UNIVERSIDADE ESTADUAL DE LONDRINA

MATHEUS MERTZ RIBEIRO

PRODUÇÃO DE *AMILASES* POR *Aspergillus welwitschiae*

Londrina
2021

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Mestre em Biotecnologia.

Orientador: Profª. Dra. Daniele Sartori
Coorientador: Profª. Dra. Maria Helena Pelegrinelli Fungaro

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Londrina, 26 de fevereiro de 2021.

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RESUMO

As *amilases* apresentam grande importância biotecnológica, podendo ser aplicadas em diversos setores industriais. Estas enzimas podem ser obtidas de fontes vegetais, animais ou principalmente por microrganismos. Entre os microrganismos, os fungos do gênero *Aspergillus* são os mais utilizados industrialmente. A utilização de fontes microbianas para geração de *amilases* apresenta algumas vantagens, como fácil manipulação e obtenção de quantidades maiores de produtos. Por outro lado, ainda há escassez quanto a disponibilidade de linhagens seguras, quanto a não produção de micotoxinas e aptas à produção de *amilases* em quantidades superiores. Neste sentido, o objetivo deste estudo foi selecionar linhagens de *Aspergillus welwitschiae* potenciais produtoras de *amilases*. As linhagens selecionadas foram avaliadas quanto aos melhores parâmetros de produção destas enzimas e posteriormente, os extratos brutos enzimáticos obtidos foram parcialmente purificados e caracterizados. Dentre um total de 24 linhagens de *A. welwitschiae*, todas foram produtoras de *amilases*, sendo que a linhagem UELAs 15.262 foi selecionada por apresentar maior capacidade de produção destas enzimas. Em pH 7,0 a 35 °C houve melhor produção de *amilases* por UELAs 15.262 e sob estas condições a fermentação submersa foi conduzida, resultando na produção de 951 U/mL no sétimo dia de fermentação. Nestas mesmas condições, a fermentação também foi realizada com a linhagem mutante de *A. welwitschiae* UELAs 15.262/35, previamente obtida por mutação induzida por luz ultravioleta, a qual produziu 580 U/mL de *amilases* no sexto dia de fermentação. A partir dos parâmetros abióticos selecionados para a produção de *amilases*, foi feita a caracterização destas enzimas obtidas dos Extratos Brutos Enzimáticos Parcialmente Purificados (EBEPP). As melhores atividades de *amilases* ocorreram em pH 5,5, e pH 5,0, a 60 °C, para UELAs 15.262 e UELAs 15.262/35, respectivamente. Quanto a estabilidade térmica, houve redução de apenas 30% (UELAs 15.262/35) e 35% (UELAs 15.262) nas atividades de *amilases* ao longo de 100 minutos de incubação. Foi detectado também que os íons metálicos Cu^{2+} e Fe^{3+} contribuíram para maior atividade destas enzimas obtidas por ambas as linhagens, resultando em 2.410 U/mL e 3.150 U/mL de atividade de *amilases* obtidas do EBEPP de UELAs 15.262 e UELAs 15.262/35, respectivamente. Os dados deste estudo, permitiram detectar maior produção de *amilases* por *A. welwitschiae* UELAs 15.262, enquanto a maior atividade de *amilases* foi obtida por UELAs 15.262/35. Este foi o primeiro estudo voltado à obtenção e caracterização de *amilases* a partir de linhagens de *A. welwitschiae,* potenciais candidatas a aplicações industriais.

Palavras-chave: *Amilases*. Fermentação Submersa. *Aspergillus welwitschiae.*

RIBEIRO, Matheus Mertz. **Production of *Amylases* by *Aspergillus welwitschiae***. 2021. 81 p. Dissertation (Master's degree in Biotechnology) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Amylases are of great biotechnological importance and can be applied in various industrial sectors. These enzymes can be obtained from vegetable or animal sources, or mainly by microorganisms. Among microorganisms, fungi of the genus *Aspergillus* are the most industrially used. The use of microbial sources to generate *amylases* presents some advantages, such as easy handling and obtaining larger quantities of products. On the other hand, there are still shortages regarding the availability of safe strains that do not produce mycotoxins and are able to produce *amylases* in larger quantities. Therefore, the objective of this study was to select strains of *Aspergillus welwitschiae* that are potential producers of *amylases*. The selected strains were evaluated for the best production parameters of these enzymes, and later, the obtained enzyme crude extracts were partially purified and characterized. Among a total of 24 strains of *A. welwitschiae*, all were producers of *amylases*, and the UELAs 15.262 strain was selected for presenting the highest production capacity of these enzymes. In pH 7.0 at 35 °C there was better production of *amylases* by UELAs 15.262 strain and under these conditions submerged fermentation was conducted, resulting in the production of 951 U/mL on the seventh day of fermentation. Under these same conditions, fermentation was also conducted with the mutant strain UELAs 15.262/35 of *A. welwitschiae*, previously obtained for induced mutation by ultraviolet light, which produced 580 U/mL of *amylases* on the sixth day of fermentation. From the abiotic parameters selected to produce *amylases*, the characterization of these enzymes obtained from the Crude Enzyme Extracts Partially Purified (CEEPP) was performed. The best *amylase* activities occurred at pH 5.5, and pH 5.0, at 60 °C, for UELAs 15.262 and UELAs 15.262/35, respectively. As for thermal stability, there was only 30% (UELAs 15.262/35) and 35% (UELAs 15.262) reduction in amylase activities over 100 min of incubation. It was also detected that the metal ions Cu^{2+} and Fe^{3+} contributed to higher activity of these enzymes obtained by both strains, resulting in 2,410 U/mL and 3,150 U/mL of *amylase* activity obtained from the CEEPP of UELAs 15.262 and UELAs 15.262/35, respectively. The data from this study, allowed detecting higher amylase production by *A. welwitschiae* UELAs 15.262, while the highest amylase activity was obtained by UELAs 15.262/35. This was the first study aimed at obtaining and characterizing amylases from *A. welwitschiae* strains, potential candidates for industrial applications.

Key-words: *Amylases*. Submerged Fermentation. *Aspergillus welwitschiae*.

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SUMÁRIO

1 INTRODUÇÃO

As enzimas do tipo *amilases* podem ser obtidas por meio de fontes animais, vegetais e por microrganismos, sendo este último a principal forma utilizada industrialmente, devido a fácil manipulação e a geração de grandes quantidades de metabólitos em curtos períodos de tempo.

