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**SELEÇÃO DE FUNGOS FILAMENTOSOS PRODUTORES DE LIPASES COM
POTENCIAL DE APLICAÇÃO PARA A PRODUÇÃO DE BODIESEL POR CATÁLISE
ENZIMÁTICA**

PALMAS – TO
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Tese apresentada à Universidade Federal do Tocantins como parte dos requisitos para obtenção do título de Doutor em Biotecnologia e biodiversidade na linha de pesquisa em Bioprospecção e Desenvolvimento de Bioprocessos e Bioprodutos

Orientador: Dr. Emerson Adriano Guarda
Coorientador: Dr. Alex Fernando de Almeida

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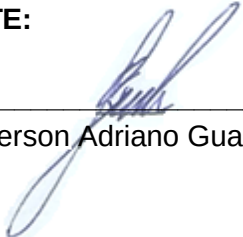
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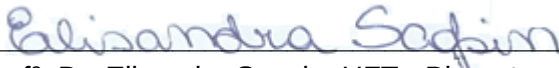
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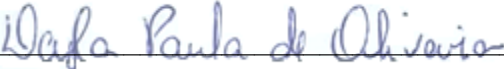


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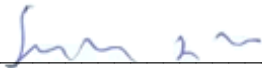
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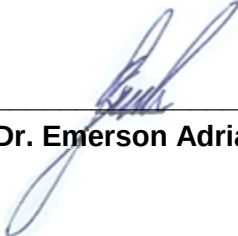


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Prof. Dr. Emerson Adriano Guarda

*Determine que algo pode e deve ser feito, e
então você achará o caminho para fazê-lo.*

Abraham Lincoln

DEDICATÓRIA

Dedico este trabalho (*in memoriam*) a minha querida mãe Telma Bezerra Mendes, minha irmã Neyla Bezerra Mendes, que DEUS esteja as iluminando. Aos meus filhos Mateus Fernandes Bezerra, Ícaro Fernandes Bezerra e minha esposa, amiga e companheira Fabiane Fernandes da Silva. Espero que eles possam desfrutar de tudo que o mundo tem a oferecer de bom, principalmente que não exista mais violência, isso em um futuro bem próximo.

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SUMÁRIO

Lista de tabelas	xi
Lista de gráficos	xii
Lista de figuras	xiii
Lista de abreviaturas	xiv
RESUMO	15
ABSTRACT	16
1. INTRODUÇÃO	17
OBJETIVO GERAL E ESPECIFICOS	18
2. REVISÃO BIBLIOGRÁFICA	19
CAPÍTULO I – PRODUÇÃO DE BIODIESEL E A CATÁLISE ENZIMÁTICA	
1. Introdução	20
2. Biodiesel: Definições e considerações gerais	21
3. Etapas do processo de produção do biodiesel	23
4. Reação de transesterificação	25
5. Fatores que influenciam a reação de transesterificação	27
6. Transesterificação enzimática	28
7. Lípases	29
8. Fontes produtoras de lípases	31
9. Estrutura e mecanismo de atuação das lípases	34
10. Fatores que influenciam a produção de lípases fungicas	36
11. Aplicações biotecnológicas das lípases	37
12. Lípases na produção de biodiesel	41
13. Considerações finais	44
Referências bibliográficas	44
CAPÍTULO II - LIPOLYTIC ENZYMES PRODUCED BY FILAMENTOUS FUNGI ISOLATED FROM DECOMPOSITION LEAVES IN AQUATIC ENVIRONMENT WITH HYDROLYTIC AND ESTERIFICATION ACTIVITIES	
ABSTRACT	49
1. INTRODUCTION	50
2. MATERIAL AND METHODS	51
2.1 Microorganisms	51
2.2 Molecular identification	51
2.3 Screening of filamentous fungi for lipolytic enzyme production	52
2.4 Submerged culture selection	52
2.5 Enzyme assays	52

2.5.1 Determination of hydrolytic activity	52
2. 5.2 Determination of esterification activity	53
2.6 Enzyme production under submerged conditions	53
2.6.1 Culture media composition	53
2. 6.2 Effect of nitrogen and carbon sources on hydrolytic enzymes production	54
2. 6.3 Time-course of cultivation	54
3. RESULTS AND DISCUSSION	54
3.1 Screening of filamentous fungi for lipolytic enzyme producing	54
3.2 Molecular identification of fungal strains	57
3.3 Medium composition for lipase production	58
3.4 Nitrogen sources on lipase production	60
3.5 Carbon sources on lipase production	61
3.6 Time-course of lipase production	63
4. CONCLUDING REMARKS	64
REFERENCES	65

CAPÍTULO III – OPTIMIZATION IN THE LIPASE PRODUCTION BY FUSARIUM SOLANI

ABSTRACT	70
1. INTRODUCTION	71
2. MATERIAL AND METHODS	72
2.1 Fungal strain used	72
2.2 Culture medium	72
2.3 Inoculum standardization and the obtaining of the crude enzymatic extract by submerged fermentation	72
2.4 Experimental design	73
2.4.1 Plackett and Burman (PB) 7 variables	73
2.4.2 Central Rotational Compound Design (DCCR) 3 variables	73
2.5 Validation of the experiment	74
2.6 Biochemical characterization	74
2.6.1 Determination of optimum temperature and pH	74
2.6.2 Determination of pH stability	74
2.6.3 Determination of thermal stability	75
2.7 Determination of activity and stability in solvents	75
2.8 Determination of pH	75
2.9 Determination of hydrolytic activity	75
2.10 Determination of the production of fungal biomass	75
3. RESULTS AND DISCUSSION	76
3.1 Delineamento experimental Plackett e Burman (PB) 7 variáveis	76
3.2 Central Rotational Compound Design (DCCR) 3 variables	79
3.3 Validation of the experiment	85

3.4 Biochemical characterization	86
3.4.1 Determination of stability in pH	90
3.4.2 Determination of termal stability	91
3.4.3 Determination of activity and stability in solvents	92
4. CONCLUSION	93
REFERENCES	94
CONCLUSÃO GERAL	95
PERSPECTIVAS DE TRABALHOS FUTUROS	95
ANEXOS	80

LISTA DE TABELAS

Capítulo I:

TABELA 1 – Micro-organismos produtores de lipases relatados da literatura	32
TABELA 2 – Fontes e origens de lipases comerciais	39
TABELA 3 – Aplicação biotecnológica de lipases	41
TABELA 4 – Principais vantagens e desvantagens dos processos químicos e enzimáticos para a produção de biodiesel	43

Capítulo II:

TABLE 1. Screening of filamentous fungi for lipolytic enzymes production with hydrolytic and esterification activities	55 e 56
TABLE 2. Molecular identification of filamentous fungi producing lipase	58
TABLE 3. Media composition for lipolytic enzyme production with hydrolytic and esterification activities for <i>Trichodrema sp</i> , <i>Penicilium sp</i> , <i>F. solani</i> and <i>T. harzianum</i>	59
TABLE 4. Source of nitrogen for lipolytic enzyme production with hydrolytic and esterification activities for <i>F. solani</i> F61	61
TABLE 5. Carbon Source for lipolytic enzyme production with hydrolytic and esterification activities for <i>F. solani</i> F61	62 e 63

Capítulo III:

TABLE 1. Range of values studied in experimental planning PB 7, optimization in the production of lipases	76
TABLE 2. First experimental design PB 7, optimization in the production of lipases	77
TABLE 3. Main effects of the variables in PB 7 for optimization in the production of lipases	78
TABLE 4. Range of values studied in the experimental design DCCR 3, optimization in the production of lipases	80
TABLE 5. Experimental planning DCCR 3, optimization in the production of lipases	80
TABLE 6. Regression coefficients for the second experimental design DCCR 3, optimization in the production of lipases	82
TABLE 7. ANOVA for the second experimental design DCCR 3, optimization in the production of lipases	82
TABLE 8. Parameters established for validation	85
TABLE 9. Target for optimization of the enzymatic activity response	85
TABLE 10. Range of DCCR experimental planning, temperature and pH	86
TABLE 11. DCCR experimental design, temperature and pH	87
TABLE 12. Regression coefficients, DCCR experimental design, temperature and pH	88
TABLE 13. ANOVA DCCR experimental design, temperature and pH	88
TABLE 14 Effect of organic solvents on free lipase of <i>Fusarium solani</i>	92

LISTA DE GRÁFICOS

Capítulo III:

Graph 1. Main effects of the significant variables in PB 7	79
Graph 2. Main effects of variables and significant interactions in the second DCCR3	83
Graph 3. Response of experimental validation in lipases production by <i>Fusarium solani</i>	86
Graph 4. Main effects of variables and significant interactions on DCCR, temperature and pH	89
Graphic 5. Effect of pH on the stability of <i>Fusarium solani</i> lipase in the crude extract.	90
Graphic. 6. Thermostability of <i>Fusarium solani</i> in the crude extract	91

LISTA DE FIGURAS

Capítulo I:

Figura 1 – Etapas do processo de produção de biodiesel	24
Figura 2 – Transesterificação de triacilgliceróis (triglicerídeos)	26
Figura 3 – Reações químicas na transesterificação de triglicerídeos	27
Figura 4 – Processo enzimático da produção de biodiesel	29
Figura 5 – Reações catalisadas por lipase do tipo não específica e 1, 3 específica	30
Figura 6 – Mecanismo de catalise enzimática por lipases	35
Figura 7 – Diferentes reações cat. por lipases em sol. aquosas e não aquosas	39

Capítulo II:

Figure 1. Growth curve for lipolytic enzyme production with hydrolytic and esterification activities for <i>Fusarium solani</i> F61	64
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Capítulo III:

Figure 1 Morphology of <i>Fusarium solani</i> (zoomed 40 times)	72
Figure 2 Response surface for enzymatic activity (Tryptone and Calcium Chloride)	84
Figure 3 Response surface for enzymatic activity (Tryptone and magnesium sulfate)	84
Figure 4 Response surface for enzymatic activity (Calcium chloride and magnesium sulfate)	84
Figure 5 Response surface for the DCCR, temperature and pH	90

LISTA DE ABREVIATURAS

Capítulo I:

ANP – Agência Nacional do Petróleo, Gás Natural e Biocombustíveis

B-100 – Biodiesel

GEEs – Gases de efeito estufa

CO₂ – Gás Carbônico

SO₂ – Dióxido de enxofre

NaOH – Hidróxido de Sódio

KOH – Hidróxido de Potássio

OFAT – *One factor at time*

DOE – *Design of experiments*

SSF – *Fermentation in solid state*

Capítulo II:

UV – *Ultraviolet*

UFT – *Federal University of Tocantins*

PDA – *Potato dextrose agar*

DNA – *Deoxyribonucleic acid*

PCR – *Polymerase chain reaction*

UFMG – *Federal University of Minas Gerais*

LCPM – *Laboratory of Cellular and Molecular Parasitology*

pNPP – *p-nitrophenyl-palmitate*

Capítulo III:

DCCR – *Rotational compound central delineation*

NaCl – *Sodium chloride*

LAPEQ – *Research Laboratory in Environmental Chemistry and Biofuels*

DMSO – *Dimethylsulfoxide*

PB7 – *Plackett-Burman*

RESUMO

As lipases microbianas são biocatalisadores muito proeminentes devido a sua capacidade de catalisar uma ampla variedade de reações em meios aquosos e não aquosos. Neste trabalho, os fungos filamentosos selecionados foram isolados de folhas decompostas no rio Buritizal, localizado em Taquaruçu no Estado do Tocantins e identificados com o gene ITS, foram rastreados para produção de lipase com atividade hidrolítica e esterificação. *Fusarium solani* apresentou a maior produção de lipase, com 2,37 U/mL e atividade de esterificação de 0,07 U/mL utilizando meio 3 contendo: KH_2PO_4 1,00 g.L⁻¹, MgSO_4 H_2O 1,123 g.L⁻¹, CuSO_4 0,06 g.L⁻¹. A suplementação deste meio de cultura com fontes de nitrogênio orgânico aumentou a produção de lipase em (461,29%) 3,48 U/mL usando triptona e (419,35%) 3,22 U/mL usando extrato de levedura, todos os resultados foram analisados sempre tendo como referencia a atividade inicial do fungo 0.62 U/mL. Entre os óleos vegetais avaliados no trabalho, o óleo de algodão degomado induziu a produção de lipase em (1212,90%), 8,14 U/mL. Um planejamento fatorial Plackett-Burman com 15 experimentos foi conduzido para avaliar a influência de sete fatores na produção de lipases por *F. solani*. Os fatores investigados foram: peptona, triptona, extrato de levedura, cloreto de cálcio, fosfato de potássio, sulfato de magnésio e sulfato de cobre, que foram mantidos em cinco variáveis fixas contendo: óleo de algodão, pH, temperatura, agitação e tempo e como resposta à atividade enzimática. A concentração de triptona, cloreto de cálcio e sulfato de magnésio tiveram um efeito significativo ($p < 0,10$) na produção de lipase e foram estudados consecutivamente através de um DCCR (delineamento central composto rotacional) completo, com o intuito de otimizar a produção das lipases do fungo *F. solani*. Após a otimização utilizando o DCCR, obtiveram-se atividades lipolíticas máximas de 24,84 U/mL com o uso de 10 g.L⁻¹ de triptona, 3,50 g.L⁻¹ de cloreto de cálcio e 0,50 g.L⁻¹ sulfato de magnésio, 1 g.L⁻¹ de fosfato de potássio e 1% de óleo de soja. O modelo estatístico mostrou uma correlação de 85,67% com os dados experimentais. A caracterização bioquímica da lipase mostrou que a enzima tem melhor atuação em pH 7 a uma temperatura de 40°C, onde o modelo estatístico mostrou uma correlação de 94,15% com os dados experimentais. Desta forma as lipases produzidas por *F. solani* tem potencial para aplicação e uso na produção de biodiesel, podendo ser uma alternativa aos processos convencionais na produção de biocombustíveis.

Palavras chave: Lipase, otimização, hidrólise, esterificação, *Fusarium solani*

ABSTRACT

Microbial lipases are very prominent biocatalysts due to their ability to catalyze a wide variety of reactions in aqueous and non-aqueous media. In this work, the selected filamentous fungi were isolated from leaves decomposed in the Buritizal River, located in Taquaruçu in the State of Tocantins and identified with the ITS gene, were screened for lipase production with hydrolytic activity and esterification. *Fusarium solani* showed the highest production of lipase, with 2.37 U/mL and esterification activity of 0.07 U/mL using medium 3 containing: KH₂PO₄ 1.00 gL⁻¹, MgSO₄ H₂O 1.123 gL⁻¹, CuSO₄ 0.6 gL⁻¹. Supplementation of this culture medium with organic nitrogen sources increased lipase production in (461.29%) 3.48 U/mL using tryptone and (419.35%) 3.22 U/mL using yeast extract, all the results were always analyzed with reference to the initial activity of the fungus 0.62 U/mL. Among the vegetable oils evaluated in the work, degummed cotton oil induced lipase production in (1212.90%), 8.14 U/mL. A Plackett-Burman factorial design with 15 experiments was conducted to evaluate the influence of seven factors on the production of lipases by *F. solani*. The factors investigated were: peptone, tryptone, yeast extract, calcium chloride, potassium phosphate, magnesium sulphate and copper sulphate, which were kept in five fixed variables containing: cotton oil, pH, temperature, agitation and time and as a response to enzymatic activity. The concentration of tryptone, calcium chloride and magnesium sulphate had a significant effect ($p < 0.10$) on lipase production and were studied consecutively through a complete DCCR (rotational composite) design, in order to optimize production of the fungus *F. solani* lipases. After optimization using DCCR, maximal lipolytic activities of 24.84 U/mL were obtained with the use of 10 gL⁻¹ tryptone, 3.50 gL⁻¹ calcium chloride and 0.50 gL⁻¹ magnesium, 1 gL⁻¹ potassium phosphate and 1% soybean oil. The statistical model showed a correlation of 85.67% with the experimental data. The biochemical characterization of lipase showed that the enzyme has a better performance at pH 7 at a temperature of 40 ° C, where the statistical model showed a correlation of 94.15% with the experimental data. In this way the lipases produced by *F. solani* have potential for application and use in the production of biodiesel, and can be an alternative to the conventional processes in the production of biofuels.

Keywords: Lipase, optimization, hydrolysis, esterification, *Fusarium solani*

1 - INTRODUÇÃO

Devido aos graves problemas ambientais que acontecem em decorrência da emissão dos gases de efeito estufa (GEEs), causados em grande parte pela utilização do petróleo e seus derivados, muitos países resolveram voltar-se para a utilização de fontes alternativas de energias limpas e renováveis em substituição aos combustíveis fósseis (FREIRE et al., 2012). Neste sentido, muitas pesquisas vêm sendo desenvolvidas com o intuito de desenvolver derivados de óleos vegetais com propriedades e desempenho próximo ou igual ao do diesel de hidrocarbonetos.

Desta forma, o biodiesel aparece como uma fonte bastante promissora, uma vez que sua utilização contribui diretamente para a diminuição dos poluentes na atmosfera, pois o CO₂ (gás carbônico) liberado durante a sua combustão é absorvido no crescimento das plantas.

O biodiesel é uma mistura de ésteres alquílicos de ácidos graxos saturados e insaturados de cadeias longas (ALBUQUERQUE et al., 2010). Essa mistura é oriunda de uma reação denominada de transesterificação onde, os óleos vegetais e ou gorduras animais são catalisados na presença de uma base, com a adição de um álcool de cadeia curta, normalmente o metanol, produzindo ésteres alquílicos e glicerol.

Para a produção do biodiesel, uma grande variedade de matérias primas pode ser utilizadas como óleos vegetais de soja, girassol, canola, algodão, mamona, pinhão manso, entre outros, gorduras de origem animal como sebos, ou ainda os óleos usados em frituras “soja”.

A reação de transesterificação pode fazer uso de vários catalisadores tais como alcalinos, ácidos, heterogêneos e biocatalisadores mais especificamente enzimas (as lípases) ou também pode fazer uso de alcoóis nos seus estados supercríticos. Dos métodos citados o mais comum ou industrialmente utilizado é o alcalino, um processo com um custo efetivo e bastante eficiente. No entanto, esse processo possui alguns inconvenientes como a separação do catalisador e o álcool colocado em excesso que não participa da reação, o que faz a necessidade de realizar repetidas lavagens para alcançar a pureza adequada ao produto.

A produção de biodiesel usando um biocatalisador supera e muito as vantagens dos outros catalisadores, pois atinge um alto grau de pureza, inexistência de rejeito aquoso alcalino, baixa presença de outros contaminantes, maior seletividade com excelentes rendimentos (BAJAJ, et al., 2010), portanto os processos enzimáticos são promissores como alternativa à rota química. Diante destas vantagens, pesquisas vêm sendo realizadas para selecionar, otimizar e diminuir o elevado custo da produção de enzimas puras.

As lipases microbianas são biocatalisadores muito proeminentes devido à sua capacidade de catalisar uma grande variedade de reações em meios aquosos e não aquosos. O comportamento químico, regio e enantio-específico dessas enzimas tem causado enorme interesse entre cientistas e indústrias (HASAN *et al.*, 2009).

As lipases são as principais enzimas usadas como biocatalisadores na reação de transesterificação enzimática. Podendo as mesmas e seus meios de culturas serem selecionados e otimizados para melhorar o desempenho e uso de tais enzimas.

A presente tese de doutorado foi desenvolvida principalmente no Laboratório de Microbiologia Ambiental e Biotecnologia da Universidade Federal do Tocantins, (LAMBIO UFT) e no Laboratório de Pesquisas em Química Ambiental e Biocombustíveis (LAPEQ UFT).

O trabalho será apresentado na forma de artigos científicos de acordo com as normas estabelecidas por cada revista ao qual o artigo será submetido. O capítulo I é uma revisão bibliográfica abordando os principais pontos do tema proposto. Nos capítulos II e III são apresentados os resultados, na forma como foram submetidos à publicação nos periódicos. Por fim, as considerações finais, com as principais conclusões obtidas e as perspectivas para trabalhos futuros.

OBJETIVO GERAL

Este trabalho teve como objetivo geral, realizar uma triagem e a seleção de fungos filamentosos produtores de lipases capazes de catalisar reações de transesterificação para a produção de biodiesel.

Os objetivos específicos foram:

- Isolar as linhagens de fungos produtores de lipases e caracterizá-las quanto aos aspectos morfofisiológicos;
- Realizar a identificação das linhagens por meio do gene ITS;
- Selecionar linhagens fúngicas produtoras de lipases com atividade lipolítica;
- Analisar quantitativamente a atividade lipolítica dos fungos isolados, em diferentes condições de cultivo
- Aplicar delineamentos experimentais no processo de otimização da atividade Enzimática;
- Otimizar a produção de lipases fúngicas;
- Caracterizar bioquimicamente a enzima obtida no processo de otimização.

2 - REVISAO BIBLIOGRÁFICA

CAPÍTULO I

CAPÍTULO PUBLICADO NO LIVRO: TOPICOS ESPECIAIS EM BIOTECNOLOGIA E BIODIVERSIDADE EM DEZEMBRO DE 2017 PELA EDITORA CVR.

Processo de produção de biodiesel e a catálise enzimática

Danylo Bezerra Mendes

Danyel Bezerra Mendes

Fabiane Fernandes da Silva

Emerson Adriano Guarda

Neste capítulo apresentaremos informações que permitirão ao leitor conhecer as características do processo de produção do biodiesel e diversos fatores que influenciam a reação, bem como conhecer as principais características da catalise enzimática.

1. Introdução

Os graves problemas ambientais surgidos em decorrência da emissão dos gases de efeito estufa devido a utilização de petróleo e seus derivados têm encorajado muitos países investir em pesquisas para a utilização de fontes renováveis de energias limpas em substituição aos combustíveis fósseis (Freire et al., 2012). Os óleos vegetais são uma boa opção como fonte de biocombustíveis, pois são recursos renováveis e tem grande produção anual, porém, para o seu uso nesta finalidade é necessário um processo de modificação química, tornando-o compatível com os motores atuais movidos a diesel (Suarez et al., 2009). Este novo produto é denominado biodiesel e possui muitas propriedades e desempenho próximo ou igual ao diesel fóssil, além de reduzir a emissão de poluentes na atmosfera, pois o CO₂ liberado durante a sua combustão pode ser absorvido durante o crescimento das plantas (Santos 2012).

O biodiesel é uma mistura de ésteres alquílicos de ácidos graxos saturados e insaturados de cadeias longas (Albuquerque et al. 2010). Essa mistura, é oriunda de uma reação denominada de transesterificação, onde os óleos vegetais e ou gorduras animais, em meio básico, com a adição de um álcool de cadeia curta, normalmente o metanol, produz ésteres alquílicos e glicerol.

Para a produção do biodiesel, uma grande variedade de matérias primas pode ser utilizada: óleos vegetais como: soja, girassol, canola, algodão, mamona, pinhão manso, entre outros, gorduras de origem animal como sebos, ou ainda os óleos usados em frituras (óleos de reuso).

A reação de transesterificação deve ser catalisada e pode fazer uso de vários tipos de catalisadores, como alcalinos, ácidos, heterogêneos e biocatalisadores, mais especificamente enzimas (as lipases) ou também pode fazer uso de alcoóis nos seus estados supercríticos. Dos métodos citados o mais utilizado industrialmente é o alcalino, que em geral, resulta em tempos de reação mais curtos com maior produção de rendimento (Amini et al., 2016). No entanto possui alguns inconvenientes, como a separação do catalisador e o álcool colocado em excesso que não participa da reação, o que torna necessária a necessidade de repetidas lavagens para alcançar a pureza adequada ao produto.

O uso de um biocatalisador na produção de biodiesel supera em muito as vantagens dos outros catalisadores, pois atinge um alto grau de pureza, inexistência de rejeito aquoso alcalino, baixa presença de outros contaminantes, maior seletividade com excelentes rendimentos (Bajaj et al., 2010), portanto os processos enzimáticos são promissores como alternativa à rota química. Diante destas vantagens, pesquisas vêm sendo realizadas para selecionar, otimizar e diminuir o elevado custo da produção de enzimas puras.

As lipases microbianas são biocatalisadores muito proeminentes devido à sua capacidade de atuar em uma grande variedade de reações em meios aquosos e não aquosos. O comportamento quimio, regio e enantio-específico dessas enzimas tem causado enorme interesse entre cientistas e indústrias (Hasan et al., 2009). As lipases são as principais enzimas usadas como biocatalisadores na reação de transesterificação enzimática e desta forma este trabalho tem como objetivo apresentar os principais aspectos relacionados ao processo de produção de biodiesel, bem como as principais características da catálise enzimática.

2. Biodiesel: definições e considerações gerais

No dia 13 de janeiro de 2005, a ANP – Agência Nacional de Petróleo, Gás Natural e Biocombustível, pela Lei nº 11.097, definiu biocombustível como sendo “Combustível derivado da biomassa renovável para uso em motores a combustão interna ou, conforme regulamento para outro tipo de geração de energia, que possa substituir parcial ou totalmente combustível de origem fóssil”. Na mesma lei definiu-se biodiesel como: “Biocombustível derivado da biomassa renovável para uso em motores a combustão interna ou, conforme regulamento para outro tipo de geração de energia, que possa substituir parcial ou totalmente combustível de origem fóssil”.

A última resolução aprovada pela ANP de nº 07 de 13 de março de 2008 define biodiesel – B100 – como um combustível composto de alquil ésteres de ácidos graxos de cadeia longa, derivados de óleos vegetais ou de gorduras animais conforme a especificação contida no Regulamento Técnico nº 1/2008, que é parte integrante da referida Resolução.

De acordo com a definição de Parente (2003), biodiesel é um combustível renovável, biodegradável e ambientalmente correto, sendo, portanto, um promissor substituto ao óleo diesel mineral, constituído de uma mistura de ésteres metílicos ou etílicos de ácidos graxos, obtidos da reação de transesterificação de qualquer triglicerídeo com um álcool de cadeia curta, metanol ou etanol, respectivamente.

Segundo (Candeia 2008), biodiesel pode ser definido quimicamente como sendo um combustível alternativo constituído por ésteres alquílicos de ácidos carboxílicos de cadeia longa, oriundos de fontes renováveis como óleos vegetais, gorduras animal e ou residual, cuja utilização está associada à substituição de combustíveis fósseis em motores de ignição por compressão.

Diante da demanda mundial por combustível como fontes de energia, surgiu a necessidade de gerar alternativas como os biocombustíveis (Amini et al., 2016). Desta forma, surge a crescente preocupação com o meio ambiente despertando a busca por fontes alternativas de energia no Brasil e no mundo. Várias pesquisas têm se concentrado no desenvolvimento de novos insumos básicos, buscando um caráter renovável para a produção de combustíveis que possam vir a substituir os derivados de petróleo. Desta forma, a biomassa encontra-se em um papel de destaque em razão da sua ampla disponibilidade, biodegradabilidade e baixo custo (Suarez et al., 2009).

O uso do biodiesel como combustível tem um importante papel nas políticas governamentais, não só na área social e ambiental, como na econômica, tendo em vista as vantagens que este combustível apresenta na economia do país (Santos et al., 2008). Dessa forma, o uso de biodiesel não é apenas uma alternativa econômica e segura diante dos problemas relacionados ao petróleo, muitas são as vantagens que podem ser destacadas no uso desse biocombustível. Entretanto, vale ressaltar que existem também algumas desvantagens. A literatura disponibiliza por meio de autores como: Parente (2003), Candeia (2008), Gomes (2009) e Aranda (2010), relatam alguns aspectos do biodiesel:

a) Vantagens:

- Ausência de enxofre e compostos aromáticos, o que proporciona uma combustão limpa e sem a formação de SO_2 (dióxido de enxofre), gás que

provoca a chuva ácida e de compostos cancerígenos (hidrocarbonetos policíclicos aromáticos);

- Tem número de cetano elevado (superior a 50) e consequentemente alto poder de auto-ignição e combustão. Este fator é refletido de modo especial na partida a frio, no ruído do motor e no gradiente de pressão nos motores a diesel;
- Possui teor médio de oxigênio em torno de 11% e composição química homogênea, favorecendo uma combustão mais completa e eficiente, além de expelir menos resíduos para a atmosfera;
- Possui maior ponto de fulgor quando comparado ao diesel convencional, de modo que, em condições normais de transporte, manuseio e armazenamento não é inflamável, proporcionando uma maior segurança;
- Apresenta expressiva melhora na lubrificação do motor, conferindo maior longevidade do mesmo e seus entornos;
- Podem ser produzidos a partir de matérias-primas renováveis;
- É seguro, renovável, não tóxico e biodegradável;
- Possui vantagens sociais e econômicas.

b) Desvantagens:

- O biodiesel possui um menor poder calorífico. Todavia, esta desvantagem é bastante pequena, em torno de 5% em relação ao diesel convencional;
- Cristalização em baixas temperaturas em regiões de clima muito frio, a viscosidade do biodiesel aumenta bastante. Assim como o diesel, podem ocorrer formações de pequenos cristais, que se unem e impedem o bom funcionamento do motor. Porém, existem diversas precauções que podem ser tomadas para contornar este problema, como por exemplo, o uso de aditivo ou de mistura biodiesel/diesel mineral, dentre outros.

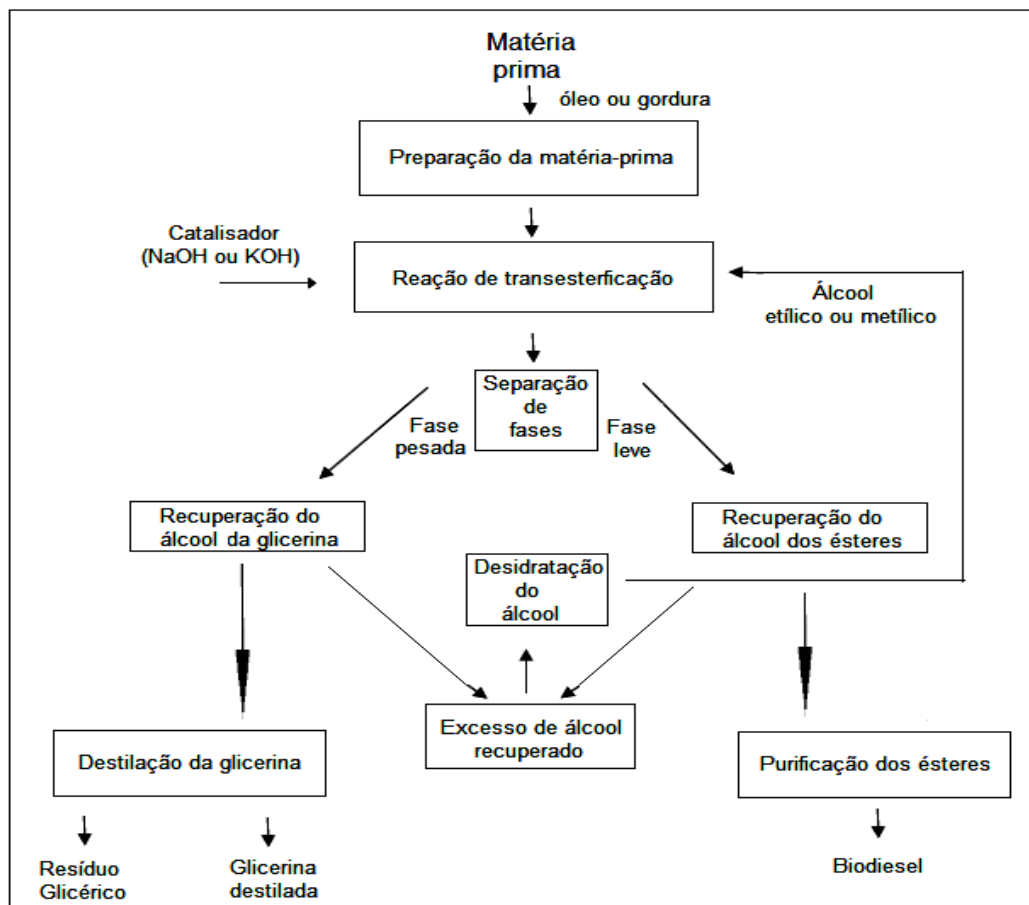
3. Etapas do processo de produção do biodiesel

De acordo com Parente (2003), o processo de produção de biodiesel é constituído de algumas etapas como a preparação da matéria-prima, reação de transesterificação, separação de fases, recuperação do álcool da glicerina,

recuperação do álcool dos ésteres, desidratação do álcool, purificação dos ésteres e destilação da glicerina.

A Figura 1 mostra o processo de produção de biodiesel, partindo de uma matéria-prima graxa qualquer, onde as etapas operacionais são descritas.

Figura 1. Etapas do processo de produção de biodiesel.



Fonte: Adaptado de Parente (2003).

