium containing ethanol/HCl resulting in the production of imidoester, which in turn reacts with the amino groups present in the enzyme to form amidino bonds (LEE; LEE, 1997). The laccase was immobilized on amidination activated nanofiber PAN and successfully applied to remove 2,4,6-trichlorophenol (TCP) from the water (XU *et al.*, 2013). Li *et al*. (2011) can also observe this functionalization of polyacrylonitrile for lipase immobilization. The support-bound protein functioned as a nucleus in which the other unbound enzymes were adsorbed onto it, forming an aggregate of proteins by molecular interactions (LI *et al.*, 2011).

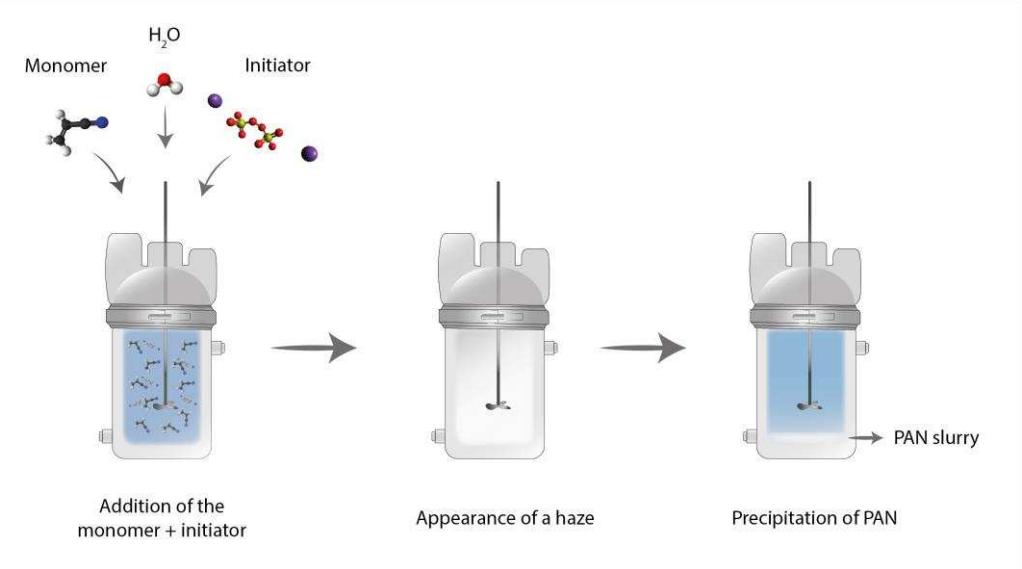
Amidoximation is another PAN functionalization process that is still little explored (Figure 5(c)). Zhao *et al*. (2015) studied the amidoximation of polyacrylonitrile fibers through the reaction with hydroxylamine. They reported that a greater degree of amidoximation of the fibers resulted in a higher conversion factor of the nitrile group, which provides a better balance between adsorption capacity and mechanical properties of PAN (ZHAO *et al.*, 2015). The use of nitrile and hydroxylamine groups allows obtaining amidoximes with good yields and reaction times between 1 to 48 hours (SAHYOUN; ARRAULT; SCHNEIDER, 2019). Nanofibers containing polyurethane (PU), amidoxime polyacrylonitrile (AOPAN), and β-cyclodextrin (β-CD) were activated using this method for laccase immobilization (WU *et al.*, 2017). The authors concluded that the amidoxime groups generated by functionalization led to significantly greater membrane hydrophilicity.

Therefore, it is imperative to reiterate the main advantages of using polyacrylonitrile as support for laccase immobilization: a synthetic polymer with interesting properties, versatile, non-toxic, among other characteristics already mentioned in this work.

2.5 ACRYLONITRILE POLYMERIZATION IN SOLUTION WITH PRECIPITATION

Acrylonitrile can be polymerized in various ways to form polyacrylonitrile in a variety of morphologies. An easy-to-apply method that allows the formation of PAN particles is solution polymerization with precipitation, also called slurry polymerization (EBDON; HUCKERBY; HUNTER, 1994a; EBDON; HUCKERBY; HUNTER, 1994b). This method is homogeneous, where the reactive phase (monomer) and the initiator are soluble in the continuous phase. The polymer formed is insoluble and, therefore, precipitates in the reactor when the polymerization reaction is completed (THOMAS; GLEASON; MINO, 1957). This polymerization process is schematized in Figure 6.

Figure 6 – Representation of particle synthesis by solution with precipitation



Source: adapted from Thomas *et al.* (1961)

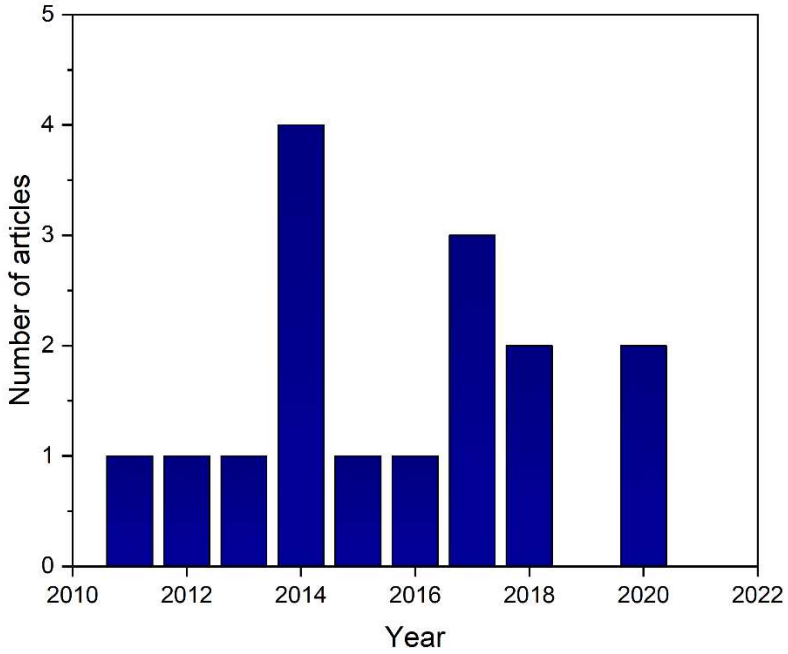
After the addition of the monomer followed by the initiator, the polymerization is evident by the appearance of a haze, followed by the precipitation of the polyacrylonitrile, manifesting itself as a white polymer. When polymerization is complete, the slurry formed can be rigid and retain approximately four times its weight in water (THOMAS, 1961). One of the initiators commonly used in this type of polymerization is potassium persulfate (KPS) since vinyl monomers such as acrylonitrile accelerate the thermal decomposition of KPS in aqueous solutions (SARKAR; BANERJEE; KONAR, 1988). It was observed by Boguslavsky *et al*. 2005 producing

PAN nanoparticles in a continuous aqueous phase in the presence of potassium persulfate and surfactant. The result of this study was the formation of PAN nanoparticles with a crystal structure of approximately 13 nm (BOGUSLAVSKY; BARUCH; MARGEL, 2005).

2.6 PAN APPLICATION AS SUPPORT FOR LACCASE IMMOBILIZATION

Many matrices have been developed for laccase immobilization to improve its stability and operating conditions. Due to the functional groups in the structure, PAN can create relatively strong interactions between laccase and support (DARONCH *et al.*, 2020; WU *et al.*, 2017). There are already some studies in the literature on applying PAN in laccase immobilization, still little explored, but with potential further investigations. In the *Scopus* database for "polyacrylonitrile AND laccase AND immobilization," 16 different articles were found (Figure 7), only one of them is a review article, and deals with recent advances in laccase immobilization in general, unlike what this article proposes, which is to discuss the potential of PAN-based materials for laccase immobilization.

Figure 7 – Number of articles per year of articles published using PAN-based materials for laccase immobilization (on Scopus)



Source: from author

Nicolucci *et al*. (2011) investigated the possible application of laccase and tyrosinase immobilized on polyacrylonitrile beads applied to biodegradation of some bisphenol (BPA) derivatives in fluidized bed reactors. The results had shown that the laccase from *Trametes versicolor* could oxidize different bisphenols. The laccase immobilized in PAN beads provided excellent biphenol oxidation results (~100%) (NICOLUCCI *et al*., 2011). Other works by the same research group have also been published using laccase immobilized on PAN beads for biodegradation of different components, such as 2- and 4-chlorophenol (CPs) (MENALE *et al.*, 2012); and to bioremediate aqueous solutions polluted by endocrine disruptors belonging to the alkylphenols (APs) class (CATAPANE *et al.*, 2013).

PAN electrospun fibrous membranes (EFMs) have also been extensively investigated for laccase immobilization. Xu *et al*. (2013) evaluated the degradation and removal of 2,4,6-trichlorophenol (TCP, a chlorophenol) by the immobilized laccase on polyacrylonitrile fibrous membranes. The results have shown that laccase loading could reach as high as 220 mg/g fibers and the immobilized laccase retained 72% of the free laccase activity after immobilization. Additionally, the degradation tests resulted in 87% of TCP's removal efficiency in 4 hours (XU *et al.*, 2013).

Other works have also investigated the incorporation of different materials in the matrix of these PAN fiber membranes. Li *et al*. (2016) evaluated the covalently immobilized laccase application in a PAN/O-MMT (organically modified montmorillonite) electrospun composite fibrous membrane in the degradation of the crystal violet dye. This study showed that the loading of laccase in the support was 276 mg/g of fiber membrane and showed a 91% efficiency in dye removal in 20 minutes (LI *et al.*, 2016). In Taheran *et al*. (2017) study, the laccase was immobilized onto PAN-biochar composite fiber membrane, and the obtained biocatalyst was investigated to remove antibiotics (chlortetracycline). The removal efficiency reached 58.3% at a flow rate of 1 mL/h.cm² (TAHERAN *et al.*, 2017b). Table 3 shows the different PAN-based materials used in laccase immobilization, the immobilization method, the enzyme load in the support, and other relevant information. The works presented show that it was possible to observe an incidence in PAN-based materials in the form of nanofiber membranes produced by electrospinning and some made with composites that use polyacrylonitrile. In addition to this type of material, PAN was also explored in the form of beads. In the following topics, each support will be discussed individually.

Table 3 – Different PAN-based materials used in laccase immobilization.

Support	Support modification	Laccase source	Immobilization method	Protein loading	Substrate	Reuse/ Enzymatic activity	Application	Reference
Polyacrylonitrile (PAN) beads	Use of Glutaraldehyde and Phenylenediamine (PDA)	*Trametes versicolor*	Covalent bonding	3.56 ± 0.4 mg/g	Bisphenol-A, Bisphenol B, Bisphenol F, and Tetrachlorobisphenol A	85% of the initial activity after 30 days	Biodegradation of BPA derivatives	(NICOLUCCI *et al*., 2011)
Polyacrylonitrile (PAN) beads	Use of Glutaraldehyde and Phenylenediamine (PDA)	*Trametes versicolor*	Covalent bonding	2.5 mg/g	2-Chlorophenol and 4-Chlorophenol	70% of initial activity after 20 days of work	Biodegradation of chlorophenols	(MENALE *et al.*, 2012)
Polyacrylonitrile (PAN) beads	Use of Glutaraldehyde and Phenylenediamine (PDA)	*Trametes versicolor*	Covalent bonding	3.33 ± 0.4 mg/g	Onylphenol and octylphenol	95% of initial activity after 50 days	Biodegradation of onylphenol and octylphenol	(CATAPANE *et al.*, 2013)
PAN electrospun fibrous membrane (EFMs)	Activation by amidination reaction	*Pleurotus ostreatus*	Covalent bonding	220 mg/g	2,4,6-Trichlorophenol (TCP)	75% of initial activity after 5 cycles	Degradation and removal of 2,4,6-trichlorophenol	(XU *et al.*, 2013)
Amidoxime polyacrylonitrile (AOPAN) fibrous membrane	Use of metal ions (Fe^{3+}, Cu^{2+}, Ni^{2+}, Cd^{2+})	*Ganoderma lucidum*	Physical adsorption	Fe–AOPAN (>60 mg/g); Cu–AOPAN (60 mg/g); Ni–AOPAN (45 mg/g); Cd–AOPAN (20 mg/g)	ABTS	Cu–AOPAN–Lac retained >65% of initial activity after 20 days	–	(WANG, *et al.*, 2014a)

Polyacrylonitrile /montmorillonite/graphene oxide (PAN/MMT/GO) composite fibers	Without chemical modification on the support surface	*Ganoderma lucidum*	Physical adsorption	–	Catechol	75% of the initial activity after 15 cycles	Catecholl Degradation	(WANG *et al.*, 2014b)
Amidoxime modified PAN (AOPAN) fiber membrane	Use of hydroxylamine hydrochloride	Commercial laccase	Covalent bonding	400 mg/g	Reactive red X-3B and cobalt acetate	60 % of initial activity after 20 days storage	Dye degradation and metal ion adsorption	(ZHANG, *et al.*, 2014)
Electroless-gold-plated fibrous membrane	Use of glutaraldehyde	*Trametes versicolor*	Covalent bonding	0.430 ± 0.051 μg protein/mm^3	ABTS	80.2 ± 4.3% of initial activity after 5 cycles	–	(SAWADA; SAKAI; TAYA, 2015)
Electrospun polyacrylonitrile (PAN)/O-MMT membranes	Alkaline hydrolysis and carboxyl activation	*Trametes versicolor*	Covalent bonding	276 mg/g	Crystal violet (CV)	85% of initial activity after 5 cycles and 70% after 10 cycles	Degradation of a triphenylmethane dye	(LI *et al.*, 2016)
AOPAN/MMT composite fiber membranes	Use of glutaraldehyde	*Ganoderma lucidum*	Adsorption/ Crosslinking	89.26 mg/g	ABTS	64.5% of initial activity after 10 cycles	–	(FENG *et al.*, 2016)
Polyacrylonitrile -biochar composite fibrous membrane	Use of HNO_3/H_2SO_4 solution, ethylenediamine, and glutaraldehyde	*Trametes versicolor*	Covalent bonding	–	Mixture of Chlortetracycline (CTC), Diclofenac (DCF), and Carbamazepine (CBZ)	94% of initial activity after 60 days	Degradation of pharmaceutically active compounds	(TAHERAN *et al.*, 2017a)
Polyacrylonitrile -biochar composite fibrous membrane	Use of hydroxylamine hydrochloride for surface modification	*Trametes versicolor*	Covalent bonding	–	Chlortetracycline (CTC)	50% of the initial activity after 7 cycles	Degradation of a pharmaceutically active compound	(TAHERAN *et al.*, 2017b)