Em função de serem produzidas por diversas espécies de microrganismos, as *amilases* apresentam múltiplas propriedades em seu mecanismo de ação, podendo atuar em amplas faixas de pH e temperatura. Devido às distintas características de atuação, as enzimas amilolíticas apresentam grande importância biotecnológica com amplo espectro de aplicação nos setores industriais como, indústrias têxteis, de papel, de detergentes, de ração animal, alimentícia e indústria farmacêutica.

Tradicionalmente, as *amilases* são produzidas industrialmente por Fermentação Submersa (FS), devido à possibilidade de melhor controle dos parâmetros fermentativos, menor risco de contaminação e facilidade no processo de recuperação enzimática.

Inserido no setor industrial, um dos principais gêneros de microrganismos utilizados para a produção de enzimas amilolíticas é o gênero *Aspergillus*, tendo como principal representante neste setor, a espécie *Aspergillus niger*. No entanto, outra espécie muito similar, *A. welwitschiae* também pode apresentar potencial para a produção de metabólitos de interesse industrial e/ou biotecnológico.

Poucos são os relatos voltados à produção de metabólitos por *A. welwitschiae*, em especial não há nenhum estudo voltado à produção de *amilases* por linhagens desta espécie. Embora, assim como *A. niger*, linhagens desta espécie também possam produzir micotoxinas como, Ocratoxina A e Fumonisina B2, alguns representantes da espécie são incapazes de produzir ambas as micotoxinas e consequentemente, a seleção de linhagens de *A. welwitschiae* com maior potencial de *amilases* por exemplo, favorece a introdução destes exemplares no setor industrial.

Nesse contexto, o presente estudo teve como objetivo selecionar linhagens de *Aspergillus welwitschiae* produtoras de *amilases* e estabelecer os melhores parâmetros bioquímicos para a produção e atuação dessas enzimas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Selecionar linhagens de *Aspergillus welwitschiae* produtoras de *amilases* e estabelecer os melhores parâmetros para a produção e atuação dessas enzimas.

2.2 OBJETIVOS ESPECÍFICOS

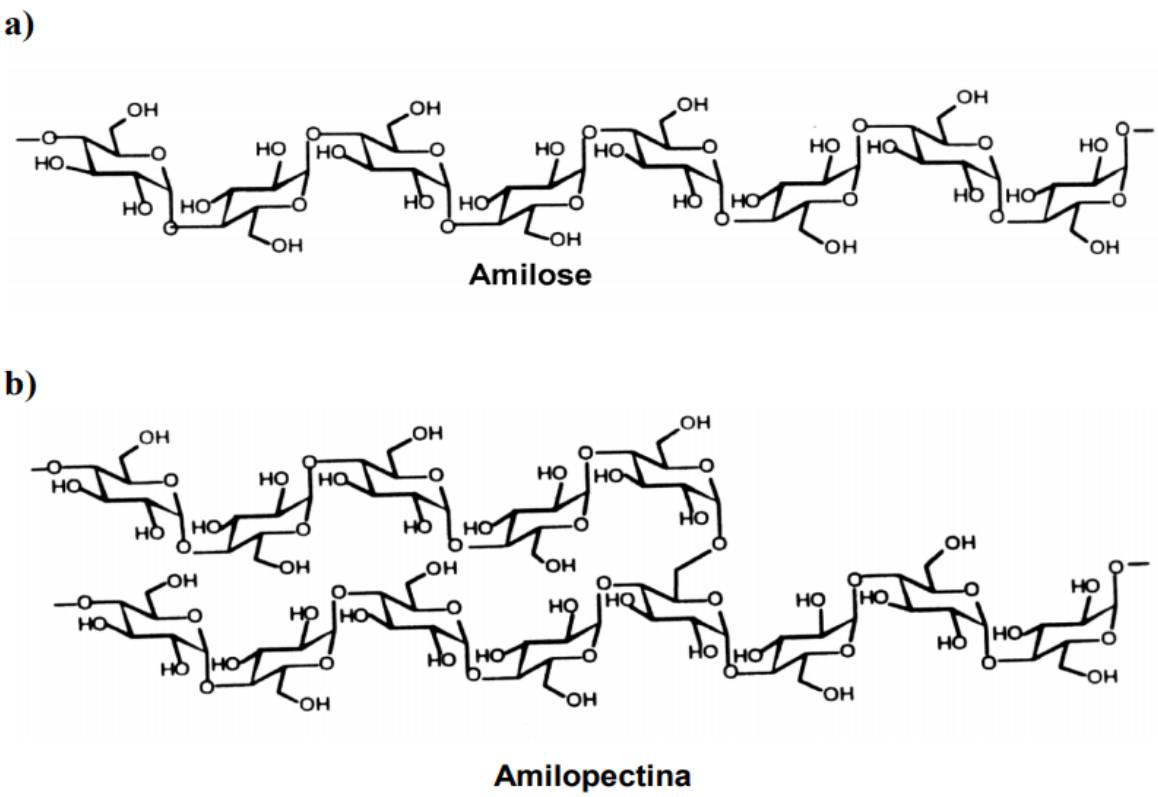
- Selecionar linhagens de *Aspergillus welwitschiae* produtoras de *amilases*;
- Otimizar os parâmetros abióticos para a produção de *amilases* em Fermentação Submersa, para as linhagens selecionadas;
- Avaliar a produção de *amilases,* pelas linhagens selecionadas e pela linhagem mutante UELAs 15.262/35;
- Determinar a atividade das *amilases* nos Extratos Brutos Enzimáticos;
- Realizar a purificação parcial e caracterização das *amilases* produzidas, quanto a pH, temperatura, estabilidade térmica e efeito de íons metálicos.

3 REVISÃO DA LITERATURA

3.1 *AMILASES* E A APLICAÇÃO NO SETOR INDUSTRIAL

A molécula de amido (Figura 1), substrato para *amilases* é formada por dois tipos de polissacarídeos: A amilose, constituída por cadeia linear, não ramificada, com cerca de 250 a 300 resíduos de D-glicopiranose, unidas por ligações glicosídicas α-1,4, representando 15-20% da molécula de amido. E a amilopectina, uma molécula ramificada, formada por cerca de 1400 resíduos de α-glicose unidas por ligações glicosídicas α-1,4, e ligações glicosídicas α-1,6, constituindo aproximadamente 80-85% da molécula de amido (COSTA et al., 2013; AMAGLIANI et al., 2016; SJÖÖ; NILSSON, 2017).