Parente (2003) descreve de forma sumária as etapas do processo de produção de biodiesel:

- Preparação da matéria prima – Essa etapa consiste em preparar a matéria graxa para uma maior conversão em ésteres por meio da transesterificação. Para essa transformação ser alcançada, a matéria-prima deve ter essencialmente o mínimo de umidade e sua acidez a menor possível;

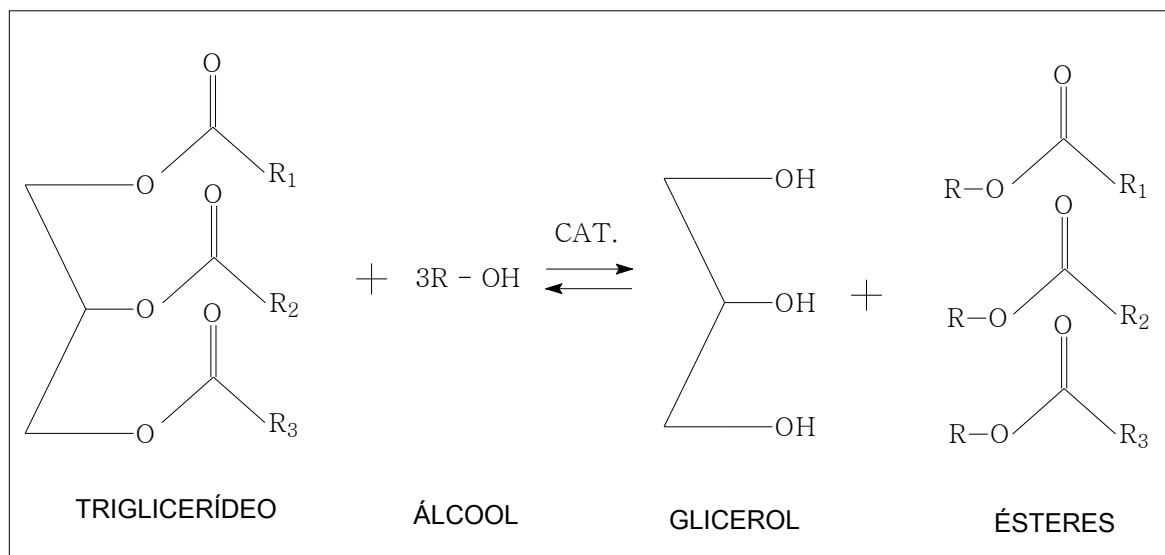
- Reação de transesterificação - Nessa etapa, a matéria prima passa por uma reação química que a converte em ésteres metílicos ou etílicos por meio da transesterificação com o uso de um catalisador;
- Separação de fases – A mistura reacional é composta de duas fases, uma densa e outra leve, que podem ser separadas por decantação ou centrifugação. A fase densa é constituída de glicerina, impurezas e excesso de álcool utilizado na reação. A fase leve é uma mistura de ésteres podendo ser metílicos ou etílicos, dependendo do tipo de álcool utilizado;
- Recuperação do álcool da glicerina – A fase pesada (glicerina) é submetida a uma evaporação para a retirada do excesso de álcool. Este é tratado posteriormente para reutilização no processo de produção;
- Recuperação do álcool dos ésteres – A fase leve (biodiesel) passa basicamente pelo mesmo tratamento como o descrito acima na recuperação do álcool da glicerina;
- Desidratação do álcool – Nessa reação, uma quantidade em excesso de álcool é utilizada para garantir o deslocamento da reação no sentido dos produtos. Esse produto passa por uma destilação onde é retirada boa parte da umidade (água) oriunda da lavagem do biodiesel. Esse processo é fácil de ser conduzido usando calor;
- Purificação dos ésteres – Após várias etapas, o biodiesel passa por uma sequência de lavagens para retirada de vestígios de catalisador e glicerina, seguido de uma desumidificação para se enquadrar nas especificações de comercialização do produto; e
- Destilação da glicerina – A purificação é feita por destilação a vácuo, resultando em um produto límpido e transparente conhecida como “glicerina destilada”.

4. Reação de transesterificação

A principal rota para obtenção de biodiesel no Brasil e no mundo é a transesterificação (ou alcoólise) alcalina, homogênea, de óleos e gorduras. Essa reação ocorre entre óleos ou gorduras que tem em sua composição tri-ésteres da glicerina com ácidos graxos, misturados a metanol ou etanol, com um catalisador

básico (Hidróxido de sódio - NaOH ou Hidróxido de Potássio - KOH), resultando em uma mistura de ésteres metílicos ou etílicos de ácidos graxos, hoje mais conhecido como biodiesel (Fig 2) (Suarez et al., 2009).

Figura 2. Transesterificação de triacilgliceróis.

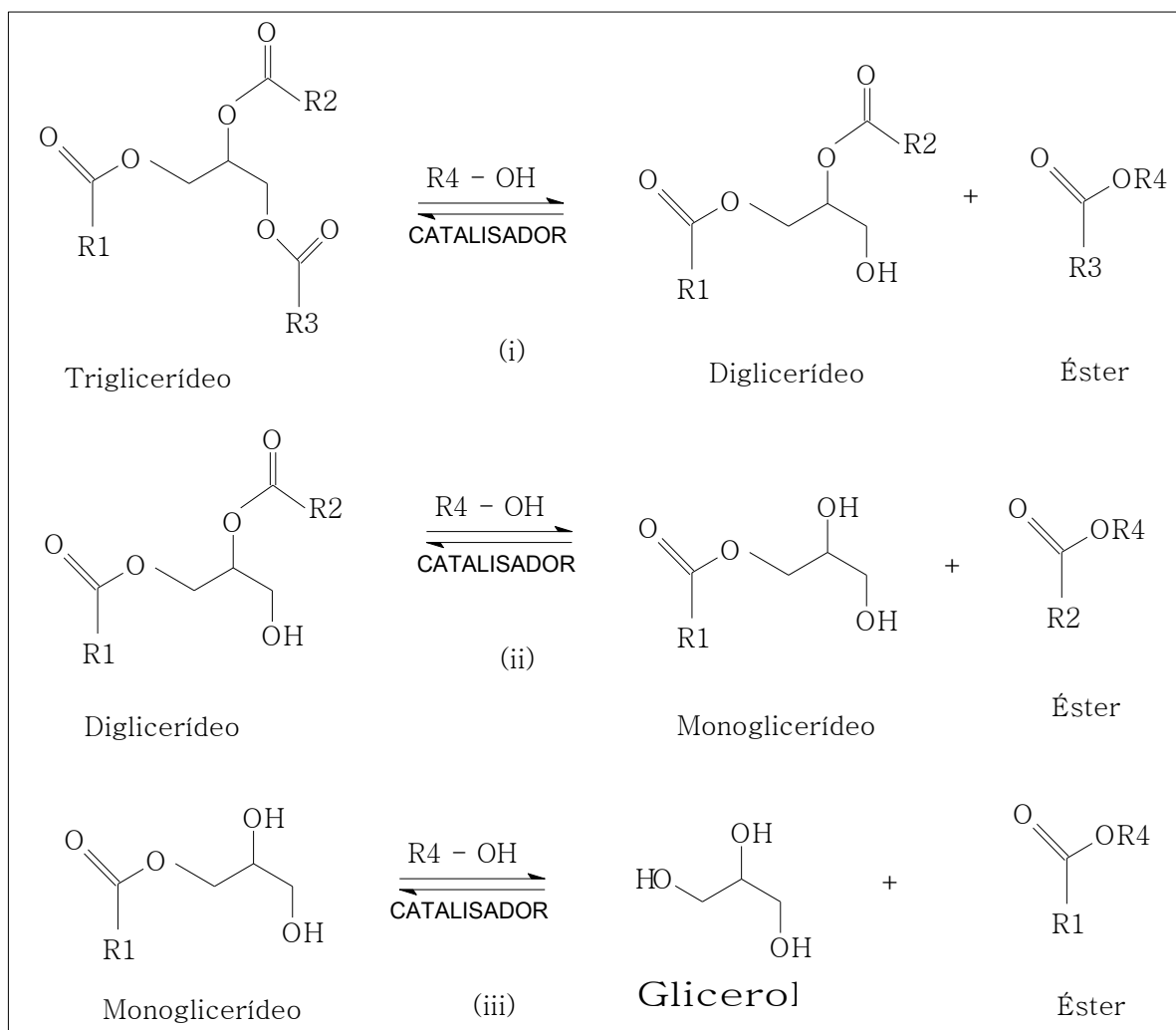


Fonte: Suarez et al. (2009)

Percebe-se pela reação que a mesma é reversível, o que faz necessário o uso de álcool em excesso para deslocar a reação no sentido da obtenção do produto (Lima 2005). Com a reação de transesterificação ocorre a separação da glicerina do óleo vegetal e cerca de 20% da massa molecular do óleo vegetal é devida a glicerina. A glicerina torna o óleo mais denso e viscoso, e durante o processo de transesterificação é removida do óleo vegetal, deixando-o menos denso reduzindo a viscosidade (Melo 2009).

A figura 3 (i, ii e iii) ilustra as reações químicas envolvidas para a transesterificação de triglicerídeos e obtenção dos ésteres. Suarez & Meneghetti (2007) reafirma a reversibilidade das reações i, ii e iii o que exige realmente um excesso de álcool no meio reacional para promover um aumento no rendimento em mono-álcoois.

Figura 3. Reações químicas na transesterificação de triglicerídeos.



Fonte: Suarez & Meneghetti (2007)

Três tipos de catalisadores podem ser usados para acelerar a reação de transesterificação: os catalisadores ácidos, básicos e enzimáticos. Por razões econômicas e pela grande disponibilidade, o hidróxido de sódio (NaOH) é o mais utilizado, sendo este mais rápido que os catalisadores ácidos (Lima 2005).

O metanol tem sido o álcool mais utilizado nas reações de transesterificação, devido a sua natureza física e química (cadeia curta e polaridade). No entanto, a utilização do etanol pode ser uma alternativa, considerando o ponto de vista ambiental e por este ser um produto menos tóxico e oriundo da cana de açúcar que é uma fonte renovável (Lima 2005).

5. Fatores que influenciam a reação de transesterificação

A transesterificação utilizando a catálise básica requer que os insumos possuam certo grau de pureza para que a reação não tenha a formação de produtos indesejados. O mínimo de umidade (água), fosfatídeos e ácidos graxos livres são essenciais para minimizar a formação de sabão (Suarez et al., 2009). Nos processos que usam óleo “in natura”, adiciona-se álcali em excesso para remover todos os ácidos graxos livres e este tratamento é conhecido como neutralização (Lima 2005).

Existe um reconhecimento na literatura científica que a catálise básica possui problemas operacionais quando o óleo vegetal apresenta altos teores de ácido graxo livre, que são formadores de sabão, além de consumir parte do catalisador durante a sua formação, dificultando assim, a separação dos produtos (ésteres e glicerina) no final do processo (Suarez & Meneghetti 2007).

Chialastri et al. (2011) realizando um estudo sobre fatores de influência no rendimento da reação de transesterificação etílica, relatou que a temperatura, agitação, tempo de reação, razão molar e tipo de catalisador são também variáveis significativas quanto ao rendimento final da reação.

Para Demirbas (2008) em estudo sobre fatores de influência no rendimento da reação de transesterificação metílica, o mesmo apresenta algumas variáveis significativas como a razão molar de álcool para óleo vegetal e a temperatura e esses são fatores primordiais que afetam quanto ao rendimento final da reação.

De acordo com Saad (2005), outra desvantagem atrelada à rota da transesterificação é a formação de água no meio reacional, decorrente da pré-solubilização dos hidróxidos no álcool para a produção do alcóxido correspondente, que atua como o verdadeiro catalisador na reação.

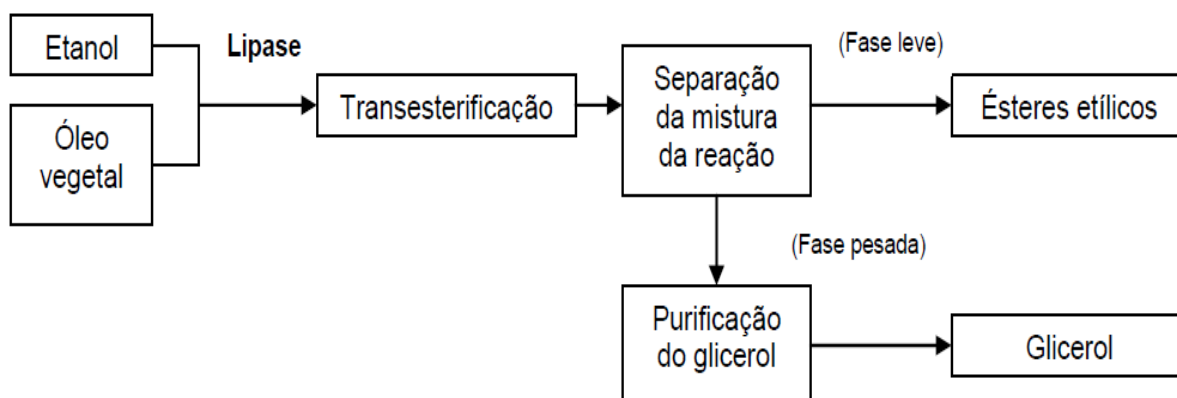
6. Transesterificação enzimática

A transesterificação enzimática consiste na modificação lipídica realizada pelas lipases e tem vantagem de permitir maior controle sobre a distribuição

posicional dos ácidos graxos no produto final, devido à seletividade e regioespecificidade das lipases (Andrade 2012).

Na reação de transesterificação enzimática, ocorrem algumas etapas como a fase contendo o glicerol, denominada de pesada, que pode ser simplesmente separada da fase com o biodiesel, denominada de leve, sendo que após a separação não há necessidade de desodorização nem neutralização do produto final, reduzindo assim o tempo de reação. Igualmente como ocorre no processo de transesterificação por catalise básica, o excesso de álcool utilizado, tende a deslocar a reação no sentido da formação do produto e desta forma, elevar o rendimento de biodiesel e o biocatalizador pode ser utilizado por várias vezes. A Figura 4 abaixo ilustra o processo enzimático da produção de biodiesel.

Figura 4. Processo enzimático da produção de biodiesel.



Fonte: Andrade (2012).

7. Lipases

As lipases constituem o grupo mais importante de biocatalisadores para aplicações biotecnológicas (Ahmed 2009). Lipases (triacylglicerol acil-hidrolase, E.C.3.1.1.3) são enzimas ubíquas produzidas pela maioria dos sistemas biológicos incluindo animais, plantas e micro-organismos (Mohanasrinivasan et al., 2009).

O interesse industrial por lipases vem aumentando gradativamente, especialmente nas áreas de engenharia de proteínas e enzimologia em meios não convencionais, as quais ampliaram consideravelmente o potencial de aplicação das enzimas como catalisadores em processos industriais (Roveda et al., 2010). Elas

catalisam a esterificação, interesterificação (acidólise, alcoólise, aminólise e transesterificação), além da atividade hidrolítica dos triglicerídeos (Hasan et al., 2009).

As lipases são amplamente utilizadas no processamento de gorduras e óleos, detergentes e formulações de desengorduramento, processamento de alimentos, síntese de produtos químicos finos e produtos farmacêuticos, fabricação de papel e produção de cosméticos (Sharma et al., 2001).

De acordo com Paques et al. (2006) as lipases são enzimas com certa especificidade e podem ser classificadas ou dítidas de acordo a sua ação da seguinte forma:

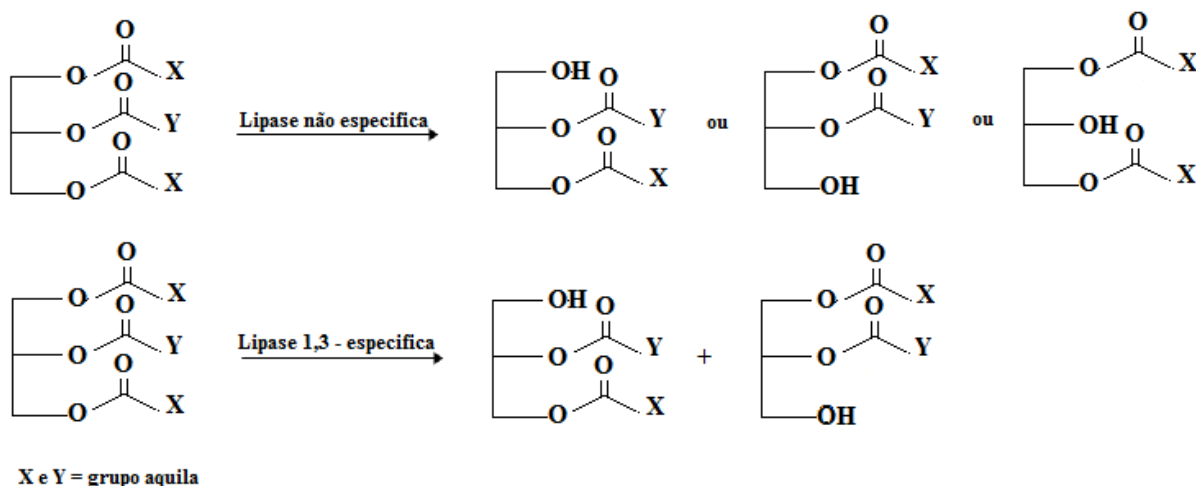
1. Regiosseletivas - subdivididas em:

- I. lipases não-específicas - hidrolisam ésteres de ácidos graxos primários ou secundários, liberando ácidos graxos na posição 1(3) ou 2;
- II. lipases 1, 3 - específicas - hidrolisam apenas ésteres de ácidos graxos primários, isto é, na posição 1 ou 3 (Figura 5).

2. Tipo-seletivas com relação ao tamanho da cadeia carbônica e/ ou ao número de insaturação do grupo acila.

3. Enantiosseletivas.

Figura 5. Reações catalisadas por lipases do tipo não específica e 1, 3 específica.



Das inúmeras fontes de obtenção de lipases, as de origem microbiana são as mais utilizadas industrialmente, pois apresentam procedimentos mais simples de isolamento, são mais estáveis e com propriedades mais diversificadas que as lipases de outras fontes (Macedo et al., 2009). Por serem biocatalisadores bastante eficazes devido à elevada atividade específica do substrato, elas possuem um baixo impacto no ambiente (Franken et al., 2010). As lipases podem ser produzidas em cultivo submerso e em estado sólido por meio de micro-organismos tais como bactérias, leveduras e fungos filamentosos (Almeida et al., 2016).

As enzimas de origem microbiana possuem muitas vantagens sobre as equivalentes de origem animal ou vegetal, como o menor custo de produção, a possibilidade de produção em larga escala em fermentadores industriais, além de oferecer um amplo espectro de características físico-químicas (Roveda et al., 2010).

Muitos trabalhos têm sido realizados na transesterificação catalisada por lipase de triglicerídeos. Pesquisadores de todo o mundo vêm tentando superar as limitações de uma produção de biodiesel catalisada por enzimas, tentando reduzir o alto custo da enzima, o baixo rendimento, o alto tempo de reação, a necessidade de solventes orgânicos e a necessidade de água na mistura de reação (Bajaj et al., 2010).

8. Fontes produtoras de lipases

As principais fontes de obtenção de lipases para aplicação industrial têm sido os micro-organismos, embora estas sejam produzidas também por eucariotos superiores (plantas e animais) (Messias et al., 2011).

Os fungos filamentosos são popularmente conhecidos como bolores e encontram-se amplamente disseminados na natureza, sendo frequentemente observados em pães, queijos velhos e frutas deterioradas. Cada filamento desses micro-organismos cresce principalmente em sua extremidade, a partir da extensão da célula terminal (Almeida 2007). Tanto micro-organismos eucariotos (leveduras e fungos) como procariotos (bactérias, incluindo-se os actinomicetos), são produtores de lipases e suas propriedades variam de acordo com a procedência (Messias et al., 2011).

As enzimas produzidas por fermentação microbiana são em sua maioria extracelulares (Gutarra et al., 2009) e este fato facilita os processos de extração e purificação. Desta forma, conferindo maior estabilidade à enzima que consequentemente permite o fácil controle das condições de cultivo que pode ser realizada em escala industrial (Mendes 2009). A fermentação de estado sólido (SSF) é uma alternativa interessante para a produção de enzimas microbianas devido à possibilidade de usar resíduos agropecuários e subprodutos como fontes de nutrientes e suporte ao desenvolvimento de micro-organismos (Rigo et al., 2010).

As lipases extracelulares são secretadas em quantidades significativas por algumas espécies de fungos filamentosos quando são cultivados em condições apropriadas, sendo facilmente separadas da massa micelial por filtração ou centrifugação (Colen 2006). A otimização da condição de cultivo para a produção de lipases microbianas é de suma importância, uma vez que os parâmetros influenciam as propriedades do micro-organismo produtor, assim como a razão de lipases extracelulares e intracelulares (Andrade 2012).

Os fungos filamentosos principalmente aqueles pertencentes aos gêneros *Aspergillus*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Rhizomucor* e *Thermomyces* são reconhecidos como os melhores agentes microbianos produtores de lipases (Cardenas et al., 2001).

Dependendo da fonte, as lipases podem ter massa molecular variando entre 20 a 75 KDa, atividade em pH na faixa entre 4 a 9 e em temperaturas que podem variar de ambiente a 70°C (Mendes 2009).

Abaixo segue a Tabela 1 com micro-organismos produtores de lipases relatados na literatura:

Tabela 1. Micro-organismos produtores de lipases relatados na literatura

Fonte	Micro-organismos	Referências
Arqueobactérias	<i>Natronococcus</i> sp.	Boutaiba et al., 2006
Bactérias	<i>Bacillus stearothermophilus</i> MC 7	Kambourova et al., 2003

(Gram-positivas)	<i>B. megaterium</i>	Lima et al., 2004
	<i>Burkholderia glumae</i>	Khattabi et al., 2003
	<i>B. cepacia</i>	Fernandes et al., 2007
	<i>Ralstonia</i> sp.	Yoo et al., 2011
	<i>Staphylococcus aureus</i>	Shah & Wilson 1965
	<i>S. epidermidis</i>	Simons et al., 1998
	<i>S. xylosus</i>	Mosbah et al., 2007
Bactérias	<i>Chromobacterium viscosum</i>	Jaeger & Reetz 1998
(Gram-negativas)	<i>Photobacterium lipolyticum</i>	Yang, Sohn & Kim 2009
	<i>Pseudomonas aeruginosa</i>	Jaeger et al., 1997
	<i>P. mendocina</i>	Jaeger & Reetz 1998
	<i>P. fluorescens</i> HU380	Kojima & Shimizu 2003
	<i>Serratia marcescens</i>	Jaeger et al., 1997
Actinomicetos	<i>Streptomyces cinnamomeus</i> Tü89	Sommer, Bormann & Götze 1997
	<i>S. coelicolor</i> A3(2)	Côté & Shareck 2008
Fungos	<i>Candida rugosa</i>	Dalmau et al., 2000
Leveduriformes	<i>C. cylindracea</i>	Brozzoli et al., 2009
	<i>Torulopsis ernobii</i>	Yoshida, Motai & Ichishima 1968
	<i>Issatchenkia orientalis</i>	Costas, Deive & Longo 2004
	<i>Yarrowia lipolytica</i>	Domínguez et al., 2003
Fungos	<i>Antrodia cinnamomea</i>	Lin, Wang & Sung, 2007
Filamentosos	<i>Alternaria</i> sp.	Tom & Crisan 1975
	<i>Aspergillus carneus</i>	Saxena et al., 2003
	<i>A. terreus</i>	Gulati et al., 1999
	<i>A. Níger</i>	Edwinoliver et al., 2010

<i>A. oryzae</i>	Toida et al. 2000
<i>Beauveria bassiana</i>	Hegedus & Khachatourians 1988
<i>Botryosphaeria rhodina</i>	Messias et al., 2009
<i>B. ribi</i>	Messias et al., 2009
<i>Botrytis cinérea</i>	Comménil et al., 1999
<i>Cunninghamella verticillata</i>	Gopinath et al., 2002
<i>Geotrichum</i> sp.	Burket et al., 2004
<i>Fusarium globulosum</i>	Gulati et al., 2005
<i>F. oxysporum</i>	Prazeres, Cruz & Pastore 2006
<i>Mucor circinelloides</i>	Szczesna-Antczak et al., 2006
<i>Penicillium aurantiogriseum</i>	Lima et al., 2003
<i>P. citrinum</i>	Miranda et al., 1999
<i>P. restrictum</i>	Cammarota & Freire 2006
<i>P. simplicissimum</i>	Gutarra et al., 2007
<i>P. verrucosum</i>	Kempka et al., 2008
<i>Rhizomucor miehei</i>	Jaeger & Reetz 1998
<i>Rhizopus arrhizus</i>	Li, Wang & Tan 2006
<i>R. chinensis</i>	Sun & Xu 2009
<i>R. delemar</i>	Açikel, Erşan & Açikel 2010
<i>R. homothallicus</i>	Diaz et al., 2006
<i>R. oryzae</i>	Essamri, Deyris & Comeau 1998
<i>Thermomyces lanuginosa</i>	Fernandes et al., 2004
<i>Trichoderma viride</i>	Kashmiri, Adnan & Butt 2006

Adaptado de (Messias et al., 2011).

9. Estrutura e mecanismo de atuação das lipases

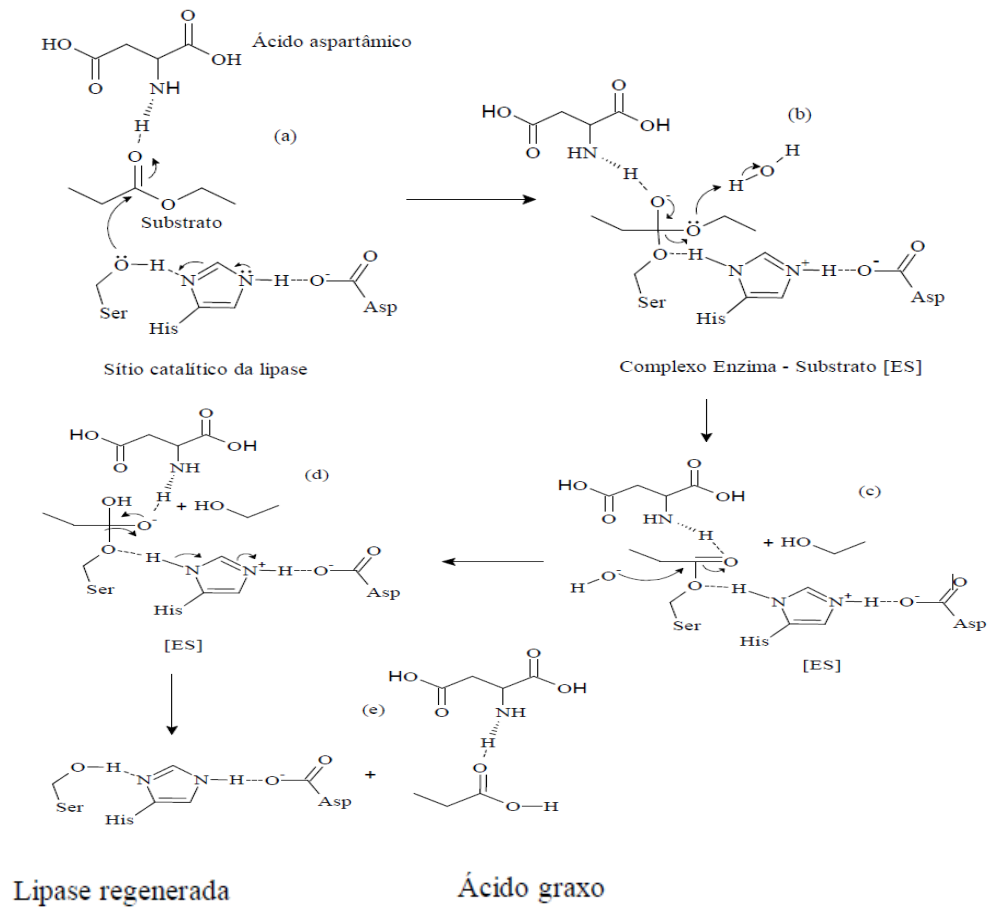
As lipases são enzimas que apresentam um mecanismo peculiar de atuação, chamado de ativação interfacial (Rodrigues 2009), que difere do clássico Michaelis-Menten. A ativação interfacial é explicada pela estrutura tridimensional e o sítio ativo da enzima. O sítio ativo da lipase é caracterizado pela tríade composta de serina, histidina e ácido glutâmico, também complexos acil-enzima. Em meios aquosos e na ausência de solventes orgânicos e interface, os sítios ativos das lipases apresentam uma conformação fechada e possuem baixa atividade. Por outro lado, no meio reacional, acessível ao substrato, o sítio ativo está aberto expondo a sua zona hidrofóbica e interage com a interface lipídica (Jaeger & Reetz 1998, Rodrigues 2009, Salvador et al., 2009).

Nas reações enzimáticas, necessariamente deve ocorrer a formação de um intermediário, denominado complexo enzima-substrato para que ocorra a reação, que acontece no sítio ativo das lipases que é coberto por uma cadeia peptídica denominada “tampa”, de característica hidrofóbica (Carvalho 2014). Quando a tampa encontra a interface água-lipídeo, ocorre interação no sistema e a tampa sofre uma alteração conformacional e o sítio ativo da enzima fica exposto, permitindo a catálise (Messias et al., 2011).

A estrutura química e as características cinéticas das lipases microbianas variam dependendo do micro-organismo, gênero, espécie e também da cepa (Messias et al., 2011).

Na Figura 6 está representado o mecanismo de hidrólise enzimática, onde a reação envolve a formação de dois intermediários tetraédricos. O mecanismo de ação de lipases se inicia com um ataque do átomo de oxigênio do grupo –OH do resíduo de Ser nucleofílico ao carbono carbonílico ativado do éster lipídico, que está ligado ao sítio ativo da enzima (Almeida 2012). Este é estabilizado pelos resíduos catalíticos de histidina e ácido aspartâmico e ocorre um rearranjo e uma molécula de álcool é liberada, após ocorre o segundo ataque nucleofílico por um íon hidroxila referente à água. Por fim, há um rearranjo na molécula e o ácido graxo é liberado e em seguida a enzima é regenerada (Jaeger & Reetz 1998).

Figura 6. Mecanismo de catálise enzimática por lipases



Fonte: Adaptado de (Jaeger & Reetz 1998).

10. Fatores que influenciam a produção de lipases fúngicas

Diversos fatores são de extrema importância para a produção de lipases por micro-organismos como as fontes de carbono, fontes de nitrogênio, pH inicial, meio mineral, inóculo, agitação/aeração e temperatura são fatores que geram estratégias, que podem ser empregadas para aumentar a eficiência na produção de lipases microbianas e estes influenciam de forma significativa na produção de lipases (Kumar et al. 2011).

Sharma et al., (2001), Colla et al., (2012) e Kumar et al., (2011) descrevem em seus trabalhos a importância dos fatores para a produção de enzimas lipolíticas, e estes são de forma sucinta apresentados abaixo.

- Efeito das fontes de carbono: É o fator mais importante para a expressão dessas enzimas. As fontes de carbono atuam tanto como fonte de energia e também meio indutor;
- Efeito das fontes de nitrogênio: apresentam importante função na síntese de enzimas, sendo utilizadas pelas células para síntese de muitos fatores de crescimento celular e aminoácidos necessários para o metabolismo celular e síntese de enzimas;
- Efeito do meio mineral: a suplementação dos meios de cultivo com uma solução de minerais ocasionou um aumento de três vezes na atividade enzimática dos meios contendo os óleos de gergelim e oliva, e de quatro vezes no meio contendo óleo de milho. Os elementos traços são geralmente componentes estruturais das enzimas e estes podem influenciar positivamente a produção de lipases;
- Efeito da temperatura: as temperaturas na faixa de 26,5°C a 32°C foram utilizadas em inúmeros trabalhos de pesquisa de produção de lipases, sendo 30°C a temperatura utilizada com maior frequência;
- Efeito da agitação/aeração: na produção de lipases via bioprocesso submerso, a implementação da transferência de oxigênio através de agitação e inserção de ar é um importante fator para a produção de lipases por micro-organismos, em especial fungos filamentosos;
- Efeito do pH: o pH desempenha um papel muito importante na produção de enzimas, bem como na sua estabilidade. O efeito do pH na produção de enzimas foi estudado e observou-se que o rendimento ótimo para lipases foi alcançado a pH 7.

Há dois métodos de se avaliar a influência desses parâmetros sobre a resposta: o método “um fator por vez”(one-factor-at-time - OFAT) analisa um parâmetro por vez, enquanto os outros estão fixos e o método “planejamento experimental” (*design of experiments* - DOE), em que se analisam todos os parâmetros de uma só vez (Videira 2014).

O uso de ferramentas como o planejamento experimental para otimizar condições de cultivo e produção é bastante utilizado, uma vez que esses programas possibilitam a análise de muitas interações entre os parâmetros utilizados com um número reduzido de experimentos, o que leva a uma avaliação e compreensão das

interações entre os diferentes parâmetros do processo, gerando assim economia de tempo e reagentes (Chennupati et al., 2009).

11. Aplicações biotecnológicas das lipases

As lipases microbianas apresentam uma grande versatilidade, podendo as mesmas promover um grande número de reações em temperatura ambiente, moderada e ou em pressões moderadas. O uso dessas enzimas em aplicações biotecnológicas como reações de hidrolises, bioconversões e sínteses, aumentou significativamente nos últimos anos (Almeida 2012).

As muitas aplicações de lipases incluem sínteses orgânicas de especialidade, hidrólise de gorduras e óleos, modificação de gorduras, melhoramento de sabor no processamento de alimentos, resolução de misturas racêmicas e análises químicas (Sharma et al., 2001).

Sob certas condições, elas também catalisam reações de esterificações, tiotransesterificações em solventes orgânicos, sistemas bifásicos e em suspensões micelares (Babicz 2009). O deslocamento do equilíbrio na reação, no sentido direto (hidrólise) ou inverso (síntese) é controlado pela quantidade de água presente na mistura reacional (Babicz 2009).

As lipases catalisam uma série de diferentes reações. Além de quebrar as ligações de éster de triacilgliceróis com o consumo de moléculas de água (hidrólise), as lipases são também capazes de catalisar a reação reversa sob condições microaquosas, como por exemplo, a formação de ligações éster, a partir de um álcool e ácido carboxílico (síntese de éster) (Castro et al., 2004).

A atividade hidrolítica da lipase pode ser diretamente relacionada com sua atividade de síntese, mas é independente de sua atividade de interesterificação. Lipases de diferentes fontes são capazes de catalisar a mesma reação, embora possam diferir no desempenho sob as mesmas condições reacionais (Castro et al., 2004).

As reações de esterificação catalisadas por lípase receberam grande importância devido a numerosos produtos que podem ser obtidos (Kiran et al.,

2000). Neste contexto, um conhecimento das atividades de esterificação de lipases de diferentes fontes, bem como preparações de lipase obtidas através de diferentes procedimentos, como a imobilização, são essenciais para explorar um grande número de reações de esterificação até então ainda não estudadas (Kiran et al., 2000).

Para a reação de esterificação é possível produzir lipases com a fermentação em estado sólido (SSF), pois, neste processo o fermentado produzido pode ser usado diretamente para catalisar as reações de esterificação e transesterificação (Rigo et al. 2010).

No caso da produção de lipases por fermentação em estado sólido, as principais vantagens, envolvem a possibilidade de usar o catalisador em processos biossintéticos sem qualquer extração e imobilização antes da sua utilização, uma vez que as enzimas produzidas desta forma consistem em um biocatalisador barato e naturalmente imobilizado, que pode ser aplicado para diversos processos, inclusive para a esterificação (Rigo et al., 2010).

Embora a síntese de éster possa ser feita quimicamente com catálise ácida ou básica, o uso da tecnologia enzimática oferece as vantagens de condições suaves, reações laterais reduzidas e com maior especificidade (Villeneuve et al., 2000).

A Figura 7 abaixo apresenta as diferentes reações catalisadas por lipase em soluções aquosas e não aquosas.

Figura 7. Diferentes reações catalisadas por lípase em soluções aquosas e não aquosas.