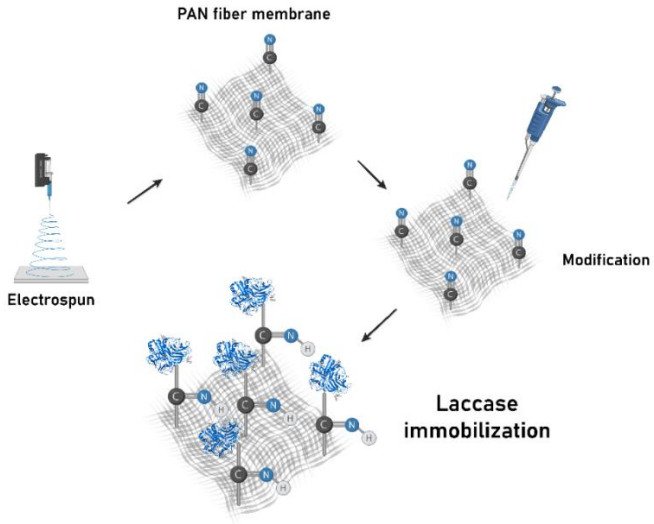
Electrospun blend fiber membrane consisting of polyurethane, polyacrylonitrile, and β-cyclodextrin	Amidoxime modification	*Ganoderma lucidum*	Ionic bonding	186.34 mg/g	ABTS	47 ± 5.2% of the initial activity after 10 cycles	–	(WU *et al.*, 2017)
Polyacrylonitrile /polyvinylidene fluoride (PAN/PVdF) electrospun fibrous membranes	Alkaline treatment with NaOH solution and use of glutaraldehyde	*Trametes versicolor*	Covalent bonding	538 mg/g	2,4,6-Trichlorophenol (TCP)	68% of the initial activity after 30 days	Degradation of 2,4,6-trichlorophenol	(XU *et al.*, 2017)
Polyacrylonitrile (PAN) ultrafiltration membrane	Modified by polyethyleneimine (PEI), metal-organic frameworks (MOFs), laccase and polydopamine (PDA)	*Trametes versicolor*	Adsorption/ Covalent bonding	–	Bisphenol A (BPA)	62% of initial activity after 7 reuse cycles	Micropollutants removal	(REN, Z.; LUO; WAN, 2018)
TiO_2 sol-gel coated polyacrylonitrile /organically modified montmorillonite (PAN/O-MMT) fibers	Functionalized by a sol-gel coating of TiO_2	*Trametes versicolor*	Covalent bonding	342 mg/g	Crystal violet (CV)	~60% of its initial activity after 8 cycles (30° C) and <50% after 2 cycles (50° C)	Removal of a triphenylmethane dye	(WANG *et al.*, 2020)

Source: from author

2.6.1 Fibers

Among the several precursors for the production of fibers, PAN is the most used polymer, mainly for its high carbon yield (up to 56%) and its flexibility to adapt the structure of these fibers' final products (NATARAJ; YANG; AMINABHAVI, 2012). There are several methods for producing fibers, but most of these processes are very expensive due to the product's low yield and the expensive equipment required. Therefore, the electrospinning method is the most widely used, capable of generating fibers with diameters from 10 nm to 10 µm from a polymer solution under the application of an electrostatic force (NATARAJ; YANG; AMINABHAVI, 2012; SCAMPICCHIO *et al.*, 2012). PAN fibers were the first high-quality carbon fibers to be produced (QIN, 2016). Figure 8 shows a scheme for obtaining PAN fibers for laccase immobilization.

Figure 8 – Scheme for obtaining PAN fibers for laccase immobilization



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The main advantage of these materials with nanometric to micrometric scale, including nanoparticles and fibrous membranes for use as supports for immobilizing enzymes, is that they have a large surface area leading to a high enzyme load and the high volumetric activity of the enzyme. In comparison, fibrous membranes have even more advantages, such as high recovery and reuse of enzymes. The surface of an electrically spun fiber membrane can have many active sites for the chemical/physical immobilization of enzymes. That is why so many works, as seen, have shown interest in using these materials as an innovative type of transporters/supports for

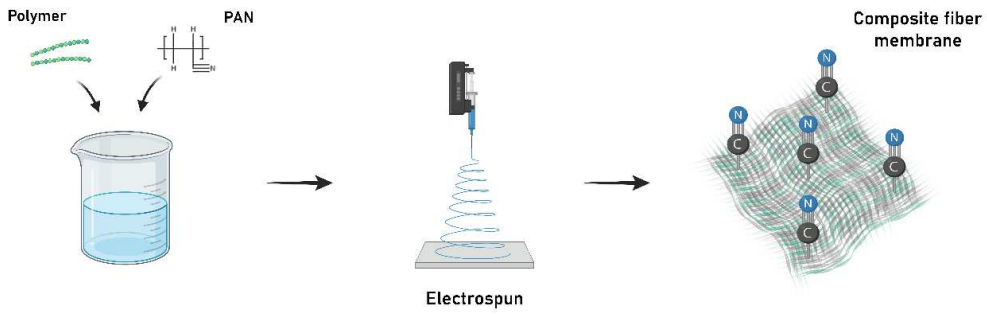
immobilizing enzymes. Besides, membranes have also been studied for many other applications, such as reactors, sensors, supercapacitors, and solar cells (WU *et al.*, 2017). Briefly, the main qualities of fibers as excellent supports are attributed to: (i) different polymers can be electrospun to meet other requirements as supports, (ii) the high porosity and the interconnectivity of the electrified supports gives them a low resistance to mass transfer and (iii) fiber surfaces can be modified to contribute to enzymatic activity (WANG *et al.*, 2009).

Although the fibers have a large proportion of surface area to volume, the immobilized enzymes form the only monolayer in each fiber, limiting the enzyme load. Also, studies using these materials are still minimal, as there are still problems in their application on a large scale. One of them is that fibers are still very difficult to be manufactured in batches. Also, a few tools can be used to evaluate the fiber surface effect on immobilized enzymes (NATARAJ; YANG; AMINABHAVI, 2012; QIN, 2016; WANG *et al.*, 2009).

2.6.2 Composite fibers

The evolution of fiber composites began when scientists discovered plastics. The first fiber-reinforced composite was developed in 1935, but the main evolution of fiber composites occurred during World War II due to the need for lighter materials. Synthetic fibers are the most common reinforcement for polymeric composites due to their unique mechanical behavior in various applications (VIGNESHWARAN *et al.*, 2020). Figure 9 shows an image of a scheme for obtaining PAN copolymer fibers for laccase immobilization.

Figure 9 – Scheme for obtaining PAN copolymer fibers for laccase immobilization



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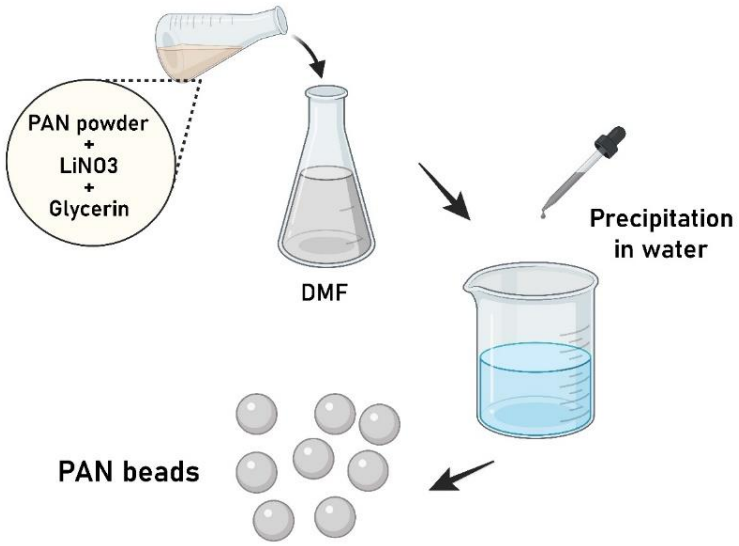
The use of other polymers in conjunction with PAN forms attractive polymeric composites to manufacture electrospun fiber membranes. For application in laccase immobilization, composites can contribute with reactive groups to improve the bonds between the enzyme and the support and the degradation of the desired substrate (ANDREESCU; NJAGI; ISPAS, 2008; WANG *et al.*, 2020). For example, Wang and co-workers used a PAN composite with montmorillonite (MMT) and graphene oxide (GO). The MMT and GO nanolayers could adsorb the laccase reaction products, and their properties improve oxidation and catechol removal from aqueous solutions that have been investigated. The catechol removal reached 39% ± 2.23% (WANG *et al.*, 2014b).

Other works used the same PAN/MMT composite laccase immobilization to degrade violet crystal dye (LI *et al.*, 2016; WANG *et al.*, 2020). Taheran *et al*. (2017a) evaluated the laccase immobilization in fibers of a PAN/Biochar composite for application in the degradation of chlortetracycline (CTC). The choice of biochar was due to its low cost, availability, interesting physicochemical properties, and role in adding value to wood waste. PAN/Biochar fiber membranes had a surface area of 12 m²/g, and the biodegradation results reached 58.3% (TAHERAN *et al.*, 2017b).

2.6.3 Beads

Polymer beads are solid polymer pellets (LIU *et al.*, 2020). This type of material is preferred to support enzymes' immobilization when it comes to bioreactors over resins and inorganic adsorbents (MENALE *et al.*, 2012). Few works in the literature investigated the use of polyacrylonitrile beads for laccase immobilization. In these works, the support was synthesized from the dissolution of polyacrylonitrile powder (18 g), $LiNO_3$ (1 g), and glycerin (3 g) in dimethylformamide (DMF), and this mixture was precipitated in water, forming the polymeric beads(CATAPANE *et al.*, 2013; MENALE *et al.*, 2012; NICOLUCCI *et al.*, 2011) (Figure 10). In these publications, laccase immobilization was performed using a diazotization process involving the phenolic group of tyrosine residues not present in the catalytic site. The activation of the support occurred from the treatment with NaOH aqueous solution and an aqueous solution of 1,2-diaminoethane, followed by treatment with aqueous glutaraldehyde solution, used as a coupling agent and, finally, to obtain amino aryl derivatives, PAN beads were also treated with phenylenediamine (PDA) solution in sodium carbonate buffer. (NICOLUCCI *et al.*, 2011).

Figure 10 – Representation of obtaining PAN beads



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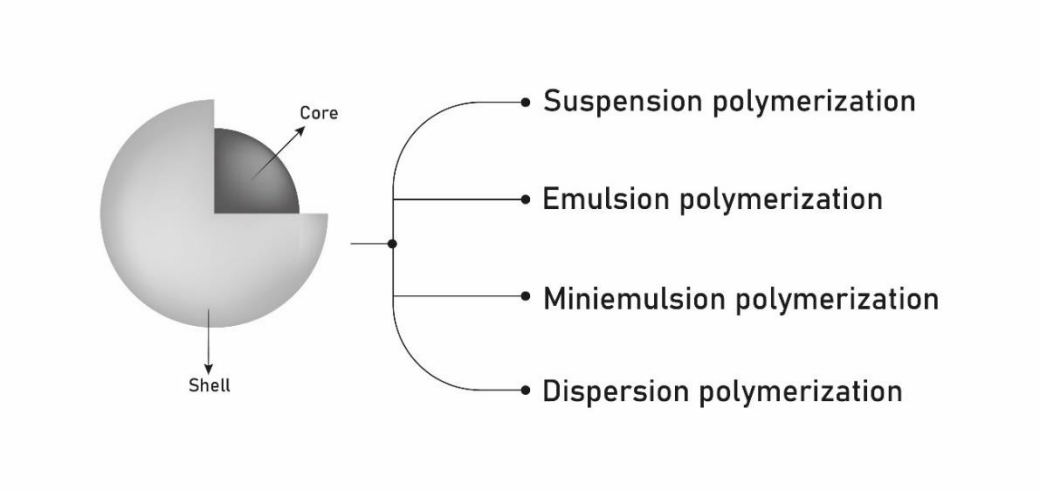
Despite the satisfactory efficiency of laccase immobilization and biocatalyst reuse (95% of the enzyme activity was maintained after 50 days) (CATAPANE *et al.*, 2013), the preparation of these supports from polyacrylonitrile beads required many steps for activation, which is not attractive. A possible solution would be using polyacrylonitrile copolymers or even the formation of hybrid particles, incorporating other functional groups on the granule surface, as was done previously for the PAN fibers (LI *et al.*, 2016; WANG *et al.*, 2020).

2.4.4 Core-shell

Core-shell morphology particles are usually composed of a core (inner material) and a shell (outer material) (Figure 11). The core and shell are, in most cases, different in composition or structure (SU *et al.*, 2020). These particles are generally synthesized by a two- or several-step process. First, the core particles are synthesized, and the shell is then formed into the core particle using different methods, depending on the type of core, the materials of the shell, and their morphologies (HAYES *et al.*, 2014). Core-shell particles are increasingly attracting attention due to their controlled structure and unique properties with biomedicine, electronics, and catalysis applications. In particular, these particles can present high porosity and, consequently, an ultra-high surface area, adjustable porous structure, and orientation, which are of great interest for bioadsorption and biocatalysis (SU *et al.*, 2020).

Figure 11 – Representation of the core-shell morphology particle and its main ways of obtaining



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There is still no investigation in the literature of laccase immobilization in PAN particles in core-shell morphology. However, there is research using core-shell particles based on other materials for laccase immobilization. Deng *et al.* (2015) developed a one-step aqueous dopamine self-polymerization strategy to immobilize laccase on the surface of silica-coated magnetic nanoparticles. Briefly, a mixture of silica-coated magnetic nanoparticles and dopamine was made in a buffer solution containing laccase. This methodology results in an enzymatic derivate with higher storage stability and immobilization efficiency than when laccase was covalently attached by glutaraldehyde, preserving 80% of its initial activity after 70 days of storage and retaining 65% of it is initial activity after reusing 10 batches (DENG *et al.*, 2015). Still, other enzymes were successfully immobilized on supports with this morphology.

When we talk about the potential of PAN-based core-shell materials, some authors have already evaluated its application as a gold nanoparticles carrier (FAN *et al.*, 2012) and for the removal of hexavalent chromium from aqueous solution (WANG *et al.*, 2013). The microtubes composed of Kapok-polyacrylonitrile were prepared by a self-assembly method assisted by cetyltrimethylammonium bromide. The influence of the AN monomer concentration on the morphology of the microtubes was investigated. Parameters as hydrophilia and specific surface area of the kapok microtubes were improved because of the outer PAN coating constructed by the aggregation of PAN nanoparticles. Additionally, gold nanoparticles have been successfully immobilized on kapok-PAN microtubes (FAN *et al.*, 2012).

Wang *et al.* (2013) prepared polyacrylonitrile/polypyrrole (PAN/PPy) core-shell structure fibers via electrospinning followed by in situ polymerization pyrrole monomer. This core-shell fiber mat was used for hexavalent chromium removal from an aqueous solution. The PAN/PPy fiber mat's adsorption capacity was 74.91 mg/g at 45 ° C, and desorption results show that the adsorption capacity can remain up to 80% after 5 times usage (WANG *et al.*, 2013).