Figura 1 - Representação da molécula de amido, amilose (A) e amilopectina (B)

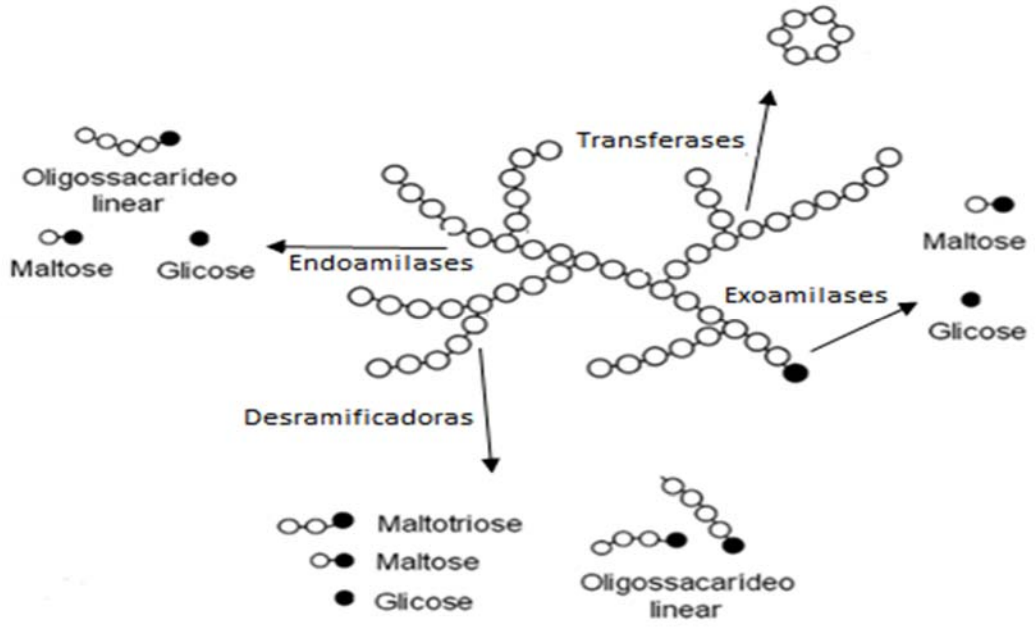


Fonte: Robyt (1998).

As *amilases* pertencem à família das Glicosil Hidrolases (GH), e são classificadas de acordo com o mecanismo de atuação sobre as moléculas de amido (GRIEBELER et

al., 2015; POLIZELI et al., 2016). Segundo Reddy et al. (2003) e Castro et al. (2011), as enzimas amilolíticas são divididas em quatro subclasses: *endoamilases*, *exoamilases*, desramificadoras e *transferases*, conforme apresentada na Figura 2.

Figura 2 - Classificação das *amilases* conforme o mecanismo de ação.



Fonte: Adaptado de Bertoldo; Antranikian (2002).

As *endoamilases*, também são conhecidas como enzimas dextrinizantes. Esse grupo de enzimas atua especificamente sob as ligações glicosídicas α-1,4 da molécula de amido. A ação destas enzimas acarreta a geração de diferentes resíduos, tais como moléculas de glicose, dextrina ou oligossacarídeos lineares (Figura 2). Uma das principais enzimas constituintes deste grupo são as α-*amilases* (EC 3.2.1.1.) (GUPTA et al., 2003; CASTRO et al., 2018).

As *exoamilases* hidrolisam ligações glicosídicas atuando sobre a extremidade não redutora da molécula de amido, produzindo carboidratos de menor massa molar como a glicose ou maltose (Figura 2). As *β-amilases* (EC 3.2.1.2, α-1,4-D-glicano-maltoidrolase) são *exoamilases* encontradas em vegetais, cujo atuação produz maltose. Outra *exoamilase* conhecida é a *glucoamilase* (EC 3.2.1.3, α-1,4-D-glicano-glicoidrolase), caracterizada por romper as ligações α-1,4 e α-1,6 do amido, levando à formação de glicose e maltose (FERNANDES et al., 2007; SANTOS et al., 2012).

As *amilases* desramificadoras como a *pululanases* e a *isoamilases* atuam sobre as ligações glicosídicas α-1,6 do amido e oligossacarídeos relacionados, formando maltose e glicose (Figura 2). As *isoamilases* diferentemente das *pululanases* são incapazes de hidrolisar o pululano (polissacarídeo constituído de moléculas de maltotriose) (ANTO et al., 2006).

Já as *transferases* são conhecidas por hidrolisarem as ligações glicosídicas α-1,4 da molécula de amido e transferir em grupos funcionais específicos para um aceptor glicosídico, com a constituição de uma nova ligação glicosídica α-1,4 ou α-1,6 (Figura 2). As principais enzimas desta subclasse são as *amilomaltases* e *ciclodextrinas* (GUPTA et al., 2003; FERNANDES et al., 2007).

As enzimas do tipo *amilases* apresentam grande importância biotecnológica, constituindo aproximadamente 25% do mercado mundial de enzimas. A capacidade de atuar sobre o amido, gerando produtos de alto valor agregado, faz com que estas enzimas desempenhem um papel fundamental em diversos processos industriais, tais como exemplo: as indústrias têxteis, de papel, de detergentes, de ração animal, alimentícia e farmacêutica, dentre outras (LUZ et al., 2016; GOPINATH et al., 2017; SOCCOL et al., 2017; CASTRO et al., 2018; COELHO et al., 2018).

No segmento têxtil e na fabricação de papel, as *amilases* são utilizadas no processo de retirada das gomas das fibras têxteis e das folhas de papel. Para impedir o rompimento do papel, e dos fios no processo de tecelagem, os materiais utilizados são engomados em produtos à base de amido, conferindo maior resistência e integridade às suas superfícies. A aplicação do amido nativo torna tais superfícies muito viscosas, e, por essa razão, as *amilases*, atuam na hidrólise das moléculas de amido da goma, reduzindo a viscosidade, e fornecendo maior qualidade para os tecidos e papéis produzidos (TIMAR-BALÁZSY; EASTOP, 1998; GUPTA et al., 2003; ORLANDELLI et al., 2012; AHMAD et al., 2019).

Na indústria de detergentes, as *amilases* também são muito utilizadas. Cerca de 90% de todos os detergentes líquidos contêm *amilases* em suas formulações (FAR et al., 2020). As *amilases* proporcionam maior capacidade de limpeza e remoção de manchas através da degradação de certos resíduos alimentícios que contêm amido (como molhos, chocolates) (KOTTWITZ et al, 1994; SOUZA; MAGALHÃES, 2010; SIMAIR et al., 2017).