REAÇÃO DE HIDRÓLISE

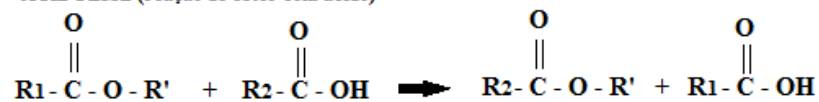


REAÇÃO DE ESTERIFICAÇÃO

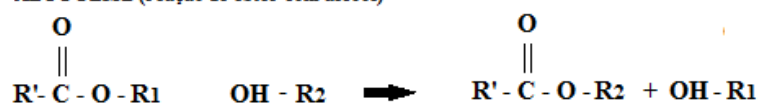


REAÇÃO DE INTERESTERIFICAÇÃO

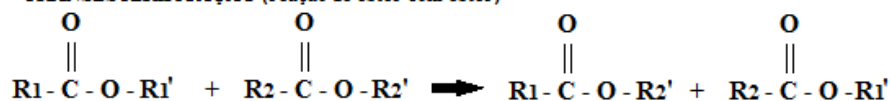
ACIDOLISE (reação de éster com ácido)



ALCOÓLISE (reação de éster com álcool)



TRANSESTERIFICAÇÃO (reação de éster com éster)



O potencial de exploração comercial de uma lípase microbiana é determinado pelo seu rendimento, atividade, estabilidade dentre outras características (Shu et al., 2010). Para a exploração comercial de uma lípase microbiana, as características de alto rendimento e alta atividade significam que o produto lípase tem maior competitividade no mercado (Li & Zong 2010). Já a alta estabilidade do produto da lípase, ajudará a expandir seu campo de aplicação, a prolongar sua meia-vida da prateleira e prolongar seus ciclos de uso (Shu et al., 2010).

A Tabela 2 a seguir apresenta uma lista com as fontes e origens de lipases comerciais.

TABELA 2. Fontes e origens de lípase comerciais

Fonte biológica	Fornecedor
<i>Achromobacter</i> sp.	Meito Sangyo
<i>Aspergillus niger</i>	Aldrich, Amano, Biocatalysts, Fluka, Novozymes, Röhm
<i>Aspergillus</i> sp.	Novozymes
<i>Candida antarctica A</i>	Boehringer, Fluka, Novozymes
<i>Candida antarctica B</i>	Fluka, Novozymes, Boehringer
<i>Candida cylindracea</i>	Meito
<i>Candida rugosa</i>	Aldrich, Altus, Amano, Biocatalysts, Boehringer, Fluka, Meito Sangyo, Sigma, Roche
<i>Chromobacterium viscosum</i>	Asahi
<i>Geotrichum candidum</i>	Amano, Biocatalysts
<i>Mucor javanicus</i>	Amano
<i>Mucor miehei</i>	Amano, Boehringer, Biocatalysts, Fluka, Novozymes
<i>Penicillium roqueforti</i>	Amano, Biocatalysts, Fluka
<i>Pseudomonas</i> sp.	Amano, Boehringer, Fluka, Mitsubishi, Röhm, Sigma
<i>Rhizopus arrhizus</i>	Biocatalysts, Boehringer, Fluka, Sigma
<i>Rhizopus oryzae</i>	Amano, Sigma
Germe de trigo	Fluka, Sigma
Pâncreas de suíno	Aldrich, Amano, Biocatalysts, Boehringer, Fluka, Röhm, Sigma

Fonte: Adaptado de (Paques et al., 2006).

As principais aplicações industriais descritas na literatura sobre o uso de lipases são nas indústrias de alimentos, detergentes, farmacêuticas, cosméticos, química, petroquímica, bebidas. (Rondhane et al., 2010); (Bussamara et al., 2010); (Li & Zong 2010) e (Contesini et al., 2010).

A Tabela 3 a seguir apresenta uma relação de indústrias e aplicações possíveis para as lipases.

Tabela 3. Aplicação biotecnológica de lipases

Indústria	Ação	Produto ou aplicação
Laticínios	Hidrólise da gordura do leite	Desenvolvimento de agentes flavorizantes em leite
Panificação	Aumento do aroma	Prolongar a vida de prateleira
Cervejaria	Aceleração da fermentação em função dos lipídios	Melhorar a qualidade das bebidas
Carnes e peixes	Desenvolvimento do aroma	Remoção do excesso de gordura
Farmacêutica	Digestão de óleos e gorduras em alimentos	Lipídios específicos e digestivos
Médica	Determinação de triglicerídeos no sangue	Realização de exames clínicos
Papel	Tratamento de polpas de celulose	Melhoria da qualidade do papel
Tratamento de resíduos	Decomposição e remoção de substâncias oleosas	Resíduos isentos de gorduras
Produção de biocombustíveis	Modificação lipídica e remoção do glicerol	Produção de biocombustíveis
Detergentes	Hidrólise de gorduras	Remoção de óleos
Couro	Hidrólise	Produtos de couro
Cosméticos	Síntese	Emulsificantes, umidificantes

Fonte: Adaptado de (Andrade 2012) e (Colla et al., 2012)

12. Lipases na produção de biodiesel

Nos últimos anos, a transesterificação catalisada por lipase tornou-se uma alternativa efetiva à catálise básica e ácida para a produção de biodiesel. A transesterificação baseada em enzimas usa menos energia que os processos quimicamente catalisados. Além disso, ao contrário da catálise básica, pode ser usado com substratos que contenham ácidos graxos livres (Canet et al., 2016).

As aplicações industriais das lipases estão contribuindo para um contínuo aumento do mercado mundial de enzimas, uma vez que as lipases estão entre as enzimas mais utilizadas em processos industriais (Almeida 2012).

A produção de biodiesel catalisada por lipases microbianas é uma excelente alternativa na tentativa de substituir as vias químicas de síntese e reduzir os custos de produção do biodiesel e das enzimas (Ramos et al., 2011). O uso de lipases para produção de biodiesel é de relevante importância, considerando-se o crescimento da utilização desse biocombustível em âmbito mundial, não somente pelo aspecto de meio ambiente, mas, principalmente, por se tratar de uma fonte de energia renovável (Castro et al., 2004).

O custo da enzima é o principal obstáculo a ser superado pelas indústrias de produção. Desta forma, o que se busca é o desenvolvimento de produtos e processos de biodiesel, para alcançar alta pureza com um processo econômico e favorável ao meio ambiente em condições moderadas de reação (Jegannathan et al., 2008).

O processo de produção comercial de biodiesel é fundamentalmente realizado por via química, mas a rota enzimática tem despertado grande interesse na comunidade científica (Tan et al., 2010). Um aspecto comum a estes processos é a busca pela otimização das condições de reação, de modo a lhes conferir características que os tornem viáveis e disponíveis para aplicações industriais (Bajaj et al., 2010). Entretanto, dentre algumas desvantagens essencialmente econômicas, o processo enzimático, uma vez otimizado, poderá apresentar vantagens muito interessantes em relação ao processo químico, tais como a facilidade de separação do catalisador a obtenção de produtos mais puros por permitir o uso de etanol hidratado na reação (Colla et al., 2012).

Embora os processos de transesterificação enzimática para obtenção de biodiesel ainda não sejam comercialmente desenvolvidos, novos resultados têm sido reportados em artigos e patentes. O aspecto comum desses estudos consiste na otimização das condições de reação como solvente, temperatura, pH, tipo de micro-organismo que gera a enzima, etc. a fim de estabelecer as características para aplicações industriais. Contudo, tanto o rendimento como o tempo de reação ainda são desfavoráveis se comparados com o sistema de reação por catálise básica (Nascimento et al., 2001).

Bajaj et al., (2010), relatou em seu trabalho que foram investigadas lipases de diferentes fontes para determinação de sua atividade de transesterificação em diferentes óleos. As características investigadas nas lipases foram sua capacidade de utilizar todos os mono, di e triglicerídeos, bem como os ácidos graxos livres na transesterificação, baixa inibição do produto, alta atividade e rendimento, menor tempo de reação, reutilização da enzima imobilizada, temperatura e resistência ao álcool.

A Tabela 4 a seguir apresenta as principais vantagens e desvantagens dos processos químico e enzimático para a produção de biodiesel.

Tabela 4. Principais vantagens e desvantagens dos processos químico e enzimático para a produção de biodiesel

Processos	Vantagens	Desvantagens
Químico		Dificuldade de separação do catalisador
		Impossibilidade de reutilização do catalisador
	Simplicidade	
	Alto rendimento	Dificuldade de utilização de etanol hidratado
	Curto tempo de reação	Obtenção de produtos com menor grau de pureza
Enzimático	Facilidade de separação do catalisador (suporte);	
	Obtenção de produtos com maior grau de pureza;	Longo tempo de reação
	Possibilidade de reutilizar o catalisador (enzima) na reação;	Elevado custo da enzimas
	Possibilidade de utilizar etanol hidratado na reação;	Rendimentos inferiores ao processo químico

Fonte: Adaptado de (Nascimento et al. 2001)

13. Considerações finais

O processo de produção de biodiesel no país já se encontra bem consolidado, uma vez que a produção atende à demanda de uso deste biocombustível e também consegue atingir todos os seus parâmetros de qualidade. A reação de transesterificação ainda é o método mais utilizado para a produção de biodiesel em grande escala, o que é economicamente e industrialmente viável quando comparado a outros processos de produção. O único inconveniente neste

processo é o uso do metanol na cadeia produtiva, pois sabemos que o mesmo é oriundo de fontes não renováveis fazendo com que o biodiesel perca seu caráter de biocombustível biodegradável.

O uso de catalisadores biológicos (enzimas) cresceu bastante, mas ainda existe o inconveniente de ser um processo de produção com um custo elevado, o que torna o uso deste processo ainda distante da realidade de nosso país. Mas é neste sentido que pesquisas vem sendo desenvolvidas para superar o obstáculo do custo das enzimas e o desenvolvimento de produtos e processos de produção de biodiesel, para alcançar alta pureza com uma viabilidade econômica favorável a indústria e ao meio ambiente.

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ARTIGO SUBMETIDO**CAPÍTULO II****Lipolytic enzymes with hydrolytic activity and esterification produced by filamentous fungi isolated from decomposing leaves in streams of the cerrado, Brazil**

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Abstract

Microbial lipases are very prominent biocatalysts because of their ability to catalyze a wide variety of reactions in aqueous and non-aqueous media. In this work, the filamentous fungi isolated from leaves decomposed in the creek of the cerrado and identified with the ITS gene were screened for lipase production with hydrolytic activity and esterification. Agar plates with Tween 20 and rhodamine B were used for selection, while culture submerged with olive oil was subsequently used to select 38 filamentous fungi. *Thichoderma harzianum*, *Fusarium solani* and sample F5, F36 were grown in 6 different culture media. *F. solani* presented the highest production of lipase 2.37 U/mL with esterification activity of 0.07 U/mL using medium 3 with (g.L⁻¹) (KH₂PO₄ 1.00, MgSO₄ H₂O 1.123, CuSO₄ 0.06). Supplementation of this culture medium with organic nitrogen sources increased lipase production by (461.29%) 3.48 U/mL using tryptone and (419.35%) 3.22 U/mL using yeast extract. Among the vegetable oils from the Amazon region evaluated, degummed cotton oil induced lipase production at (1212.90%), 8.14 U/mL. In this way the application of lipases produced by filamentous fungi in the hydrolysis and esterification of vegetable oils can be an alternative to the conventional processes in the production of biofuels.

Keywords: Lipase, filamentous fungi, hidrólise, esterification, *Fusarium solani*.

1. Introduction

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) have emerged as key enzymes in swiftly growing biotechnology, owing to their multifaceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences (Gupta et al., 2004). These enzymes are able to catalyze many reactions on ester bonds with preference on water-insoluble substrates being activated when absorbed to an oil-water interface to generate free fatty acids, monoacylglycerols, diacylglycerols and glycerol (Kumar et al., 2017). Lipases are organic solvent-tolerant which open an infinity option of application in chemical industry because esterification reactions to produce food flavor, fragrance, cosmetic and pharmaceutical, as well as transesterification to produce biodiesel (Kuperkar et al., 2014; Kumar et al., 2016; Tacias-Pascacio et al., 2017).

Lipases production has been developed mainly by submerged fermentation due to the dominated and developed engineering aspects; however they can also be produced by solid state fermentation (GUTARRA et al., 2009; RODRIGUES et al., 2016), in which insoluble substrates with low percentages of water in their composition are used (PANDEY, 2003).

There are several methods of selection of lipase-producing organisms, lipolysis being observed directly by changes in the appearance of the substrate (HASAN et al., 2009), where lipase production is indicated by the formation of light halos around cultured colonies on agar plates containing tributyrin, triolein and tweens (Hankin & Anagnooskis 1975). The lipolytic activity in solid media can also be determined using dyes such as rhodamine B (RODRIGUES et al., 2016) and Vitoria B blue (COLEN, 2006), in which a drop in pH occurs due to the fatty acids released by the hydrolysis of the substrate, causing the formation of fluorescent halos around the colony, being observed the color change of the indicators used (NOBRE, 2012). The combination with rhodamine B proved to be a rapid method in which this compound binds fatty acids, mono- and diglycerides and develops fluorescence under ultraviolet (UV) light. Thus, this technique is very convenient for the rapid selection of lipase-producing microorganisms (PÉREZ et al., 2018).

Lipases are inducible enzymes, i.e. produced by microorganisms in the presence of an inducer, which may be the substrate itself or the product of its hydrolysis. The inducer can be added to the culture medium to stimulate production

(ROVEDA et al., 2010), require sources of lipid carbon in the form of oils for higher enzyme yield (SALIHU et al., 2011). The mechanisms that regulate lipase biosynthesis vary significantly among the different producing microorganisms. In some cases, lipase production appears to be constitutive and independent of the addition of lipid substrates in the culture medium (CORTEZ et al., 2017). However, their presence may increase the level of lipolytic activity. There are several reports of the use of tested vegetable oils to evaluate their induction power in the production of lipases by filamentous fungi, such as palm oil (PENHA et al., 2016), canola oil, corn oil, sunflower oil (SANTOS et al., 2014), castor oil (TOSCANO et al., 2013) and cotton oil (CASTRO et al., 2016).

The aim of this work was to select strains of filamentous fungi isolated in aquatic environments in tropical ecosystems, producers of lipolytic enzymes with hydrolytic activity and esterification, and to evaluate the nutritional conditions to produce this enzyme under submerged conditions.

2. Material and methods

2.1. Microorganisms

Filamentous fungi were obtained from Microbial Culture Collection “Carlos Augusto Rosa” from Federal University of Tocantins (UFT). The filamentous fungi strains were isolated from decomposition leaves harvested in Buritizal river located in -10 15' 51,91040" latitude and -48 06' 54,7474" longitude, Palmas city, Tocantins, Brazil. The microorganisms were cultured in potato dextrose agar (PDA), 28° C for 7 days. Storage of the filamentous fungi were carried out in Castellani method (Castellani, 1939).

2.2. Molecular identification

Total DNA from the filamentous fungal strains was extracted from cultures grown on PDA medium using the Illustra™ Nucleon PhytoPure, plant and fungal DNA extraction kits (GE Healthcare, Amersham, England). The DNA was quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Uniscience, Brazil), later diluted to a concentration of 50 ng for direct use in the polymerase chain reaction (PCR) using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3 ') (INNIS et al., 1990).

Amplifications were performed on Mastercycler® nexus (Eppendorf) thermocycler using the GoTaq® DNA Polymerase kit (Promega Madison-USA) with final reaction volume of 25 µL. After the amplifications, PCR products were checked by 1% (w/v) agarose gel electrophoresis (SAMBROOK et al., 2001) and visualized under ultraviolet light in an LPIX-EX photodocumentator (Loccus Biotechnology São Paulo - Brazil). The 1 Kb DNA Ladder (Promega Canada - USA) was used as molecular weight marker. The amplified products were purified with Exonuclease I and Alkaline Phosphatase (ExoSap-IT®) solution (USB Corporation Austin-USA) followed by sequencing using the same PCR primers on an ABI 3500 xl automatic sequencer (Life Technologies) according to the Dideoxy or chain termination method (SANGER et al., 1977) using the BigDye Terminator v 3.1 sequencing kit (Life Technologies Foster city - USA). This stage was carried out at the Laboratory of Polar Microbiology and Tropical Connections of the Federal University of Minas Gerais (UFMG) and Laboratory of Cellular and Molecular Parasitology (LCPM) of the Oswaldo Cruz Foundation - Fiocruz / René Rachou Institute.

2.3. Screening of filamentous fungi lipolytic enzyme production

Lipase-producing filamentous fungi strains were screened using high-throughput method in agar plate containing PDA medium supplemented with 1% (w/w) olive oil with Rodamine B (0.01%, w/w). The lipolytic activity was indicated by the appearance of orange fluorescence halos around the colonies which were observed under UV light at 350 nm (Kumar et al., 2012). A second medium method was according to Hankin and Anagnostakis (1975) composed of (g.L⁻¹): peptone, 10; NaCl, 5; CaCl₂*2H₂O, 0.1, agar, 20, supplemented with 1 % (w/v) Tween 20. The final pH was adjusted to 7.4 and the medium was autoclaved at 121 °C, for 20 min. The lipolytic activity was indicated by appearance of opaque área due to calcium salts precipitation halos around the colonies. Cultures were incubated at 28° C and daily examined for 7 days. The enzymatic activity was measured by an enzymatic index.

2.4. Submerged culture selection

Liquid medium was prepared using Vogel's medium (Vogel 1956). Trace elements solution (solution A) was prepared containing (g.L⁻¹): citric acid *H₂O, 50, ZnSO₄*7H₂O, 50, Fe(NH₄)₂(SO₄)₂*6H₂O, 10; CuSO₄*5H₂O, 2.5; MnSO₄*H₂O, 0.05;

H₃BO₃, 0.05; Na₂MoO₄*2H₂O, 0,05. Salt solution (solution B) was prepared containing (g.L⁻¹): sodium citrate*5H₂O, 150; KH₂PO₄, 250; MgSO₄*7H₂O, 10; CaCl₂*2H₂O, 5 and biotin solution (0.1 mg/mL), 5 mL; solution A, 5 mL; 0.2 mL chloroform. The solutions were maintained at 4° C. Medium preparation consisted in 50-fold dilution of the solution B, replacing glucose by other carbon sources and the final pH was adjusted to 6.0.

Cultures were performed in Erlenmeyer flasks (250 mL) containing 25 mL of culture medium supplemented with 1% (w/v) olive oil and 0.2% (w/v) yeast extract. Media were autoclaved at 121 °C for 20 min. Inocula were prepared using 5-day old cultures. Medium was inoculated with 1 mL conidia suspension (10⁷ spores per mL) and incubated at 28 °C, 180 rpm for 7 days. Biomass was separated from the fermentation broth by filtration (membrane cellulose acetate 0.45 µm cut-off) and dried at 105° C until constant weight. Broth was used for hydrolytic and esterification activities assays.

2.5. Enzyme assays

2.5.1 Determination of hydrolytic activity

Lipase activity was assayed with *p*-nitrophenyl-palmitate (pNPP) as substrate (Almeida et al., 2013). The pNPP was firstly solubilized in 0.5 mL of dimethyl sulfoxide, then diluted to 50 mM with 50 mM sodium phosphate buffer pH 7.0, containing 0.5 % Triton X-100. Lipase activity was determined by following the hydrolysis of pNPP at 37° C. The reaction was stopped at different intervals by heat shock (90 °C, 1min), followed by the addition of 1 mL saturated sodium tetraborate solution. The *p*-nitrophenolate formed was measured spectrophotometrically at 405 nm. The molar extinction coefficient for *p*-nitrophenol (pNP) at 405 nm is 1.8 x 10⁴ m/cm⁻¹. Controls were prepared without enzyme. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of pNP per min. Results were presented as total units or specific activity in units by milligram of protein.

2.5.2 Determination of esterification activity

The esterification activity (EA) catalyzed by the crude enzymatic extracts was followed by modified titrimetric assays (Rigo et al., 2010). The esterification activity of crude enzymatic extracts was measured by the consumption of acid in the esterification reaction with ethanol and oleic acid (1:1 equimolar ratio). Experiments

were carried out in Erlenmeyer flasks (125 mL) containing 5 mL of reaction medium. The reaction was initiated by addition of 1 mL of the enzymatic extract. All experimental runs, a control assay (blank) was always carried out. After incubation in shaker for 120 min at 40° C and 180 rpm, the content of fatty acids remaining in the aliquot were extracted by the addition of 15 mL of an acetone/ethanol solution (1:1, v/v). The amount of oleic acid was then titrated with 0.1 M NaOH until pH 11. One unit of lipase activity was defined as the amount of dry enzyme preparation necessary to the consumption of 1 $\mu\text{mol}\cdot\text{min}^{-1}$ of acid under assay conditions. All assays were performed in duplicate, and the results are expressed in terms of units per gram of dry substrate and calculated as follows:

$$EA = \frac{(Va - Vb) \times M \times 100}{E \times t}$$

Where: Va = volume of NaOH used after 120 min (mL); Vb = volume of NaOH used at zero time of reaction (mL); M = molarity of NaOH solution; t = time (min).

2.6. Enzyme production under submerged conditions

2.6.1 Culture media composition

Medium composition was evaluated for lipolytic enzymes production by filamentous fungi selected according with each mineral formulation ($\text{g}\cdot\text{L}^{-1}$):

Medium 1 (M1): $\text{MgSO}_4\cdot\text{H}_2\text{O}$ 0.14; K_2HPO_4 0.50; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 7.20 (Kashmiri et al., 2006);

Medium 2 (M2): NaH_2PO_4 12.00; K_2HPO_4 2.00; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.03; $\text{ZnSO}_4\cdot\text{H}_2\text{O}$ 0.03; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ 0.005 (Ülker et al., 2011);

Medium 3 (M3): KH_2PO_4 1.00; $\text{MgSO}_4\cdot\text{H}_2\text{O}$ 1.123; CuSO_4 0.06 (Pera et al., 2006);

Medium 4 (M4): $\text{MgSO}_4\cdot\text{H}_2\text{O}$ 0.28; KCl 0.50; K_2HPO_4 2.00; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ 0.005; NaH_2PO_4 2.00; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.50 (Adham and Ahmed, 2009);

Medium 5 (M5): KH_2PO_4 1.00; $\text{MgSO}_4\cdot\text{H}_2\text{O}$ 0.28; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.13; NaCl 0.10; H_3BO_3 0.0005; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ 0.00004; KI 0.00001; $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ 0.00023; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.0004; $\text{MnSO}_4\cdot\text{H}_2\text{O}$ 0.0004 (Olson and Johnson, 1948);

Medium 6 (M6): $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 0.28; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.40; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1.83; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.12 (Gochev et al., 2012).

Submerged cultures were carried out using the media supplemented with 1% (w/v) olive oil and 0.2% (w/v) yeast extract, at 28° C, 180 rpm for 5 days.

2.6.2 Effect of nitrogen and carbon sources on hydrolytic enzymes production

Nitrogen sources were evaluated using organic sources (peptone, yeast extract, corn steep liquor, tryptone and urea) and inorganic sources (ammonium sulfate and ammonium chloride) in different concentrations (0.2, 1 and 2%, w/v).

Hydrophobic carbon sources such as castor oil (*Ricinus communis*), pequi oil (*Caryocar brasiliense*), babassu oil (*Attalea speciosa*), copaiba oil (*Copaifera langsdorffii*), buriti oil (*Mauritia flexuosa*), degummed soybean oil (*Glycine max*), degummed cotton oil (*Gossypium hirsutum*) and soybean oil fried in concentration of 1.0% (w/v) were used in replaced to olive oil.

2.6.3 Time-course of cultivation

Time-course of cultivation of filamentous fungi was carried out in Erlenmeyer flasks (250 mL) containing 25 mL of selected culture medium, carbon and nitrogen sources for 10 days, at 28° C and 180 rpm. Cultures were inoculated with 1 mL conidia suspension (10^7 conidia per mL). Samples were withdrawing each 24 h and biomass and lipolytic enzymes were assayed.

3. Results and discussion

3.1. Screening of filamentous fungi lipolytic enzymes producing

Thirty-eight filamentous fungi isolated from decomposition leaves were firstly evaluated to lipolytic enzymes in agar plate by enzymatic index formed by rhodamine B and calcium precipitation halos (Table 1). Potential enzymes production by filamentous fungi strains were evaluated in submerged cultivation and biomass, hydrolytic activity and esterification activity were also evaluated. Among microorganisms strains evaluated, 66% presented enzymatic activity positive in agar plate using olive oil or Tween 20 as carbon sources. Solid agar plate has proved to be the best direct method in examining fungal growth as well as the ability for production of extracellular enzymes. Furthermore, it is a useful rapid method of

screening to evaluate individual fungi of genetic variants for either presence or absence of enzymes such as lipases (Geoffry and Ahur, 2018). Smitha et al. (2014) screened 181 marine fungi cultures for their ability to secrete hydrolytic enzymes using nutrient agar supplemented with Tributyrin as substrate and found that 60.2% to be having lipolytic activity. The high sensitive of agar plate method to identify active and true lipase involve several factors, e.g. microorganism growth, production and diffusion of the enzymes, activity and specificity of the enzyme to substrate, and enzyme concentration. Other factors that influence the halo formation by enzyme fungi producer are medium composition, temperature growth, chemical and biological environment (Bentubo and Gompertiz, 2014; Sadati et al., 2015; Naz and Jadahy, 2015). Agar plate method was used to screened lipase fungi producer with activity on long-chain triacylglycerides. Winayanuwattikun et al. (2011) selected 39.17% strains with fluorescence halo on palm oil hydrolyze, indicating the lipase production with specificity on long-chain fatty acids. About the enzyme production and halo formation, Colen (2006) related that halo formation by degradation of lipid substrates and the enzyme produced do not present relation to the volumetric enzyme production under submerged conditions.

Table 1. Screening of filamentous fungi for lipolytic enzymes production with hydrolytic and esterification activities

Strains	Enzymatic Index*	Biomass (g.L ⁻¹)	Hydrolytic activity (U/mL)	Esterification (U/mL)	activity
F2	-	-	-	-	
F3	+	4.25	0.05	n.d.	
F4	-	-	-	-	
F5	+	3.79	0.07	0.09	
F7	+	3.89	0.04	n.d.	
F8	-	-	-	-	
F11	-	-	-	-	
F14	+	3.44	0.03	n.d.	
F15	+	3.42	0.02	n.d.	
F16	+	2.40	0.08	n.d.	
F20	+	1.66	0.02	n.d.	
F21	+	0.94	0.01	n.d.	
F22	+	9.16	0.05	0.02	
F23	-	-	-	-	
F24	+	10.76	0.07	n.d.	
F25	-	-	-	-	
F26	+	9.51	0.06	n.d.	
F28	-	-	-	-	
F29	-	-	-	-	
F30	+	7.00	0.07	n.d.	
F31	+	9.40	0.19	0.0	

Continua na (primeira)...

F32	+	8.91	0.08	n.d.
F33	+	8.39	0.12	0.06
F35	+	4.06	0.12	n.d.
F36	+	8.54	0.19	0.11
F37	+	8.76	0.19	0.08
F59	-	-	-	-
F61	+	5.12	0.62	0.09
F77	-	-	-	-
F85	-	-	-	-
F91	-	-	-	-
F104	+	8.51	0.18	0.04
F116	+	9.14	0.06	n.d.
F120	+	10.14	0.05	n.d.
F125	+	9.68	0.05	n.d.
F130	+	9.08	0.07	n.d.
F137	-	-	-	-
F307	+	9.68	0.16	0.10

Culture conditions: *agar plate was carried out at 28°C for 7 days; submerge cultivation were carried out in Vogel medium with 1% olive oil at 28°C, 180 rpm for 5 days. Legend: (+) presence of activity, (-) absence of activity; n.d. not activity detected under the assay conditions.

Filamentous fungi that presented positive enzymatic index on agar plate were cultivated under submerged conditions with olive oil as sole carbon source. In this experiment, the cultivations which presented hydrolytic activity on p-nitrophenyl palmitate were also evaluated to esterification activity. The culturing of fungus on liquid medium supplemented with inducer substrate has been widely used to screen potential lipase producers. This technique has helped to reveal the low producers of lipase that could not be identified in case of solid agar plate method. Further, variability of lipase activity has been experienced in the solid agar plate method with some strains showing intensive growth and the halos produced upon lipolysis of substrates could be covered by mycelium (Kotogán et al., 2014; Geoffry and Achur, 2018). Cognisant of such problem of visibility leading to difficulty in data comparison, it is important to grow the strains in liquid medium and further compare the lipase activity of the filtrates. Twenty-six strains that presented enzymatic index also presented hydrolytic activity, which the highest enzyme activity was observed with strain F61 (0.62 U/mL). Intermediary enzyme activity was observed with seven filamentous fungi (0.12 – 0.19 U/mL) and lowest enzyme activity (0.01 – 0.08 U/mL) was observed for seventeen filamentous fungi strains. Goldbeck and Maugeri Filho (2013) observed that from 372 strains studied, 207 displayed a hydrolysis halo around the colonies, representing 55.65% of the total. At this stage, from 44 strains were initially pre-selected and cultivated in liquid medium to the lipolytic activity

production, 11 strains were considered to show potential to lipase produce, since they produced the highest lipolytic activities. Almeida et al. (2013) evaluated the screening of 11 filamentous fungi and 8 yeast strains on agar plate, in which produced an opaque halo around them and were selected as lipase producers, representing 21 % of the microorganisms. The cultivation of these strains in liquid medium containing olive oil showed that 63% presented lipase activity on p-NPP hydrolysis. In other study, Griebeler et al. (2011) observed that among the 203 isolated strains, the filamentous fungi presented higher potential for lipase production both in tributyrin and hydrolytic activity; so, some microorganisms that presented hydrolysis halos did not present hydrolytic activity when cultivated in soybean bran.

Among the fungal strains that produced hydrolytic activities, the esterification activity was observed in 36% of total isolates. The highest esterification activity (0.11 U/mL) was observed with F36 strain, followed by F307 (0.10 U/mL), F5 and F61 (0.09 U/mL) and F37 (0.08 U/mL). Lipase in organic synthesis is very prominent and the screening of lipase that work adequately in organic medium for ester synthesis, converting triglycerides to alkyl esters and triacylglycerol interesterification has tremendous application particularly in food industry (Geoffry and Achur, 2018). Cardenas et al. (2001) selected fungal lipase that work adequately in organic medium for heptyl oleate synthesis which observed that *M. mucoroides*, *F. poae*, *F. solani* and *O. sulphureo-ochraceum* presented highest yield after a short reaction time of 4–8h. In this study, it was observed that lipases from *F. poae* and *F. solani*, which had displayed a low hydrolytic activity, were very active in the synthesis of heptyl oleate.

The amount of biomass produced by filamentous fungi indicate that each isolated strain could grow on liquid medium supplemented with olive oil and no correlation was found to exist between lipase and biomass production (Table 1). Maximal biomass production was observed for F24 and F120 strains (10.76 g.L⁻¹ and 10.14 g.L⁻¹, respectively) what produced 0.07 and 0.05 U/mL of hydrolytic activity, respectively. In the case of F61 strain, this isolated produced higher lipase activity (0.62 U/mL) and intermediary biomass (5.12 g.L⁻¹) compared to the other fungal isolates. Messias et al. (2009) also observed no correlation between lipase and biomass production by *Botryosphaeria* isolates on different oils and glycerol carbon sources. The authors observed that *Botryosphaeria ribis* EC-01 grown on glycerol

produced higher lipase titres and less biomass compared to the other vegetable oils evaluated.

3.2. Molecular identification of fungal strains

Ten filamentous fungi that presented potential to produce lipase with hydrolytic and esterification activities were identified by microcultivation technique, based on shape of fructification body, and by molecular technique, based on ribosomal targets ITS-1 and ITS-4 (Table 2). The number of ITS sequences available in public databases has increased significantly, and the expanding database may improve the quality and accuracy of fungal identification (Goldbeck and Maugeri Filho, 2013). By morphological classification, the filamentous fungi strains were identified as belonging to the *Trichoderma* spp. (strains F5, F37 and F307), *Penicillium* sp. (F36) and *Fusarium* sp. (F61). A BLAST search in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the ITS-1 and ITS-4 sequenced fragment showed 99% sequences identity with *Trichoderma harzianum* for F37 and 307 strains and *Fusarium solani* F61. Genera *Fusarium* have been widely recognized as lipase producers (Maia et al., 2001; Liu et al., 2009; Oliveira et al., 2013). *Fusarium solani* is among well-known lipase producers. Studies on fermentation conditions to produce extracellular lipase from *F. solani* showed variations among different strains of the fungus.

Table 2. Molecular identification of filamentous fungi producing lipase

Strains	Species	Similarity index (%) %	GenBank accession numbers
			ITS
F03	<i>Aspergillus calidoustus</i>	99	MK649917
F21	<i>Chaetomium aureum</i>	99	MK649918
F37	<i>Trichoderma harzianum</i>	99	MK649919
F61	<i>Fusarium solani</i>	99	MK649920
F104	<i>Aspergillus flavus</i>	99	MK649921
F116	<i>Aspergillus flavus</i>	99	MK649922
F120	<i>Penicillium citrinum</i>	99	MK649923
F125	<i>Aspergillus</i> sp	99	MK649924
F130	<i>Aspergillus oryzae</i>	98	MK649925
F307	<i>Trichoderma harzianum</i>	99	MK649926

3.3. Medium composition for lipase production

Minerals in the medium composition affected cell wall permeability or buffered the medium and hence increased the enzyme production. The quantity and quality of

nutrients available and the ability to assimilate successfully are the major determinants of microbial nature and its metabolic activity. In this work, the effect of mineral composition was evaluated to lipolytic enzyme production by *Trichoderma* sp. F5, *T. harzianum* F37, *T. harzianum* F307, *Penicillium* sp. F36 and *F. solani* F61 under submerged conditions, using olive oil as sole carbon source (Table 3). The highest lipase production was observed using medium M3 with *F. solani* F61 (2.37 U/mL), which presented esterification activity of 0.07 U/mL. Yield fermentation ($Y_{P/S0}$) was 218.04 U/g of substrate. Medium M3 is composed by potassium, magnesium, phosphorus, copper and sulfur. Lipase production was increased in 3.8-fold using this culture medium. Pera et al. (2006) developed this culture medium to produce lipase by *Aspergillus niger* under submerged conditions. Lipases from *Fusarium* spp. are known to exhibit some interesting properties, such as their stability in polar organic solvents like ethanol, acetone, and n-propanol. This characteristic is an important prerequisite for using such enzymes in transesterification reactions in the presence of short-chain alcohols to produce biodiesel (Oliveira et al., 2013).