Given the works presented above, it is interesting to observe the potential of polyacrylonitrile forming shell-core materials as carriers for the immobilization of enzymes, especially laccase. The exploration of different polymers to form the core of this type of structure, using PAN-based shells, should be the differential in applying the biocatalyst to be developed.

2.5 CONCLUSIONS AND FUTURE OUTLOOK

Polyacrylonitrile-based materials have been proven to be excellent supports for laccase immobilization. They can provide large proportions of surface area to volume, pore sizes adapted to the enzyme's dimensions, functionalized surfaces, multiple sites for interaction or binding, and low mass transfer resistance. In addition, polyacrylonitrile has the advantage of being a low-cost synthetic polymer with a molecular structure rich in nitrile groups, interesting due to ease of modification for application as enzymatic support. The materials of core-shell morphology showed great potential for application in the immobilization of the enzyme because they allow the synthesis of particles with porous shells and specific components on their surface, allowing greater interaction with the enzyme. Nevertheless, the studies on PAN-based materials are still limited and summarized in fibrous materials produced by electrospinning and functionalized beads. From this review, due to the wide advantages and the great potential of applying polyacrylonitrile in the enzyme immobilization, it is expected that the other forms of PAN-based materials, in addition to different functionalization methods, can verify the needs for exploring this material to achieve consistent and low-cost support for laccase immobilization.

CHAPTER 3

In this chapter, the methodologies concerning the immobilization of laccase on polyacrylonitrile particles have been carefully described.

 [3]

[3] Bring the reader closer to the QR code to access the articles that served as a reference for the construction of this methodology.

3. MATERIALS AND METHODS

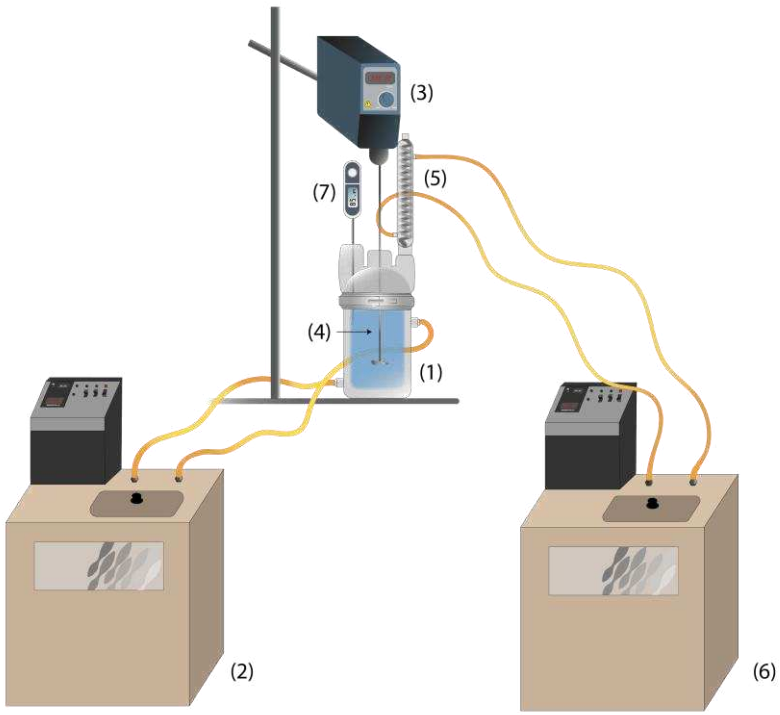
3.1 CHEMICALS

The commercial laccase from *Trametes versicolor* (EC 1.10.3.2), 1 U/mg (catechol, pH 4.5 and 25 °C) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ≥ 98.0 %) were provided by Sigma-Aldrich (USA). To obtain the polyacrylonitrile, it was used acrylonitrile monomer (Vetec, 99.0 %) and Potassium persulfate – KPS (Vetec, 99.0 %) as an aqueous phase initiator. Sodium hydroxide (Neon, ≥ 97.0 %), sulfuric acid (Nuclear, ≥ 95.0 %), nitric acid (Synth, 54.0%), ethylenediamine (Neon, 99.0%), carbodiimide (N-(3-Dimethylaminopropyl)-N'-ethyl carbodiimide hydro-chloride, Sigma Aldrich, ≥ 98.0%) and glutaraldehyde (Neon, 50.0 %) were used to activation of PAN support. All analyzes were performed at the Biological Engineering Laboratory (LiEB), Carlos Henrique Neves Polymerization Process Control Laboratory (LCP) and Analysis Center of the Chemical and Food Engineering Department of the Federal University of Santa Catarina (UFSC).

3.2 PREPARATION AND CHARACTERIZATION OF PAN PARTICLES

The particles were prepared by solution polymerization with precipitation in a jacketed borosilicate glass reactor with a capacity of 1 L. The temperature was controlled by a thermostatic bath (Microquímica, MQBMP-01) with water as circulating fluid for temperature control at 85 °C in the reactor and under-regulated agitation at 460 rpm by mechanical stirrer (IKA®RW20 digital) and a pitched three-blade turbine impeller with a pitch angle of 45 °. For cooling the glass condenser coupled to the reactor (used to avoid the loss of acrylonitrile vapor phase to the atmosphere), another thermostatic bath (Microquímica, MQBMP-01) with the controlled temperature at 5 °C was used. Still coupled to the reactor, a thermoresistance thermocouple (PT100) (Kasvi, K29-5030) was placed to check the temperature inside the reactor (Figure 12). In this order, 750 mL of distilled water, 80 g (~10 % m/m) of acrylonitrile monomer, and 3.8 g of KPS initiator were added into the reactor. It is worth mentioning that this formulation is based on previously published works concerning the acrylonitrile polymerization in the aqueous phase (EBDON; HUCKERBY; HUNTER, 1994a; EBDON; HUCKERBY; HUNTER, 1994b). After 4 h of polymerization, the reactor was cooled, and the particles were collected, vacuum filtered, and washed with distilled water. After drying in an incubator (Tecnal, TE-424) with a controlled temperature at 50 °C for 24 h, the particles were separated by size on sieves (1.20, 1.40, 1.70 mm).

Figure 12 – Reactor diagram for solution polymerization with precipitation. (1) Jacketed borosilicate glass reactor; (2) thermostatic bath at 85 °C; (3) mechanical stirrer at 450 rpm; (4) pitched three-blade turbine impeller with a pitch angle of 45 °; (5) glass condenser ;(6) thermostatic bath at 5 °C; (7) thermoresistance thermocouple.



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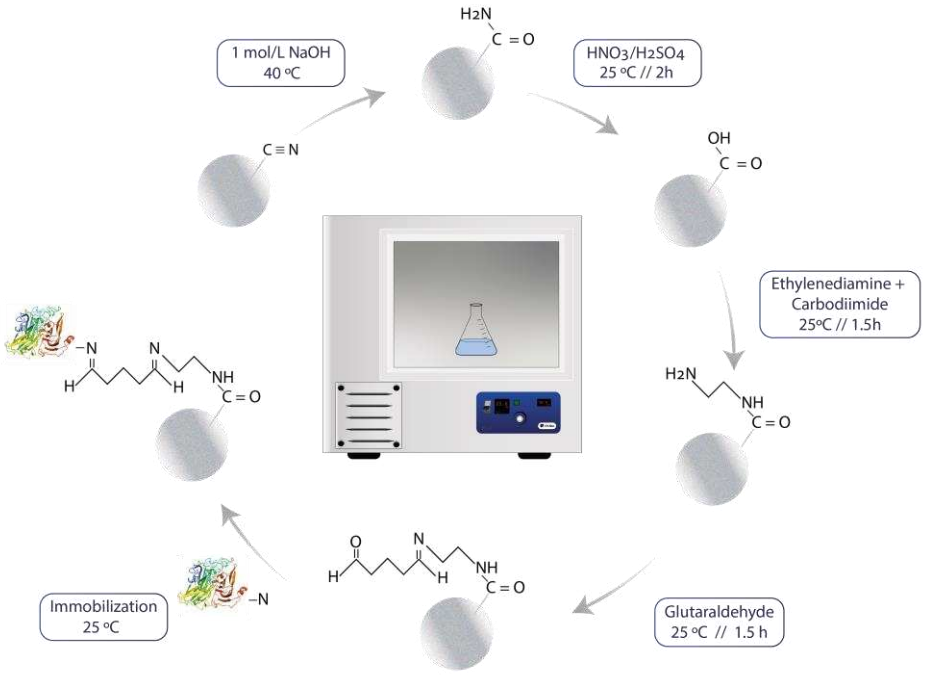
The morphology of PAN particles was analyzed by Optical microscopy (Binocular stereomicroscope, Tecval, 10x). The measurement of the surface area and the porosity of the PAN particles were performed by nitrogen physisorption isotherms (-195.8 °C), under a critical pressure of 33.5 atm and relative pressures (P/P_0) of approximately 0.99. These analyzes were performed on surface area analyzer (Quantachrome Instruments - Autosorb-1). A sample of approximately 100 mg of particles was used to perform the analysis. The surface area, diameter, and pore volume were obtained from the data treatment through the method of Brunauer, Emmett, and Taller (BET) and Barrett, Joyner e Halenda (BJH).

3.3 PAN PARTICLES FUNCTIONALIZATION

After synthesis, drying and separation, 100 mg of PAN particles were weighed and functionalized in four steps. At first, they were immersed in 50 mL of 1 mol/L NaOH in an incubator (Tecnal, TE-424) kept at 40 ºC for different times (1, 2, and 3 h). After this time, the

samples were washed with distilled water and directed to the second step, where they were immersed in 50 mL of 10 % v/v HNO_3/H_2SO_4 (50:50 v/v) solution for 2 h at 25 °C. These first two processes were adapted from the methodology proposed by Taheran *et al.* (2017), and their main objective was the formation of –COOH groups, which will be essential in the next step. The functionalized PAN particles were washed again with distilled water and immersed in 50 mL of 1 mol/L ethylenediamine (pH 4.7)/ 0.1 mol/L carbodiimide solution at 25 °C for 1.5 h. This step was previously described by Henriques *et al.* (2018) for the amination of carboxylic groups present in proteins. It was tested to generate the same free primary amino groups on the polyacrylonitrile-based support. After the new washing in distilled water, the fourth step consisted of immersing the particles in a solution containing different amounts of glutaraldehyde (2, 4, 6, 8, and 10 % v/v) for 1.5 h at 25 ºC. The efficiency in immobilization, when using this functionalization step, occurs by the formation of the Schiff base between the amine group of the enzyme and the chemical group available on the support (ASLAM *et al.*, 2021). In Figure 13, the processes involved in functionalization and immobilization are illustrated.

Figure 13 – Laccase Immobilization on functionalized PAN particle



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Furthermore, to determine the polymer chain modifications achieved by the functionalization of the support, samples were inlaid in KBr pellets and analyzed by Fourier Transform Infrared Spectroscopy - FTIR (Shimadzu, prestige-21) with a resolution of 4 cm^{-1} and 32 scans, by the transmittance in the region 4000–400 cm^{-1}.

3.4 IMMOBILIZATION OF LACCASE ONTO PAN PARTICLES

The functionalized PAN was treated with 8 mL of laccase in acetate buffer solution (0.1 mol/L, pH 4.5) (0.5 mg/mL) for up to 6 h. The heterogeneous catalysts were then removed and thoroughly rinsed. This same treatment with laccase solution (0.5 mg/mL) was tested with PAN without any previous functionalization step to evaluate the ability of support to immobilize the enzyme by physical adsorption. The immobilization yield was determined from the decay of enzymatic activity in the supernatant after 6 h of immobilization.

3.5 LACCASE ACTIVITY ASSAY

The determination of laccase activity was monitored by the oxidation rate of ABTS (GARCÍA-MORALES *et al.*, 2018; RAHMANI *et al.*, 2015). For the activity from free laccase, 0.3 mL of aqueous ABTS (5 mmol/L) and 0.3 mL of enzymatic solution (0.5 mg/mL) were added to 2.4 mL of phosphate-citrate buffer (0.1 mol/L, pH 6) in a glass cuvette. As a blank, 0.3 mL of Milli-Q water was used instead of the free enzyme. To determine the activity of immobilized laccase, 100 mg of the heterogeneous catalyst was added into 0.3 mL of 5 mmol/L ABTS solution and 2.4 mL phosphate-citrate buffer solution (pH 6). The reaction occurred at 25 °C for 2 min. The change in absorbance at 420 nm was observed using a UV/Vis spectrophotometer (U-2900, Hitachi High-Technologies, Japan), and the ABTS molar extinction coefficient (ε_{420} = 3.6 L^{-1} μmol^{-1} cm^{-1}) was used to calculate the laccase activity, presented by Equation 1 (CHILDS; BARDSLEY, 1975).

$$EA = \frac{\Delta Abs \,.\, V}{\varepsilon . d . t . v} \qquad \text{Equation 1}$$

Where:

EA: enzymatic activity $\left(\frac{U}{L}\right)$;

ΔAbs: absorbance variation;
V: total reaction volume (L);
ε: molar extinction coefficient (L^{-1} μmol^{-1} cm^{-1});
d: step length (cm);
t: reaction time (min);
v: sample volume (L); or (g) in the case of PAN+Lac.

One unit of laccase activity was determined as the amount of enzyme needed to oxidize 1 μmol of substrate per minute. All analyzes were performed in triplicate.

3.6 PROPERTIES OF FREE AND IMMOBILIZED LACCASE

3.6.1 Enzyme derivative thermostability test

The thermal stability of the catalyst was evaluated added 100 mg of the enzymatic derivative to 2.4 mL of 0.1 mol/L phosphate-citrate buffer solution (pH 6.0) and placing at different temperatures (30, 50, 60, and 70 °C) in a water bath (Dist, DE-911). The enzymatic activity of the derivative was measured according to the methodology described in Section 3.5 at each time interval (0.08, 0.25, 0.5, 1, 2, 4, 6, and 8 h). Similarly, for comparison, 0.3 mL of laccase solution (0.5 mg/mL) was added to 2.4 mL of 0.1 mol/L phosphate-citrate buffer solution (pH 6.0) and kept at the same conditions tested for immobilized laccase.

3.6.2 Stability test of immobilized laccase at different pHs

The stability of the catalyst at different pH values was performed by adding 100 mg of enzymatic derivative in 0.1 mol/L phosphate-citrate buffer solutions with different pH values (3, 5, and 8) and left at 25 °C for different periods (0.08, 0.25, 0.5, 1, 2, 4, 6 and 8 h). Suspension samples containing the enzymatic derivative were collected at each interval, following its oxidation activity through the methodology described in Section 3.5. Additionally, the same procedure was performed for the laccase in its free form, in which laccase solution (0.5 mg/mL) was added in 0.1 mol/L solutions of phosphate-citrate buffer with the same pH values, for the same pH values, as previously described.