Além disso, produtos que contém *amilases* podem ser utilizados também na assepsia de tanques, compostagem, tratamentos de resíduos e efluentes (RUBIO et al., 2007; EDC, 2021).

Na indústria alimentícia, como de sucos de frutas, as *amilases* atuam com a finalidade de evitar turvações e gelatinizações durante o processamento. Já nas cervejarias, as *amilases* atuam contribuindo com a formação de dissacarídeos e oligossacarídeos facilitando o processo fermentativo pelas leveduras (D''AVILA et al., 2012; OLIVEIRA; SILVA, 2017). Na sacarificação do amido, isto é, na conversão do amido em açúcares fermentáveis, a utilização das *amilases* é na produção de amido hidrolisado na forma de monossacarídeos do tipo glicose e frutose. Por possuir propriedades adoçantes, a glicose e a frutose são utilizadas como adoçantes na indústria de bebidas carbonatadas (refrigerantes). O amido também pode ser convertido em xaropes de milho com alto teor de frutose (VAN DER MAAREL et al, 2002; SIMAIR et al., 2017).

Nos processos de panificação, as *amilases* ao promoverem a hidrólise do amido, levam à geração de açúcares fermentáveis, como maltose e glicose, proporcionando melhor aproveitamento destes açucares pelas leveduras, resultando em maior volume e textura dos pães. No entanto, neste tipo de indústria, é comumente utilizado *amilases* bacterianas, por apresentarem maior estabilidade durante o processo de panificação (ORLANDELLI et al., 2012; AHMAD et al., 2019).

Relativo a vasta utilização das *amilases* no setor alimentício, as *amilases* são usadas no processamento de cereais para consumo como misturas de cereais, arroz ou produção de derivados como mingau de milho e farinha láctea. Neste processo as *amilases* são adicionadas com o intuito de reduzir a viscosidade da mistura durante o processo de gelatinização, processo que consiste em aquecer os grânulos de amido até o rompimento das cadeias de amilose e amilopectina. Esse procedimento é utilizado para facilitar o escoamento da mistura pela tubulação. Posteriormente, as enzimas amilolíticas são desnaturadas pelo aquecimento durante a secagem dos cereais, deste modo são conceituadas como coadjuvantes tecnológicas de fabricação (CEREDA et al., 2003; ALLBRANS, 2004). E, ainda no setor de alimentação animal, o uso de *amilases* contribuem com a maior digestibilidade das rações pelo sistema digestivo dos animais

(CAMPESTRINI; SILVA; APPELT, 2005).

Na indústria farmacêutica, as *amilases* obtidas de pâncreas de suínos são utilizadas para o tratamento de inflamações crônicas do pâncreas e nas deficiências de secreção do suco pancreático em humanos, entre outros benefícios (FAULKS; BAILEY, 1990; ELMARZUGI et al., 2014). No desenvolvimento de cosméticos, as enzimas estão presentes principalmente em produtos de esfoliação de pele, higiene pessoal e anti-sinais, sendo a *glucoamilase*, a mais utilizada para a remoção ou prevenção da placa dentaria (SIM et al., 2003; MONTEIRO, 2009).

A extensa utilização de *amilases* no setor industrial se deve ao fato dessas enzimas apresentarem vantagens quanto à atuação, como alta seletividade, condições brandas de pH, temperatura e pressão, evitando condições abióticas extremas, e contribuindo parcialmente com a redução de problemas ambientais e toxicológicos (COURI, 2020).

3.2 PARÂMETROS ABIÓTICOS PARA A ATIVIDADE DE *AMILASES*

As *amilases* apresentam algumas particularidades quanto a sua atuação, que podem proporcionar melhor desempenho funcional destas enzimas e, consequentemente, sua aplicação na indústria biotecnológica. Dentre as características favoráveis a um melhor mecanismo de ação estão: pH, temperatura, estabilidade térmica, presença de cofatores, entre alguns outros fatores.

O pH ideal de ação de *amilases*, pode variar desde ácido até a pHs alcalinos, a depender da origem do microrganismo (SARANRAJ; STELLA, 2013; SAINI et al., 2017). Algumas *amilases* de origens fúngicas, em especial do gênero *Aspergillus,* apresentam pH ideal de ação, principalmente em condições ácidas entre pH 4,0 e 6,0 (RIAZ et al., 2012; PARASHAR; SATYANARAYANAXIAN, 2017; PASIN et al., 2017; XIAN; FENG, 2018; AISIEN; IGBINOSA, 2019; OLUWABUNMI et al., 2019; WANG et al., 2020), entretanto, alguns *amilases* de origem bacteriana apresentam melhor ação em condições neutras em pH 7,0 (LINCOLN et al., 2019) ou alcalinas em pH 8,0 e 9,0 (AFRISHAM et al., 2016; HAMMAMI et al., 2018).

Outro parâmetro fundamental para a atividade enzimática é a temperatura de ação, podendo variar entre 25°C a 80°C (DEY; BANERJEE, 2015; BARRADAS-

DERMITZ; AGUILAR-USCANGA, 2018). Algumas enzimas provenientes de espécies de *Aspergillus* apresentam melhores atividades enzimáticas em torno de 40°C a 50°C (SAHNOUN et al., 2012; DEY; BANERJEE, 2015; KARIM et al., 2018; XIAN; FENG, 2018). No entanto, é conhecido que a maioria das *amilases* apresenta melhor atuação em altas temperaturas entre 55°C a 80°C (NGUYEN et al., 2002; GOMES et al., 2005; DOSS; ANAND, 2012; BAGHERI, HODARAHMI; MOSTAFAIE, 2014; DEL MORAL, BARRADAS-DERMITZ; AGUILAR-USCANGA, 2018).

Aliado à temperatura e pH adequados, tem-se também a variação do parâmetro termoestabilidade ou estabilidade térmica. Grande parte das *amilases* produzidas por espécies do gênero *Aspergillus* são estáveis entre 40 e 120 minutos de reação enzimática (SAHNOUN et al., 2012; DEL MORAL et al., 2017; PASIN et al., 2017).

Ainda, acerca dos parâmetros para melhor atividade de *amilases*, sabe-se que estas enzimas são classificadas como metaloenzimas, isto é, apresentam ao menos um íon metálico ligado ao seu sítio ativo, favorecendo a atividade enzimática (GUPTA et al., 2003). Alguns cátions metálicos, em especial, íons de Ca^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+} e Cu^{2+}, tem sido descrito como cofatores, contribuindo para o aumento da atividade de *amilases* (PATEL et al., 2005; DEY; BANERJEE, 2015; SELIM, 2016).