Table 3. Media composition for lipolytic enzyme production with hydrolytic and esterification activities for *Trichoderma* sp, *Penicillium* sp, *F. solani* and *T. harzianum*.

Linhagens	Media compositions	Hydrolytic activity (U/mL)	$Y_{P/S0}$ (U/g substrate)	Esterification activity (U/mL)
<i>Trichoderma</i> sp F5	M1	0.71	59.64	n.d
	M2	0.06	5.04	0.05
	M3	0.03	2.40	0.03
	M4	0.05	4.00	n.d
	M5	0.03	2.52	n.d
	M6	0.03	2.52	n.d
<i>Penicillium</i> sp F36	M1	0.63	52.92	n.d
	M2	0.24	22.08	n.d
	M3	0.10	8.00	n.d
	M4	0.18	15.12	n.d
	M5	0.19	17.48	0.04
	M6	0.02	1.76	0.09
<i>T. harzianum</i> F37	M1	0.42	36.96	0.03
	M2	0.02	1.68	0.08
	M3	0.16	15.36	n.d
	M4	0.10	8.80	n.d
	M5	0.24	20.16	n.d
	M6	0.08	7.04	0.05
<i>F. solani</i> F61	M1	0.73	67.16	n.d

Continua (na primeira)...

	M2	0.35	32.2	n.d
	M3	2.37	218.04	0.07
	M4	0.45	46.06	0.03
	M5	0.65	57.20	0.01
	M6	0.02	1.84	n.d
<i>T. harzianum</i> F307	M1	0.42	40.32	0.05
	M2	0.03	2.88	0.18
	M3	0.06	5.76	0.05
	M4	0.04	3.52	n.d
	M5	0.24	23.04	0.19
	M6	0.04	3.36	0.02

Culture conditions: culture were carried with 1% olive oil and 0,2% yeast extract, 28°C, 180 rpm for 5 days. Legend: n.d. not activity detected under the assay conditions.

In this work, the medium composition influenced strongly on lipase production by *F. solani* F61 where presence of calcium (CaCl_2), zinc (ZnSO_4), iron (FeSO_4) and manganese (MnSO_4) on the media decrease sharply the enzyme production. On the other hand, for *Trichoderma* sp. F5, *Penicillium* sp. F36, *T. harzianum* F37, *T. harzianum* F307 the highest lipase production was observed using Medium 1 (M01) (0.42 – 0.73 U/mL), what is composed by $\text{MgSO}_4 \cdot \text{H}_2\text{O}$; K_2HPO_4 and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Esterification activity under these conditions were only observed to *T. harizianum* F37. The highest esterification activity was observed with *T. harzianum* F307 (0.18 U/mL) using medium 2 (M2). Filamentous fungi strains presented different behavior against the medium composition indicating that the better conditions for lipase production need to be optimized for each fungi strain to determine the ideal nutrient concentration in the cultures. Sharma et al. (2001) reported that trace elements are generally structural components and stabilizers of enzymes, whereas ions such as Mg, Fe, Ca, Cu, Co, Na, K, Mn and Zn appear to positively influence lipase production. The effect of the salts on the properties of the proteins is perceived by the activity, conformational stability and solubility (Pacheco et al., 2015). These effects may appear due to ionic bonds at specific sites of the proteins, altering their level of hydration (Yu et al., 2005). Magnesium salt, for example, is required by most microorganisms because of their ability to perform some regulatory functions associated with increased metabolism (Bankar et al., 2009). Other micro-nutrients needed to regulate these functions are potassium, considered essential for osmoregulation (Salihu et al., 2012), iron which in turn is essentially used for

synthesis of heme and cytochrome (Venkateshwar et al., 2010) and the calcium, considered necessary for the effective stabilization of lipases (Snellman and Colwell 2004). These characteristics can be associated with structural changes of the enzyme and the fact that the salts increase the dispersion of enzymatic molecules, facilitating mass transfer during the reaction (Pacheco et al., 2015).

3.4. Nitrogen sources on lipase production

The effect of nitrogen sources on lipase production by *F. solani* F61 was carried out using the medium M3 supplemented with 1% (w/v) olive oil (Table 4). The concentrations organic and inorganic nitrogen sources in the culture medium promoted an increased of lipase production; but the maximum enzyme production was observed with 2% (w/v) tryptone (3.22 U/mL), followed by yeast extract at 1% and 2% (3.22 U/mL and 3.14 U/mL, respectively). Under these conditions were also observed the highest biomass values (10.48 – 15.19 g.L⁻¹). Corn steep liquor and peptone provided intermediary values of lipase production, while urea, ammonium chloride and ammonium sulfate provided the lowest values of enzyme production. It was observed that enzyme production was not associated to the biomass formation, indicating that the nitrogen sources can modulate the lipase secretion to the extracellular medium. Fermentation parameters showed that yield of fermentation ($Y_{P/SO}$) with tryptone was 278.40 U/g; esterification activity was highest when ammonium chloride (0.23 U/mL), followed by ammonium sulfate (0.17 U/mL) at 2% (w/v). Nitrogen sources, including organic nitrogen and inorganic nitrogen sources, play an important role in the synthesis of enzymes. Because inorganic nitrogen sources can be used quickly, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. Therefore, both organic sources and inorganic sources are used in lipase fermentation (Tan et al. 2004). Beside this, in the industrialization of fermentation processes, the nutrient cost of the medium is one of the most important factors, and supplementation of nitrogen source is too costly for commercial production of enzymes and biofuels, because it could govern 50% of the overall medium cost (Edwinoliver et al., 2009).

Table 4. Source of nitrogen for lipolytic enzyme production with hydrolytic and esterification activities for *F. solani* F61

Nitrogen sources	Concentration (%)	Biomass (g.L ⁻¹)	Hydrolytic activity (U/mL)	$Y_{(P/SO)}$ (U/g)	Esterification activity (U/mL)
Organic sources					
Corn steep liquor	0.2	5.52	0.05	5.25	n.d
	1.0	7.54	0.49	44.10	n.d
	2.0	9.45	1.10	71.50	n.d
Peptone	0.2	6.86	0.21	22.05	n.d
	1.0	8.21	2.15	204.25	0.04
	2.0	9.43	1.34	127.30	n.d
Continua na (primeira)...					
Tryptone	0.2	4.45	0.96	91.20	n.d
	1.0	10.48	2.89	231.30	0.05
	2.0	15.19	3.48	278.40	0.02
Urea	0.2	6.54	0.11	11.50	0.01
	1.0	7.72	0.41	45.10	n.d
	2.0	7.64	0.57	62.70	n.d
Yeast extract	0.2	3.18	2.37	237.00	0.03
	1.0	10.88	3.22	241.50	0.03
	2.0	14.85	3.14	251.20	n.d
Inorganic sources					
Ammonium chloride	0.2	3.54	0.05	5.00	n.d
	1.0	0.10	0.10	10.50	0.06
	2.0	0.17	0.16	17.60	0.23
Ammonium sulfate	0.2	7.06	0.06	6.00	0.02
	1.0	3.01	0.11	12.10	0.10
	2.0	6.78	0.21	24.15	0.17

Culture conditions: submerge cultivation were carried out in medium containing g.L⁻¹ - KH₂PO₄ – 1, MgSO₄.H₂O – 1,123 e CuSO₄ – 0,06. Supplemented with 1% olive oil at 28°C, 180 rpm for 5 days.

Legend: n.d. not activity detected under the assay conditions.

3.5. Carbon sources on lipase production

Several vegetable oils from Amazonian region were used as carbon sources in substitution to olive oil. Cultures were carried out using tryptone and ammonium chloride at 2% (w/v) as nitrogen source (Table 5). Cotton oil used as carbon source by *F. solani* F61 presented the highest lipase production value (8.14 U/mL) followed by soybean oil (6.17 U/mL) using tryptone as nitrogen source. Cotton oil is composed by palmitic acid (17-29%), stearic acid (1-5%), oleic acid (14-44%) and linoleic acid (33-58%), while soybean oil is composed by palmitic acid (2-13%), stearic acid (2-6%), oleic acid (18-31%), linoleic acid (49-57%) and linolenic acid (2-10%). The

production of lipases in cotton and soybean oils may be related to oleic acid, since it is known as an inducer in the production of lipase and is present in the composition of cotton and soybean oils in greater quantity than the other oils used (exception , and may be responsible for the higher enzymatic production, in addition to having been cited in the literature as a good inducer of lipase production (LI; WANG; TAN, 2006). Parameter fermentation for lipase production were $Y_{P/SO} = 732.60$ U/g of substrate using cotton oil and $Y_{P/SO} = 524.45$ U/g of substrate for soybean oil. On the other hand, the usage of Amazonian oil with ammonium chloride as nitrogen source presented the lowest value for lipase production (0.12 – 0.88 U/mL); however, the highest esterification activities were observed with copaiba oil (0.43 U/mL), babassu oil (0.41 U/mL) and castor oil (0.40 U/mL). Biomass production by *F. solani* F61 was also higher when the organic nitrogen with different Amazonian oils were used (9.0 - 14.5 g.L⁻¹), while for inorganic nitrogen source present the lowest biomass production (1.7 – 8.6 g.L⁻¹) Although copaiba oil has a large amount of oleic acid, the enzymatic production of lipase was not high, as well as its biomass production, showing that in high amounts oleic acid concentrations can also inhibit lipase production (WANG; XU, SHAN, 2008).

Table 5. Carbon Source for lipolytic enzyme production with hydrolytic and esterification activities for *F. solani* F61

Carbon sources	Biomass (g.L ⁻¹)	Hydrolytic activity (U/mL)	$Y_{(P/SO)}$ (U/g)	Esterification activity (U/mL)
Tryptone				
Castor oil	13.99	3.77	339.30	0.09
Pequi Oil	13.63	2.88	273.60	0.10
Babassu Oil	14.55	3.50	192.50	0.03
Copaiba Oil	8.98	0.29	29.00	0.07
Buriti oil	13.14	4.51	451.00	0.14
Degummed Soybean Oil	13.95	6.17	524.45	0.07
Cotton Oil Degomado	13.94	8.14	732.60	0.05
Frying Oil	13.00	5.02	351.40	0.05
Ammonium choride				
Castor oil	8.64	0.18	19.80	0.40
Continua na (primeira)...				

Pequi Oil	5.70	0.12	14.40	0.30
Babassu Oil	4.59	0.88	105.60	0.41
Copaiba Oil	3.03	0.12	13.80	0.43
Buriti oil	5.98	0.24	27.60	0.36
Degummed Soybean Oil	5.10	0.14	14.00	0.36
Cotton Oil Degomado	4.02	0.39	42.90	0.28
Frying Oil	1.74	0.14	16.10	0.29

Culture conditions: submerge cultivation were carried out in medium containing g.L^{-1} - KH_2PO_4 – 1, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ – 1,123 e CuSO_4 – 0,06. Supplemented with 1% carbon source, 2% Triptone and 2% Ammonium choride at 28°C, 180 rpm for 5 days. Legend: n.d. not activity detected under the assay conditions.

3.6. Time-course of lipase production

F. solani F61 was cultured for 240 hours to verify the time-course for lipase production using tryptone 2% (w/v), cotton oil 1% (w/v), 28 °C and 180 rpm (Figure 1). In the first 24 hours of cultivation, lipase production was observed in 0.80 U/mL with continuous increase up to 72 hours of culture (10.68 U/mL) and yield of fermentation was 1,158.00 U/g of substrate. Under these conditions, the enzyme production was associated to microbial growth once the maximum lipase production was observed in the end of log phase. After this period the enzyme production decreased continuously reaching 240 hours. Winaynuwattikun et al. (2011) evaluated the time-course for lipase production by *F. solani* strain and the maximum enzyme production was observed after 72 hours (1.26 U/mL) during the initial stationary phase. Salihu et al. (2011) evaluated the lipase production by several microorganisms isolated from palm oil factory effluents. In this study, the *C. cylindracea* was observed to produce maximum enzyme activity after 144 h (2.1 U/mL). The decrease of enzyme production observed after 72 hours of cultivations for *F. solani* F61 can be associated to nutrient depletion of protease activity. Mahanta et al. (2008) observed these conditions during the solid-state cultivation of *Pseudomonas aeruginosa* in that the decrease in enzyme activity was associated to nutrient depletion or denaturation of the enzyme caused by interaction with other components in the environment or alteration in the pH of the medium. Long et al. (2007) reported that several factors are known to influence the expression of lipase activity among the components of the medium, such as carbon sources, nitrogen, as

well as the inducers in the form of oils, fatty acid and esters. these factors contribute most to the production of lipases.

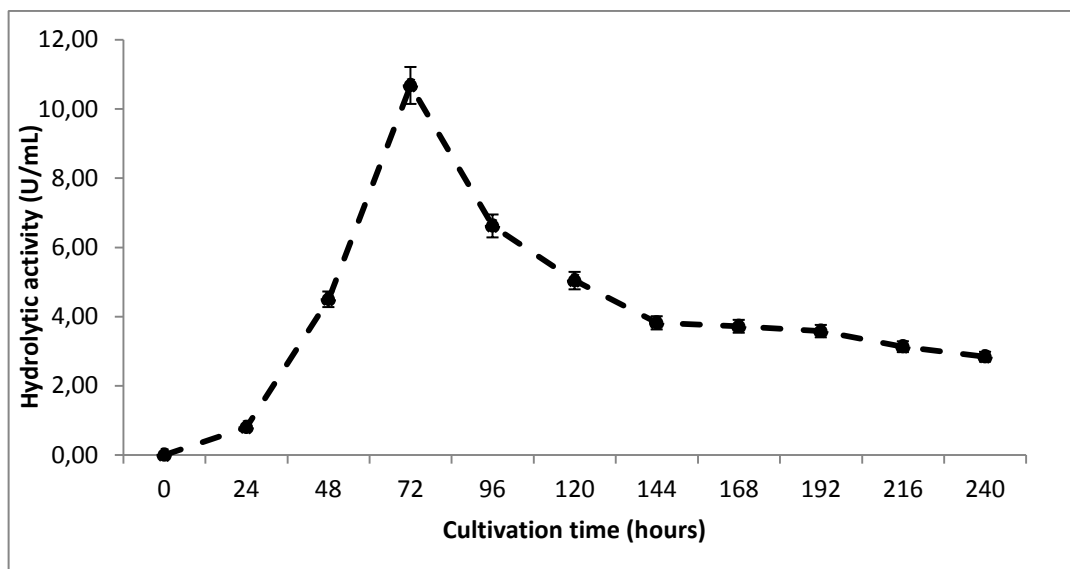


Figure 1 kinetics for production lipolytic enzyme production with hydrolytic and esterification activities for *F. solani*.F61

4. Concluding remarks

The selection of lipolytic filamentous fungi isolated from senescent plant material in Cerrado streams showed that, even under conditions not suitable for the induction of lipases, it is possible to find microorganisms with potential for enzymatic production in this habitat, which are probably from the vegetation, which use the oils produced by the plant as an energy source. It is evidenced in this work the potential of the numerous "wild" strains tested for the production of lipases using unconventional oils, requiring the same of a more in-depth study as to the optimization of its production and activity. The basal composition of the culture medium was essential to increase lipase production, and its supplementation with tryptone (2% w/v) and degummed cottonseed oil (1% w/v) increased production by 13.3-fold (8.14 U/mL). The growth curve of the fungus showed that the highest production of lipases was observed after 72 hours of culture (10.68 U/mL). Under these conditions, there was a 17.22-fold increase in enzyme production. The enzyme produced by *F. solani* F61 showed potential to be used in hydrolysis and esterification processes in organic medium to produce fatty acid esters and transesterification.

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ARTIGO SUBMETIDO**CAPÍTULO III****OPTIMIZATION IN THE LIPASE PRODUCTION BY *Fusarium solani***

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Abstract

A Plackett-Burman factorial design with 15 experiments was conducted to evaluate the influence of seven factors on lipases production by *Fusarium solani*. The factors investigated were peptone, tryptone, yeast extract, calcium chloride, potassium phosphate, magnesium sulfate and copper sulphate. Five fixed variables (cotton oil, pH, temperature, agitation and time) were maintained and as a response the enzymatic activity. The concentration of tryptone, calcium chloride and magnesium sulphate had a significant effect ($p < 0.10$) on lipase production and was studied consecutively through a complete DCCR (central rotational compound design), in order to optimize lipase production of the fungi *F. solani*. After optimization using DCCR, maximum lipolytic activities 24.84 U/ml were obtained, with the use of 10 g.L⁻¹ tryptone, 3.50 g.L⁻¹ calcium chloride and 0.50 g.L⁻¹ magnesium sulfate, 1 g.L⁻¹ potassium phosphate and 1% soybean oil. The statistical model showed a correlation of 85.67% with the experimental data. The biochemical characterization of lipase showed that the enzyme has a better performance at pH 7 at a temperature of 40 °C, where the statistical model had a correlation of 94.15% with the experimental data. In this way, lipases produced by *F. solani* have potential for application and use in biodiesel production.

Keywords: Lipase, Otimização, Hidrólise, esterificação, *Fusarium solani*.

1. Introduction

Currently, enzymes have several industrial applications (HAACK et al., 2006) and among them, lipases are widely used (COLLA et al., 2016). Their applications result from the ability to catalyze reactions, especially hydrolysis and inter and transesterification of lipids, making these enzymes useful in several sectors, including the production of lipids with high levels of unsaturated fatty acids (RESHMA et al., 2008) and methyl- esters of fatty acids (biodiesel) (PARK et al., 2006).

Industrial enzymes are mainly produced by submerged fermentation in batch and fed-batch cultures (HAACK et al., 2006) using filamentous fungi. Filamentous fungi, especially those belonging to genera *Aspergillus*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Rhizomucor* and *Thermomyces* are known as the best microbial agents producing lipases (MESSIAS et al., 2011). Due to the wide versatility of action of this group of filamentous fungi in producing lipases, its industrial and technological use has grown significantly (MACIEL et al., 2010). These industrial applications stimulated interest in the isolation of new lipases from new sources (DHEEMAN et al., 2011). Submerged processes have some advantages over solid-state processes, such as higher homogeneity of the culture medium and easier control of parameters, such as temperature and pH.

Coradi et al. (2013) mentioned that lipases produced by submerged fermentation have a better recovery of extracellular enzymes and of the biomass determination is facilitated by simple filtration or centrifugation. Other factors, such as nutrient types and concentrations, pH, agitation, and the presence and concentration of inducers may affect the productivity of these bioprocesses. Researches using microorganisms isolated from new environments and using agro-industrial waste in the composition of mediums are necessary to obtain high yields at lower costs.

The statistical optimization of the processes has advantages over the classical practice of changing one variable at a time (BOX et al., 1978; KAUSHIK et al., 2006) with a smaller number of experiments and the possibility of evaluating the effects of interaction between the variables. Many researchers have reported the use of these techniques for the production of lipases by microorganisms (BURKERT et al., 2004; WANG et al., 2008). An efficient and widely used approach is the application of Plackett-Burman projects that make possible the efficient triage of key-variables for additional optimization rationally. The objective of this work was to optimize lipase production by the fungus *F. solani*, evaluate the significant variables for lipase

production through submerged fermentation and optimize these variables through the response surface methodology.

2. Material and methods

2.1. Fungal strain used

The fungal strain selected was *F. solani* obtained from a previous work where, after the shaker incubation, the isolate was tested for lipase activity, so this strain was selected because it had the highest enzymatic activity.

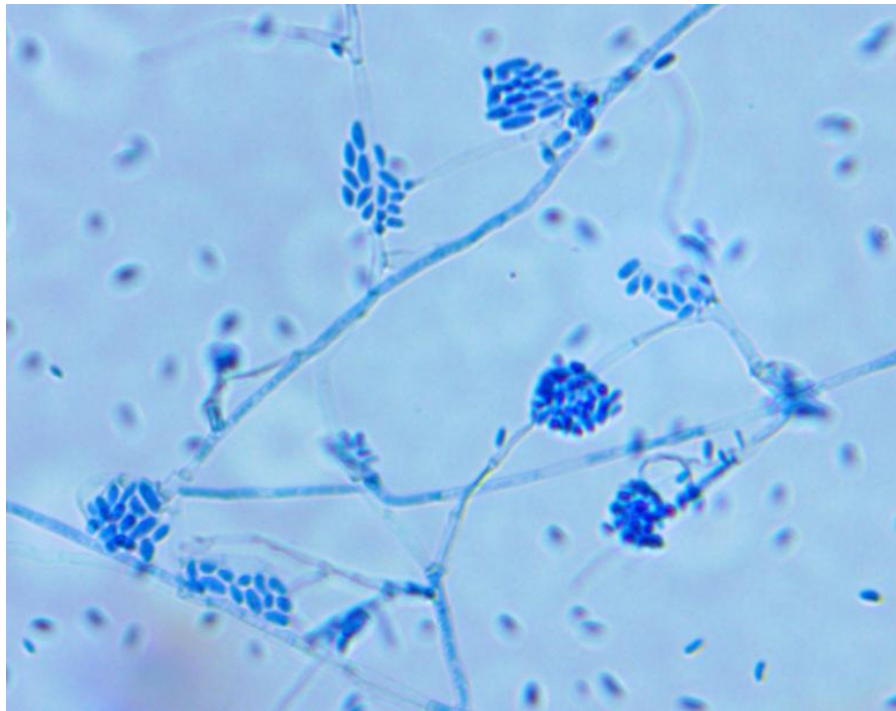


Figure 1. Morphology of *Fusarium solani* (zoomed 40 times)
(Foto: Danylo Mendes)

2.2. Culture medium

The selected *F. solani* strain was tested for the ability to produce enzymes (lipases) by submerged fermentation in the basal medium containing the following composition: KH_2PO_4 1,00 g.L^{-1} ; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 1,123 g.L^{-1} ; CuSO_4 0,06 g.L^{-1} (PERA et al., 2006), supplemented with 1% cotton oil as carbon source, 2% tryptone, 2% peptone and 2% yeast extract as a nitrogen source.

2.3. Inoculum standardization and the obtaining of the crude enzymatic extract by submerged fermentation

The selected *F. solani* strain was grown in culture medium prepared by diluting 39 g.L⁻¹ potato dextrose agar (BDA) in distilled water, plus 0.2% chloramphenicol. The pH was adjusted to 5. The medium was autoclaved at 121 °C for 15 min at 1 atm pressure.

The inoculation was performed punctually in the center of the Petri dishes containing the culture medium. Cultures were kept in a BOD greenhouse at 28 °C for 7 days. The surface of all the plates was added 5 mL of 0.85% NaCl saline, and was later gently scraped with sterile stiletto to obtain spores. The suspension was filtered and the spore concentration of the filtrate was determined in Neubauer bright-line Optik Labor chamber. The suspension, when necessary, was adjusted so that the spore count was close to the concentration of 1x10⁷ spores.mL⁻¹, concentrating it with more inoculum or diluting it with saline.

The crude enzymatic extract was obtained by culturing the lineages in *Shaker Cienlab* CE-725 R, for 5 days at 28 °C and 180 rpm. The medium used is described in item 2.2. After the culture, the medium was subjected to filtration of the solids, the filtrate was used as an enzymatic extract for analytical tests and the crude extracts were stored in glass flasks at -10 °C in the Laboratory of Environmental Chemistry and Biofuels Research (LAPEQ - UFT).

2.4. Experimental design

2.4.1. Plackett and Burman (PB) 7 variables

The experimental design carried out in the first stage of the work aims to evaluate the variables effect for optimizing the composition of the culture medium used for lipase production. This was obtained from a Plackett and Burman type design with fifteen experiments, with three central points, having seven independent variables analyzed (peptone, tryptone, yeast extract, calcium chloride, potassium phosphate, magnesium sulphate and copper sulphate), five fixed variables (cotton oil, pH, temperature, agitation and time) and as a response, the enzymatic activity was obtained.

This experimental design presents an effect table and a Pareto chart, where it is possible to analyze the influence of the main effects, indication of the variables that

should be included in the subsequent planning and the new ranges that should be studied for each variable.

2.4.2. Central Rotational Compound Design (DCCR) 3 variables

Through the analysis of the first experimental design, it was possible to carry out a central rotational compound type design with the culture medium used for lipase production. Each parameter was studied in four different levels (-1.68, -1, 0, +1, +1.68). All parameters were taken as a central coded value, considered as an ideal point of production, and a matrix of 17 experiments with three factors was generated by using the program Protimiza (2014). In the DCCR, three central points were used to verify the process reproducibility, whose three independent variables (tryptone, calcium chloride and magnesium sulfate) and six fixed ones (potassium phosphate, cotton oil, pH, temperature, agitation and time) were analyzed and obtained the enzymatic activity as a response.

This experimental design aimed to study the calculation of the effects, to statistically analyze the experiment and to evaluate the surface response graphs, thus obtaining the optimization of the culture medium for lipase production.

2.5. Validation of the experiment

An experimental design was carried out using the Minitab 18 statistical software, where the results of the central rotational compound design (DCCR) 3 variables were added to validate the lipase production experiment. The optimum condition for lipase production was determined with the program from the strain analyzed and the experiment was conducted to verify the results. Six experiments were carried out with the same conditions to verify the reproducibility of the results, and three independent variables (tryptone, calcium chloride and magnesium sulphate), six fixed variables (potassium phosphate, cotton oil, pH, temperature, agitation and time) and the enzymatic activity as a response.

2.6. Biochemical characterization

2.6.1 Determination of optimum temperature and pH

A DCCR was performed with eleven experiments, of which three central points, having two independent variables analyzed (pH and temperature) and

obtaining as response the enzymatic activity. Each parameter was studied in four different levels (-1.41, -1, 0, +1, +1.41). The objective of this experimental design was to study the effects, statistically analyze the experiment and evaluate the response surface graphs, thus obtaining the definition of the best temperature and pH for the optimization experiments for lipase production.

2.6.2 Determination of pH stability

The determination of pH stability was performed according to Jinaporn et al. (2016), with modifications.

Optimum pH of lipase activity was determined under lipase assay conditions using different pHs buffers. The McIlvaine buffer (Na_2HPO_4 and citric acid pH 3.0 - 8.0) was used. *P*-nitrophenyl palmitate (p-NPP) was used as the substrate at a reaction time of 1 min at 40 °C. The stability effect on pHs on lipase activity was studied over 0, 24, 48 and 72 hours of incubation.

2.6.3 Determination of thermal stability

The determination of lipase thermal stability was defined by incubating it at optimum pH and different temperatures: 35, 40, 50 and 60 °C throughout the times to its half-life (0, 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 60 and 80 minutes). The effect of temperature on lipase activity was studied using the p-NPP test as substrate in McIlvaine buffer for 1 min reaction at 40 °C.

2.7. Determination of activity and stability in solvents

The effect of various organic solvents on the lipase activity at 10% v / v (50 µL of organic solvents plus 450 µL of crude enzyme) was investigated using several water immiscible organic solvents (toluene, hexane, xylol) and soluble-in-water organic solvents (methanol, ethanol, glycerol, butanol, propanol, 2-propanol, iso-butyl, acetone, acetonitrile and dimethylsulfoxide (DMSO)). The mixture was incubated in tubes and the lipase activity was studied using the p-NPP assay as a substrate in McIlvaine buffer for 1 min reaction at 40 °C. The activity at incubation was determined at 0, 8, 12, 24, 32, 36 and 48 hours or until its half-life. A sample without addition of organic solvents was taken as control.

2.8. Determination of pH

The determination of pH was performed by reading the samples in a pH meter of the brand *Tecnal*, model TEC – 5.

2.9. Determination of hydrolytic activity

Lipase activity was determined with *p*-nitrophenyl palmitate (*p*-NPP) as substrate, according to Almeida et al. (2013). The *p*-NPP was dissolved in 0.5 mL of dimethylsulfoxide (DMSO) and then diluted to 50 mM with 50 mM sodium phosphate buffer pH 7.0 containing 0.5% (w / v) Triton X-100. The hydrolysis of *p*-NPP was determined discontinuously at 40 °C by measuring the *p*-nitrophenol release. After 5 min of preincubation of 0.9 mL of the substrate and addition of 0.1 mL of properly diluted enzyme sample, the reaction was started. The reaction was then stopped at different intervals by thermal shock at 90 °C for 1 min, followed by the addition of 1 mL of saturated sodium tetraborate solution. The absorbance was measured at 410 nm, which corresponds to the molar extinction coefficient for *p*-NP: $1.8 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$. Controls were prepared without enzyme. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP per minute.

2.10. Determination of the production of fungal biomass

After cultivation for inoculum standardization, the mycelium was vacuum filtered by using *Büchner's* funnel and *Wathman* No. 1 filter paper. After the solids filtration, the moist mycelium was oven dried at 65 °C for 1 day and then weighed with the analytical balance *Shimadzu*, model AX 200, for determining biomass production according to Berovic et al. (2003).

3. Results and discussion

3.1. Delineamento experimental Plackett e Burman (PB) 7 variáveis

The chosen experimental design of the type Plackett and Burman was important, because the independent variables were studied to obtain the best formulation for the production of lipases by *F. solani*. The actual decoded values used for this planning are set out in Table 1 with the encoded levels. The experimental design PB 7 is presented in Table 2.

Table 1. Range of values studied in experimental planning PB 7, optimization in the production of lipases.

Variable name	-1	0	+1
Peptone*	0	10	20
Yeast extract*	0	10	20
Tryptone*	0	10	20
CaCl₂.2H₂O*	0	3,50	7
KH₂PO₄*	0	1	2
MgSO₄.7H₂O*	0	0.50	1
CuSO₄*	0	0.06	0.12

* Unitis g.L⁻¹

Table 2. First experimental design PB 7, optimization in the production of lipases.

Experiments	Coded variables							Real variables*							Enzymatic activity (Y1)
	(x1)	(x2)	(x3)	(x4)	(x5)	(x6)	(x7)	Peptone	Yeast extract	Tryptone	CaCl ₂ .2H ₂ O	KH ₂ PO ₄	MgSO ₄ .7H ₂ O	CuSO ₄	
1	1	-1	1	-1	-1	-1	1	20	0	20	0	0	0	0.12	8.04
2	1	1	-1	1	-1	-1	-1	20	20	0	7	0	0	0	0.55
3	-1	1	1	-1	1	-1	-1	0	20	20	0	2	0	0	7.87
4	1	-1	1	1	-1	1	-1	20	0	20	7	0	1	0	0.54
5	1	1	-1	1	1	-1	1	20	20	0	7	2	0	0.12	1.47
6	1	1	1	-1	1	1	-1	20	20	20	0	2	1	0	6.27
7	-1	1	1	1	-1	1	1	0	20	20	7	0	1	0.12	1.09
8	-1	-1	1	1	1	-1	1	0	0	20	7	2	0	0.12	2.04
9	-1	-1	-1	1	1	1	-1	0	0	0	7	2	1	0	0.07
10	1	-1	-1	-1	1	1	1	20	0	0	0	2	1	0.12	0.48
11	-1	1	-1	-1	-1	1	1	0	20	0	0	0	1	0.12	0.77
12	-1	-1	-1	-1	-1	-1	-1	0	0	0	0	0	0	0	0.05
13	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.64
14	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.69
15	0	0	0	0	0	0	0	10	10	10	3.5	1	0.5	0.06	1.67

* Unitis g.L⁻¹

Experiment 1 obtained the highest activity index 8.04 U/mL after the fifth day of fermentation due to the influence of the interactions between the variables. Experiment 3 also had a result of 7.87 U/mL, close to the maximum found in this step.

Observing the results at the central points (experiments 13, 14 and 15), it is possible to notice that there was no great variation in the values of lipolytic activity, indicating, therefore, a good reproducibility in the data of the process. Burkert et al. (2004), report that central points provide additional degrees of freedom for error estimation, which increases power by testing the significance of effects. The estimate of a main effect is obtained by evaluating the difference in process performance caused by a change from the low level (-1) to the high level (+1) of the corresponding factor.

The lipolytic activity was evaluated at three different pHs: 5, 6 and 7. The major activities were determined at pH 7, which was defined as a parameter for other stages of this work. The analysis of the statistical data obtained from the experimental planning PB 7 allows us to obtain an effect table and a Pareto chart, where it is possible to verify which variables are statistically significant to the process.

Table 3 shows the main effects of the variables in PB 7 planning after the fifth day of fermentation. It was verified that only three of the variables studied had a statistically significant effect on the lipolytic activity when the concentration changed from level -1 to level +1.

Table 3. Main effects of the variables in PB 7 for optimization in the production of lipases.

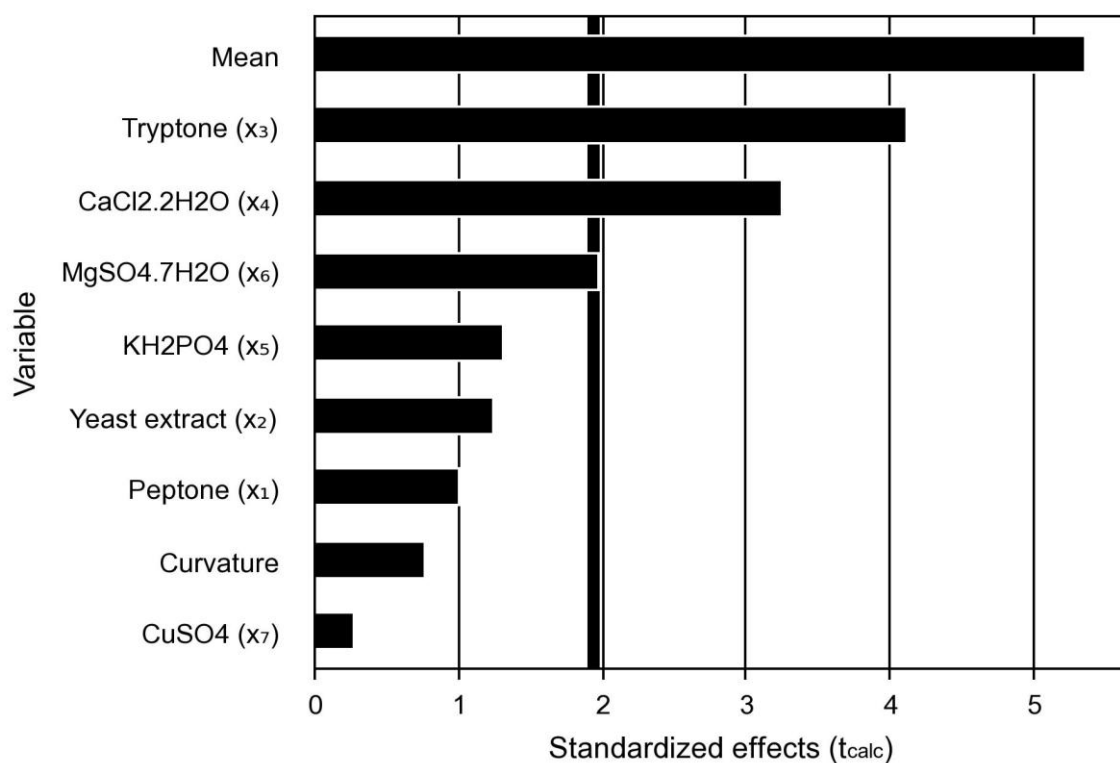
Name	Effect U/mL	Standard error	Calculated t	p-value
Mean	2.44*	0.45*	5.36*	0.0017*
Curvature	-1.54	2.03	-0.76	0.4773
Peptone (x1)	0.91	0.91	1.00	0.3554
Yeast extract (x2)	1.13	0.91	1.25	0.2589
Tryptone (x3)	3.74*	0.91*	4.12*	0.0062*
CaCl ₂ .2H ₂ O (x4)	-2.95*	0.91*	-3.25*	0.0175*
KH ₂ PO ₄ (x5)	1.19	0.91	1.31	0.2372
MgSO ₄ .7H ₂ O (x6)	-1.80*	0.91*	-1.98*	0.0950*
CuSO ₄ (x7)	-0.24	0.91	-0.27	0.7979

* Statistically significant values at 90% confidence ($p < 0.10$)

The calculated t-value measures the effect amplitude in relation to its standard error. This value was obtained by dividing each effect value by its standard error. The low “p” values were associated with the larger “t” value because they imply that the effects (or coefficients) were much larger than the standard error.