3.6.3 Storage stability test

Storage stability was monitored over 30 days. The immobilized derivative and free enzyme were stored at 5 °C, with no use of reagents. Every 5 days, samples were collected, and the enzymatic activities for ABTS oxidation were monitored. Storage stability was then plotted as the residual activity, considering the activity on the first day as 100 %.

3.6.4 Operational stability test

The operational stability of the immobilized laccase was determined by testing consecutive ABTS oxidation cycles. The residual activity assay was performed at 50 °C in a water bath (Dist, DE-911) using 0.1 mol/L phosphate-citrate buffer (pH 3) and was measured as previously described. The enzymatic activity obtained in the first cycle was considered 100 %. Then, the derivative was washed five times with 0.1 mol/L phosphate-citrate buffer (pH 3). Finally, the residual activity was calculated according to Equation 2.

$$RA = \left[\frac{C_i \times 100}{C_1}\right] \qquad \text{Equation 2}$$

Where:

RA: Residual activity (%);

C_1: Enzymatic activity at the first cycle ($U.g^{-1}$);

C_i: Enzymatic activity at the succeeding cycles ($U.g^{-1}$), $i > 1$.

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CHAPTER 4

In this chapter, the results and discussions concerning the characterization of polyacrylonitrile particles and the immobilization of laccase on them have been carefully described. Furthermore, the final heterogeneous biocatalysts were characterized physically and chemically, and the operational behavior was investigated.

 [4]

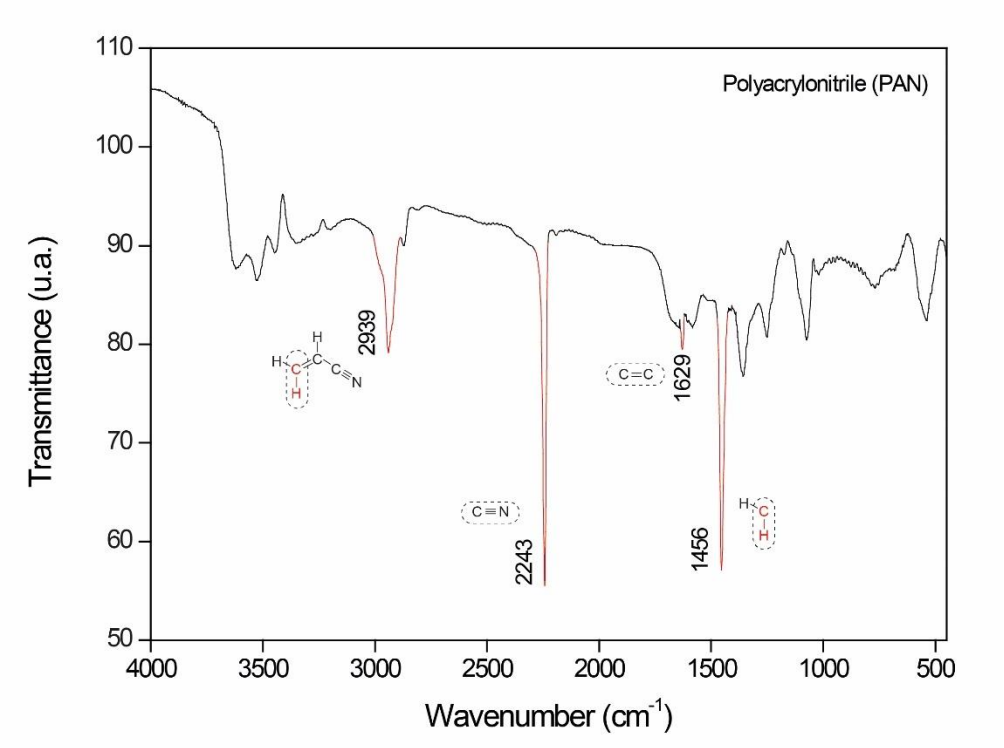
[4] Bring the reader closer to the QR code to access N_2 adsorption/desorption isotherms data - BET and BJH method in full.

4. RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF THE POLYACRYLONITRILE PARTICLES

Fourier Transform Infrared Spectra (FTIR) was used to characterize the structure of PAN particles formed from a solution polymerization process with precipitation (Figure 14). The samples showed a peak of elongation vibration absorption in the range of 2243 cm^{-1}, thus indicating the clear presence of the nitrile group (C≡N), and another peak at 2939 cm^{-1}, representing a C–H bond of $–CH_2$ in acrylonitrile, characteristics of this polymer (BAJAJ; SREEKUMAR; SEN, 2001; WANG *et al.*, 2022). The stretching vibration in the range of 1456 cm^{-1} also indicates a C–H bond of the $–CH_2$ group, values close to these are found in the literature (ASHRAFI; FIROUZZARE, 2021; KARBOWNIK *et al.*, 2019). It is also possible to point to a peak in the range of 1629 cm^{-1}, which denotes a low-intensity carbon-carbon (C=C) double bond (XUE; LIU; LIANG, 2013) or bands attributed to deformation vibrations in the N–H plane (SHEN *et al.*, 2011). According to the analysis, the applied polymerization method corresponded to the successful formation of polyacrylonitrile particles.

Figure 14 – Fourier Transform Infrared Spectra (FTIR) of PAN particles



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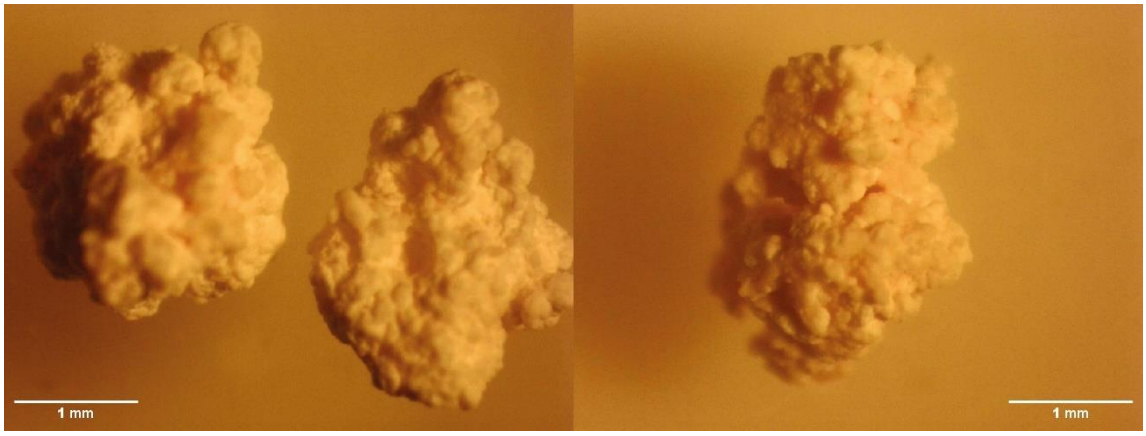
The PAN particles were separated by size on sieves (1.20, 1.40, 1.70 mm) to determine the granulometry of the particles to be used in the immobilization step. The objective of this preliminary step was to find a suitable particle size in which the heterogeneous catalyst (PAN+Lac) could be easily recovered after use. Particle morphology plays an important role in obtaining supported biocatalysts (KHANMOHAMMADI *et al.*, 2020). Furthermore, the diffusion phenomena linked to the size and shape of the particles are affected by the support morphology (GASCÓN *et al.*, 2014). Therefore, the average size (D_p) of the particles selected for laccase immobilization was $1.2 \leq D_p \leq 1.7$ mm. Optical microscopy images (Figure 15) were obtained to investigate the surface of the particles and their morphological characteristics in magnification of 10x. From these images, it is possible to observe that there is no uniformity in the exact shape and size of the particles. However, an irregular surface is observed in the polymeric particles, with the presence of few pores, which will be discussed through the results from nitrogen physisorption.

Figure 15 – Optical microscopy of PAN particles



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The main textural properties of the PAN particles, i.e., surface area, volume, and average diameter of the pores, were calculated according to the BET and BJH models. The values obtained are shown in Table 4.

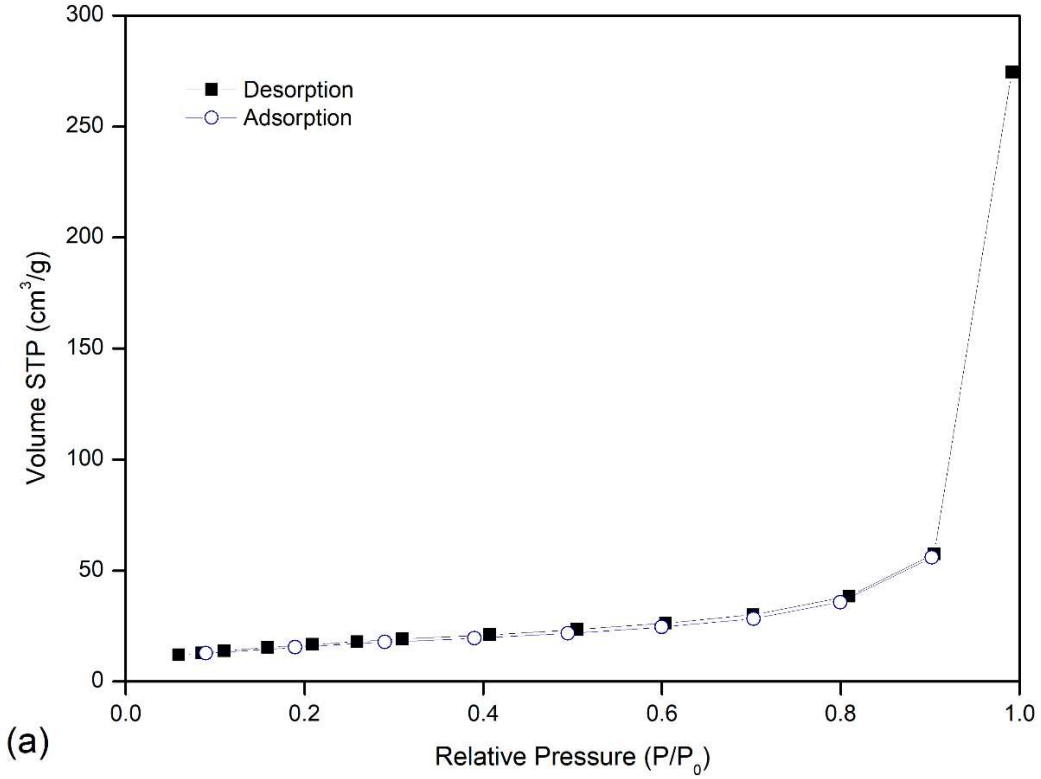
Table 4 – Porous characteristics of PAN particles using BET and BJH methods

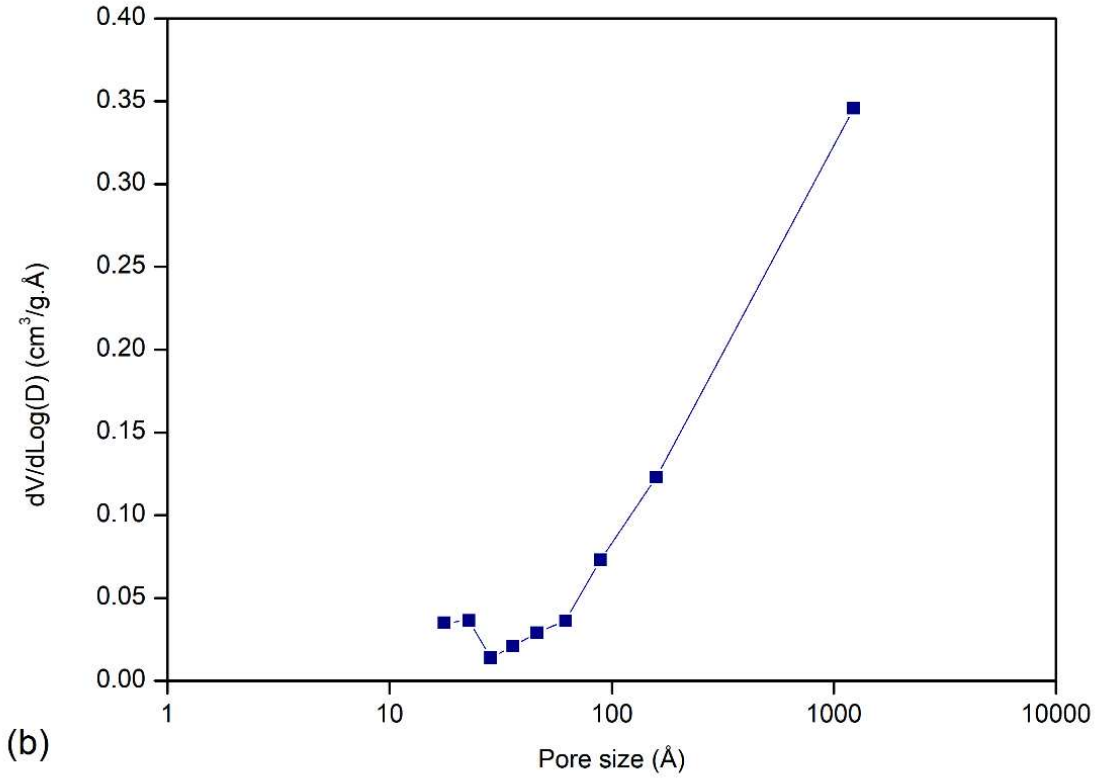
Surface area (BET)	Total Pore Volume (BJH)	Average Pore Diameter
6.07 m²/g	0.42 cm³/g	28.05 Å

Source: from author

The data obtained by these models indicate that the average value of the pore diameter in the sample of PAN particles was 28.05 Å, which implies the classification of the material as mesoporous, according to IUPAC - 20 Å to 500 Å (HWANG; BARRON, 2011). This is reinforced by the N_2 adsorption and desorption isotherms for PAN particles, shown in Figure 16(a), classified as type II, typical of mesoporous structures (SING *et al.*, 1985). At pressures below 0.3, the micropores are filled with N_2. The formation of monolayers occurs in the region called the knee, while at medium pressures, monolayer-multilayer adsorption occurs, accompanied by capillary condensation of the adsorbate in the mesopores at higher pressures, which has already been described in the literature for other materials (HWANG; BARRON, 2011). From the graph and according to data reported in the literature, it is possible to observe that when the relative pressure is equal to 1, the thickness of the adsorbed multilayer appears to increase immensely (THOMMES *et al.*, 2015).