Neste sentido, a caracterização da atividade de *amilases* é fundamental para direcionar estas enzimas ao ramo industrial. A Tabela 1 demonstra os parâmetros abióticos, referentes à caracterização da atividade de *amilases* provenientes de espécies do gênero *Aspergillus.*

Tabela 1 - Caracterização da atividade de *amilases* provenientes de espécies do gênero *Aspergillus*

Microrganismos	pH	Temperatura	Estabilidade térmica	Cofatores	Referências
Aspergillus japonicus	5,0	65°C	60 minutos	Co^{2+}/Mn^{2+}	Pasin et al. (2017)
Aspergillus niger	6,0	70°C	5 minutos	-	Bagheri et al. (2014)
Aspergillus flavus	4,0	65°C	540 minutos	-	Gomes et al. (2005)
Aspergillus oryzae S2	5,6	50°C	60 minutos	Ca^{2+}	Sahnoun et al. (2012)
Aspergillus oryzae IFO-30103	5,5	50°C	90 minutos	Ca^{2+}/Co^{2+}	Dey; Banerjee. (2015)
Aspergillus niger	4,3	80°C	40 minutos	Ca^{2+}	Del Moral et al. (2017)
Aspergillus flavus NSH9	4,0	50°C	120 minutos	Ca^{2+}	Karim et al. (2018)
Aspergillus sp.	9,0	30°C	50 minutos	-	Alva et al. (2007)
Aspergillus niger	5,0	70°C	30 minutos	Cu^{2+}/Fe^{3+}	Selim (2016)

Fonte: o próprio autor

Grande parte das *amilases* produzidas industrialmente são obtidas por meio de diferentes processos fermentativos, como Fermentação Estado Sólido (FES) e principalmente Fermentação Submersa (FS).

3.3 PROCESSOS FERMENTATIVOS PARA OBTENÇÃO DE *AMILASES*

No processo fermentativo em estado sólido, os microrganismos se desenvolvem e produzem seus metabólitos, a partir de substratos sólidos e sem a presença de água livre. A utilização da FES apresenta algumas vantagens, como alta produtividade e menor repressão catabólica. No entanto, também apresenta dificuldades importantes, principalmente relacionadas ao escalonamento do processo e à separação e purificação do produto. Este processo permite o aproveitamento de resíduos ou subprodutos da agroindústria, contribuindo para processos industriais mais sustentáveis e a redução nos custos de produção (SINGHANIA et al., 2009; KAREEM et al., 2010; SADH et al., 2018). A FES é muito utilizada para produção de diversos compostos que são de interesse para vários segmentos industriais (PANDEY et al., 2003; COUTO et al., 2006; ORLANDELLI et al., 2012).

Outra alternativa para a produção de *amilases* é a FS, sendo o processo fermentativo mais utilizado industrialmente, representando aproximadamente 90% dos processos industriais para a produção de enzimas, devido a facilidade do crescimento dos microrganismos, condições controladas de pH e temperatura, além da fácil recuperação e purificação das enzimas (RODRÍGUEZ-ZÚÑIGA et al., 2011; SANTOS et al., 2018).

Os possíveis reatores que podem ser utilizados na Fermentação Submersa, são os fermentadores agitados ou em torre. Além disso, o processo fermentativo pode ser realizado de quatro formas no reator: forma descontínua, semicontínua, descontínua alimentada (ou batelada alimentada) e contínua. Geralmente as indústrias utilizam o processo em batelada com tanque agitado, também conhecido como sistema de lotes, porém há possibilidade de utilizar sistemas contínuos em escala industrial (SCHMIDELL, et al., 2001). A Fermentação Submersa em escala laboratorial pode ser realizada por meio de frascos Erlenmeyer sob agitação em shakers ou biorreatores de bancada, utilizados principalmente para realizar otimizações dos parâmetros fermentativos (SHOW et al., 2015).

O desempenho do processo fermentativo é afetado por fatores químicos como o pH e componentes do meio de cultivo e fatores físicos, dentre eles estão a temperatura, agitação e aeração.

O potencial hidrogeniônico (pH), é um fator químico significativo para as fermentações industriais, podendo variar numa ampla faixa de acordo com cada produto a ser obtido. Sua importância é tamanha, que algumas respostas do meio podem ser alteradas de acordo com o pH, como por exemplo: controle da contaminação, efeito sobre o crescimento do microrganismo, taxa de fermentação e a formação de subprodutos (NAVES et al., 2010; SHOW et al., 2015).

A fermentação também depende da concentração de elementos químicos que compõem o meio de cultivo, sendo eles: fonte de carbono, fonte de nitrogênio, sais metálicos como, manganês, zinco, ferro, cobre, magnésio e outros componentes que podem ser adicionados ao meio como, por exemplo, lipídios, álcoois e fosfatos (SOCCOL et al., 2006; SHOW et al., 2015).

A temperatura é um parâmetro físico que pode influenciar a atividade do

microrganismo, afetando o crescimento e consequentemente seu metabolismo, a capacidade fermentativa e a viabilidade celular, além disso, outros dois fatores que estão diretamente relacionados a produção de metabólitos são a aeração e a agitação (NAVES et al., 2010).

A combinação dos fatores abióticos é crucial para o aumento da produção enzimática, dessa forma a Tabela 2 demonstra alguns estudos realizados para a produção de *amilases* por espécies do gênero *Aspergillus* sob Fermentação Submersa, utilizando diferentes condições abióticas.