According to the effects presented in Table 3, it was found that tryptone concentration was the only one with a positive effect on lipolytic activity, that is, there was an increase in enzymatic activity when the concentration of this variable went from level -1 to 1. The tryptone concentration had a positive effect of 3.74 U/mL, being statistically significant at 90% confidence. On the other hand, the concentrations of calcium chloride and magnesium sulfate had a negative effect of -2.95 U/mL and -1.80 U/mL respectively, statistically significant at 90% confidence, since there was a decrease in activity when the concentration went from level -1 to +1.

Graph 1 represents the values reported in table 3 where it is possible to observe the three variables that were statistically significant. Considering the results presented and observed in the Pareto graph, potassium phosphate, yeast extract, peptone and copper sulfate are excluded for the next planning because they are not statistically significant to the process.



Graph 1. Main effects of the significant variables in PB 7.

From the results obtained in this experimental design, tryptone, calcium chloride and magnesium sulfate were selected as variables to be studied in a complete experimental design, with aiming to optimize the culture medium for the lipases production.

3.2. Central Rotational Compound Design (DCCR) 3 variables

This experimental design of the DCCR type was chosen to optimize the culture medium for the lipases production by *Fusarium solani*. Based on the results obtained from the previous experimental planning PB 7, only 3 variables were analyzed, tryptone, calcium chloride and magnesium sulfate, standing fixed potassium phosphate, cotton oil, pH, temperature, agitation and time, obtaining as response the enzymatic activity.

In this stage of the work, yeast extract, peptone and copper sulfate were not included, as these were not statistically significant as seen in Pareto graph 1 in the previous step. Potassium phosphate even though having a negative effect and not being statistically significant in the process, such a micronutrient is biologically essential for the development and production of enzymes by the evaluated strain as this is the only source of phosphorus available to the microorganism. As the purpose of this experiment is to optimize the culture medium in order to obtain maximum lipolytic activity, the addition of potassium phosphate was maintained as a necessary micronutrient to the culture medium.

The minimum and maximum ranges of the parameters were investigated and the complete experimental design with their values in real and codified form are listed below in Table 4. The average maximal lipase activity was taken as the dependent variable or response (Y1). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured in the independent parameters Eq. (1). Only the central point was made in triplicates. For a three-factor system, the equation of the model is as follows:

$$Y_1 = 24,15 + 1,57 x_1^1 - 6,14 x_1^2 - 1,54 x_2^2 - 2,23 x_2^2 + 0,67 x^3 - 3,45 x_3^2 + 2,08 x_1 x_2 - 0,12 x_1 x_3 + 0,51 x_2 x_3 \quad \text{Eq.(1)}$$

where, Y_1 is the predicted response, X_1 , X_2 , X_3 are the linear coefficients; X_1^2 , X_2^2 , X_3^2 are the quadratic coefficients and $X_1 - X_2$, $X_1 - X_3$, $X_2 - X_3$ are the interaction coefficients.

The actual decoded values used for this planning are arranged in Table 4 with the encoded levels. The experimental design DCCR 3 is presented in Table 5.

Table 4. Range of values studied in the experimental design DCCR 3, optimization in the production of lipases.

Variable name *	-1,68	-1	0	+1	+1,68
Tryptone	0	4.05	10.00	15.95	20
CaCl₂.2H₂O	0	1.41	3.50	5.58	7
MgSO₄.7H₂O	0	0.20	0.50	0.79	1

* Unitis g.L⁻¹

Table 5. Experimental planning DCCR 3, optimization in the production of lipases.

Experiments	Coded variables			Real variables *			Enzymatic activity (Y1)
	(x1)	(x2)	(x3)	Tryptone	CaCl ₂ .2H ₂ O	MgSO ₄ .7H ₂ O	
1	-1.00	-1.00	-1.00	4.05	1.41	0.20	13.29
2	1.00	-1.00	-1.00	15.95	1.41	0.20	12.55
3	-1.00	1.00	-1.00	4.05	5.58	0.20	9.44
4	1.00	1.00	-1.00	15.95	5.58	0.20	9.96
5	-1.00	-1.00	1.00	4.05	1.41	0.79	17.00
6	1.00	-1.00	1.00	15.95	1.41	0.79	8.75
7	-1.00	1.00	1.00	4.05	5.58	0.79	8.16
8	1.00	1.00	1.00	15.95	5.58	0.79	15.27
9	-1.68	0.00	0.00	0.00	3.50	0.50	0.76
10	1.68	0.00	0.00	20.00	3.50	0.50	14.30
11	0.00	-1.68	0.00	10.00	0.00	0.50	22.26
12	0.00	1.68	0.00	10.00	7.01	0.50	14.96
13	0.00	0.00	-1.68	10.00	3.50	0.00	13.58
14	0.00	0.00	1.68	10.00	3.50	1.00	16.68
15	0.00	0.00	0.00	10.00	3.50	0.50	24.30
16	0.00	0.00	0.00	10.00	3.50	0.50	23.06
17	0.00	0.00	0.00	10.00	3.50	0.50	24.84

* Unitis g.L⁻¹

Analyzing the results shown in Table 5, we can verify that the highest result of lipolytic activity was achieved in experiment 17 with 24.84 U/mL carried out with 10 g.L⁻¹ tryptone, 3.50 g.L⁻¹ calcium chloride and 0.50 g.L⁻¹ magnesium sulfate, 1 g.L⁻¹ potassium phosphate and 1% cotton oil at pH 5. Muralidhar et al. (2001), when

optimizing the extracellular production of *Candida cylindracea* lipases, found lower results than this 17.30 U/mL of enzymatic activity, using glucose as carbon source. Salihu et al., 2011, also optimizing lipase production by *C. cylindracea*, found 19.90 U/mL of enzymatic activity, using palm oil as a carbon source.

The results found in experiments 15, 16 and 17, which are the central points, do not present a great difference characterizing the good reproducibility of the process.

When comparing the maximum lipolytic activities obtained at the higher and lower tryptone concentrations, it seems that increasing the concentration of 10 g.L⁻¹ to 20 g.L⁻¹ caused a decrease in lipolytic activities. Furthermore, the increase in calcium chloride concentration from 3.5 g.L⁻¹ to 7 g.L⁻¹ caused a decrease in lipolytic activities. With respect to magnesium sulfate, when an increase in concentration of 0 g.L⁻¹ to 0.5 g.L⁻¹ caused an increase in lipolytic activities.

According to the results obtained in the DCCR experimental design, the regression coefficients were calculated and a complete statistical analysis was obtained with ANOVA, Pareto graph and response surface graphs. The results are presented in Tables 6 and 7.

Table 6. Regression coefficients for the second experimental design DCCR 3, optimization in the production of lipases.

Regression				
Name	Coefficient	Standard Error	Calculated t	p-value
Mean	24.15	2.12	11.38	0.0000*
x^1	1.57	1.00	1.57	0.1596
x_1^2	-6.14	1.10	-5.60	0.0008*
x^2	-1.54	1.00	-1.55	0.1660
x_2^2	-2.23	1.10	-2.03	0.0821*
x^3	0.67	1.00	0.67	0.5227
x_3^2	-3.45	1.10	-3.15	0.0162*
$x_1 \cdot x_2$	2.08	1.30	1.60	0.1546
$x_1 \cdot x_3$	-0.12	1.30	-0.09	0.9321
$x_2 \cdot x_3$	0.51	1.30	0.40	0.7044

* Statistically significant values at 90% confidence ($p < 0.10$)

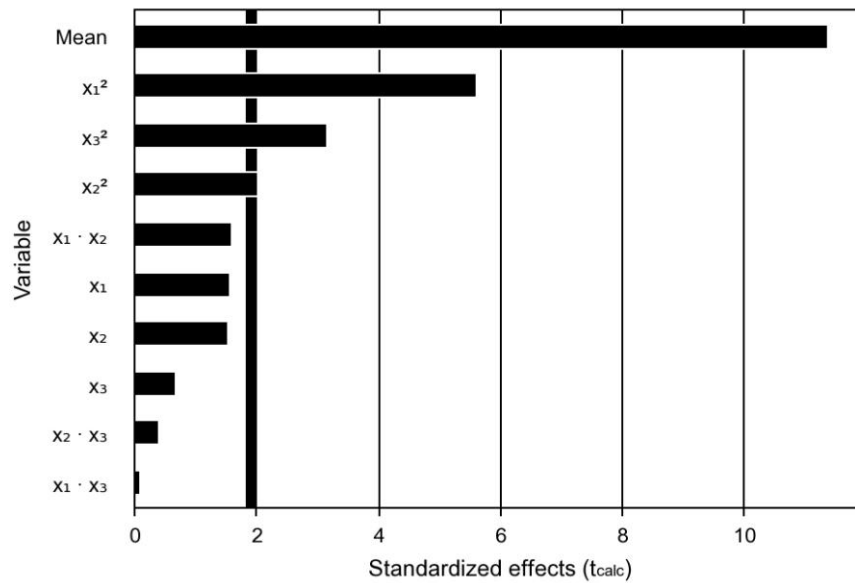
Table 7. ANOVA for the second experimental design DCCR 3, optimization in lipase production.

Anova					
Variation source	Sum of squares	Degrees of freedom	Mean square	f calc	p-value
Regression	567.3	9	63.0	4.65	0.02757
Residuals	94.9	7	13.6		
Lack of fit	93.2	5	18.6	22.4	0.04330
Pure error	1.7	2	0.8		
Total	662.3	16			

% Variation explained (R^2) = 85.67

Table 6 shows the regression coefficients for the second DCCR 3 experimental planning. The results found for the variables x_1^2 , x_2^2 and x_3^2 are statistically significant at the 90% confidence level. However, they had a negative coefficient -6.14, -2.23 and -3.45, respectively. It is noteworthy that the R^2 value of this, explains 85.67% of the experiment. Kaushik et al. (2006) report that the R^2 value provides a measure of how much the variability in observed response values can be explained by the experimental factors and their interactions. This implies that the sample variation of 85.67% for lipase production was attributed to the independent variables and only 14.33% of the total variation was not explained by the model. Burkert et al. (2004) describe in their work that a model with values above 0.9 are considered good, explain most of the variation in response and the closer the value of the R-squared is to 1.00, the stronger is the model and the better are the predictions of response.

In the same table, it is possible to see that the effect of the variable x_3 and the interaction of the variables $x_1 - x_3$ and $x_2 - x_3$ was very small, since they are smaller than the standard error. The value of the interaction effect of variables $x_1 - x_3$, in addition to being low, had a negative effect. This means that increasing the concentration of magnesium sulfate and the interaction between tryptone X magnesium sulfate, calcium chloride X magnesium sulfate did not influence the value of enzymatic activity, at 90% confidence.



Graph 2. Main effects of variables and significant interactions in the second DCCR 3.

Graph 2 shows which of the variables and interactions analyzed presented statistically significant results. It is possible to observe that only the quadratic variables were significant x_1^2 , x_2^2 and x_3^2 , and the others would not influence the process if an increase of its concentration from the level -1.68 to the level +1.68 occurred.

Thus, a readjustment was made in the range of values studied in this experiment with the objective of increasing the enzymatic activity and attending to a higher production.

Figures 2, 3 and 4 below show the response surface found for the optimization of lipase production by the fungus *F. solani*, generated in the prothymatics program. Kaushik et al. (2006) report that the three-dimensional response surface curves plotted by a statistically significant model serves to understand the interaction of the medium components and the optimal concentration of each component required for optimum lipase production. The three-dimensional graphs shown were based on the function of concentrations of two variables, in this way, Muralidhar et al. (2001) report that the significance of the interactions between the corresponding variables is indicated by an elliptical or saddle characteristic of the surface and contour graphs.

Thus, Figure 2 presents the response surface generated by the enzymatic activity obtained from the interaction of tryptone with calcium chloride, which means that at higher levels of both tryptone and calcium chloride did not result in higher

enzyme yields. The shape of the response surface curves showed a moderate interaction between these variables tested.

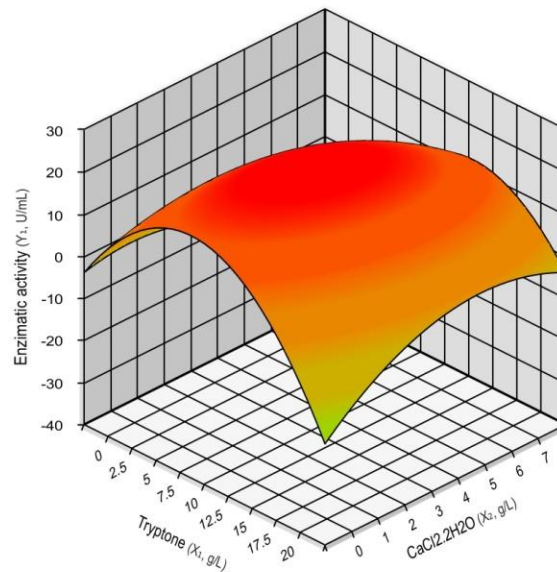


Figure 2. Response surface for enzymatic activity
(Tryptone and Calcium Chloride)

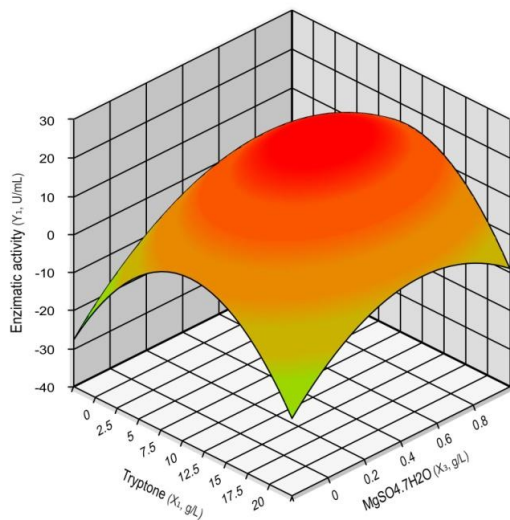


Figure 3. Response surface for
enzymatic activity
(Tryptone and magnesium sulfate)

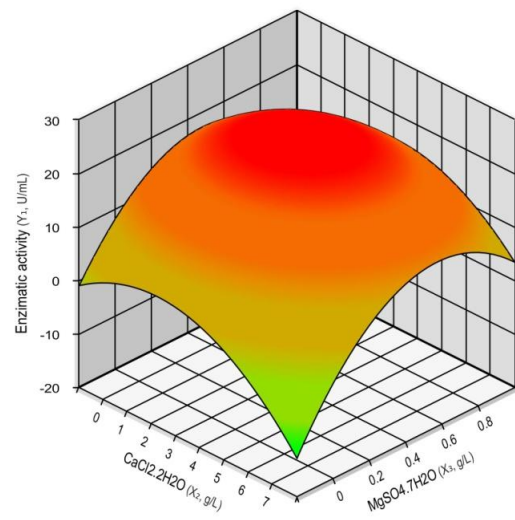


Figure 4. Response surface for
enzymatic activity
(Calcium chloride and
magnesium sulfate)

However, Figure 3 shows the response surface generated by the enzymatic activity obtained from the interaction of tryptone with magnesium sulfate, where the best production is in the range of 10 g.L⁻¹ to tryptone and 0.5 g.L⁻¹ to magnesium sulfate, what means that at lower and higher levels of both tryptone and magnesium sulfate did not result in higher enzyme yields.

Finally, Figure 4 shows the response surface generated by the enzymatic activity obtained from the interaction of calcium chloride with magnesium sulfate, where the best production is in the range of 2.7 g.L⁻¹ for calcium chloride and 0.5 g.L⁻¹ for magnesium sulfate, that is, lower and higher levels of both calcium chloride and magnesium sulfate would also not result in higher enzyme yields.

3.3. Validation of the experiment

In view of the optimization achieved, a validation of the experiment was proposed to verify the production of lipases by *F. solani*. Based on the results obtained from the previous experimental design DCCR 3, the enzymatic activity and the effectiveness of the compounds (tryptone, calcium chloride and magnesium sulphate) were analyzed in the validation process. The conditions established in the validation experiment are described in Table 8.

Table 8. Parameters established for validation.

Variable	Concentration *
Tryptone	10
CaCl ₂ .2H ₂ O	1.75
MgSO ₄ .7H ₂ O	0.5

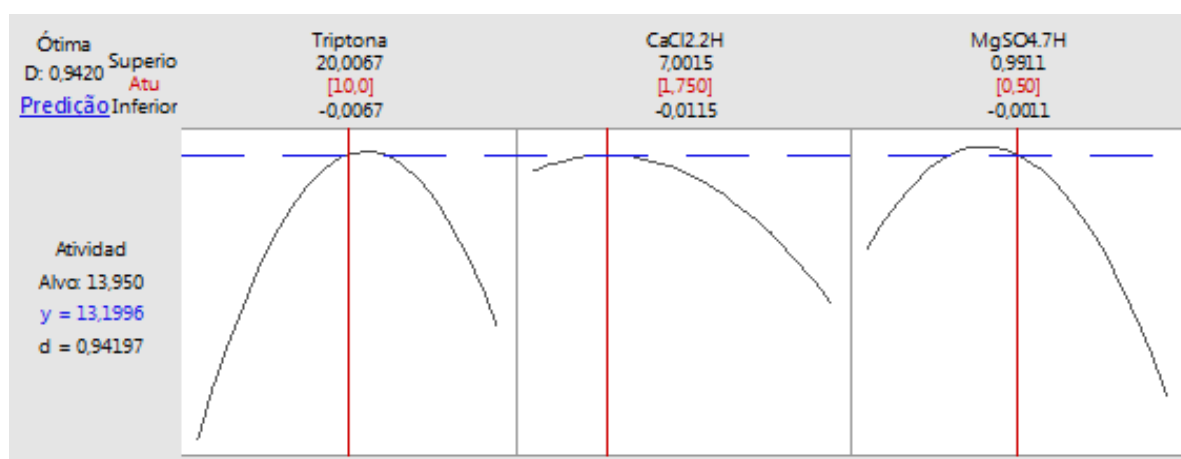
* concentracion in g.L⁻¹

Table 9 shows the optimization target plotted by the *minitab18* program, which establishes an enzymatic activity target of 13.95 U/mL within the indicated parameters. After the validation process, the maximal enzymatic activity obtained was 13.20 U/mL, which is understood to be promising along the optimization process. In order to ensure reproducibility, 6 experiments were run under the same conditions.

Table 9. Target for optimization of the enzymatic activity response

Answer	Goal	Bottom	Target	Higher
Enzimatic activity	Target	1.02	13.95	15.34

Graph 3 shows the behavior profile of the enzymatic activity against the compounds evaluated for the optimization. It can be seen in Figure 3 that the target established by Minitab 18 for calcium chloride was reached, but for tryptone and magnesium sulphate, there was a small deviation from the target, making it impossible in the validation process that the enzymatic activity was the maximum defined by it.



Graph 3. Response of experimental validation in lipase production by *Fusarium solani*.

3.4. Biochemical characterization

Table 10 shows the ranges of values studied for the DCCR experimental design, temperature and pH. An array of 11 experiments with two factors was generated using the protimiza program. The minimum and maximum ranges of the parameters were investigated and the complete experimental design with their values in real and codified form are listed in Table 11. The average maximal lipase activity was taken as dependent variable or response (Y1). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured in the independent parameters Eq. (2). All tests were performed where only the central point was performed in triplicates. For a three-factor system, the equation of the model is as follows:

$$Y_1 = 13,20 + 3,28 x_1 - 4,11 x_1^2 - 1,75 x_2 - 4,62 x_2^2 - 1,25 x_1 x_2 \quad \text{Eq.(2)}$$

where, Y_1 is the predicted response, X_1 and X_2 , are the liner coefficients; X_1^2 and X_2^2 are the quadratic coefficients and $X_1 - X_2$ are the interaction coefficients.

The experimental design was conducted with 2 variables in 11 experiments under the conditions described in Table 10. The actual decoded values used for this planning are arranged in Table 11, with the coded levels as well as the values of the respective enzymatic activities of each experiment.

Table 10. Range of DCCR experimental planning, temperature, and pH.

Variable name	Units	-1,41	-1	0	+1	+1,41
pH		5.5	6.0	7.0	8.0	8.5
Temperature	° C	25	30	40	50	60

Table 11. DCCR experimental design, temperature and pH.

Experiments	Coded variables		Real variables		Enzimatic activity (Y1)
	(x1)	(x2)	pH	Temperature	
1	-1.00	-1.00	6.0	30	0.87
2	1.00	-1.00	8.0	30	8.26
3	-1.00	1.00	6.0	50	1.04
4	1.00	1.00	8.0	50	3.41
5	-1.41	0.00	5.5	40	0.23
6	1.41	0.00	8.5	40	11.88
7	0.00	-1.41	7.0	25	8.34
8	0.00	1.41	7.0	60	1.74
9	0.00	0.00	7.0	40	13.04
10	0.00	0.00	7.0	40	13.35
11	0.00	0.00	7.0	40	13.20

When analyzing the results presented in Table 11, we can verify that the highest result of lipolytic activity was reached in experiment 10, with 13.35 U/mL performed at pH 7 and temperature 40 °C. The lowest result was found in experiment 5, with 0.23 U/mL of lipolytic activity performed at pH 5.5 and 40 °C. The results found in the experiments 9, 10 and 11, which are the central points, do not present much difference, characterizing the good reproducibility of the process.

Comparing the minimum and maximum lipolytic activities obtained at the higher and lower pHs, it seems that the increase in lipolytic activity caused an increase in the lipolytic activity, when it increased from the -1.41 level to the 0 level.

However, the increase in temperature caused a decrease in lipolytic activities, probably because of the denaturation of the enzyme at such temperatures.

Relating pH and optimum temperature in this work, KEMPKA et al. (2008) have characterized lipase from *Penicillium verrucosum* and found a pH 7.0 and temperature of 44 °C. Kamini et al. (1998) characterized the lipase from *Aspergillus niger* and found a value of pH 7.0. Similar result was obtained by BENJAMIN & PANDEY, 2000, who reported that one of three different forms of lipases produced by *Candida rugosa* presented optimum activity at pH 7.0 and 40 °C. This temperature value was also found as optimal by Pastore et al. (2003), who studied the characterization of lipase obtained from *Rhizopus* sp. and observed an optimum pH between 6.0 and 6.5. The same temperature was reported as optimal by SHU et al. (2006), for lipids of *Antrodia cinnamomea*, with pH 8.0. Ülker et al. (2010), characterizing the lipase of *Trichoderma harzianum*, found the optimal activity at pH 8.5 and temperature of 40 °C.

According to the results obtained in the DCCR experimental design, the regression coefficients were calculated and a complete statistical analysis was obtained with ANOVA, Pareto graph and response surface graph. The results are presented in Tables 12 and 13.

Table 12. Regression coefficients, DCCR experimental design, temperature and pH.

Regression				
Name	Coefficient	Standard Error	Calculated t	p-value
Mean	13.20	1.09	12.16	0.0001*
x_1	3.28	0.66	4.94	0.0043*
x_1^2	-4.11	0.79	-5.20	0.0035*
x_2	-1.75	0.66	-2.64	0.0462*
x_2^2	-4.62	0.79	-5.84	0.0021*
$x_1 \cdot x_2$	-1.25	0.94	-1.34	0.2393

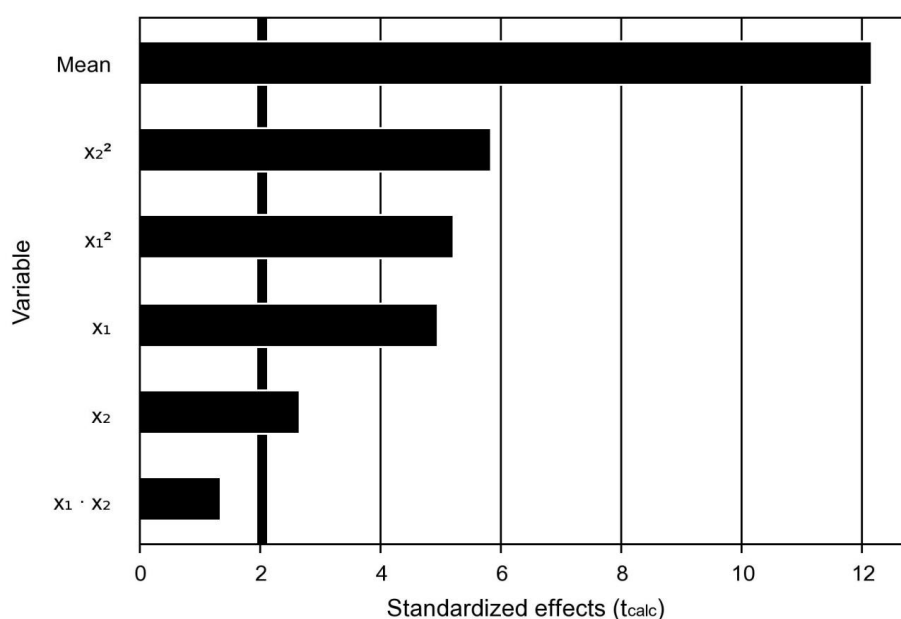
* Statistically significant values at 90% confidence ($p < 0.10$)

Table 13. ANOVA DCCR experimental design, temperature and pH

Anova					
Variation source	Sum of squares	Degrees of freedom	Mean square	f calc	p-value
Regression	284.0	5	56.8	16.08	0.00422
Residuals	17.7	5	3.5		
Lack of fit	17.6	3	5.9	244.26	0.00408
Pure error	0.05	2	0.0		
Total	301.7	10			

% Variation explained (R^2) = 94.15

Table 12 shows the regression coefficients for the DCCR experimental design, temperature and pH. The results found for the variables x_1 , x_2 , x_1^2 and x_2^2 are statistically significant at a 90% confidence level. However, the variables x_2 , x_1^2 and x_2^2 presented a negative coefficient of -1.75, -4.11 and -4.62 respectively. It is worth mentioning that the R^2 value explains 94.15% of the experiment. Kaushik et al., 2006 report that the R^2 value provides a measure of how much the variability in observed response values can be explained by the experimental factors and their interactions. This implies that the sample variation of 94.15% for lipase production was attributed to the independent variables and only 5.85% of the total variation was not explained by the model. In the same Table, it is possible to see that the effect of the interaction of the variables $x_1 - x_2$ had the negative coefficient -1.25, which was the lowest one found, not being statistically significant with a p -value of 0.2393. This means that the increase in pH or temperature for the interaction did not influence the value of enzymatic activity, at 90% confidence.



Graph 4. Main effects of variables and significant interactions on DCCR, temperature and pH.

Graph 4 shows which of the variables and interactions analyzed presented statistically significant results. It is possible to verify that the linear and quadratic variables, x_1 , x_2 , x_1^2 and x_2^2 , were significant and the interaction of linear variables $x_1 - x_2$ would not influence the process if its concentration increased from level -1.41 to level +1.41.

Figure 5 below shows the response surface found for the DCCR temperature and pH, which seeks to verify the best range for lipases production by the fungus *F. solani*. Fan et al. (2015) reported that the response surface represents a mathematical and statistical method to optimize an operational condition that is influenced by some independent variables in order to obtain the maximum yield through an economical procedure.

The response surface plot was generated from the six experiments performed, where the best production is in the 40 °C temperature range at pH 7. Through this analysis, it is evident that temperature extremes tend to decrease the lipase production, since low temperatures lower metabolism and high temperatures can inactivate lipase.

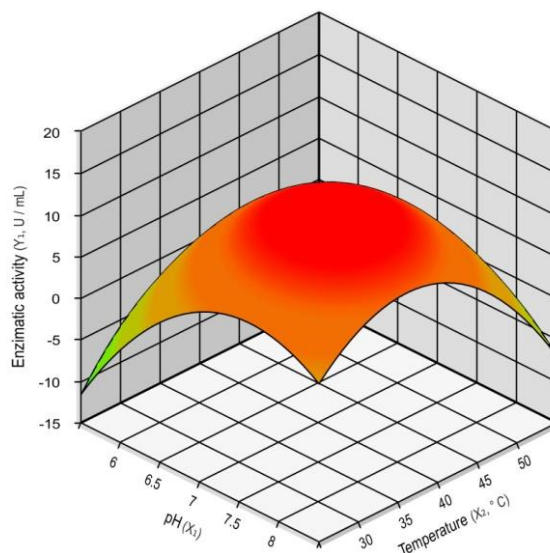
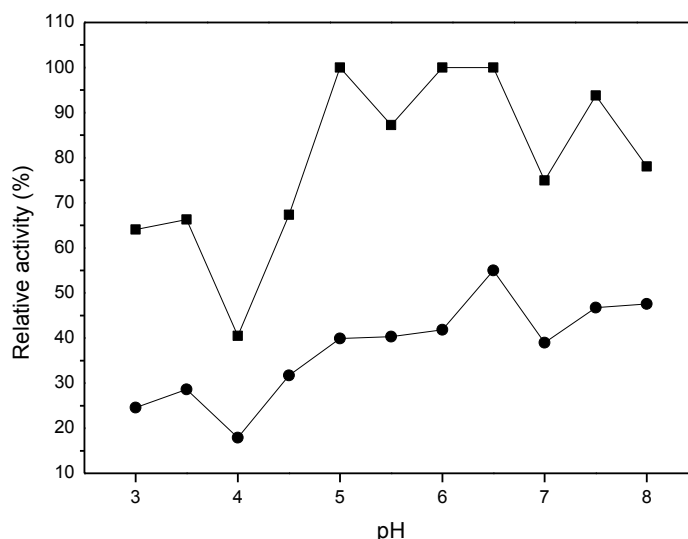


Figure 5. Response surface for DCCR, temperature and pH.

3.4.1. Determination of stability in pH

When lipase activity and stability in the crude extract were evaluated in the pH range from 3.0 to 8.0 (Graph 5), both times 24 and 48 hours presented similar profiles with the highest activity at pH 6.5.



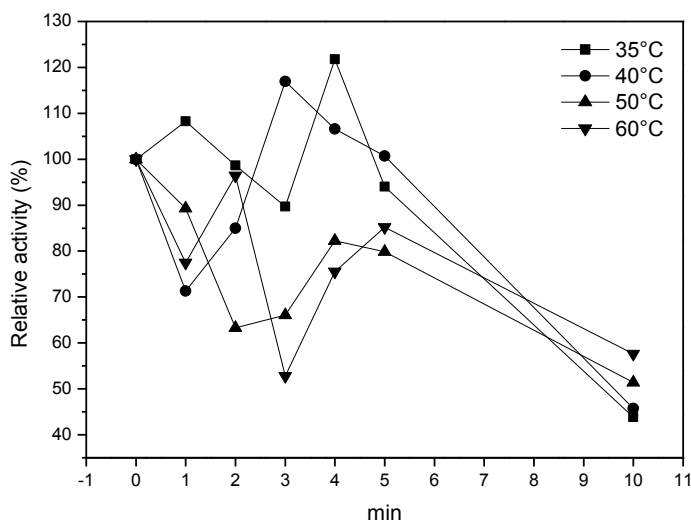
Graph 5. Effect of pH on the stability of *Fusarium solani* lipase in the crude extract. Activity was determined in McIlvaine buffer for pH between 3.0 to 8.0. Lipid activity was determined in McIlvaine buffer pH 7.0 at 40° C (■) crude extract lipase 24 h and C (●) crude extract lipase 48 h.

During incubation at different pHs, lipase started to stabilize at 65% activity in the range of pH 3.0 to 3.5, becoming stable over 24 hours in the pH range of 6.0 to 6.5 with 100% activity. At the same time, lipase maintained more than 90% activity in the range of pH 7.5 and with a decrease to 80% in activity at pH 8. Almeida et al. (2018) evaluating the stability at free and immobilized lipase pH of *C. viswanathi*, found for the same pH range, 90% activity for immobilized lipase and between 60-90% activity for free lipase. Üker et al. (2010), when evaluating the lipid pH stability of *Trichoderma harzianum*, found for the same time range a retention of 80% activity at pH 6.5 and around 90% activity at pH 8. Lima et al. (2004), evaluating the activity and stability of a crude lipase of *Penicillium aurantiogriseum*, reported that residual activity remained above 80% between pHs 5 and 9.

At 48 hours, lipase reached its half-life, with its initial activity retained in more than 70% in the range of pH 3.0 to 3.5. The crude lipase of *Fusarium solani* presented maximum stability in 48 hours, between pHs 7.5 and 8.0 with more than 55% of retained activity. It retained 50% of its activity in the range of pH 6.5.

3.4.2. Determination of thermal stability

When the thermal stability of the lipase in the crude extract was analyzed, the enzyme maintained about 120% activity up to 4 minutes at 35 °C and the half-life was in the time of 10 min (Graph 6). ÜLKER et al. (2010), evaluating the thermal stability of lipase of *Trichoderma harzianum*, found a stable profile with a retention of 100% activity at temperatures of 20, 30 and 40 °C.



Graph 6. Thermostability of *F. solani* in the crude extract. Lipase activity was determined in McIlvaine buffer pH 7.0 at 40 °C. Enzyme was incubated for 10 min.