Figure 16 – Plot of (a) N_2 adsorption and desorption isotherms for PAN particles; (b) pore size distribution of PAN





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The BJH method helps to accurately calculate the pore size distribution based on the adsorption isotherms (BARDESTANI; PATIENCE; KALIAGUINE, 2019). The theory behind this method explains that the amount of adsorbed gas results from both capillary condensation in the mesopores and physical adsorption on the pore walls (BARRETT; JOYNER; HALENDA, 1951). From these data, a graph, shown in Figure 16(b), was obtained, in which it is possible to observe the pore size distribution in terms of volume. With this graphic demonstration, the predominance of mesopores in the order of 20 to 200 Å in the sample is clear. Also, the measured maximum pore volume in desorption and the surface area were 0.4236 cm^3/g and 6.07 m^2/g, respectively.

It is possible to find reports that explain that mesoporous structures with pore sizes slightly larger than the size of the enzymes influence the processes of enzymatic immobilization (HOU *et al.*, 2014). It has been addressed previously with silica, zeolites, and bentonite-based supports (AMERI *et al.*, 2021; WEN *et al.*, 2019; YANG *et al.*, 2021; ZHANG *et al.*, 2021). There is then a correlation between the laccase immobilization yield and the volume of the mesoporous structure; that is, the greater the volumes of mesopores, the greater the immobilization yields by physical adsorption (RAMÍREZ-MONTOYA *et al.*, 2015). In the case

of the PAN particles, the total volume of pores found by the BJH method can be considered low, wich can, in turn, unfavor the immobilization by physical adsorption. On the other hand, immobilization by covalent bonding cannot be affected by textural properties, as will be presented in the next sections.

4.2 FUNCTIONALIZATION EFFECT

The functional group on the support surface can determine the appropriate immobilization method. If, for example, on its surface, there are groups such as –COOH, –OH, $–NH_2$, –C=O, or –SH, methods such as physical adsorption or covalent bonding are recommended (DARONCH *et al.*, 2020). Although PAN does not have these functional groups, the nitrile group present in its structure can be easily modified, allowing the bond with the enzyme surface (VIEIRA *et al.*, 2021). In this sense, the methodology applied to functionalize the PAN particles studied here allowed obtaining the high immobilization yields, as will be presented in the next sections.

4.2.1 Reaction time with NaOH and Acid step

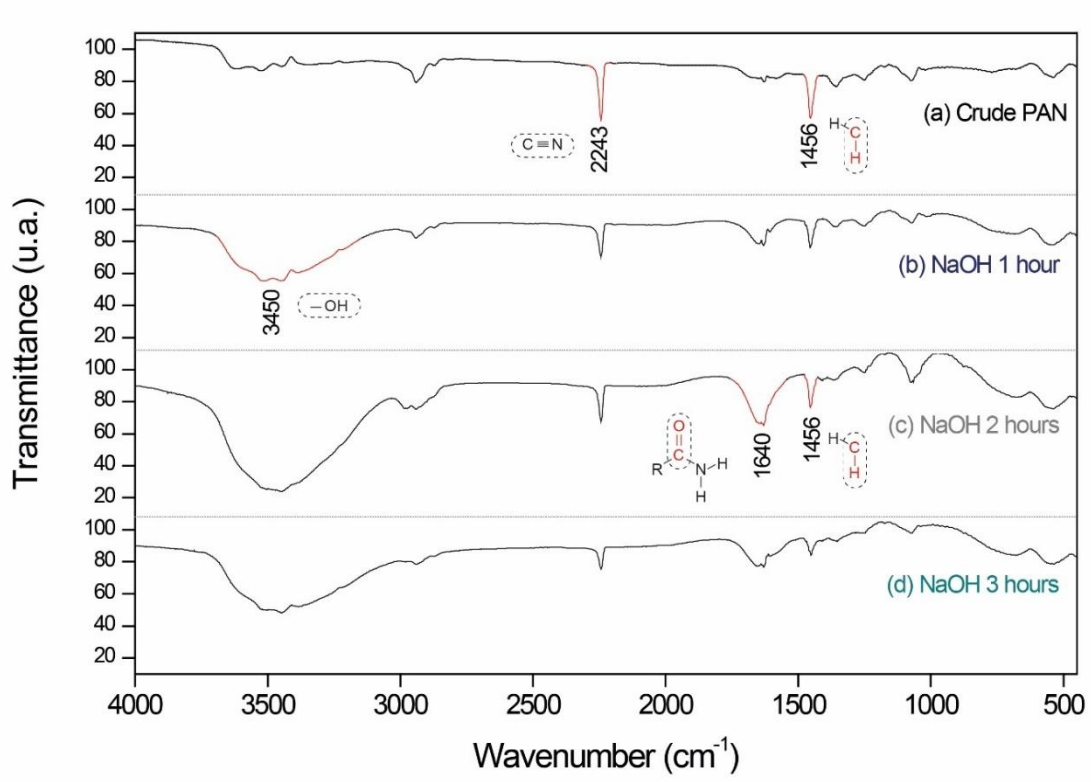
The first step of activating the polyacrylonitrile particles was immersing them in 1 mol/L NaOH solution under heating at 40 °C for different sampling periods (1, 2, and 3 h). At this first moment, the reaction time required for the nitrile groups to be converted through a hydrolysis reaction was tested. FTIR was used to understand what changes occurred after the NaOH functionalization step, as shown in Figure 17.

Hydrolysis under alkaline conditions is a widely discussed and used method for polyacrylonitrile-based materials due to the chemical changes caused in the polymer, which can be easily manipulated in subsequent reactions. Alkaline hydrolysis in solution occurs from the conversion of –CN groups on the surface of particle initially into $–CONH_2$ and, from there, at high concentrations of NaOH, into –COOH groups (ZHANG; MENG; JI, 2009). In other words, NaOH hydrolyzes the –CN group partially or completely. The effects resulting from NaOH functionalization depend on four main factors: the basicity and size of the reactant molecules and the concentration and reaction time (POPESCU; MURESAN, 2013).

Through Figure 17, it is possible to identify that from the hydrolysis, there was a gradual decrease over the reaction time of the relative intensity of peaks related to –CN groups and the CH bond of $–CH_2$, manifested in the peaks around 2243 cm^{-1} and 1456 cm^{-1}, respectively. This

decrease becomes more evident after 2 h of reaction. In addition, the intensification of the peak around 3450 cm^{-1} points to concrete evidence of the formation of the –OH group in the sample. However, the formation of the carboxylic group was not clear, as no C=O stretch peak was observed in the range of 1710 - 1780 cm^{-1} (OH; JEGAL; LEE, 2001). This same bond formed can be observed in the range from 1680 cm^{-1} to 1630 cm^{-1}. However, it refers to the C=O of the primary amides (AMIR *et al.*, 2013). The same elongation in the range of 3500 cm^{-1} to 3100 cm^{-1} may indicate the formation of NH_2 (CHEN *et al.*, 2021).

Figure 17 – Fourier Transform Infrared Spectra (FTIR) of PAN particles crude and after different times of reaction with NaOH (1; 2; 3 h)



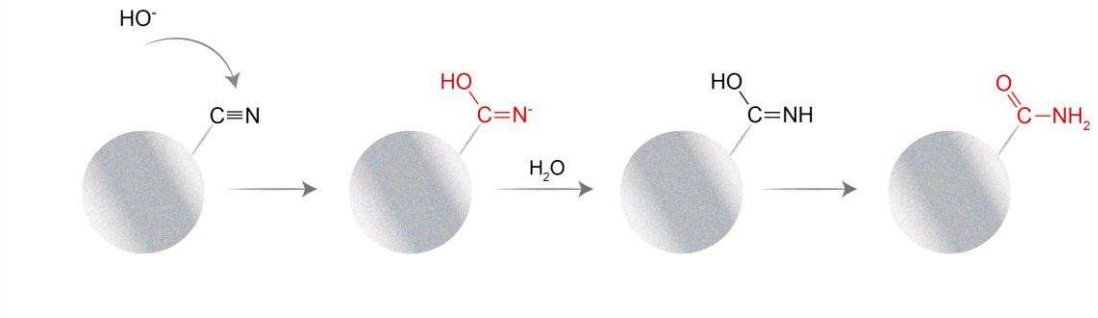
Source: from author

What is not clear is whether this strong and broad peak could be an overlap between the –OH and $–NH_2$ peaks of the amide, which would explain the first step of converting the –CN group by reaction with NaOH. As a complement, Figure 18 presents a theoretical scheme of the bonds that occur during the activation of PAN by NaOH, based on the results presented here and what is available in the literature.

The spectra presented at 2 and 3 h were similar from a chemical point of view; that is, no major changes were observed in the intensity or location of the main peaks. Therefore, the

activation time of 2 h was considered ideal for the purposes for which alkaline hydrolysis of PAN with NaOH was proposed.

Figure 18 – Possible route of the partial alkaline hydrolysis reaction of PAN



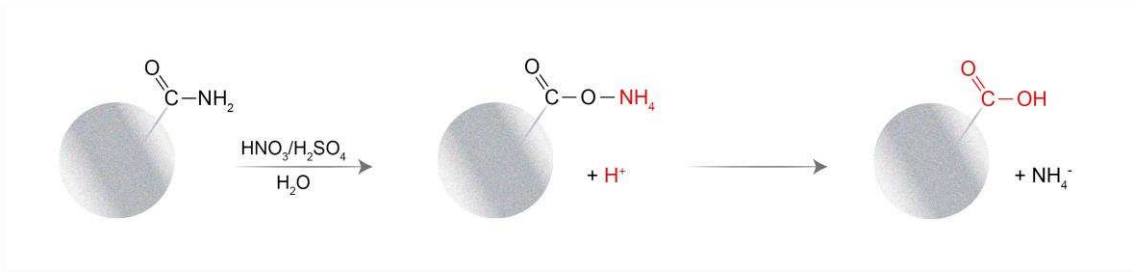
Source: Adapted from Yang *et al*. 2021

This activation step then worked to form primary amides, which will later be converted to carboxylic acid in the acid step. Paitaid and H-Kittikun (2021) performed the hydrolysis of PAN nanofibrous membranes with NaOH. They then added HCl and ethylenediamine to functionalize the membrane to immobilize lipase from *Aspergillus oryzae* ST11 by covalently bonding with glutaraldehyde, similar to the procedures performed here. The functionalization provided good immobilization yields since the recovered activity of the immobilized lipase reached up to 86.9 % (PAITAID; H-KITTIKUN, 2021). Other reports in the literature support the need for activation with NaOH followed by the acid step to form the carboxylic group on the surface of polyacrylonitrile (CHEN *et al.*, 2022; TAHERAN *et al.*, 2017).

As visualized in Figure 18, the hydrolysis in alkaline medium provided by NaOH involves a sequence of conjugated reactions that culminate in the formation on the particle surface, initially of an imidic acid and, by subsequent hydrolysis, of poly(acrylamide) (LITMANOVICH; PLATÉ, 2000). The acid hydrolysis performed in this work after alkaline hydrolysis involved immersion of PAN particles in an aqueous medium containing 10 % v/v HNO_3/H_2SO_4 (50:50 v/v) for 2 h at 25 °C. The acid hydrolysis of PAN reported in the literature, in turn, leads to the formation of poly(acrylonitrile-co-acrylamide). (KRENTSEL *et al.*, 2001). The association of the amino group of the amide with the water of the solution forms oxyammonium groups. Because they are more electropositive than the ammonium cation, the hydronium released in the second phase of the reaction displaces this ion and thus forms the carboxylic acid (ZAHN, 2004). The ion exchange resulting from this step is responsible for the

formation of the carboxylic groups necessary for subsequent amination (CHEN *et al.*, 2022). Figure 19 presents a theoretical scheme of the ion exchange allowed by acid hydrolysis.

Figure 19 – Possible route of the acid hydrolysis reaction of amide in PAN

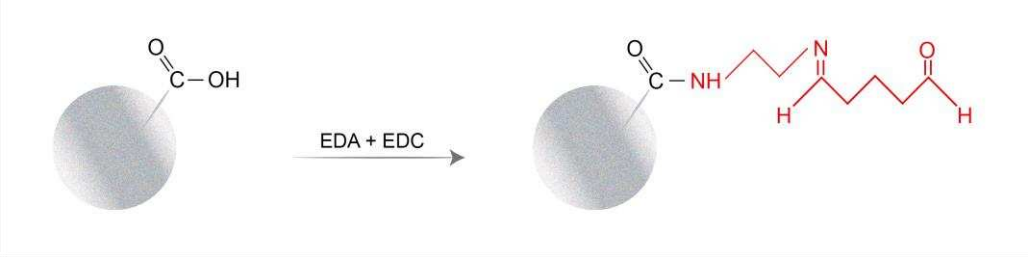


Source: adapted from ZAHN, 2004; CHEN *et al*. 2022

4.2.2 Amination

The amination step was performed by immersing the hydrolyzed PAN particles in a solution containing 1 mol/L ethylenediamine (EDA) (pH 4.75)/0.1 mol/L carbodiimide (EDC) at 25 °C for 1.5 h. This methodology has been widely used for activating carboxylic groups on the surface of proteins for immobilization or co-immobilization (HENRIQUES *et al.*, 2018). The carboxylic groups activated in the previous steps were used to react with the EDA amino groups to generate a free primary amino group. This group, in turn, could be used for a possible multipoint binding between the support and the enzyme, facilitated by glutaraldehyde, which will be discussed in the next section. Functionalization by the process of amination of the carboxylic groups of activated PAN is demonstrated by the scheme shown in Figure 20.

Figure 20 – Possible route of the amination of carboxyl group in PAN activated



Source: adapted from Henriques *et al.* 2018; Awad *et al*. 2021

The use of EDA was previously employed for the amination of nanofibrous PAN-graphene hydroxide membranes after alkaline hydrolysis in NaOH. Through FTIR analysis of

the membranes before and after functionalization, the authors noticed the disappearance of peaks at 3271 cm^{-1} and 1650 cm^{-1} and the concomitant appearance of a new peak in the range of 1572 cm^{-1} referring to the amide group, which indicated a reaction between the free amino group and the carboxyl group formed by the hydrolysis of PAN (AWAD *et al.*, 2021). The same was observed by Wang, Chen and Wang (2015) when performing amination on polyacrylonitrile fibers in another work (WANG; CHEN; WANG, 2015).

The aqueous solution of ethylenediamine is highly alkaline due to the cationic properties of the amino group (ZHANG *et al.*, 2021). To reduce the pH to 4.75, the addition of 0.1 mol/L carbodiimide and titration with HCl was necessary. This pH reduction was fundamental for the process of PAN functionalization and enzymatic immobilization because when adding only EDA solution for amination, proposed by other authors (AWAD *et al.*, 2021; WANG, X.; CHEN; WANG, 2015), laccase immobilization did not occur.