Tabela 2 - Produção de *amilases* por espécies de *Aspergillus*, por Fermentação Submersa

Microrganismos	**Condições do cultivo**	**Atividade amilolítica (U/mL)**	**Referências**
Aspergillus niger J26	50°C / pH 6,0 / 120h	6,10	Stroparo et al. (2012)
Aspergillus oryzae IIB-30	30°C / pH 5,0 / 64 h / 160 rpm	335	Abdullah et al. (2011)
Aspergillus terreus	30°C / pH 6,0 / 120h	345	Ahmed et al. (2020)
Aspergillus oryzae S2	24°C / 72h	770	Naili et al. (2016)
Aspergillus niger	40°C / pH 4,5 / 96h (sob agitação)	1.800	Sreelakshmi et al. (2014)
Aspergillus niger WLB42	30°C / 48h / 200 rpm	2.189	Wang et al. (2016)

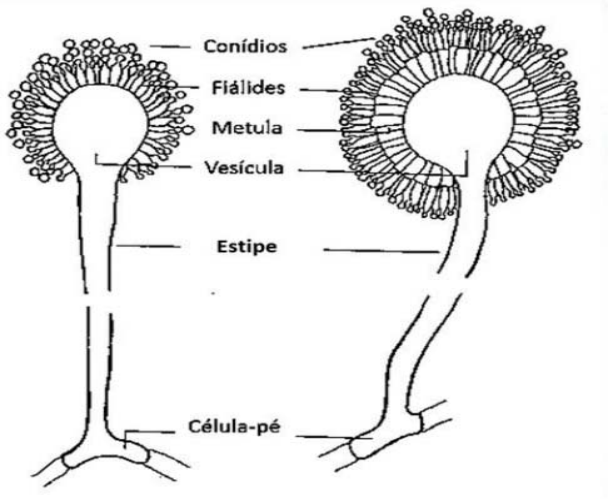
Fonte: o próprio autor

Além dos parâmetros abióticos serem fundamentais nos processos fermentativos, os fatores bióticos, por exemplo, as espécies de microrganismos utilizados, são de suma importância. O crescimento dos microrganismos é influenciado conforme a composição do meio de cultivo, o substrato ou matéria prima utilizada, ou até mesmo a própria quantidade de microrganismos inseridos no início do processo fermentativo (NAVES et al., 2010). Grande parte dos microrganismos aplicados industrialmente para produção de *amilases* são do gênero *Aspergillus* (PANDEY et al., 2005).

3.4 PROPRIEDADES GERAIS DO GÊNERO *ASPERGILLUS*

Fungos filamentosos, como os do gênero *Aspergillus,* possuem grande distribuição ao redor do mundo e importância econômica, pois são utilizados amplamente em fermentações na área biotecnológica para produção de várias enzimas extracelulares. As espécies que compõem esse gênero, tipicamente apresentam um estipe asseptada, onde em sua extremidade há formação de uma vesícula, local de crescimento das fiálides, responsáveis pela origem dos conídios, conforme mostrado na Figura 3. Uma das principais características desse gênero são as diferentes ornamentações e pigmentações (verde, amarelo, branco, marrom, cinza e preto) que seus conídios podem apresentar (SAMSON et al., 2014; FRISVAD, 2015; PARK et al., 2017).

Figura 3 – Conidióforo de *Aspergillus*



Fonte: Klich; Pitt (1988).

O gênero *Aspergillus* pertence à classe dos hifomicetos, filo *Ascomycota*, ordem *Eurotiales*, e a família *Trichocomaceae* (KLICH, 2002).

Constituem esse gênero mais de 300 espécies (VIDAL-ACUÑA et al., 2019), divididas em quatro subgêneros: *Aspergillus, Circumdati, Fumigati, Nidulantes*, distribuídos em 20 seções (SAMSON et al., 2014; HUBKA et al., 2014).

O subgênero *Circumdati* seção *Nigri* possui 32 espécies, conforme a Comissão Internacional de *Penicillium* e *Aspergillus* (ICPA) (HOUBRAKEN et al., 2020). Nesta seção é descrito um grupo de espécies com características muito similares e

morfologicamente indistinguíveis, denominado de “agregado *niger*”, sendo composto de um total de 10 espécies (SAMSON et al., 2014; SUSCA et al., 2016; D'HOOGE et al., 2019).

Algumas linhagens desta seção são descritas como produtoras das micotoxinas Ocratoxina A e/ou Fumonisina B2 (VARGA et al., 2011; FUNGARO et al., 2017), mas por outro lado, as espécies constituintes desta seção apresentam grande importância no setor biotecnológico, sendo muito utilizado na produção de enzimas (WANG et al., 2016; KARIM et al., 2018; AHMED et al., 2020).

Dentro da seção *Nigri*, mais especificamente pertencente ao “agregado *niger”*, Hong et al. (2013) renomearam a espécie *A. awamori* a *A. welwitschiae*. Esta espécie, apresenta características morfológicas indistinguíveis quando comparado a *Aspergillus niger*, sendo diferenciadas somente por caracteres moleculares e bioquímicos (MELIKOGLU et al., 2013; ADEDEJI; EZEKIEL, 2019).

A espécie *A. welwitschiae* foi relatada em castanha de caju (LAMBONI et al., 2016), amêndoas, uvas passas, figos, milho, pistache e nozes (SUSCA et al., 2016), castanha do Brasil (MASSI et al., 2016), bem como em bulbos de alhos (VANZELA et al., 2020), sementes de mostarda (HANIF et al., 2016) e cebolas (GHERBAWY et al., 2015; MASSI et al., 2016; OH et al., 2016).

Alguns estudos, destacam ambas espécies (*Aspergillus niger* e *Aspergillus welwitschiae*) da seção *Nigri* quanto à produção de enzimas hidrolíticas. Castro et al. (2015), estudando a espécie *A. awamori* IOC-3914 sob Fermentação Estado Sólido utilizando resíduos da cervejaria e farinha de babaçu como substrato, obtiveram a produção de *xilanases* (835 U/g), *amilases* (120 U/g), *celulases* (7,1 U/g) e *proteases* (2,3 U/g), nas condições de 30 °C por 120 horas. Aliyah et al. (2017), utilizando a espécie *A. niger* e sabugo de milho como substrato para produção de *amilases*, obteve 95 U/mL nas condições de 30 °C, pH 7,0 e 6 dias.

Estudos de isolamento como de Karam et al. (2017), relataram a presença de espécies do gênero *Aspergillus* com potencial para produção de *amilases*, tendo como destaque a espécie *A. awamori*. Esta espécie foi utilizada para produção de *amilases* sob Fermentação Estado Sólido, obtendo atividade amilolítica de 230 U/g, empregando o resíduo de óleo de oliva como substrato, nas condições de 30 °C por 5 dias.

Vanzela et al. (2020a), produzindo *lipases* pela espécie *A. welwitschiae* nas condições de cultivo de 40 °C, pH 9,0, 115 rpm e 24 horas, obteve a atividade lipolítica de 4,16 U/mL.

4 MATERIAIS E MÉTODOS

4.1 MATERIAL BIOLÓGICO

Este estudo utilizou 24 linhagens de *Aspergillus,* isoladas de alhos comercializados em estados brasileiros. As linhagens foram identificadas como *Aspergillus welwitschiae* e caracterizadas quanto ao não potencial de produção de Ocratoxina A e Fumonisina B2 por Vanzela et al. (2020a). Adicionalmente, a linhagem de *A. welwitschiae* UELAs 15.262/35 também foi utilizada neste estudo e se trata de uma linhagem mutante de *A. welwitschiae* UELAs 15.262, obtido por mutação aleatória induzida por luz ultra violeta (dados não publicados).