The half-lives of the enzyme at 40, 50 and 60 °C were all in the time of 10 min. Lipase did not have a desired profile for thermal stability, but maintained 110% activity in the time of 3 min at 40 °C, 90% and 80% activity in the times of 1 min and 4 min respectively at 50 °C and 95% activity in the time of 2 min at 60 °C. Hiol et al. (2000) studied the thermal stability of *R. oryzae* lipase, where it was detected that its activity remained on average with 80% at 40 °C, obtaining intermediate activity of 60% at 45 °C, and in the same thermostability study the enzyme presented half-life with 20% activity at 50 °C. Lima et al. (2004), evaluating the thermal stability of the crude extract of a *Penicillium aurantiogriseum* lipase, reported that there was only 32% residual activity after incubation at 50 °C, 45% after incubation at 45 °C and 77% after incubation at 37 °C. Ozenl et al. (2004) reported in their study that the decrease in the percentage of residual activity at high temperatures resulted first in some conformational changes in the tertiary structure of enzyme, and then its almost complete inactivation.

3.4.3 Determination of activity and stability in solvents

Stability in organic solvents is an important feature of lipases because it can determine whether the enzyme is ideal for use in synthetic catalysis reactions and also to predict which solvent would be best to perform the reaction.

Table 14 shows the effect of organic solvents on the stability of *F. solani* lipase. The solvents are listed according to their hydrophobicity (Log P) varying from -1.67 to 3.90 and according to their polarity (GHAMGUI et al., 2007). The negative hydrophobicity values (Log P) indicate that the solvent is soluble in water, whereas positive values indicate that they are insoluble, with the separation of the aqueous phase from the organic phase (SANGSTER, 1989).

Table 14 Effect of organic solvents on free lipase of *F. solani*.

Organic solvents	Log P	Free lipase	
		Relative activity (%)	t _{1/2} (h)
Control		100,00	16
Glycerol	-1.67	84,71	36
DMSO	-1.38	220,04	36
Methanol	-0.76	111,36	48
Acetonitrile	-0.40	55,79	36
Ethanol	-0.24	60,95	48
Acetone	-0.23	85,12	36
2-propanol	0.07	61,16	36
1-propanol	0.25	17,77	36
Tertiary Butanol	0.60	48,14	24
Iso-butyl	0.79	23,97	48
Toluene	2.50	309,92	12
Xylol	3.15	26,86	48
Hexane	3.90	77,69	24

Assay conditions: experiments were conducted in sealed flasks using 10% (v/v) organic solvent in McIlvaine buffer pH 7.0, 40 °C, 180 rpm. Lipase activity was determined using the same buffer at 40 °C. Activities were carried out using 100µL of protein for the free lipase. Relative activity was expressed in relation to the control (absence of organic solvent in the reaction medium). Half-lives were determined by incubating the enzyme in each organic solvent without substrate. Log P logarithm of the partition coefficient of a particular solvent n-octanol and water (SANGSTER, 1989).

Table 14 shows that the enzyme was very stable in most organic solvents, however, the activity was determined in more than 80% for the enzyme in glycerol,

DMSO, methanol, acetone and toluene. As for the enzyme in 1-propanol, iso-butyl and xylol, the smallest activities were detected. This important feature allows several industrial applications such as transesterification reactions, synthesis and resolution of racemic esters (DANDAVATE et al., 2009).

The lipase half-lives were greater than 40 hours using methanol, ethanol, iso-butyl and xylol. Half-lives greater than 30 hours were observed when using glycerol, DMSO, acetonitrile, acetone, 2-propanol, 1-propanol. Intermediate half-lives were observed with tertiary butanol and hexane, while the shorter half-life was observed with toluene.

Enzyme activity was considerably stimulated after 8 hours of incubation with DMSO, methanol and toluene (220, 111 and 309%, respectively). Intermediate activities were achieved with acetone, glycerol, hexane, 2-propanol and ethanol (85, 84, 77, 61 and 60%, respectively). 1-propanol did not significantly affect enzyme activity, with 17%) In addition, the enzyme showed good stability in water-immiscible organic solvents with a residual activity of 113.6% for n-heptane, 92, 3% for hexane, 91.0% for isooctane and 82.3% for toluene.

4. Conclusion

The optimization of lipase production by *F. solani* in submerged fermentation was possible with the use of sequential experimental design, *Plackett-Burman* and Central Rotational Compound Design, respectively. The optimum conditions were obtained using the fungus *F. solani*, obtaining maximum lipolytic activities of 24.84 U/mL with the use of 10 g.L⁻¹ of tryptone, 3.50 g.L⁻¹ of calcium chloride and 0.50 g.L⁻¹ magnesium sulfate, 1 g L⁻¹ potassium phosphate and 1% cotton oil. With the biochemical characterization of the enzyme, a pH and temperature optimization using a central rotational compound design led to a mathematical model with a correlation coefficient of 94.15% with the experimental data, showing that the enzyme has better performance at pH 7 at a temperature of 40 °C.

Acknowledge

The Federal University of Tocantins and the Postgraduate Program in Biodiversity and Biotechnology - BIONORTE. The present work was carried out with the support of the Coordination of Improvement of Higher Education Personnel Brazil - (CAPES) - Financing Code [001].

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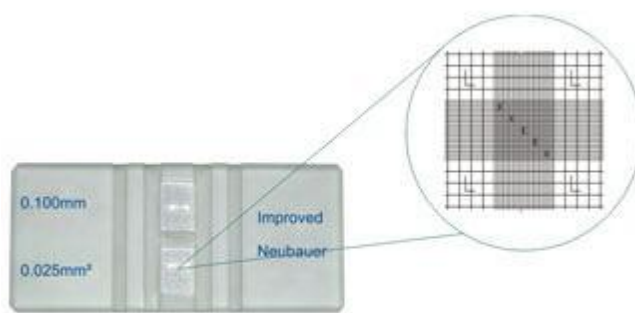
ANEXOS

ANEXO A

Cálculo da Contagem de Esporos/g de meio

Considerando-se que a câmara de Neubauer contém 25 quadrículos. Entre a câmara e a lamínula, forma-se um filme líquido da diluição preparada de 0,1 mm de espessura. A dimensão de cada quadrículo é de 0,2 mm x 0,2 mm, portanto o volume de cada quadrículo é de $4 \times 10^{-3} \text{ mm}^3$, que multiplicado pelo número total de quadrículos (25) é igual a 10^{-4} cm^3 .

Câmara de Neubauer



$$C = \frac{\text{nº de esporos} * D * 25 * 10^4}{5}$$

Onde:

C= concentração de esporos/ mL

nº de esporos = quantidade de esporos contados.

D= diluição.

ANEXO B

Fotomicrografia, foto e estruturas dos fungos.

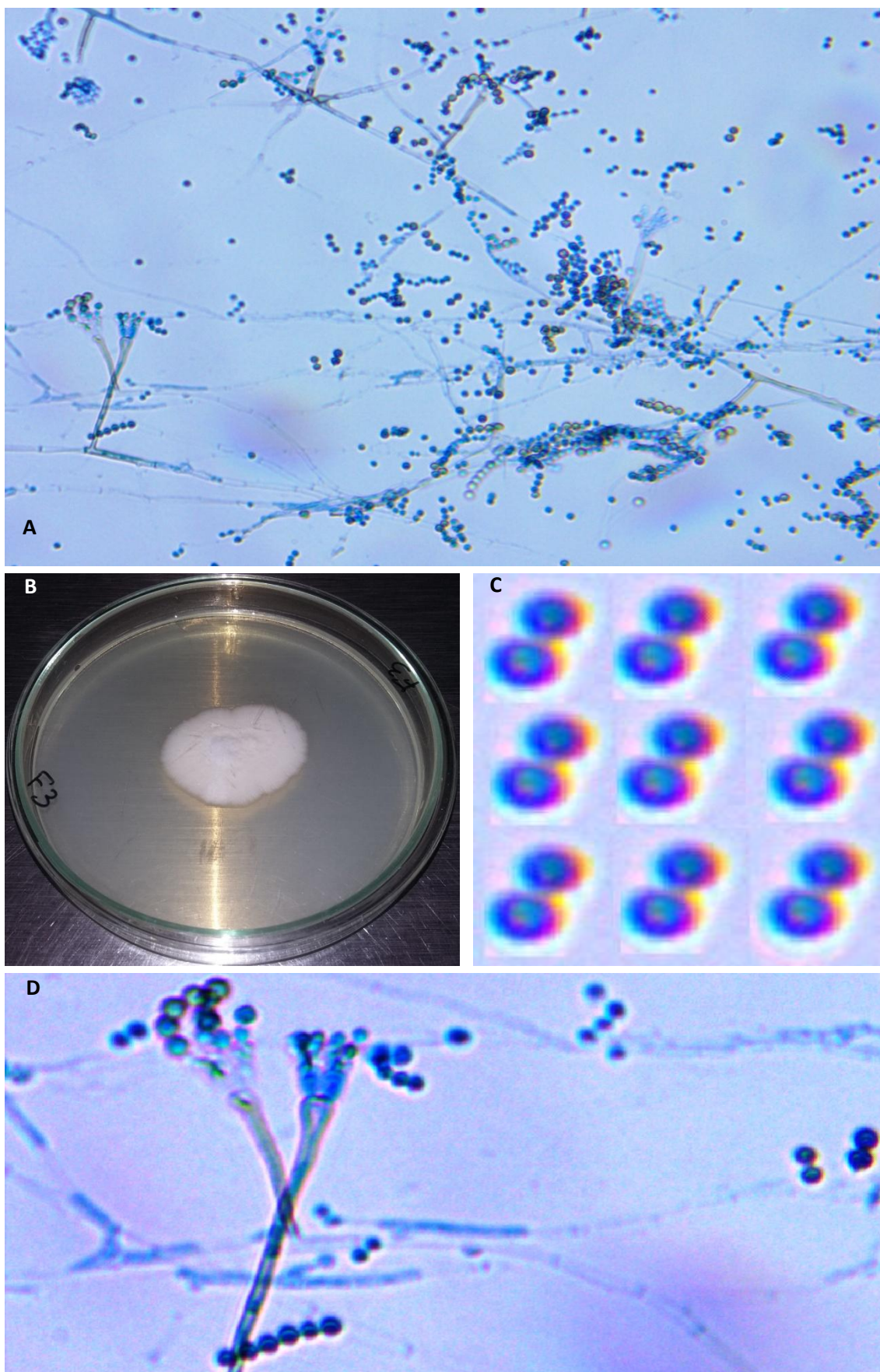


Figura 2. *Aspergillus calidoustus*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C – Conídio; D - Conidióforo.

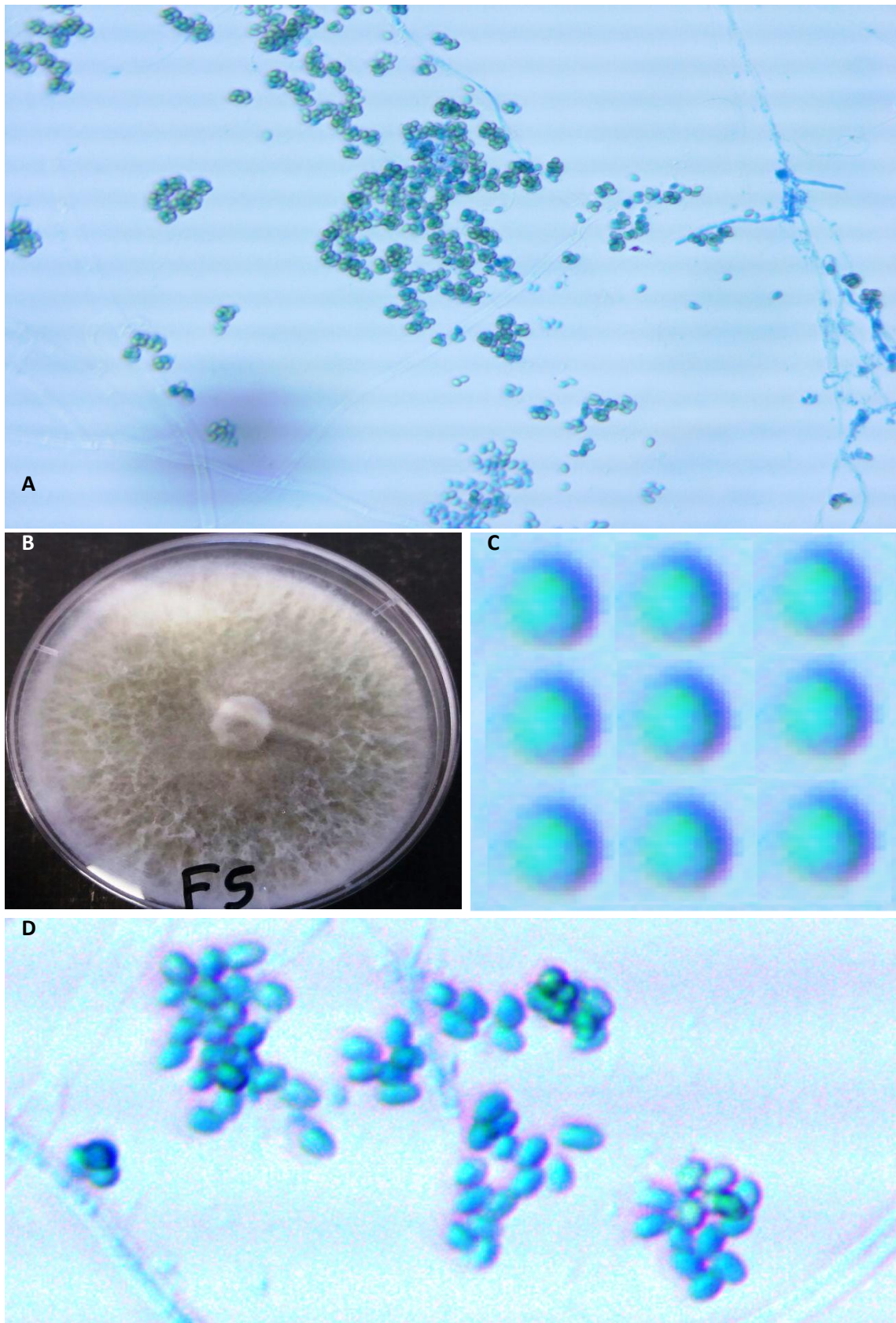


Figura 3. *Trichoderma* sp: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C – Conídio; D – Conídios e hifas.

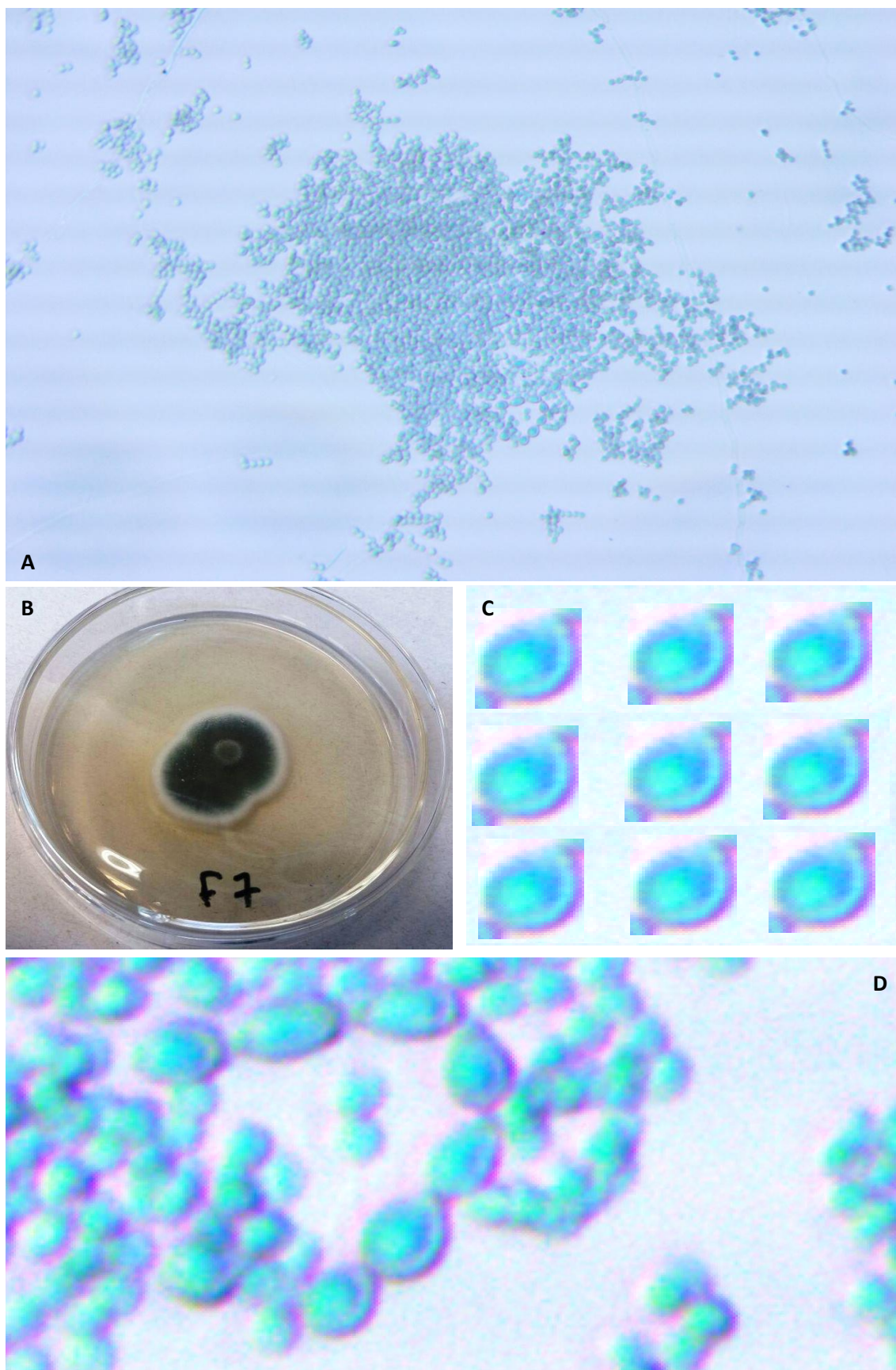


Figura 4. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C – Conídio; D – Agrupamento de Conídios.

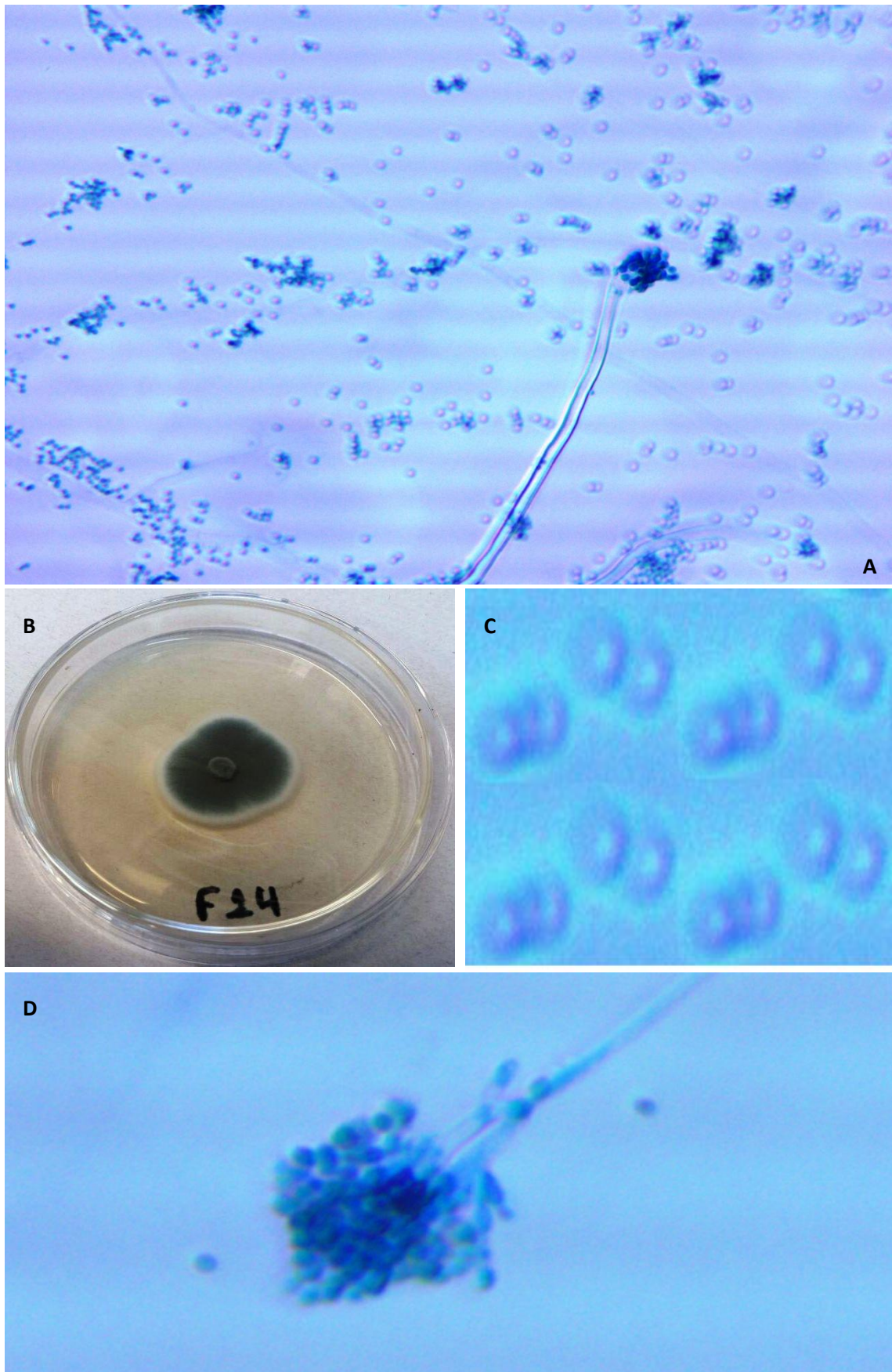


Figura 5 Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Conídio; D – Conidióforo e fiálides.

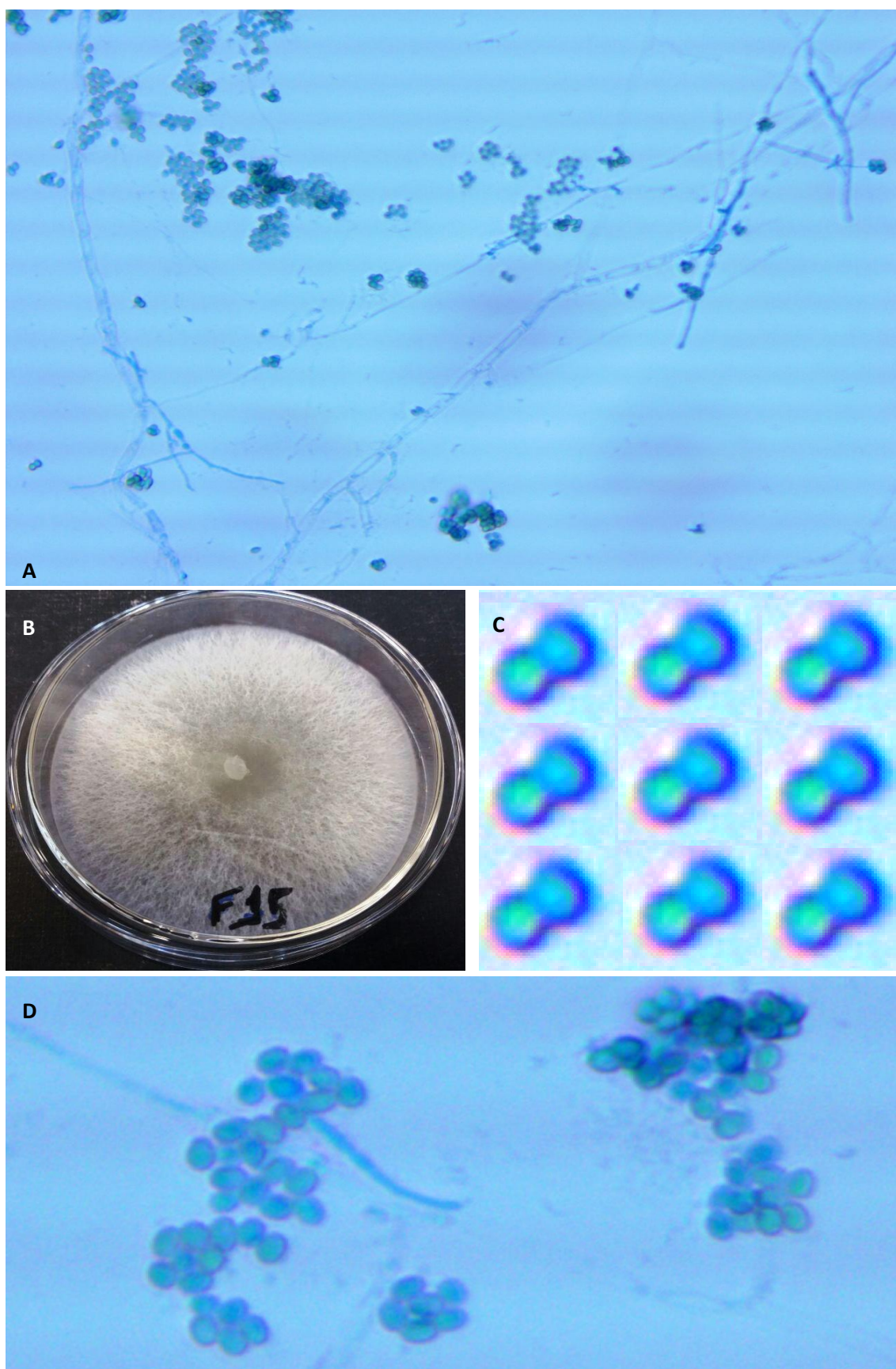


Figura 6. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C – Conídio; D – Agrupamentos de Conídio e hifas.

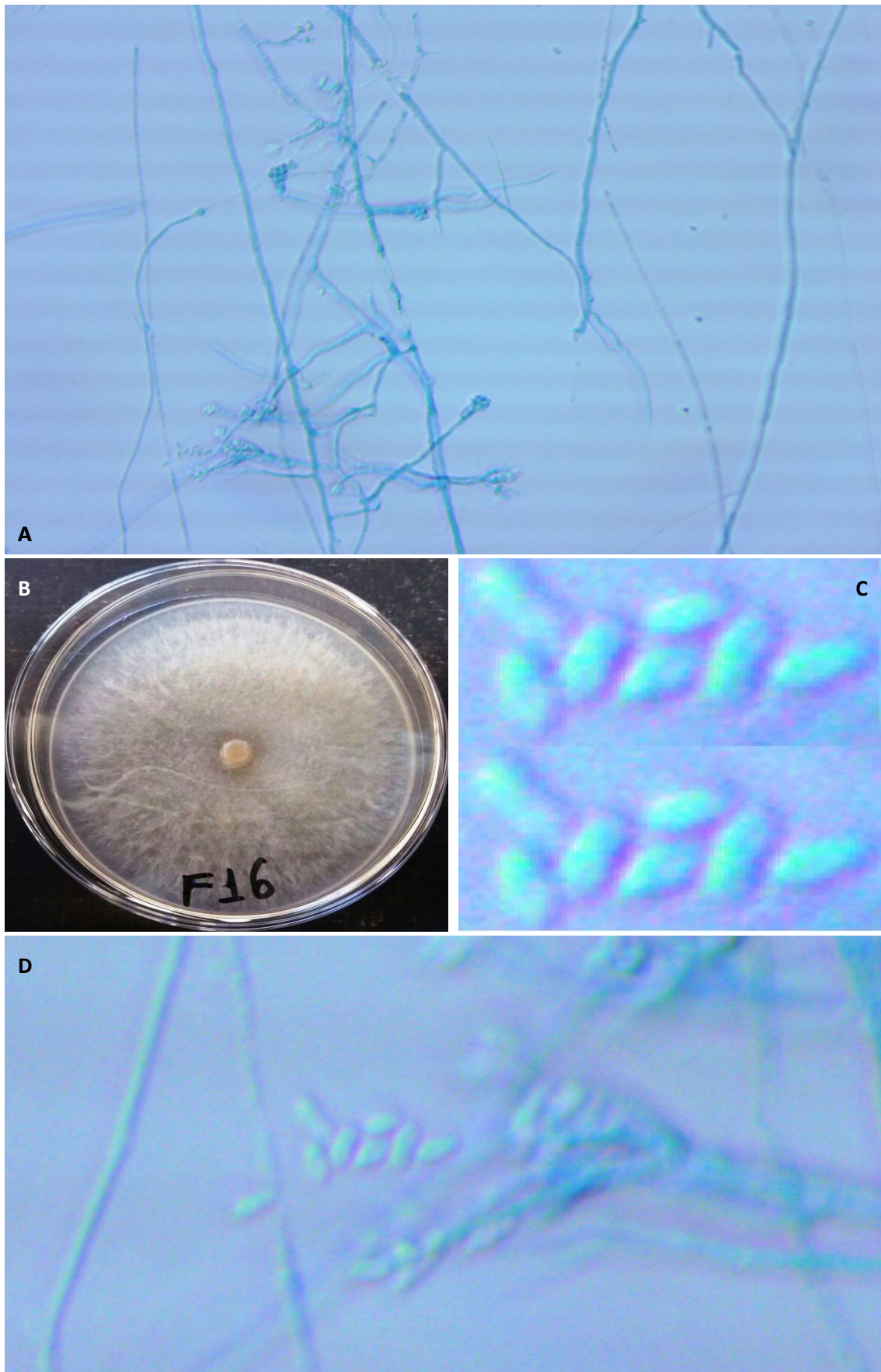


Figura 7. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Conídio; D - Conidióforo e hifas.

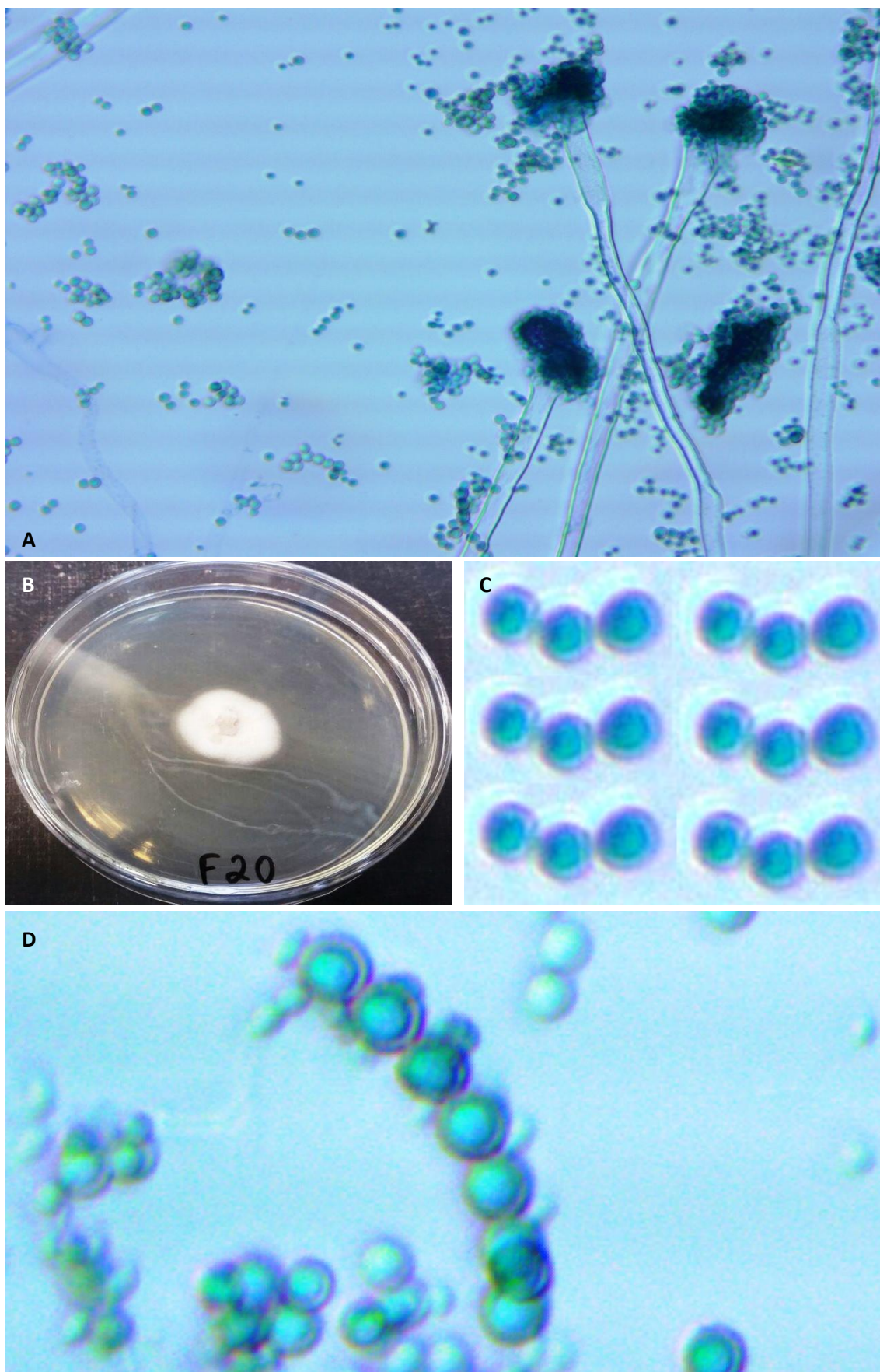


Figura 8. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Conídio; D – Agrupamentos de Conídios

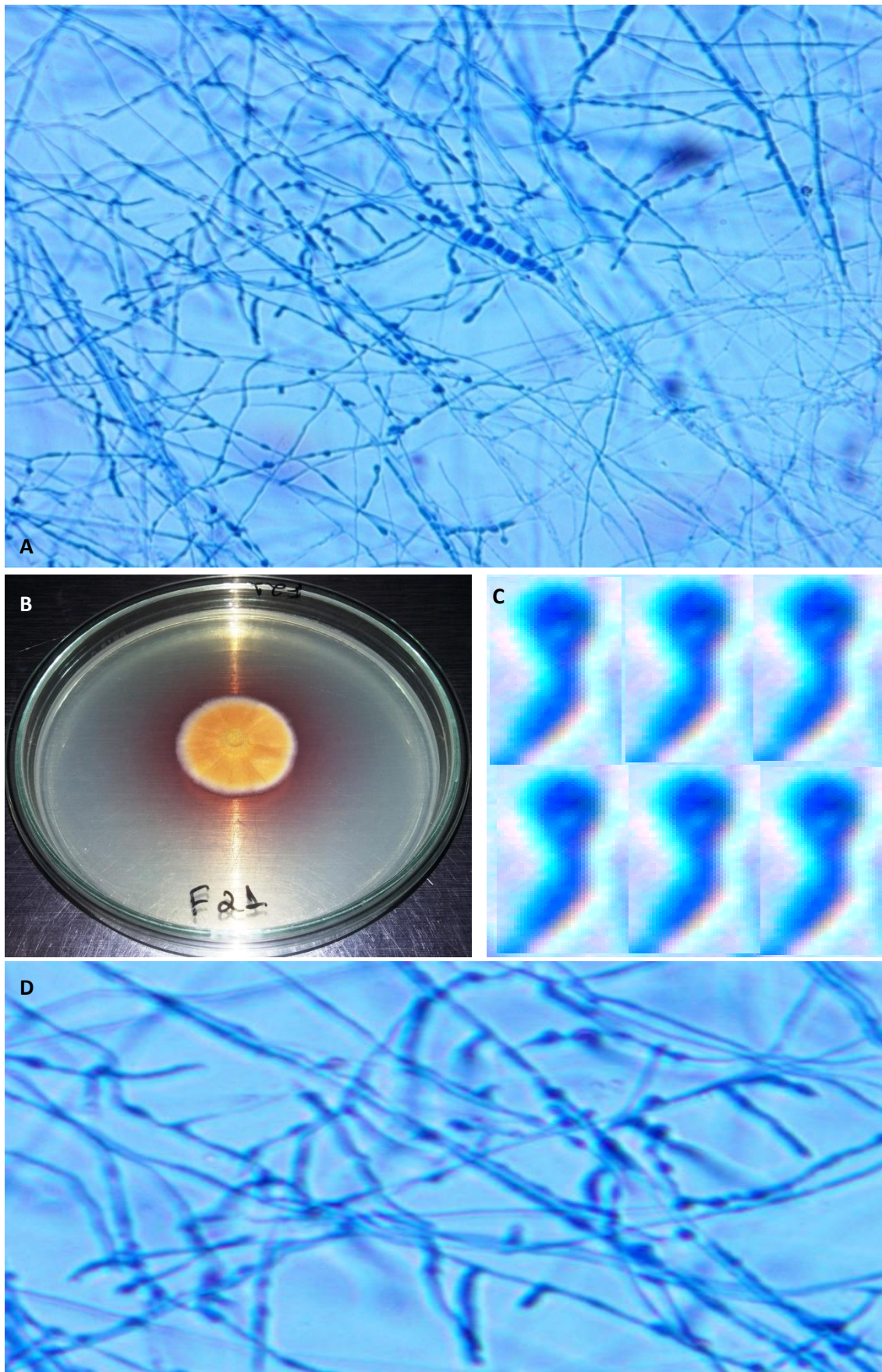


Figura 9. *Chaetomium aureum*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C - Tubo germinativo; D - Hifas.