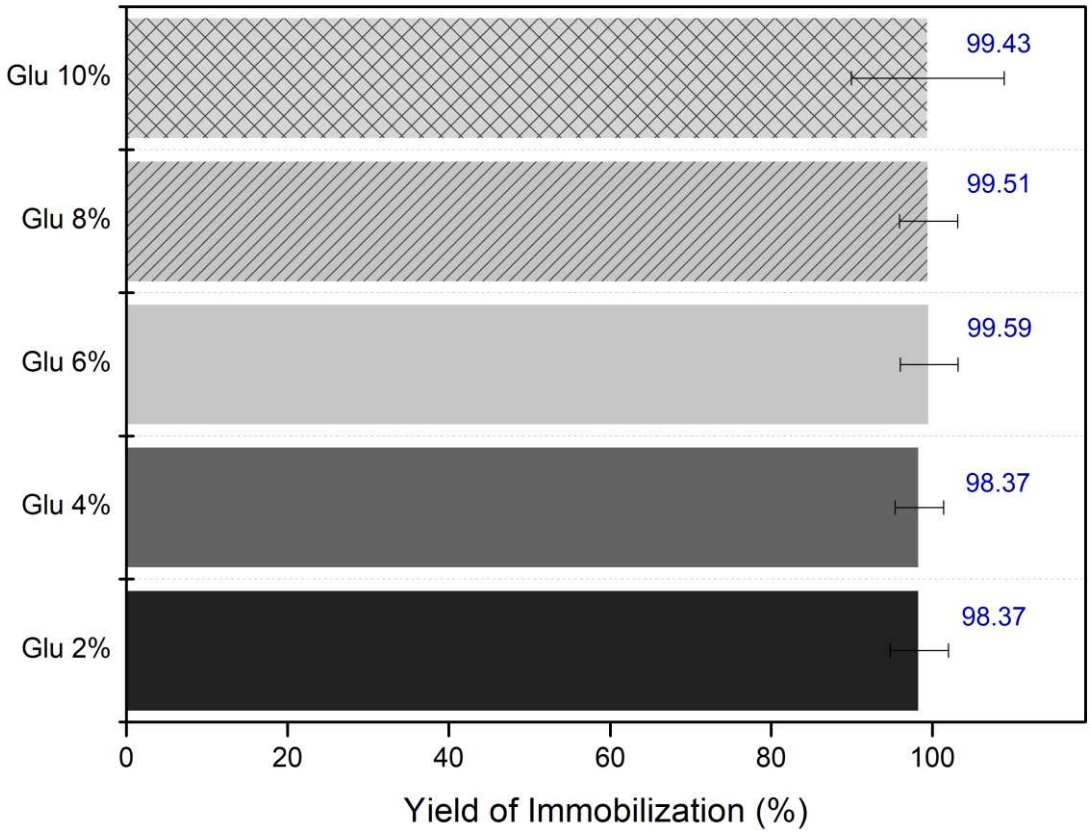
4.2.3 Glutaraldehyde concentration

Glutaraldehyde has been used as one of the main crosslinking agents, being commercially available and cheap (ZHU *et al.*, 2020). The use of this crosslinking reagent is generally combined with covalent immobilization techniques to obtain a biocatalyst with high rates of enzymatic activity, which depend on the degree of crosslinking (EŞ; VIEIRA; AMARAL, 2015). Furthermore, its use in the functionalization of polymeric matrices for immobilization has been widely discussed. To observe the effect of glutaraldehyde concentration on immobilization, laccase was covalently linked to PAN particles functionalized with different concentrations of glutaraldehyde (2, 4, 6, 8, and 10 % v/v). The temperature, pH, and immobilization time were fixed at 25 °C, 4.5 in 0.1 mol/L acetate buffer solution, and 6 h. The influence of glutaraldehyde concentration was then observed from the immobilization yield at the end of 6 h, and, as shown in Figure 21, the measured values were 98.37 ± 3.60, 98.37 ± 3.0; 99.59 ± 3.61; 99.51 ± 3.61, and 99.43 ± 3.47 %, respectively..

Therefore, the immobilization yield was not affected by glutaraldehyde concentration in the investigated range, considering the experimental error. Particularly, it is expected that increasing the concentration of the bifunctional reagent promotes an increase in the amounts of free aldehyde groups on the polymeric surface, which also increases the load capacity of the laccase. On the other hand, the reflex of excessive crosslinking promotes a steric effect where the laccase active sites are blocked by direct contact with the support or with the active site of another laccase, which other authors have previously observed (ABDULLAH *et al.*, 2007;

LONAPPAN *et al.*, 2018; ZHENG *et al.*, 2016). Taheran *et al*. (2017) report glutaraldehyde concentration focused on immobilized laccase activity. According to the authors, the interaction between macromolecules and aldehyde groups on the surface of the functionalized polymer matrix may be responsible for the conformational change of the enzyme and, therefore, for the reduction of enzymatic activity (TAHERAN *et al.*, 2017b). In this sense, the possibility of using concentrations above 6 % for subsequent experiments was discarded.

Figure 21 – Comparison of laccase immobilization on PAN particles with different glutaraldehyde concentrations (2; 4; 6; 8; 10 % v/v)



Temperature and pH set at 25 °C and 4.5 in 0.1 mol/L acetate buffer solution

Source: from author

As discussed in some works previously published in the open literature, the amount of laccase immobilized on polymeric microspheres with different concentrations of glutaraldehyde was determined by the Bradford method. The results showed the same yield profile presented here, that is, the amount of immobilized enzyme increases as the concentration of glutaraldehyde increases from 0.001 to 0.01 % (v/v), when it then starts to decrease until the concentration of 0.03 % (v/v) (MAZLAN; HANIFAH, 2017). The amount of enzyme and the

amount of glutaraldehyde are factors that, when correlated, strongly influence the specific activity of heterogeneous biocatalysts (GASSER *et al.*, 2016).

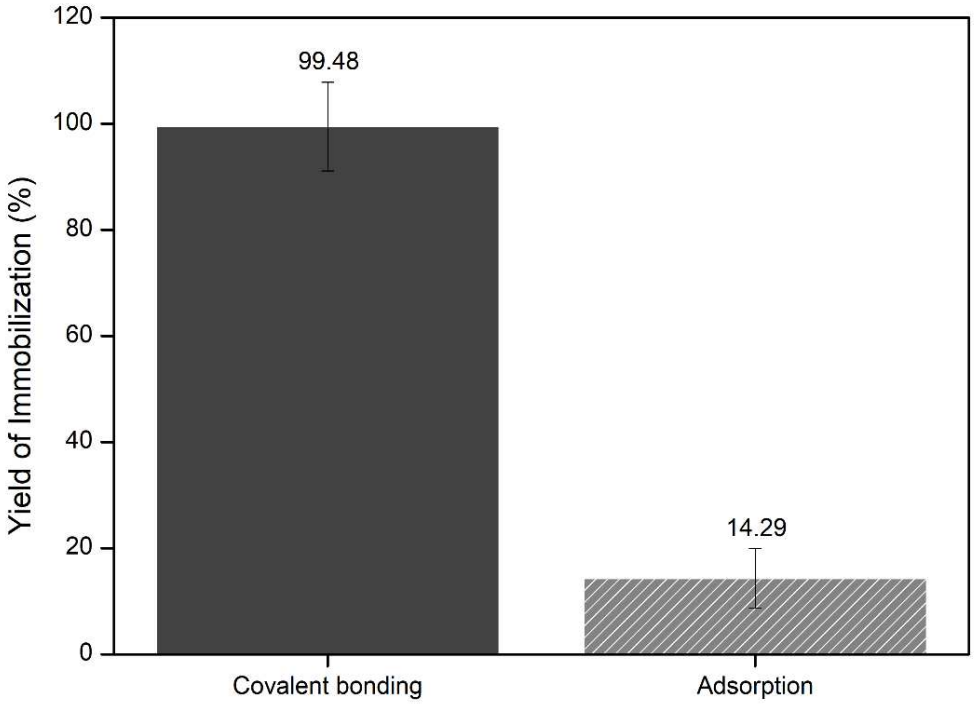
Although glutaraldehyde is widely discussed in the literature as an efficient and inexpensive crosslinking agent, its chemical composition is toxic to the environment, and its handling is dangerous (ADAMIAN *et al.*, 2021; MA *et al.*, 2018). Due to this and because the immobilization yields were very close, the concentration of glutaraldehyde selected as ideal for this work was 2 % (v/v), since this concentration is sufficient for the reagent to form the Schiff base, which will serve as a bridge between the functionalized PAN and the amino group of the protein (MIGNEAULT *et al.*, 2004).

4.3 ENZYME IMMOBILIZATION

The laccase was immobilized on functionalized and non-functionalized polyacrylonitrile particles. To simplify the discussion, we will use the term physical adsorption to refer to the method of immobilization on non-functionalized PAN particles, even if this method consists of weak chemical bonds, such as hydrogen or ionic bonds. This was not proven by the results presented here. The immobilization yield after 6 h was 99.48 ± 8.33 and 14.29 ± 5.62 % by covalent binding and physical adsorption, respectively (Figure 22). The four-step functionalization of the nitrile groups allowed a covalent bond with the amine groups at the most superficial amino acid residues of the enzyme. Despite being a more complex method, the covalent bond between enzyme and support is more stable and, therefore, more difficult to allow enzyme to leach (SIMÓN-HERRERO *et al.*, 2019).

The low yield of immobilization in the physical adsorption can be explained mainly by the reduced surface area of the polymer and the presence of inert chemical groups. In addition, the low pore volume of the particles produced did not allow intraparticle diffusion to sites where the enzyme could be adsorbed onto the support. It is possible to find reports that explain that mesoporous structures with pore sizes slightly larger than the size of the enzymes influence the processes of enzymatic immobilization. There is then a correlation between the laccase immobilization yield and the volume of the mesoporous structure; that is, the greater the volumes of mesopores, the greater the immobilization yields by physical adsorption (RAMÍREZ-MONTOYA *et al.*, 2015). Several works presented in the literature point to adsorption as the most appropriate method; however, the supports used have a large volume of pores and chemical groups available for intermolecular bonds or even fillers for ionic adsorption (ZHOU; ZHANG; CAI, 2021).

Figure 22 – Laccase immobilization on PAN particles by adsorption (non-functionalized PAN) and by covalent bonding (functionalized PAN)



Temperature and pH set at 25 °C and 4.5 in 0.1 mol/L acetate buffer solution. In 0.5 mg/mL of enzyme solution

Source: from author

On the other hand, this particle textural properties do not influence the covalent bond, as seen in the high immobilization yield. The functionalized groups of PAN were duly linked to the amino groups present in the laccase structure and, as shown later, did not generate steric impediment of contact with the substrate. Zhang *et al*. (2018) showed that there are four covalent binding sites available around the active site of the laccase, which allow a strong interaction of the enzyme with the support. It allows a high protein load on the support but does not guarantee the best performance of the supported biocatalyst. Particularly, covalent bonding is a delicate method as it can generate conformational changes in the enzyme. This can occur in a negative way, being responsible for the denaturation of the enzyme (DAS; DWEVEDI; KAYASTHA, 2021; JI; HOU; CHEN, 2016), or in a positive way, improving the catalytic activity and decreasing the diffusional effects in relation to the products or substrate (RAFIEE; REZAEE, 2021).

However, the results presented here are encouraging. The immobilization of laccase on PAN particles by covalent bonding proved to be superior to the same particle without functionalization. This methodological answer follows what is found in the scientific literature

regarding laccase immobilization. Adsorption and covalent bonding were tested for laccase immobilization on Poly(ethylene) Terephthalate Grafted with Maleic Anhydride Nanofiber Mat (PET-g-MAH). By physical adsorption, the authors could immobilize only 26.02 %, while covalent bonding reached 38.94 % immobilization. Furthermore, by introducing glutaraldehyde as a cross-linking agent in the covalent bonding, the immobilization yield increased to 48.37 % (MOHD SYUKRI *et al.*, 2020).

The results presented here make clear the need for functionalization of PAN particles for laccase immobilization. The surface of pure PAN was shown to be little reactive with the groups available on the surface of the laccase, in this sense, the four activation steps presented above allowed the formation of groups available for binding with the enzyme. Due to the better results for the method using functionalized PAN particles for immobilization by covalent bonding, the following experiments were carried out from this point onwards, no longer using the non-functionalized particles to immobilize the laccase.

4.4 EFFECT OF ENZYME CONCENTRATION

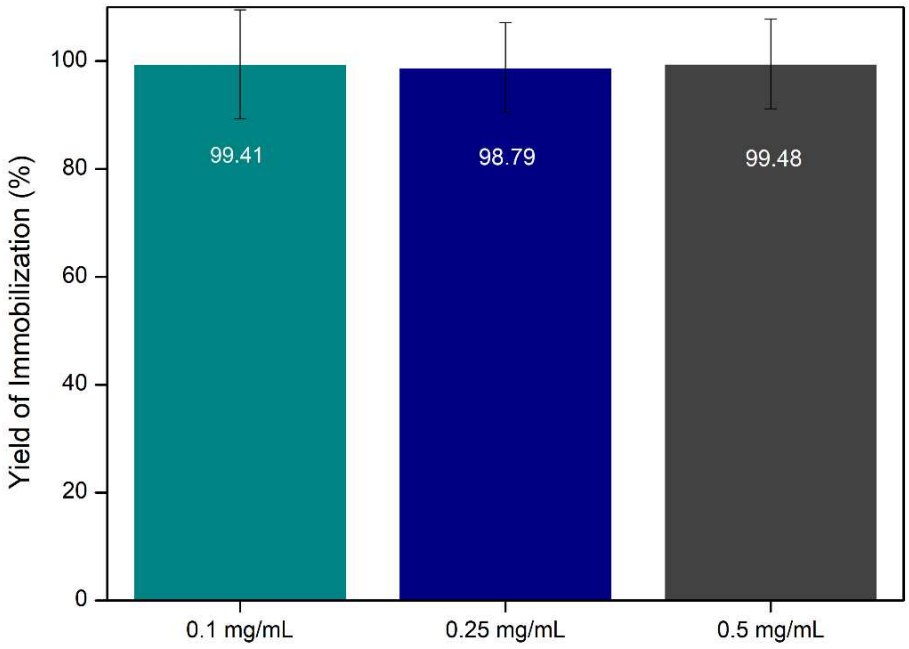
The enzyme concentration is an important parameter in the immobilization yield, since, if any effect provided by the accumulation of enzymes on a support is found, it is possible that the exact conditions of the heterogeneous preparation of the biocatalyst can exert an important influence on the final properties of the immobilized enzyme, e.g., stability (FERNANDEZ-LOPEZ *et al.*, 2017). It is recommended that the support carry as much protein as possible to obtain higher values of recovered activity (CRISTÓVÃO *et al.*, 2011). However, a high protein concentration in the immobilization can reduce the available surface area and the induction of multilayer adsorption. It can block the contact of the enzymes of the lower layer of the support with the substrate, thus decreasing the activity in the supported biocatalyst (GU; XUE; SHI, 2020). This work tested the effect of three different concentrations of laccase (0.1, 0.25, and 0.5 mg/mL) for immobilization by covalent bonding. The reactions took place over 6 h, and the immobilization yield was obtained from the enzymatic activities in the supernatant at the initial and final time. Results suggest that immobilization yield was as maximum as possible: 99.41 ± 10.09, 98.79 ± 8.33 and 99.48 ± 8.33 %, respectively for three tested concentrations (Figure 23), and not affected by increased laccase concentration.