4.2 SELEÇÃO DE LINHAGENS DE *ASPERGILLUS WELWITSCHIAE* PRODUTORAS DE *AMILASES*

As linhagens de *A. welwitschiae* foram inoculadas pontualmente em placas de Petri, contendo meio Czapeck-Dox ágar (amido solúvel 20 g/L; $NaNO_3$ 1 g/L; K_2HPO_4 1 g/L; $MgSO_4.7H_2O$ 1 g/L; $FeSO_4$ 0,01 g/L; Ágar 15 g/L e pH 4,5) para seleção do potencial de produção de *amilases*. As placas foram incubadas a 28 ± 2 ºC por 5 dias e coradas com iodina (KI 1 g/100 mL; I_2 0,5 g/100 mL), em seguida as placas foram incubadas novamente por 10 dias nas mesmas condições. O potencial de produção de *amilases* foi avaliado pelo Índice Enzimático (IE) expresso pela relação entre o diâmetro do halo de degradação + colônia e diâmetro do crescimento da colônia (HANKIN; ANAGNOSTAKIS, 1975).

As 24 linhagens de *A. welwitschiae* também foram avaliadas quanto ao potencial de produção de *amilases* em meio Czapeck-Dox líquido. Foram preparadas suspensões de 10^5 conídios/mL de cada linhagem e estas foram inoculadas em 5 mL de meio Czapeck-Dox líquido, a 28 ± 2 ºC por 4 dias. Após o período fermentativo, o Extrato Bruto Enzimático (EBE) foi coletado e o potencial de produção de *amilases* foi indiretamente avaliado pela quantificação de açúcares redutores pelo 3,5 – ácido dinitrosalicílico (DNS), conforme Miller, (1959). Ambos os experimentos foram realizados em triplicata experimental, a média e desvio padrão foram realizados com o auxílio do programa R.

4.3 AVALIAÇÃO DOS PARÂMETROS ABIÓTICOS PARA PRODUÇÃO DE *AMILASES* POR LINHAGENS DE *A. WELWITSCHIAE*

As linhagens de *A. welwitschiae* selecionadas no item 4.2, foram avaliadas quanto aos parâmetros abióticos como temperatura e pH para a produção de *amilases*. A avaliação dos parâmetros abióticos foi conduzida conforme o Delineamento Composto Central Rotacional (DCCR), demonstrado na Tabela 3. As temperaturas avaliadas foram de 28 °C, 30 °C, 35 °C, 40 °C e 42 °C e os pHs avaliados foram de 4,2, 5,0, 7,0, 9,0 e 9,8, em meio Czapeck-Dox líquido por 4 dias. Todos os experimentos foram realizados em triplicata experimental, a média, desvio padrão, DCCR foram realizados com o auxílio do programa R.

Tabela 3 - Delineamento Composto Central Rotacional (DCCR)

Ensaios	Variáveis decodificadas		Variaveis codificadas	
	Temperatura ºC **(X_1)**	pH **(X_2)**	**X_1**	**X_2**
1	30	5,0	-1	-1
2	30	9,0	-1	1
3	35	7,0	0	0
4	35	7,0	0	0
5	35	7,0	0	0
6	40	5,0	1	-1
7	40	9,0	1	1
8	35	4,2	0	-1,4
9	35	9,8	0	1,4
10	28	7,0	-1,4	0
11	42	7,0	1,4	0

4.4 PRODUÇÃO DE *AMILASES* POR *A. WELWITSCHIAE* SOB FERMENTAÇÃO SUBMERSA

As linhagens de *A. welwitschiae* selecionadas sob melhores parâmetros abióticos para a produção de *amilases* foram submetidas a cinética de produção de *amilases*, sob Fermentação Submersa, em meio Czapeck-Dox. A linhagem mutante de *A. welwitschiae* UELAs 15.262/35 foi avaliada nas mesmas condições para a análise da cinética de produção de *amilases*. Cada dia, uma porção (um frasco com a repetição) da cinética foi retirada para determinar a atividade amilolítica, até ao oitavo dia.

4.5 DETERMINAÇÃO DA ATIVIDADE AMILOLÍTICA

Decorrido o período fermentativo, o cultivo foi interrompido por filtração (papel Whatman n°1) para a obtenção do Extrato Bruto Enzimático (EBE). Após a coleta do EBE foi realizado a avaliação da atividade amilolítica, conforme Sperotto, (2014).

A determinação da atividade amilolítica foi conduzida por meio da reação enzimática (200 µL de tampão citrato-fosfato (0,05M, pH 6,0), 300 µL de amido solúvel (1%) e 100 µL do Extrato Bruto Enzimático), a mistura foi incubada a 40 °C por 30 minutos. Para a interrupção da reação enzimática, a cada amostra foi adicionado 1,5 mL de DNS, seguido da incubação a 100 °C por 5 minutos, com subsequente adição de 17,9 mL de água destilada. A quantidade de açúcares redutores foi avaliada a A_{550nm} (biochrom Libra S22). Sob tais condições, uma unidade (U) de atividade de amilase é definida como a quantidade de enzima que libera 1 µmoL de açúcares redutores por mL de amostra por minuto.

4.6 PURIFICAÇÃO PARCIAL E CARACTERIZAÇÃO DO EXTRATO BRUTO ENZIMÁTICO PARCIALMENTE PURIFICADO DE *A. WELWITSCHIAE*

4.6.1 Precipitação Das *Amilases* Contidas No Extrato Bruto Enzimático De *A. Welwitschiae*

Em um frasco, 680 mL de cada extrato bruto enzimático obtido pela linhagem de *A. welwitschiae* selecionada e a linhagem mutante UELAs 15.262/35, foram separadamente precipitados pela adição da solução saturada de sulfato de amônio 80% (1:1 v/v), sob agitação durante 2 horas a 4 °C. O precipitado resultante foi coletado por centrifugação a 9.000 rpm, por 15 mim a 4 °C. O precipitado foi solubilizado com 12 mL de tampão citrato-fosfato (0,05 M, pH 6,0). O precipitado solubilizado foi dialisado por 24 horas diante de três mudanças de água destilada.