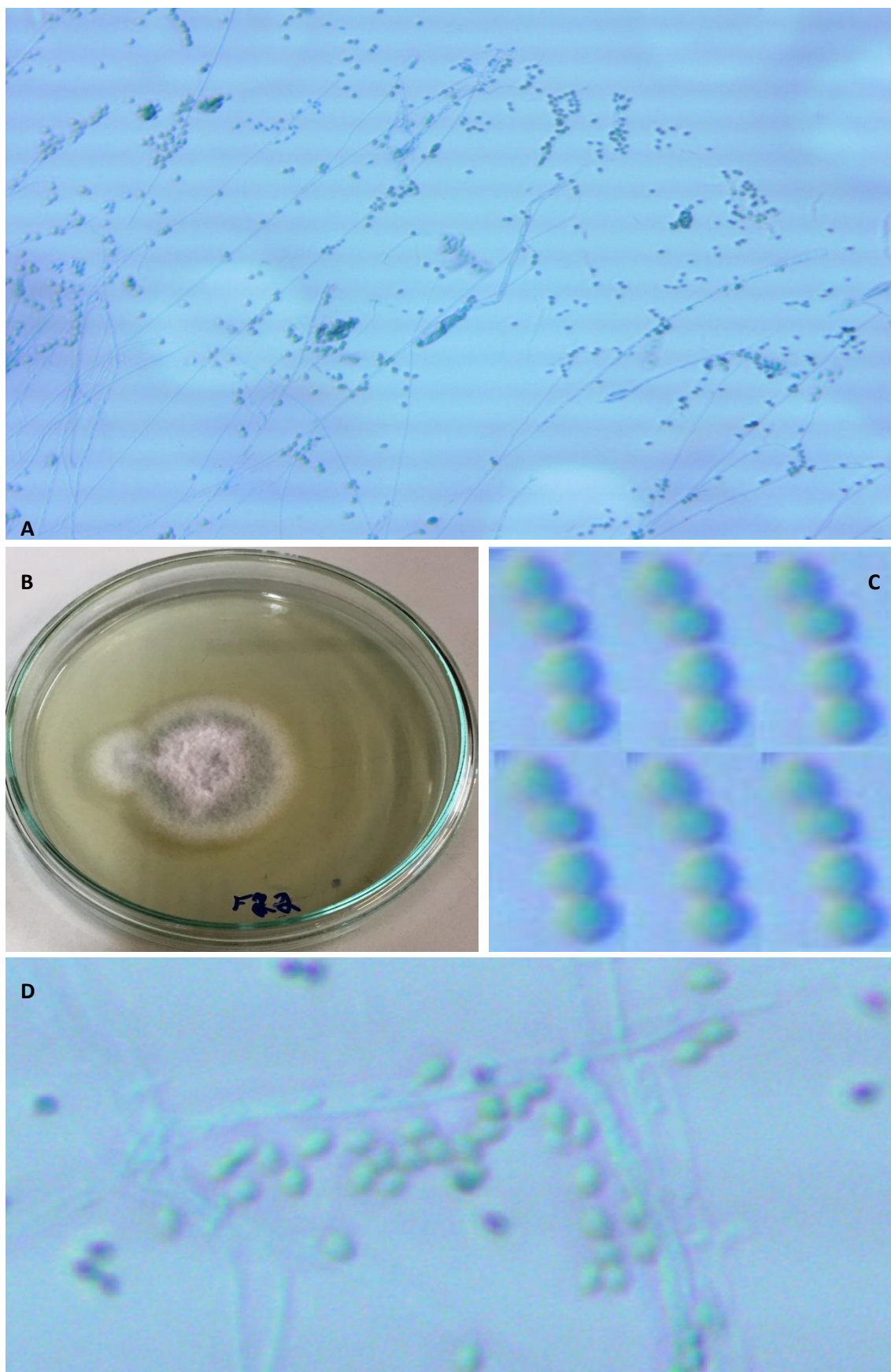


Figura 10. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com Agar Batata dextrose; C – Conídio; D – Agrupamentos de Conídios e hifas.

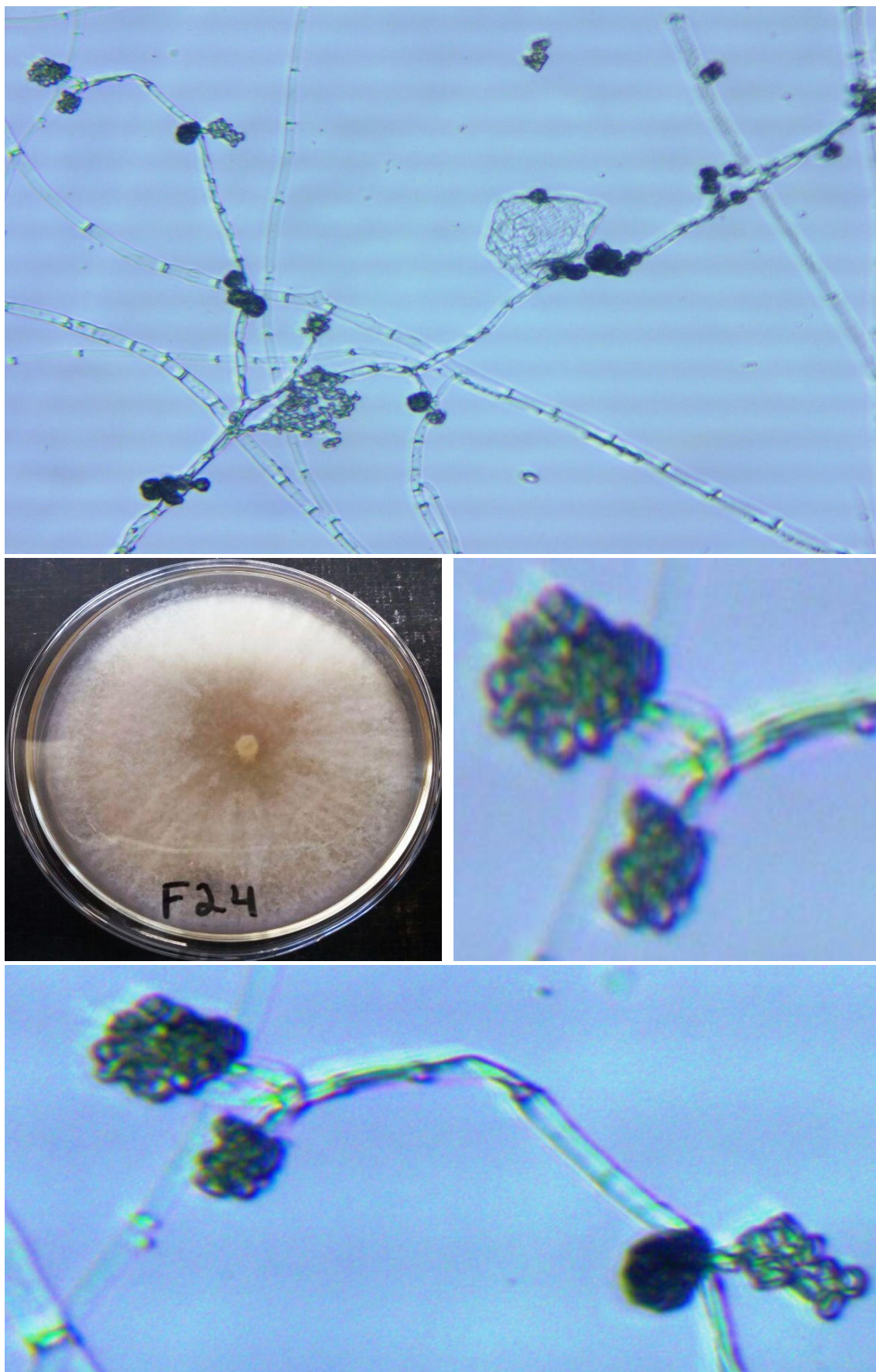


Figura 11. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Fiálides e hifas.

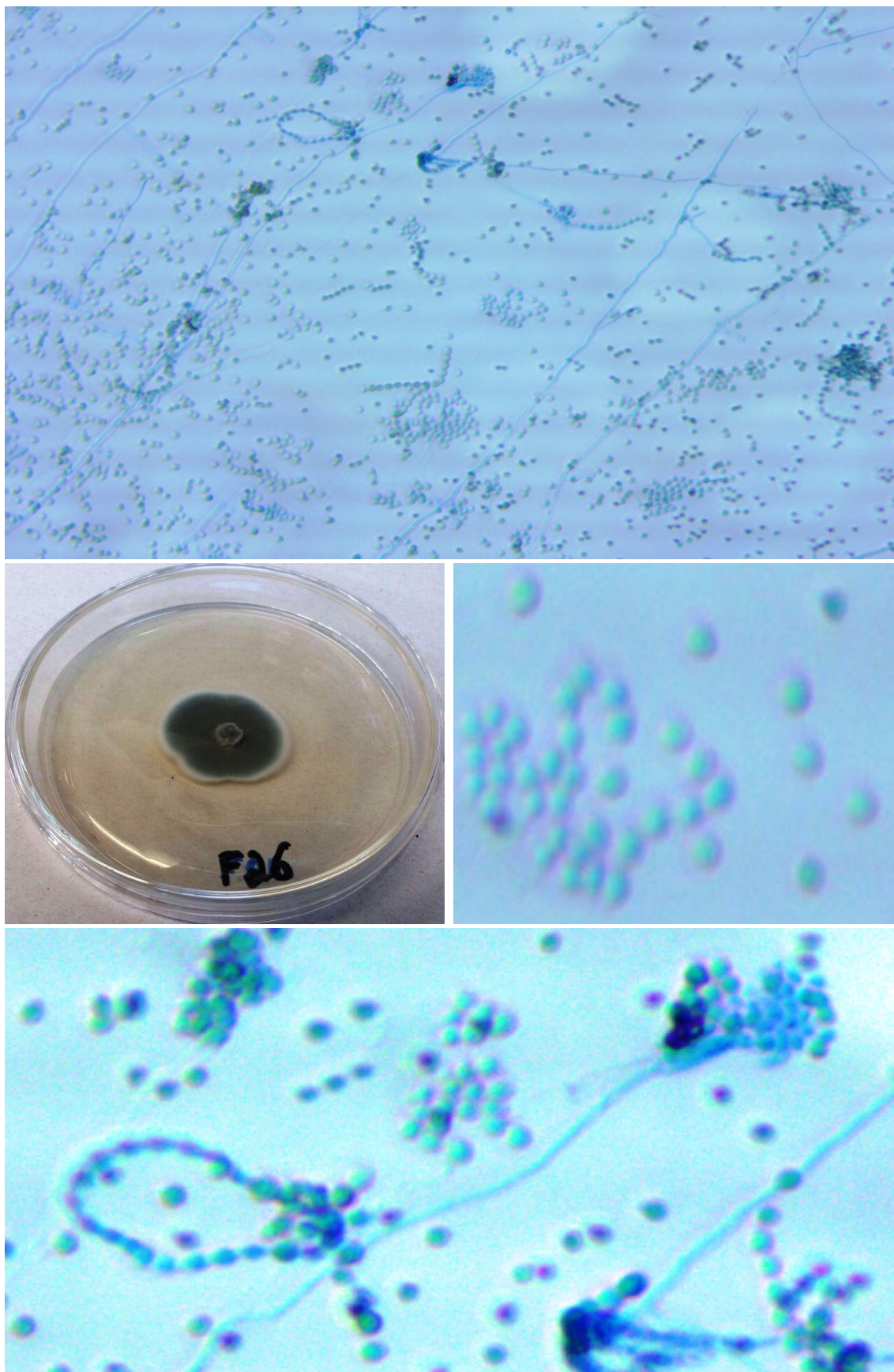


Figura 12. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Conidióforo e hifas.

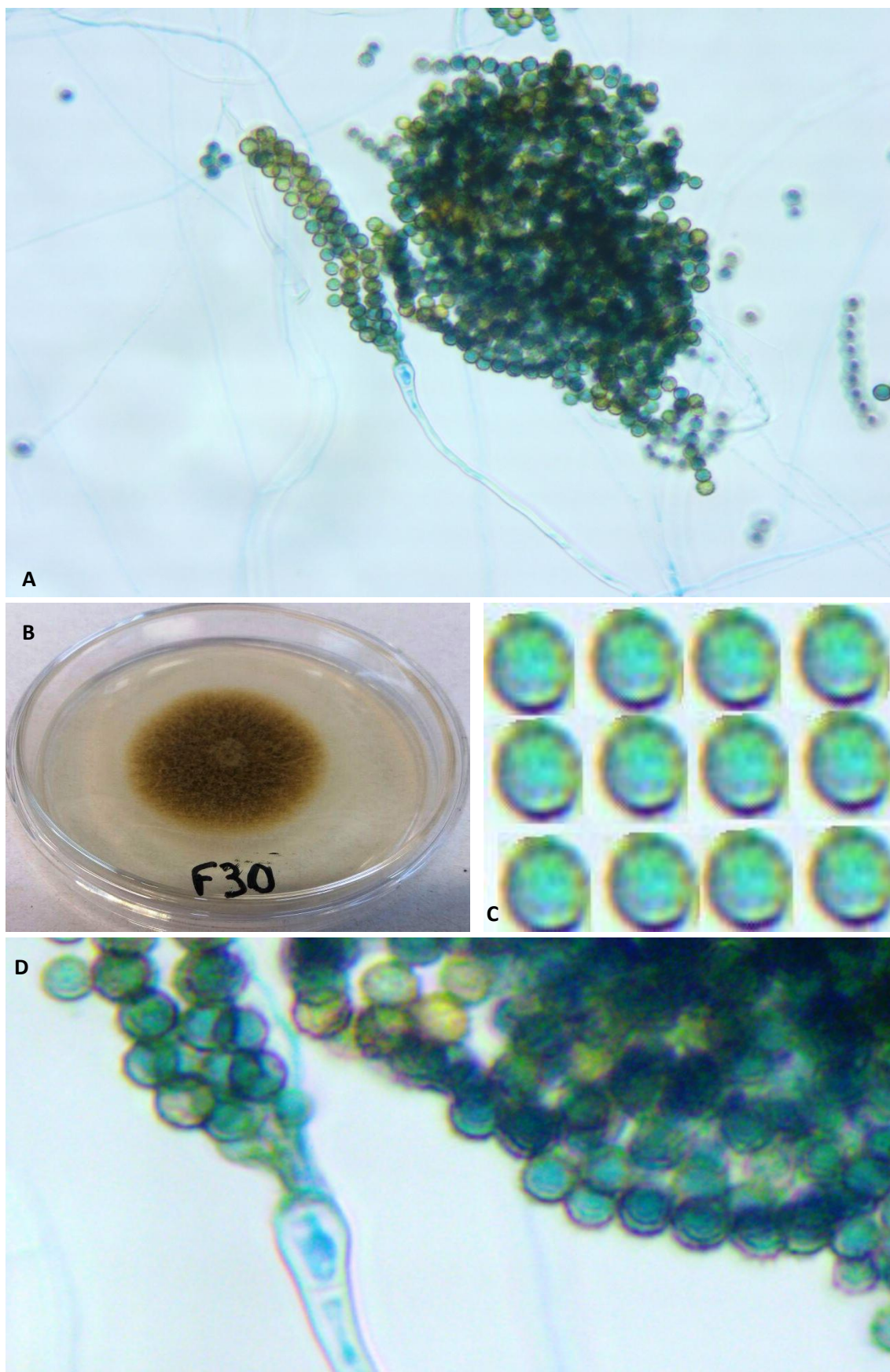


Figura 13. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Conidióforo e agrupamento de conídios.

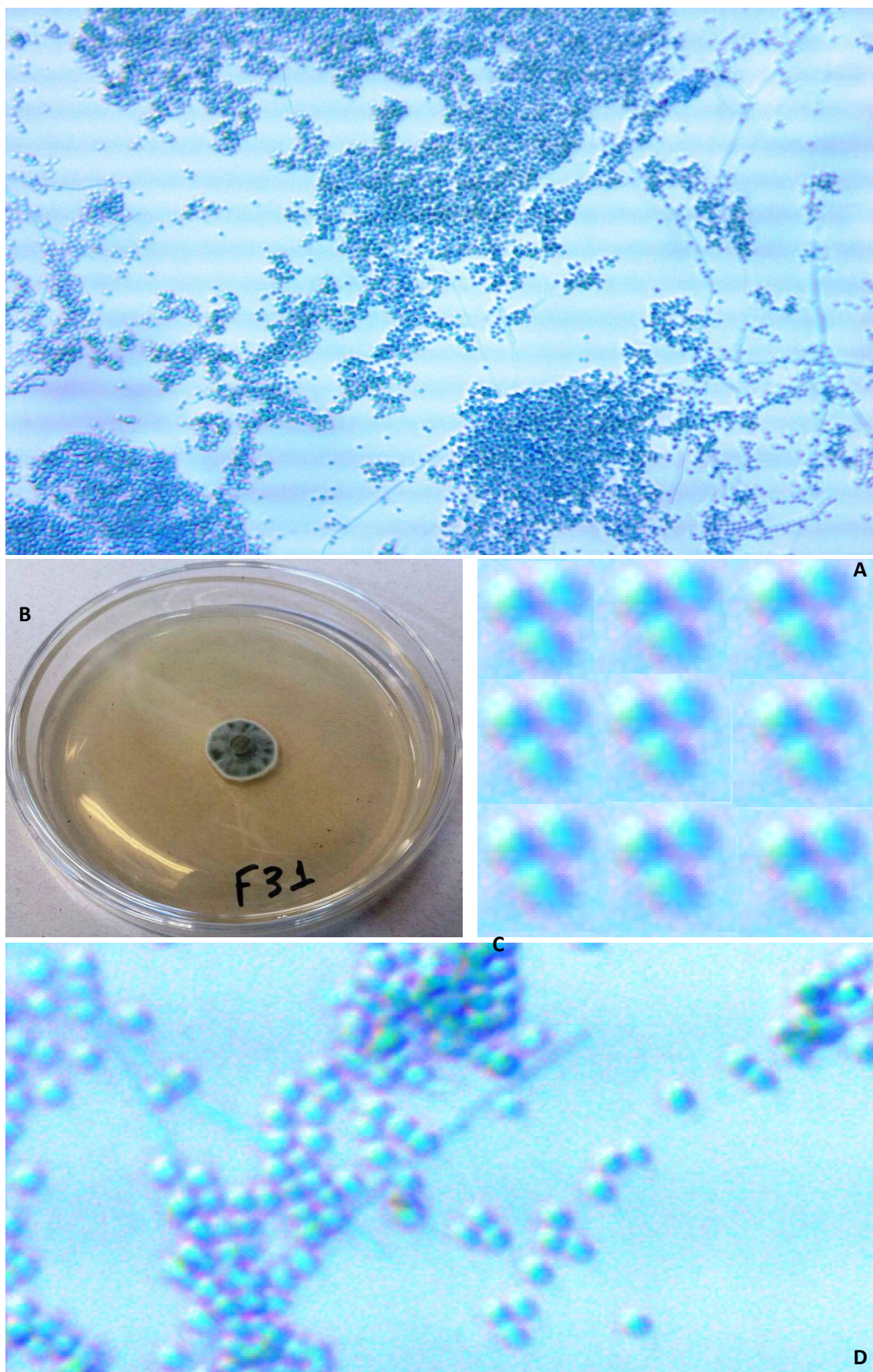


Figura 14. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D – Hifas e conídios.

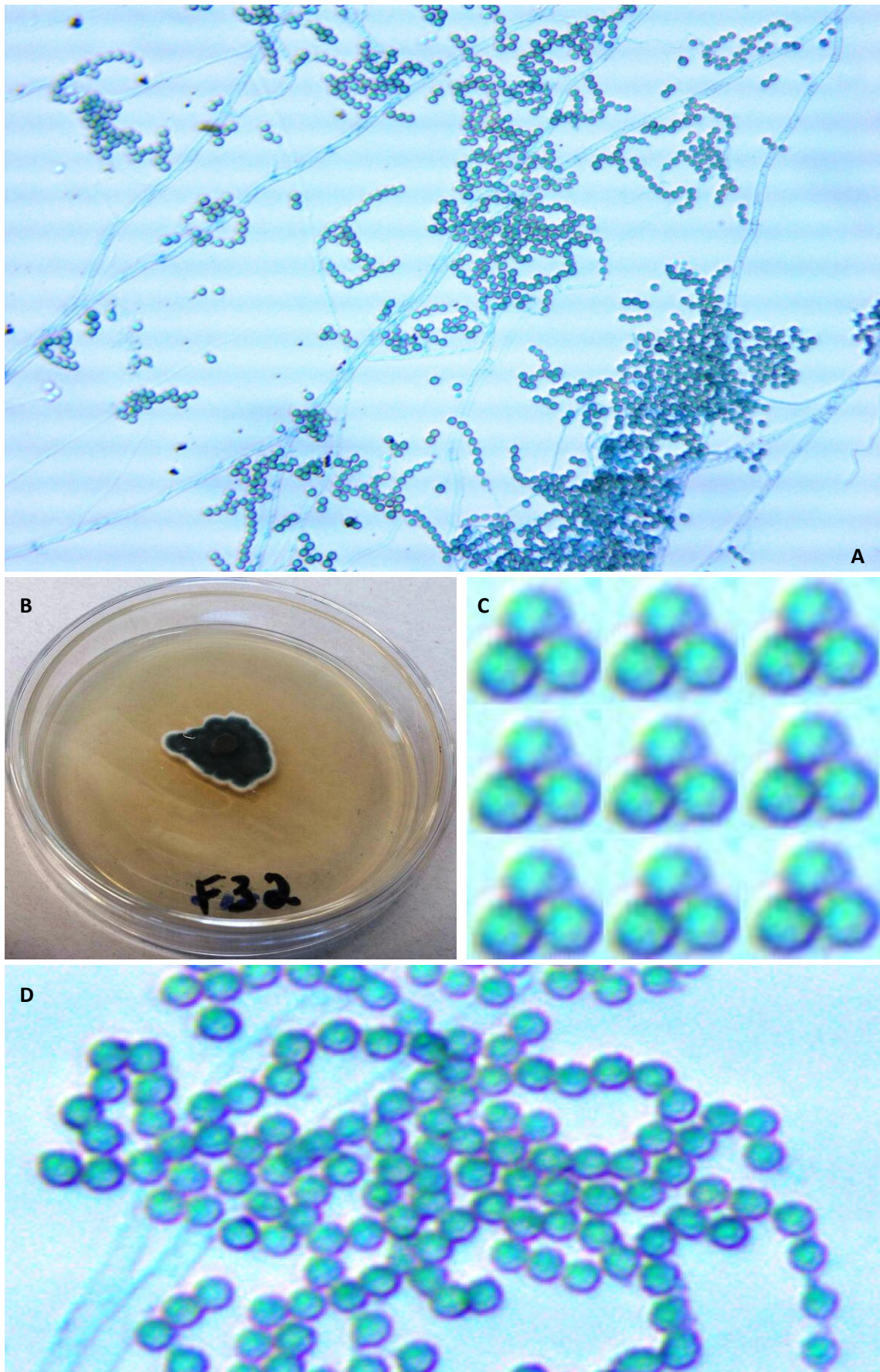


Figura 15. Fungo não identificado: A - Micrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D – Hifas e conídios.

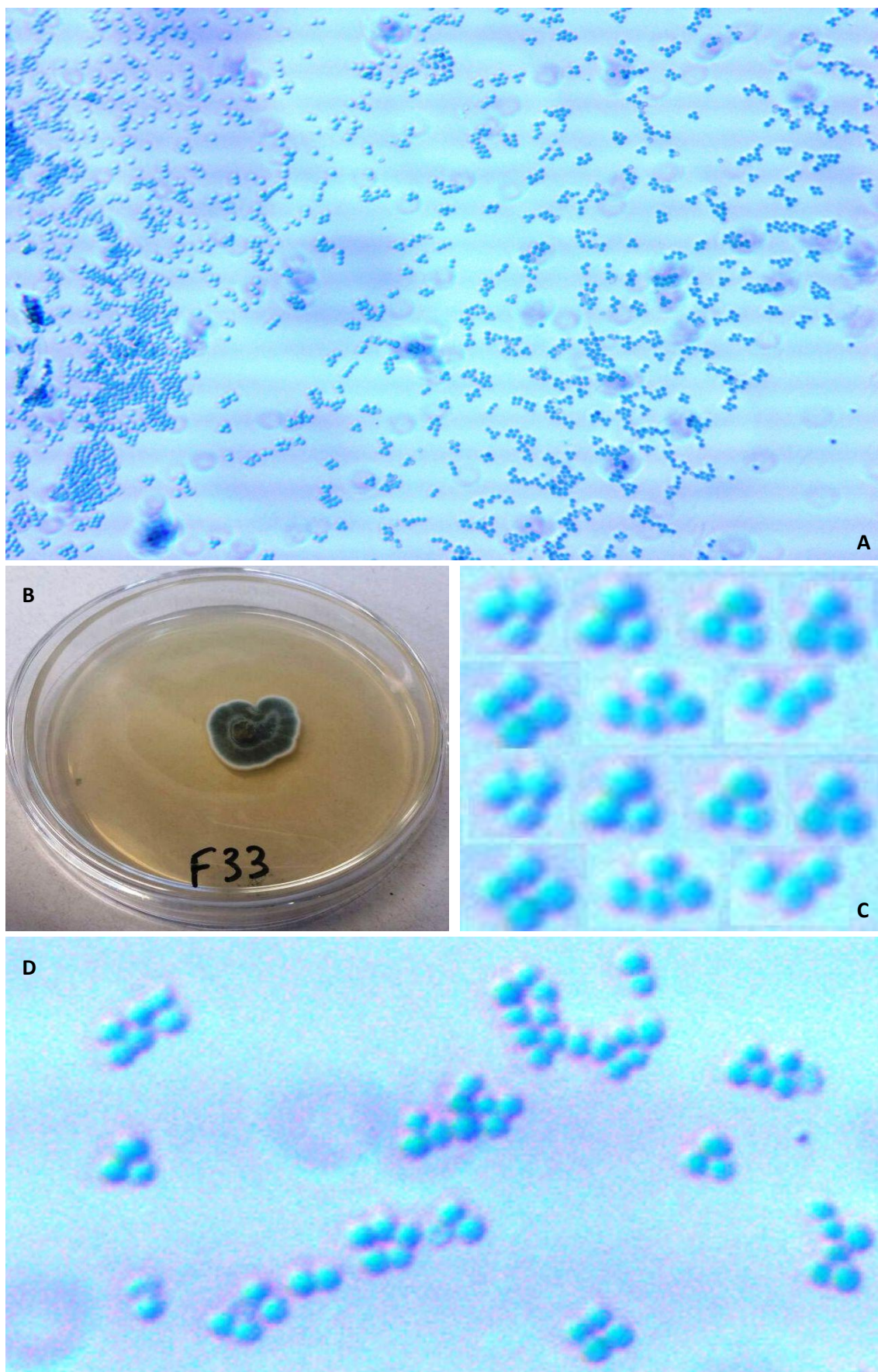


Figura 16. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D – Agrup. de conídios.

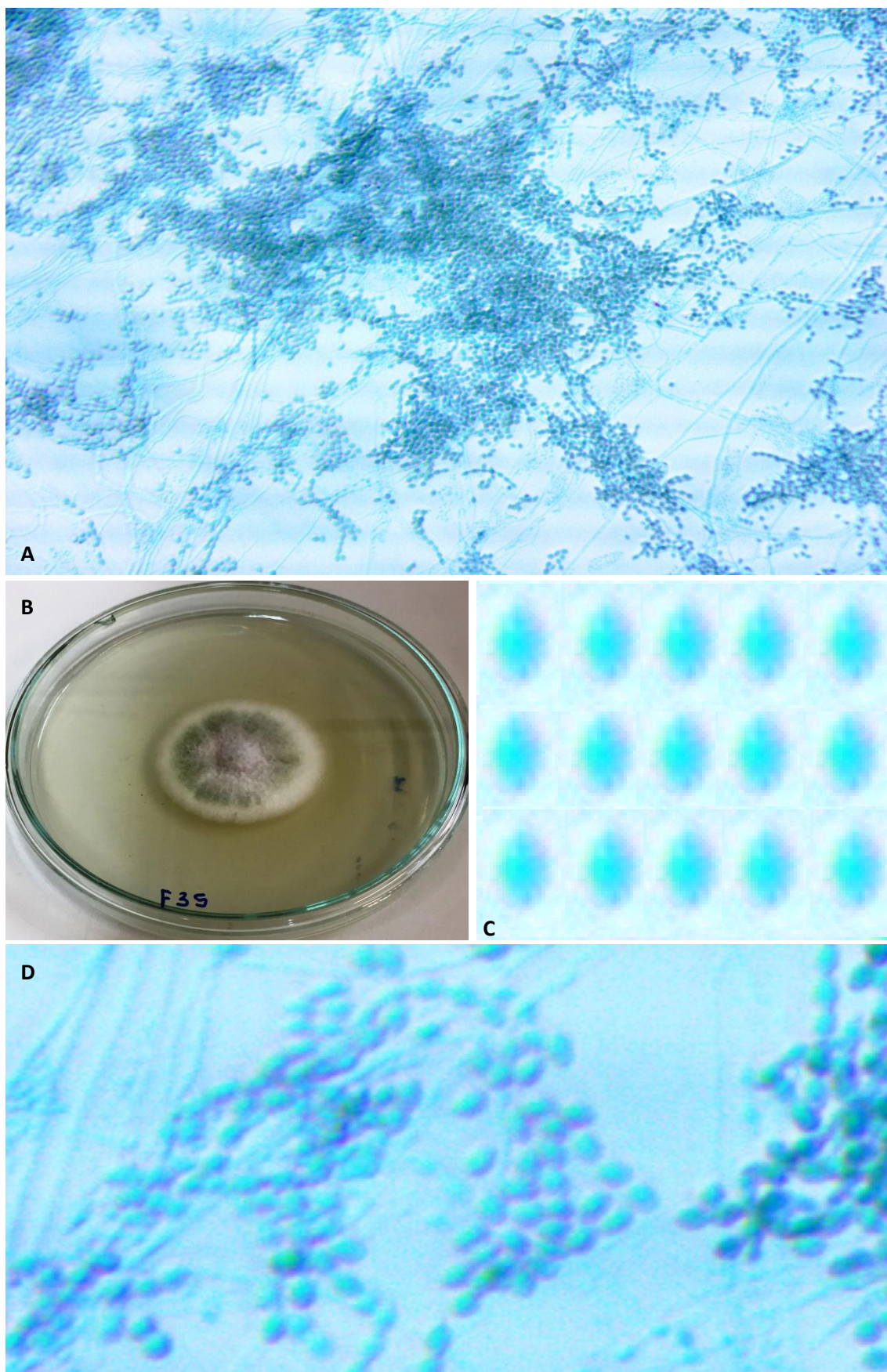


Figura 17. Fungo não identificado: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Hifas e conídios.