This result shows a significant relationship between immobilization yield and a high laccase concentration. This higher concentration of laccase can facilitate the covering of the entire surface of the support (BAUTISTA; MORALES; SANZ, 2010; LI *et al.*, 2018). It would

be expected that the enzyme overload would generate diffusional effects by blocking the active sites of the laccase, which would culminate in the reduction of the biocatalyst activity (XU *et al.*, 2013). However, for all concentration ranges studied, the immobilization yields were satisfactory.

Similar results were observed by Jankowska *et al*. (2020), who studied the effect of laccase concentration on immobilization in electrospun poly(methyl methacrylate)/polyaniline fibers. The researchers tested different concentrations of laccase (0.5, 1.0, 1.5, 2.0 mg/mL) in immobilization by covalent bonding and adsorption. They found that both the immobilization yield and the recovered activity increased as the laccase concentration increased to a concentration of 1 mg/mL, and then decreased due to the blockage generated by the accumulation of enzyme on the support (JANKOWSKA *et al.*, 2020). Additionally, the laccase was adsorbed onto poly(ester-amino) microspheres at concentrations of 0.5 to 3.0 mg/mL. The amount of laccase obtained at a concentration of 2.0 mg/mL was the one that represented the best results. However, the immobilization yield was in the opposite direction, indicating that all laccase in the 0.5 mg/mL concentration adhered to the support. At the same time, approximately 20% was immobilized at the highest concentration (SUN *et al.*, 2021).

Figure 23 – Laccase immobilization on PAN at different enzyme concentrations (0.1; 0.25 and 0.5 mg/mL)



Temperature and pH set at 25 °C and 4.5 in 0.1 M acetate buffer solution

Source: from author

The values found show intense similarity among themselves in the immobilization yield. With this, it is necessary to raise two discussions here: the first refers to the effectiveness of the method used in the functionalization of polyacrylonitrile, which allowed the loading of relevant amounts of laccase on the support. The second would be the best concentration since the yields did not differ. Although there was no statistical difference in immobilization yields for the three concentrations, the theoretical amount of enzyme bound to the support was higher at the concentration of 0.5 mg/mL. In this sense, this concentration was selected to continue the work as mentioned above because, in comparison with the other concentrations tested, it resulted in a greater amount of immobilized laccase. Furthermore, although the tests did not prove the saturation of the support by the laccase concentration, it will be noticeable during the results presented below that this amount of bound protein can contribute positively to the activity of the final biocatalyst..

4.5 PROPERTIES OF FREE AND IMMOBILIZED LACCASE

4.5.1 Enzyme derivative thermostability test

The thermal stability of the PAN+Lac derivative was evaluated at temperatures of 30, 50, 60, and 70 °C for 8 h. Enzyme activity was evaluated by the rate of ABTS oxidation at each time interval (0.08, 0.25, 0.5, 1, 2, 4, 6, and 8 h). From Figure 24(a), it is possible to identify an approximately 2-fold increase in enzymatic activity at 50 °C, for free and immobilized laccase, concerning their initial activities, which were measured at 25 °C. However, at 60 °C, the laccase immobilized on PAN was more stable than its free counterpart, maintaining its relative activity at 50% up to 6 h of incubation. At 70 °C, it is possible to observe the thermal deactivation profile of both enzymes after 2 h of reaction, shown in Figure 24(b).

There are two main ways in which temperature can affect an enzyme derivative. First, the increase in temperature generates greater molecular activity, and thus the rate of the reaction also increases (ASHKAN *et al.*, 2021). In this study, the relative activity increased when the temperature reached 50 °C, thus increasing the turnover number between the enzymes and ABTS. Generally, enzyme-support, enzyme-enzyme, and enzyme-substrate interactions improve with increasing temperature, directly influencing the optimal temperature of the enzyme and increasing its thermal resistance to denaturation (HÜRMÜZLÜ; OKUR; SARAÇOĞLU, 2021; SILVEIRA *et al.*, 2020).

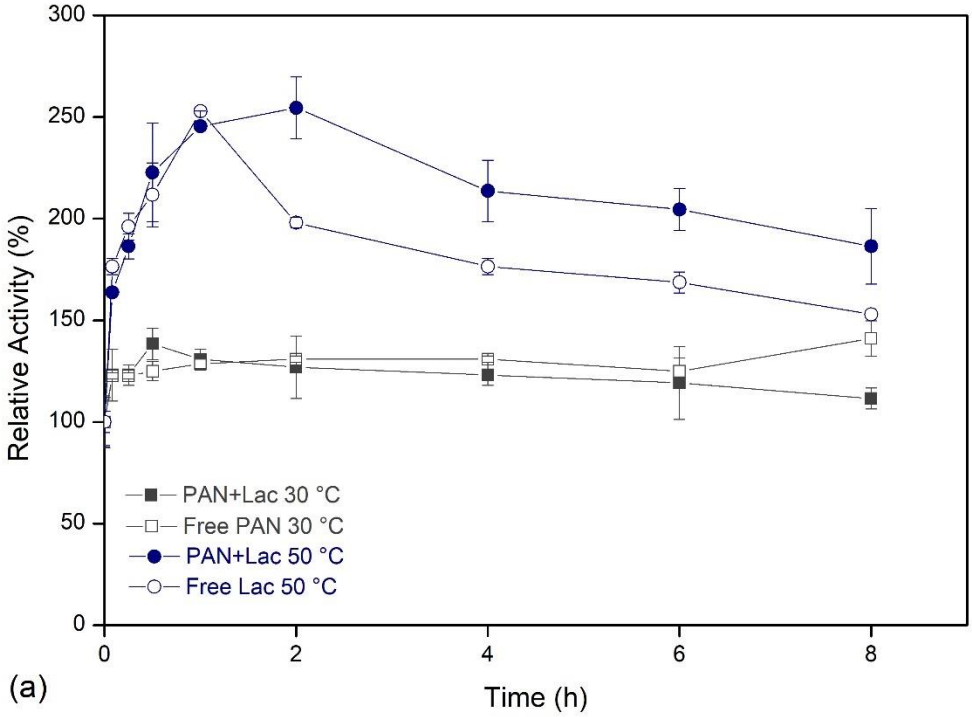
The second form of influence concerns increasing this temperature to levels that exceed the ability fo the enzyme to retain its activity, causing denaturation followed by enzyme inactivation. Therefore, upon reaching the temperature of 60 °C, denaturation of the laccase in its two forms is already noticed, explained by the decrease in the relative activity to approximately 50 and 30 % for the immobilized and free laccase, respectively, after 6 h of incubation. This advantage pointed out by the laccase immobilized on PAN can be given by certain conformational changes in its structure caused by the immobilization, which are not harmful to the active site and can protect the enzyme from direct exposure to the reaction medium with very high temperatures (MOHD SYUKRI *et al.*, 2021).

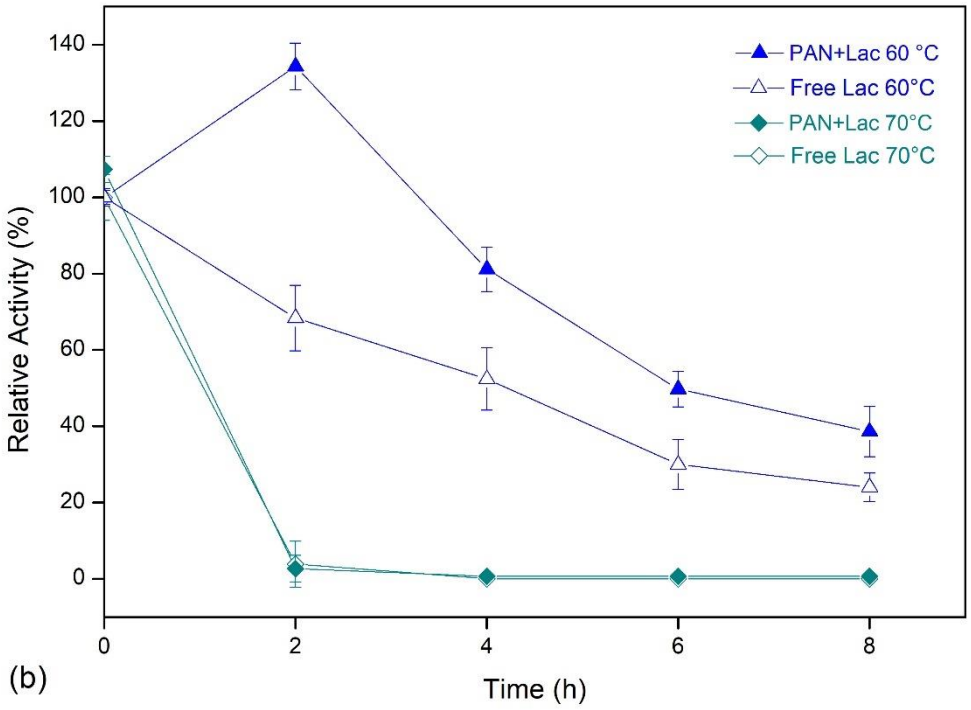
On the other hand, the covalent bond formed between the amino groups of the laccase and the functionalized groups of the support can result in protection against conformational changes caused by high temperature. This provides an increase in activation energy so that the molecule can reorganize themselves into a suitable arrangement for the contact between the active site and the substrate (MAZLAN; HANIFAH, 2017). As already reported in the literature, the covalent bonds between enzyme and support decrease the conformational flexibility of the laccase under the effect of temperature and, thus, increase its thermal stability (SADIGHI; FARAMARZI, 2013).

By comparison with other polymeric matrices used as laccase support, similar and different reports point to the effect of temperature on the enzyme's thermostability. Laccase from *Trametes hirsuta* was covalently bonded to a polyvinylidene fluoride (PVDF) membrane modified with carbon nanotubes. The enzymatic activity of the heterogeneous biocatalyst peaked at 25°C. However, at all temperatures tested, the relative activity reduction was less severe, proving that immobilization made the laccase more thermally stable. As in our study, the increase in temperature beyond 60 °C culminated in the deformation of the enzyme structure (MASJOUDI *et al.*, 2021). On the other hand, laccase from *Trichoderma asperellum* immobilized on modified chitosan showed better results when subjected to a temperature of 80 °C, as well as its free form, but maintained its residual activities higher at all temperatures tested when compared to free laccase (SHANMUGAM *et al.*, 2020).

As pointed out earlier, the PAN+Lac derivative presented the best results when subjected to incubation at 50 °C. Thus, this temperature was used later in the operational stability tests.

Figure 24 – Stability of free and immobilized laccase under several temperature conditions and reaction time of 8 h (a) data at 30 and 50 °C (b) data at 60 and 70 °C



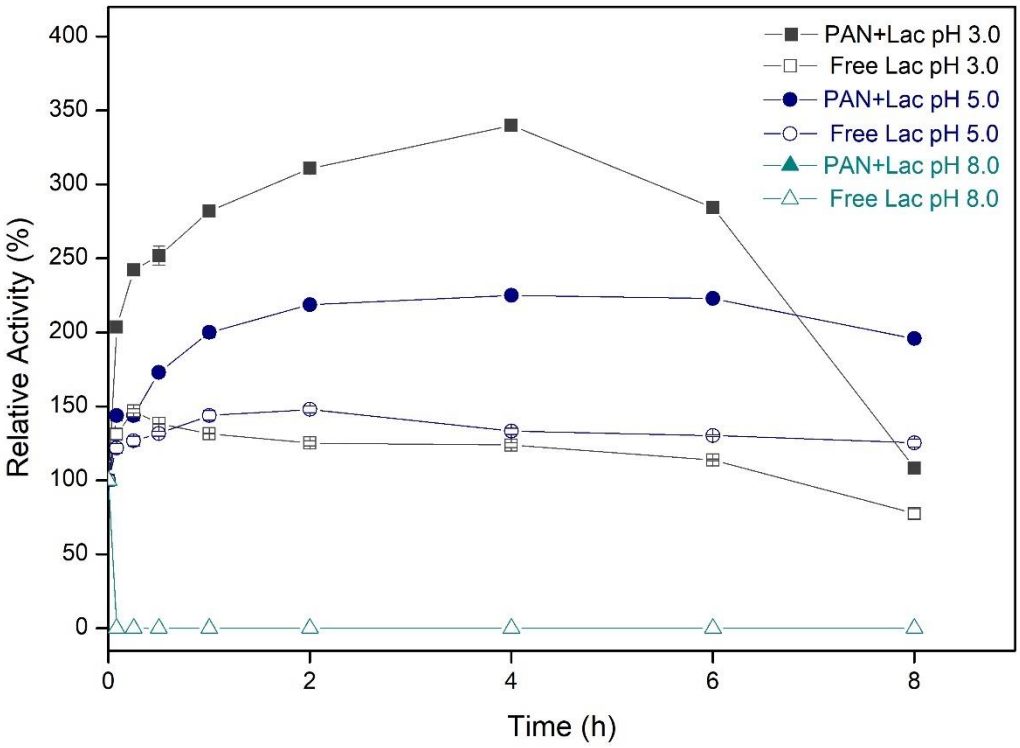


Source: from author

4.5.2 Stability test of immobilized laccase at different pHs

As with temperature, the catalytic activity of the laccase is considerably affected by the pH of the solution. For 8 h, free and immobilized laccase were evaluated for stability at different pH values (3; 5 and 8) at 25 °C. Figure 25 shows the results of the biocatalyst evaluation and the relative activity values as a function of time for each pH studied so that the activity at the initial time was established as 100%. The best results for the laccase immobilized in PAN were obtained at pH 3, where hyperactivation is observed in the first 0.08 h of immersion in buffer solution. Its relative activity increased about two times. After 4 h, this activity reaches its peak, increasing by approximately 4 times. At pH 5, the immobilized enzyme still presents better results than the free enzyme, reaching its maximum point at 2.25 times the value of the initial activity after 6 h of incubation.

Figure 25 – Stability of free and immobilized laccase under conditions of pH 3; 5 and 8, at 25 °C, and reaction time of 8 h



Source: from author

Hyperactivation under favorable pH conditions is reported in the literature. Calcium alginate granules were used to entrap the laccase from *Coriolopsis gallica,* and the results showed that immobilization allowed for a 30% hyperactivation in acid values compared to a free enzyme (DAÂSSI *et al.*, 2014). A profile of laccase activity in different pH ranges is reported in the literature, depending on the nature of the substrate. When the substrate is a non-phenolic compound, such as ABTS, the laccase generally shows maximum activity at pH in the most acidic range, followed by a continuous decrease as the pH tends to basic (CHONG-CERDA *et al.*, 2020).

However, it is not possible to say that immobilization made the laccase more stable in terms of pH variation since the free enzymes also showed stability at pH 3 and 5. However, the results bring an interesting discussion about the activity of free and immobilized enzymes under similar pH conditions, which would be interesting from the point of view of industrial applications for long periods. Free laccase was also stable at both pH 3 and pH 5 until approximately 6 h, with similar results. The activity peak occurred in the first times evaluated for both pH values, with no significant changes until approximately 6 h. After this time, results suggest enzyme activity loss. At pH 8, both free and immobilized enzymes lost their activities after the first 0.08 h of incubation.

Immobilization by covalent bonding can generate certain changes in the three-dimensional structure of the enzyme. In addition, the degree of ionization of the functional groups present in the laccase active site can also change with the change in the pH of the microenvironment. As a direct result, the affinity between the enzyme and the substrate may increase or decrease, as the degree of ionization of the active site may change and new ionic interactions may occur (AKKAYA; OZSEKER, 2019). The secondary interactions developed between the support matrix and the enzyme can be favorable regarding the amplitude in the pH range of the immobilized enzyme (ASGHER; NOREEN; BILAL, 2017).

At pH 3, the improvement in enzyme performance after immobilization in PAN is clear, while for free laccase, the most relevant results occurred at pH 5. Unequal concentrations of H+ and OH– in the reaction medium where the immobilized enzyme is inserted can cause a preferential movement from the optimal pH to more acidic values (HUANG *et al.*, 2017; MARTINO; PIFFERI; SPAGNA, 1996). In this work, it was easy to observe that pH 3 was more favorable for the immobilized enzyme to present the highest activities, while pH 5 favored the free enzyme in the same sense. In line with the results found here, the stability of the laccase immobilized on PAN-biochar nanofibers was tested for different pH values. The results showed that the bonds between the enzyme and the polymeric fibers were responsible for protecting the

enzyme from deactivation by changes in pH. Therefore, the immobilized laccase was less sensitive at lower pH values (TAHERAN *et al.*, 2017b). At alkaline pH, there is a tendency for hydroxyl anions to bind to the active site of the laccase, more specifically in type 2 and 3 coppers, this inhibits the binding of O_2, which is the terminal electron acceptor (GU; XUE; SHI, 2020), the interactions multipoint between laccase and polymer matrix did not prevent the occurrence of this phenomenon in that study. Another very close report was presented by laccase immobilization by entrapment in calcium alginate. The researchers showed that pH 5 was optimal for the stability of the free laccase, and at more acidic pH, the immobilized enzyme remained highly stable. Moreover, when subjected to higher pH values, both the free and immobilized enzyme decreased their activities vertiginously (LASSOUANE *et al.*, 2019).

As noted above, the PAN+Lac derivative presented better results at pH 3. Therefore, this pH range was used later in the operational stability tests, presented below.

4.5.3 Storage stability

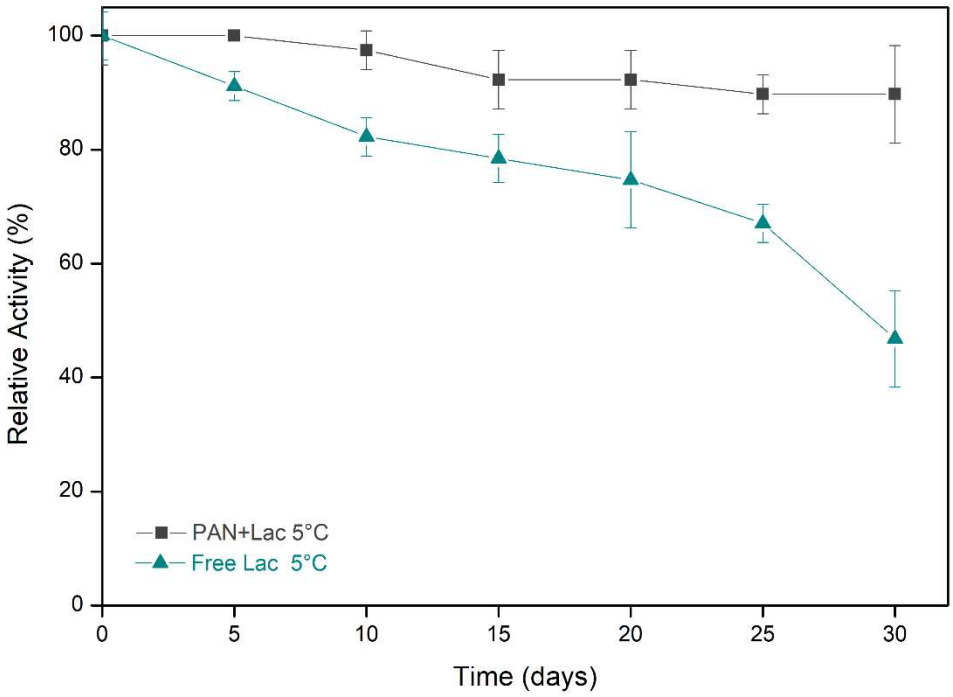
Industrial applications of enzymes depend on their shelf life and retention of enzyme activity (AGGARWAL; CHAKRAVARTY; IKRAM, 2021). Therefore, storage stability becomes a crucial factor to be credited when designing a robust biocatalyst (WANG *et al.*, 2021). Thus, it is expected that the heterogeneous biocatalysts maintain their catalytic activity even over the days of storage (RANIMOL; PAUL; SUNKAR, 2021). In this work, the immobilized and free laccase stability was measured from storage at a temperature of 5 °C for 30 days. Figure 26 shows the performance of the tested two enzymes.

The relative activity of free laccase after 30 days of storage was approximately 46 %, while the immobilized enzyme retained approximately 89 % of its initial activity. These results show better storage stability by the immobilized laccase compared to the result presented by its free counterpart. It is known that enzymes are indeed susceptible to denaturation or inactivation after prolonged storage (RAHMANI *et al.*, 2015). However, an improvement in laccase stability became clear with the immobilization of the laccase on PAN particles.

Results close to this one are discussed in the literature regarding polymeric supports. Free and immobilized laccase in alginate granules were tested for 15 days at 4 °C and pH 5. At the end of this period, the immobilized enzyme maintained 66.19 % of its initial activity, while its free form maintained only 46.18 % under the same conditions (LATIF *et al.*, 2022). When immobilized on Zein-polyvinylpyrrolidone nanofibrous membranes, laccase maintained its relative activity at approximately 64.91 % after 30 days stored in sodium acetate buffer (pH

4.5) at 25 °C, while free laccase maintained 28.64 % (RANIMOL; PAUL; SUNKAR, 2021). The results found in this work, in line with what has been developed in the literature for PAN nanofibers (TAHERAN *et al.*, 2017a), indicating that immobilization strategy does not depend on support morphology, as expected. On other hand, the use of PAN particles is an excellent method to maintain laccase stability over time. Furthermore, unlike nanofibers, PAN particles represent interesting support for industrial applications, due to the ease of recovery.

Figure 26 – Effect of storage time on the activities of immobilized laccase from *Trametes versicolor*



Source: from author

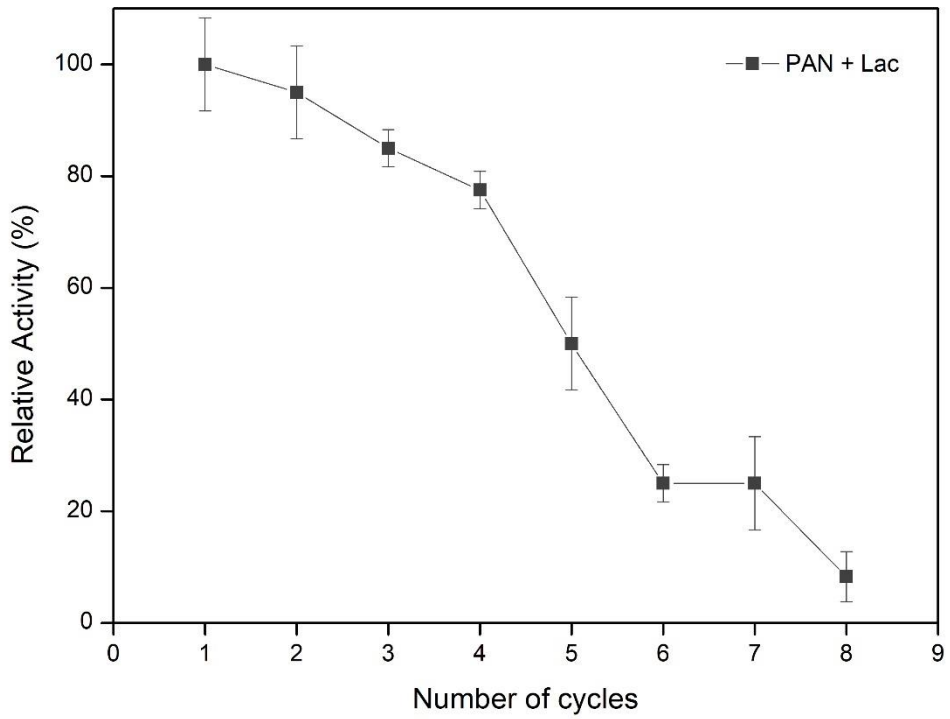
4.5.4 Operational stability of immobilized laccase

It is known that the immobilization of laccases can enable their use on an industrial scale, as it brings greater stability and the possibility of easy recovery and reuses, which contributes efficiently to green remediation approaches (BRUGNARI *et al.*, 2021; SHARMA *et al.*, 2021). Operational stability is the biggest advantage of immobilized enzymes over free enzymes and is decisive for practical applications (ZDARTA *et al.*, 2019). In this sense, the knowledge of the operational stability of the heterogeneous biocatalyst is of fundamental importance in evaluating the strength of the enzyme-support interaction. Reusability of laccase

immobilized on PAN particles was realized at pH 3.0 and 50 °C. Figure 27 shows the catalytic behaviour in ABTS degradation during 8 cycles.

Figure 27 shows that laccase immobilized on PAN by covalent bonding retains its catalytic activity using ABTS as substrate at approximately 50 % of the initial activity after 5 cycles. From the sixth cycle onwards, it is possible to observe a loss of approximately 92 % of the initial activities. This decrease in enzyme activity can occur for several reasons. A possible explanation already reported in the literature is the inactivation of the laccase throughout the process, during the washing operations between one cycle and another (BAYRAMOGLU; KARAGOZ; ARICA, 2018).

Figure 27 – Operational stability of the immobilized laccase in PAN particles in 0.1 M citrate-phosphate (pH 3.0) buffer containing 0.5 mM ABTS



Source: from author

Furthermore, during the experimental procedure, the support pores were filled with the products of the oxidation of ABTS. In this case, a steric hindrance between the laccase bound to the surface of the mesopores and the substrate of the following cycle can occur. This became more evident from the fourth cycle onwards when the relative activity decreased from

approximately 80 to 50 % in the fifth cycle. It has already been reported in the literature that steric hindrance can cause diffusional effects related to the activity of immobilized enzymes, either by the large accumulation of enzymes adsorbed on the surface of the support (XIA *et al.*, 2016; ZDARTA *et al.*, 2021) or by substrate/product mass transfer limitations between the pores of the enzyme support, which prevents the substrate from accessing the enzyme's active site (PANG; LI; ZHANG, 2015). Although the particles produced in this work are classified as mesoporous material, the average diameter is close to what is understood by microporous particles (approximately 20 Å), which explains the results discussed here. A good alternative to overcome the diffusion-controlled enzymatic reaction rates is using strategies to increase the pore size and thus improve the mass transfer of the substrate and its oxidation product (QIU *et al.*, 2009).

The results presented here agree with what has been recently published in the open literature. A polymer engineered structure was used to provide stability to a cross-linked enzymes aggregate (CLEA's) composed of laccase. The results showed that after 5 cycles, the relative activity of CLEA without the polymeric network dropped to 17 %, while the enzyme-polymer derivative maintained the activity at 67 % under the same conditions (ARISTE *et al.*, 2021). Laccase from *Trametes versicolor* immobilized on bentonite-based mesoporous support retained only 36.21 % of the initial activity after 5 cycles of ABTS oxidation (WEN *et al.*, 2019). The same laccase immobilized via covalent bonding in polyimide aerogels lost 78 % of initial activity after 7 cycles (SIMÓN-HERRERO *et al.*, 2019).

CHAPTER 5

In this chapter, the main conclusions drawn from this study are presented, as well as suggestions for future work.

 [5]

[5] Bring the reader closer to the QR code to access the proof of submission of the last co-authored Review Article, in the journal Advances in Colloid and Interface Science.

5. CONCLUSIONS

Laccase from *Trametes versicolor* was successfully immobilized by covalent bonding on polyacrylonitrile particles produced by solution polymerization with precipitation. The particles produced showed N_2 type II adsorption and desorption isotherms, characteristics of mesoporous materials; however, the pore volume was relatively low. Enzymatic immobilization by the covalent bond method on functionalized particles showed a much higher immobilization yield than immobilization on non-funtionalized PAN (99.48 and 14.29 %, respectively). The functionalization of the support in four steps (alkaline hydrolysis, acid hydrolysis, amination, and cross-linking) enabled the immobilization by the covalent bond in a satisfactory way. However, under conditions tested, the concentration of laccase and glutaraldehyde did not influence the immobilization. In general, the PAN+Lac derivative showed good stability at different pHs and temperatures, in addition to retaining approximately 89 % of the initial activity after 30 days of storage. It was also possible to use immobilized laccase, maintaining its initial activity at up to 50 % for 5 cycles. From the acquired data, it is clear that the immobilization yield by covalent bonding was independent of tge e=textural properties of support, as expected. The use of PAN particles proved to be excellent in maintaining the stability of the laccase under different conditions and over time and reuse (operational stability). Furthermore, the potential of this heterogeneous biocatalyst for applications of industrial interest became clear, mainly due to the ease of recovery of millimeter particles in bioreactors.

5.1 SUGGESTIONS FOR FUTURE WORKS

- Produce polyacrylonitrile particles with more interesting textural properties for immobilization by adsorption (with the possibility of copolymerization) and test other polymerization methods;
- Develop a simpler, more economical, and environmentally appropriate PAN functionalization technique;
- Apply laccase immobilized on polyacrylonitrile-based particles in the oxidation of emerging contaminants;
- Develop and test the application of biocatalysts obtained in bioreactors.

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