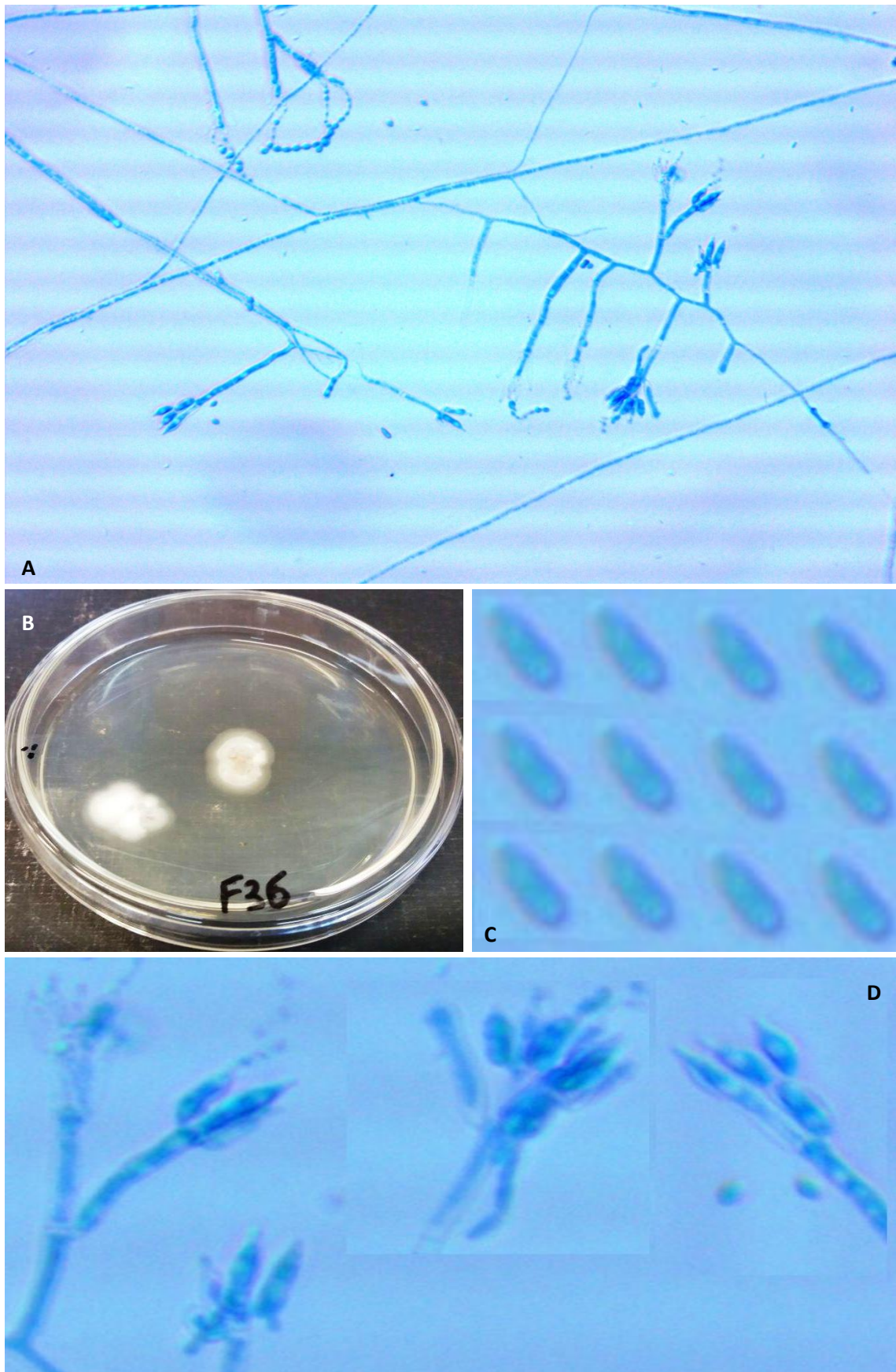


Figura 18. *Penicilium* sp: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Conidióforo e fiáldes.

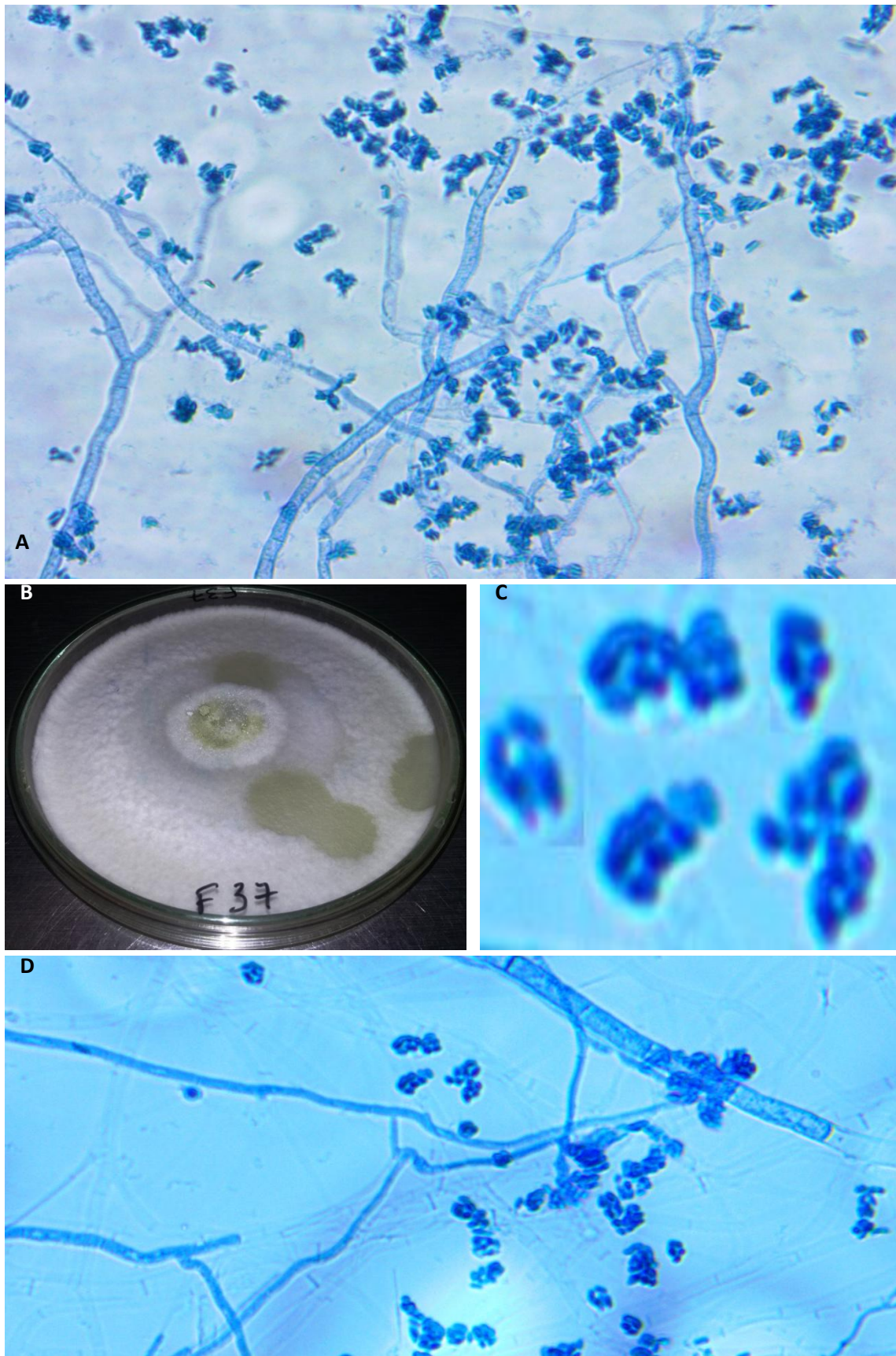


Figura 19. *Trichoderma harzianum*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Hifas, aneloconidio e agrupamento de conídios.

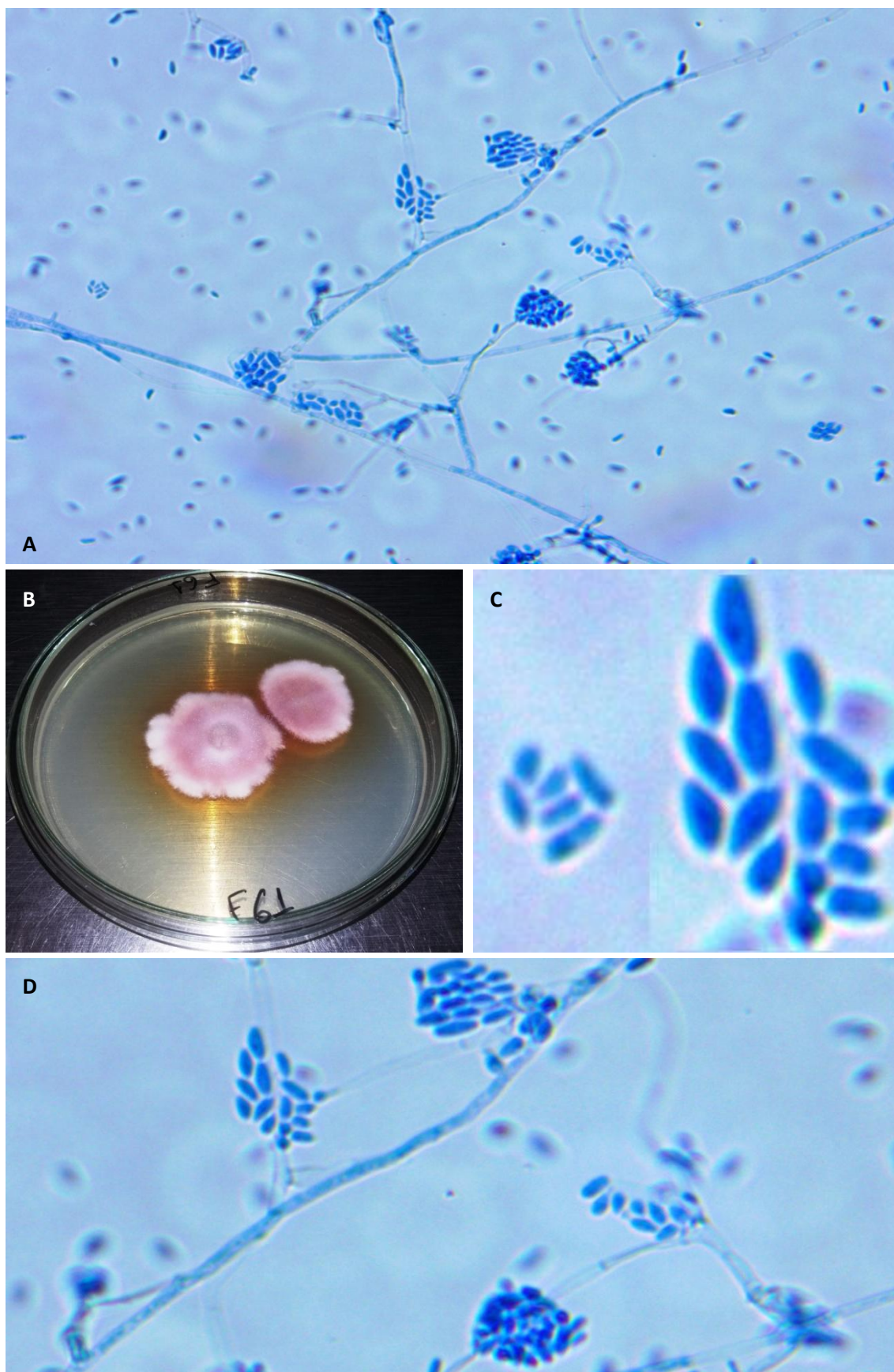


Figura 20. *Fusarium solani*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Microconídios e Macroconídios; D - Hifas e agrupamento de Conidióforos.

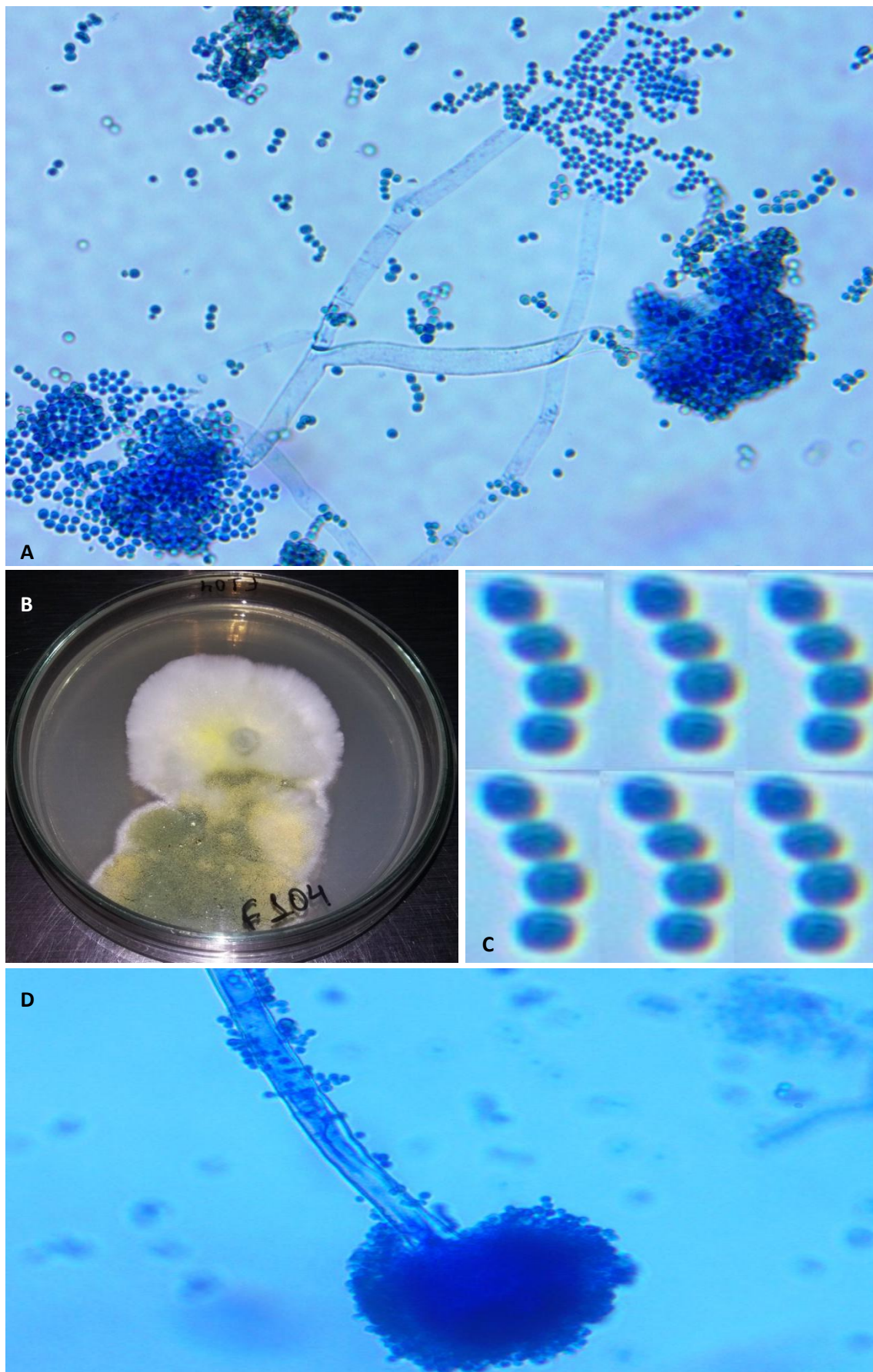


Figura 21. *Aspergillus flavus*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Conidióforo e Cleistotécio.

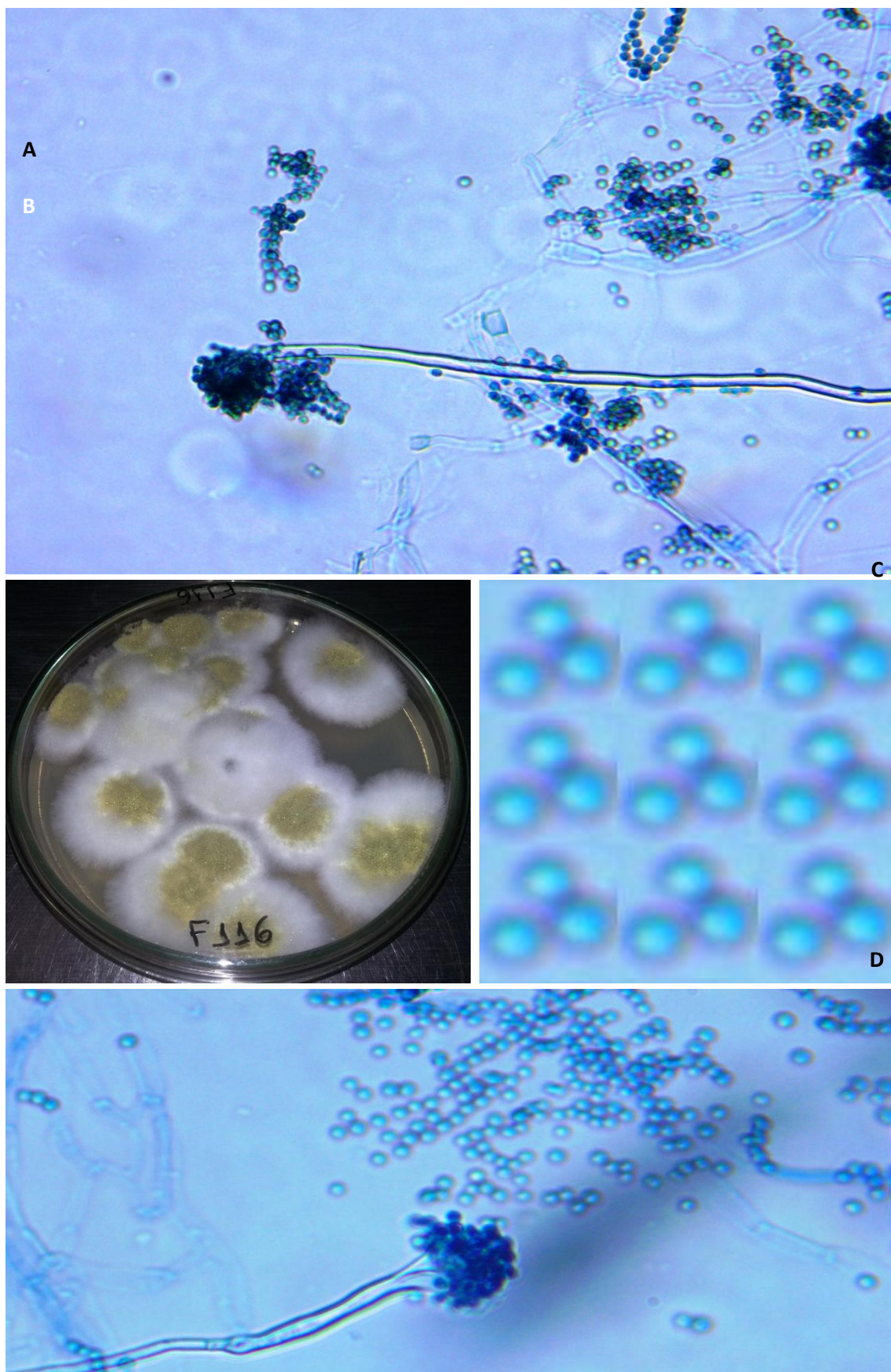


Figura 22. *Aspergillus flavus*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D - Conidióforo, fiálides e agrupamento de conídios.

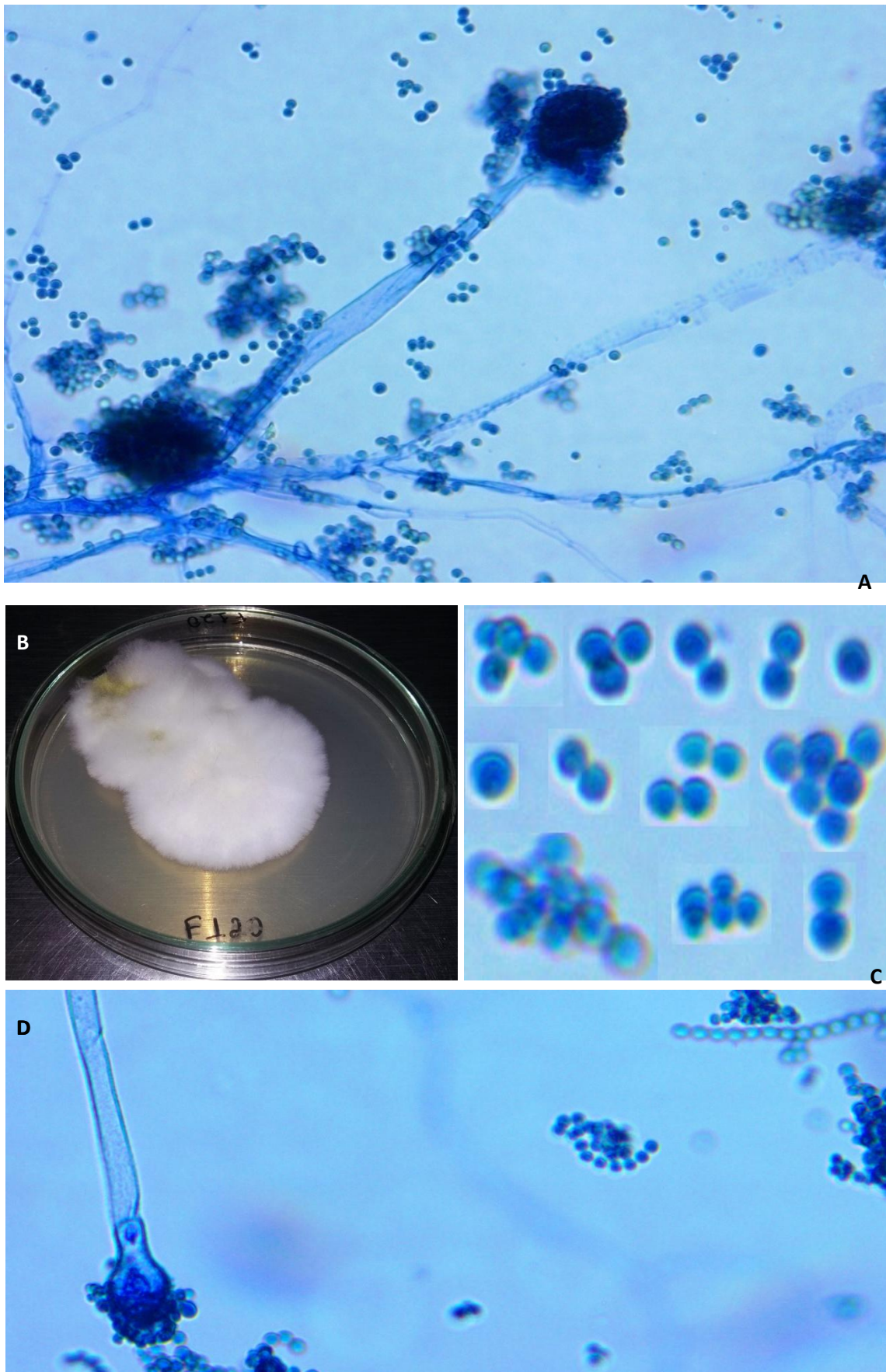


Figura 23. *Penicillium citrinum*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C - Conídios; D – Conidióforo e vesícula.

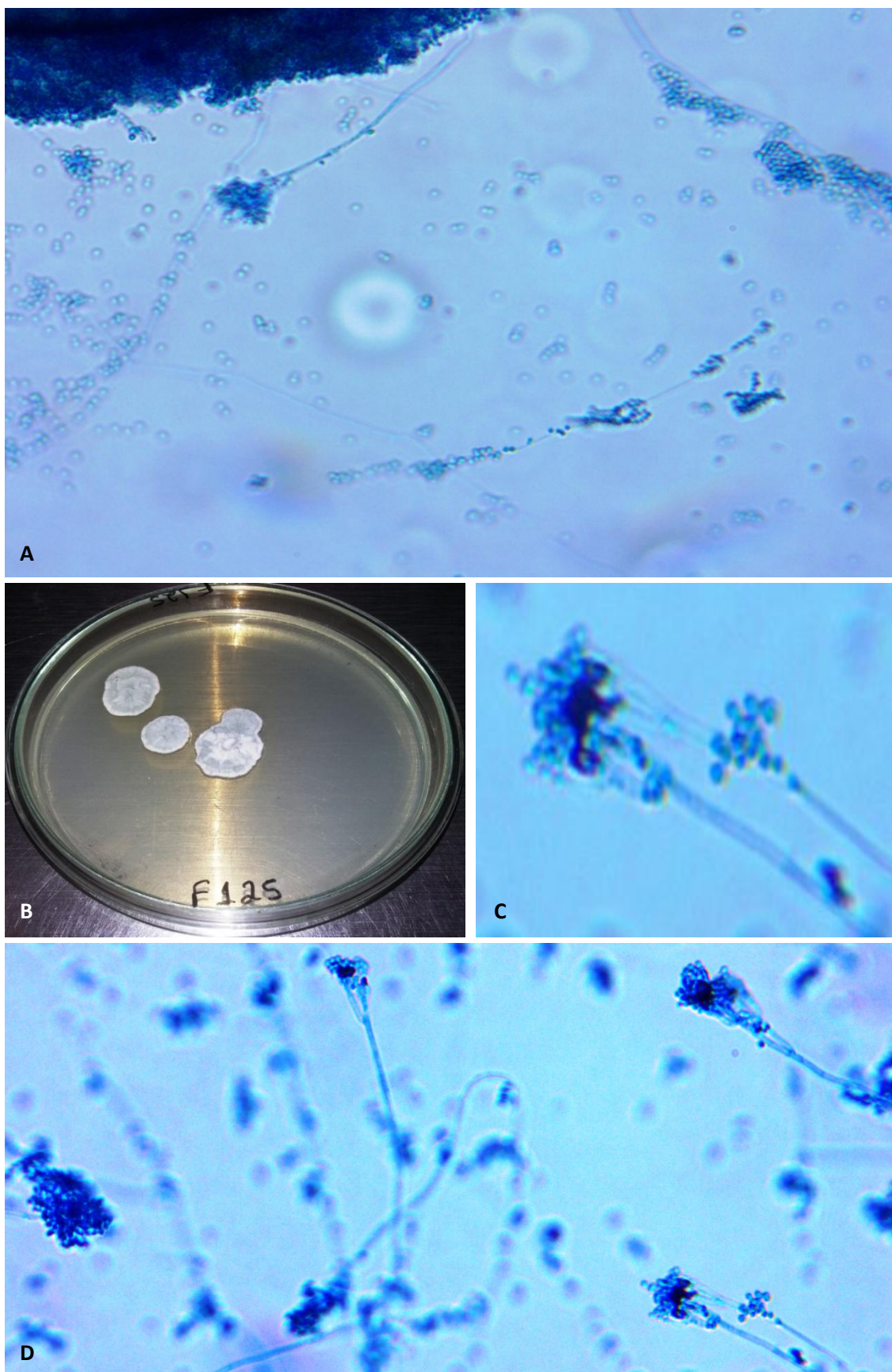


Figura 24. *Aspergillus* sp: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Conídios e fiálides; D - Conidióforo, fiálides e agrupamento de conídios.

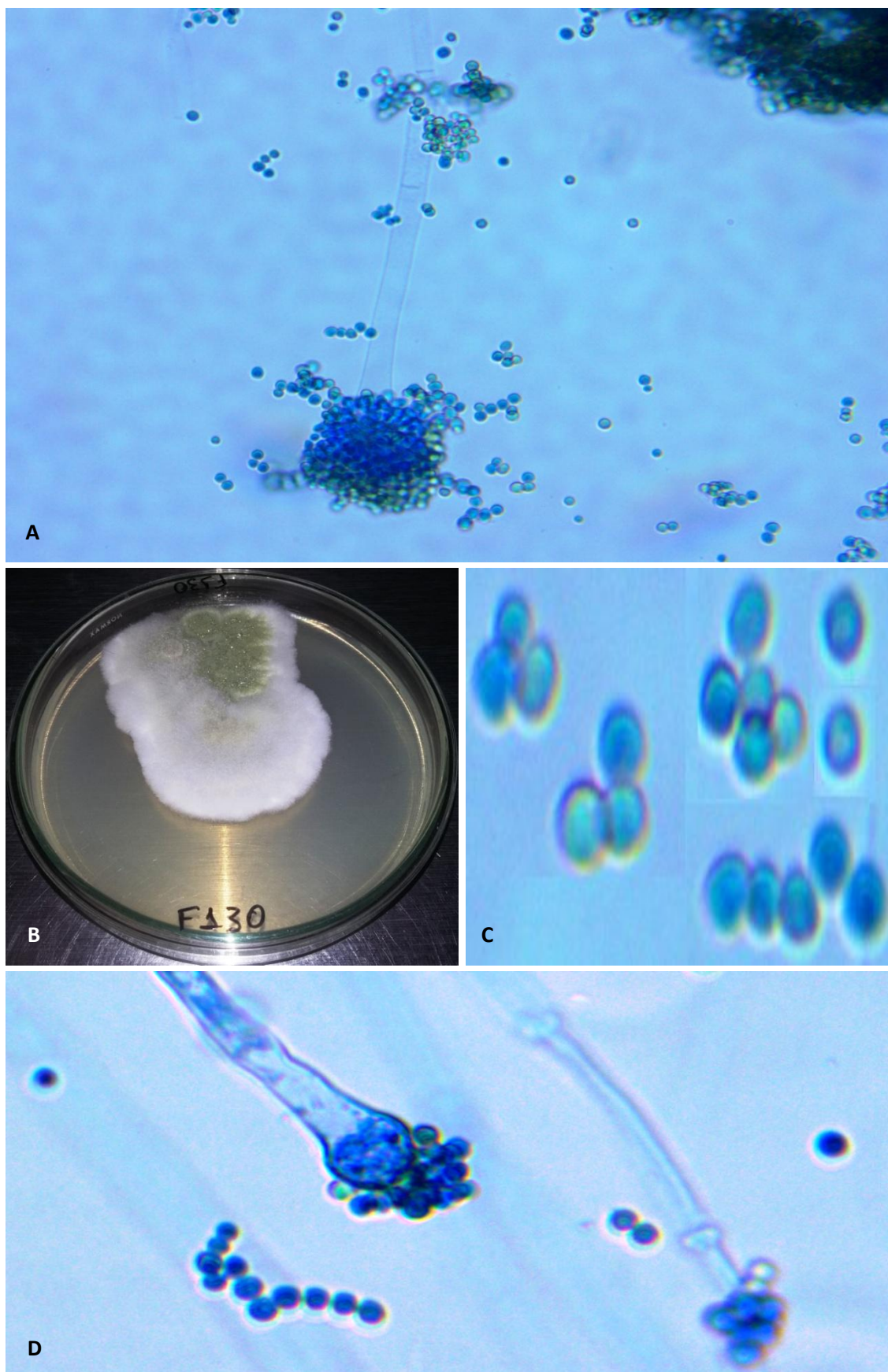


Figura 25. *Aspergillus oryzae* A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Conídios; D - Conidióforos vesícula e agrupamento de conídios.

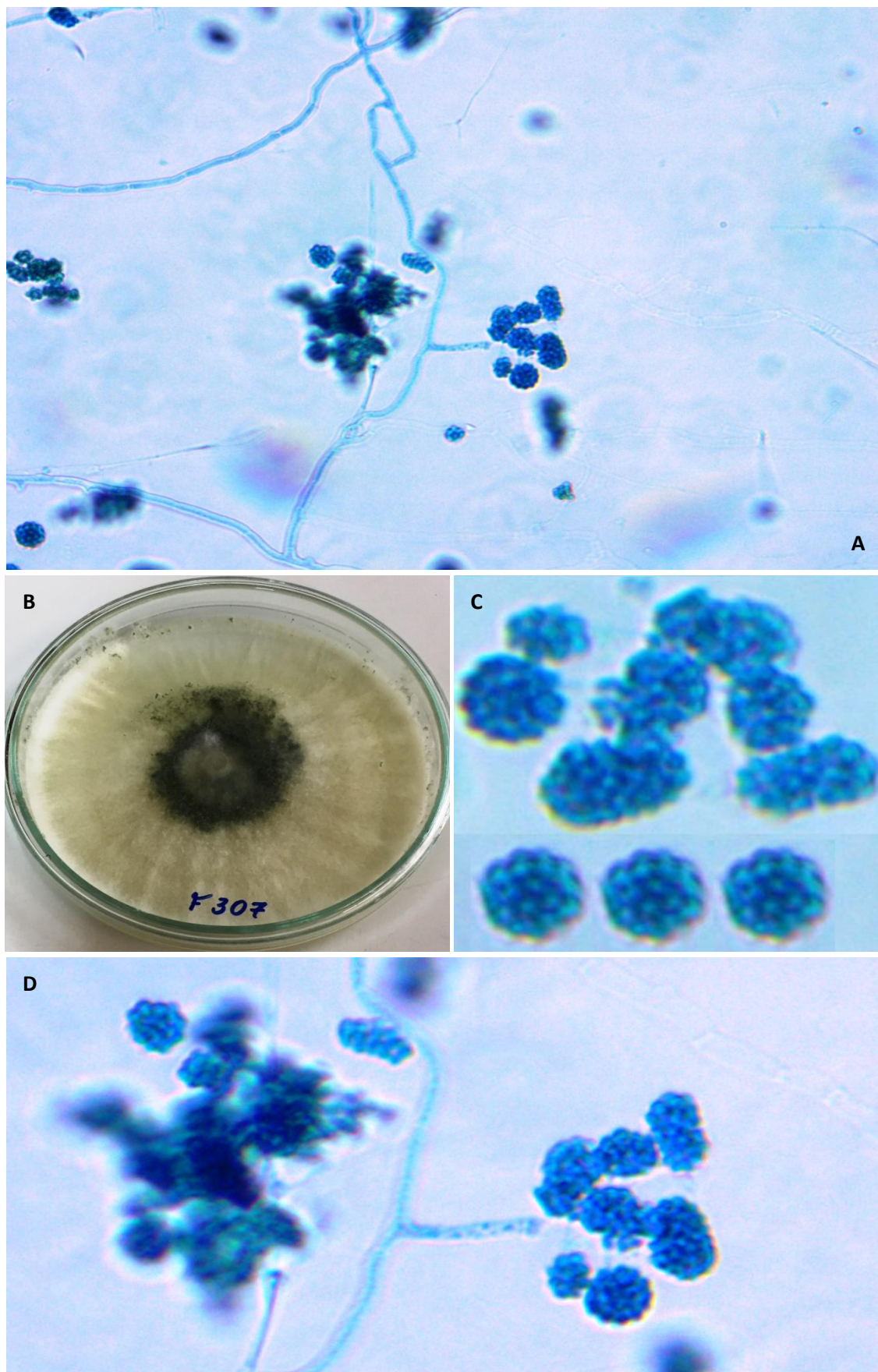


Figura 26. *Trichoderma harzianum*: A - Fotomicrografia do microcultivo; B - foto do fungo em placa de petri, cultivado com (PDA); C – Ampliação do Conidióforo; D - Hifas e Conidióforos.