4.6.2 Caracterização Bioquímica Das *Amilases* Do Extrato Bruto Enzimático Parcialmente Purificado

O Extrato Bruto Enzimático Parcialmente Purificado (EBEPP), foi avaliado quanto ao efeito do pH na atividade amilolítica. O EBEPP, foi incubado por 30 minutos, 40 °C em tampão citrato-fosfato 0,05 M nos pHs 5,5, 6,0, 6,5, 7,0 e tampão tris/HCl nos pHs 7,5 e 8,0. Em seguida, a atividade enzimática foi determinada como descrito no item 4.5.

Da mesma forma, o efeito da temperatura na atividade amilolíticas foi realizado utilizando 100 µL de EBEPP. O intervalo de temperatura verificado foi de 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C e 70 °C, durante 30 minutos de incubação, e o pH utilizado foi selecionado no item 4.6.2. A atividade amilolítica foi avaliada conforme o item 4.5.

Em condições de pH e temperatura favoráveis a uma maior atividade amilolítica, foi analisada a estabilidade térmica do EBEPP. Desta forma, 700 µL de EBEPP foi incubado sob as melhores condições (pH e temperatura) estipuladas no item 4.6.2, por diferentes períodos de tempo (0, 20, 40, 60 e 100 minutos). Para cada período a atividade amilolítica foi avaliada conforme o item 4.5.

Para a avaliação dos efeitos dos íons metálicos, 500 µL do EBEPP foram incubados junto à diferentes soluções de sais metálicos (1:1 v/v), dos cátions: Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+} na concentração de 5 mM a 30 °C por 1 hora. Em seguida, alíquotas de 100 µL foram coletadas e a atividade amilolítica foi avaliada conforme o item 4.5.

5 RESULTADOS E DISCUSSÃO

Os resultados e discussão obtidos neste trabalho estão representados na forma de artigo científico, a ser enviado a Brazilian Journal of Microbiology.

Artigo 1: *Aspergillus welwitschiae*: a potential *amylases* producer

5.1 ARTIGO CIENTÍFICO

Aspergillus welwitschiae: a potential _amylases_ producer

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Amylases are of great biotechnological importance and can be applied in several industrial sectors. In this sense, the present study aims to select *Aspergillus welwitschiae* strains with potential to produce amylolytic enzymes. The selected strains were evaluated for the best amylase production parameters under Submerged Fermentation (SmF). The Crude Extract Enzymatic of *amylases* were partially purified and characterized. In this study, the 24 strains presented potential amylase production, with the UELAs 15.262 strain as the highlight. And from the Rotational Central Composite Design conditions of pH 7.0 and temperature of 35 °C, provided better performance in the production of *amylases*. Submerged Fermentation was conducted under the selected conditions, where the UELAs 15.262 strain produced 951 U/mL on the seventh day of fermentation, while the UELAs 15.262/35 strain produced 580 U/mL on the sixth day of fermentation. After the standardization of abiotic conditions to produce *amylases*, the characterization of the Crude Enzymatic Extract Partially Purified (CEEPP) was performed. The best enzymatic activities were obtained in the abiotic combination of pH 5.5, 60 °C and pH 5.0, 60 °C for UELAs 15.262 and UELAs 15.262/35, respectively. Both CEEPP showed great thermal stability at a temperature of 60 °C, with reduction in amylolytic activity of 35% for UELAs 15.262 and 30% for UELAs 15.262/35. The effect of metal ions Cu^{2+} and Fe^{3+} contributed to an increase in enzymatic activity in both strains.

Key-words: *Amylases*, Submerged Fermentation, *Aspergillus welwitschiae*

1. INTRODUCTION

Amylases have great biotechnological importance and can be applied in textile industries, detergents, paper and cellulose, bakery, distilled beverages, brewery, cereals for food, liquefaction and saccharification of starch, animal feed, chemical and pharmaceutical industry [10, 14, 15, 37, 39]. This class of hydrolytic enzymes can be produced by different microorganisms, being the *Aspergillus niger* specie widely used in industrial processes [19, 35]. However, other close species like *A. welwitschiae* can also present potential for industrial and/or biotechnological applications.

The *Aspergillus welwitschiae* specie is inserted within the *Aspergillus* section *Nigri* [25]. In this section, there is a group of fungi with very similar morphological characteristics that are difficult to identify, denominated as "*niger* aggregate", with the species *A. welwitschiae* as constituent [20, 25, 47]. This species has already been isolated from different sources such as coffee, cocoa, onion bulbs, garlic and several other products [13, 20, 21, 48, 52]. Although the production of Fumonisin B2 and Ochratoxin A has been reported in *A. niger* and *A. welwitschiae*, part of these strains does not produce these toxins [20, 47].

The species *A. welwitschiae* has a large distribution in Brazil, being reported in several Brazilian states [47, 50]. Some strains of *A. welwitschiae* have been isolated in almonds, raisins, figs, corn, pistachios and walnuts [47], Brazil nuts [21], as well as in garlic bulbs [50].

Filamentous fungi are responsible for producing approximately 60% of all industrial enzymes [8]. This class of microorganism presents some advantages in enzymatic production such as development in wide ranges of pH and temperature, production of high concentrations of enzymes and growth in both liquid and solid medium [18, 22]. Traditionally, amylolytic enzymes are produced industrially by Submerged Fermentation (SmF), representing 90% of world production [27]. The great use of SmF in the industrial sector, is directly linked to the facility of sterilization and better monitoring of the fermentation process.

The use of mutant strains to produce *amylases* is increasingly being used in studies and industrial applications. The mutant strains can enable the production of larger quantities of metabolites, in addition, the *amylases* produced by mutant strains can act in different abiotic conditions and have differences in their structures, providing better resistance and performance in extreme conditions of temperature and pH, besides increase the enzymatic activity with the use of different cofactors [2, 3, 36].

In view of this, this work aimed at the selection of *Aspergillus welwitschiae* strains of potential amylolytic enzyme producers, as well as the production and optimization of *amylases* by Submerged Fermentation, followed by a partial purification and characterization of the Crude Enzymatic Extract Partially Purified, this is the first work that reports the production of *amylases* by *A. welwitschiae* strains.

2. MATERIALS AND METHODS

2.1. BIOLOGICAL MATERIAL

This study used 24 strains of *Aspergillus*, isolated from garlic marketed in different Brazilian states. The strains were molecularly identified as *Aspergillus welwitschiae* and characterized as non-producing potential Ochratoxin A and Fumonisin B2 by [50]. In addition, a UELAs 15.262 mutant strain obtained by randomized mutation

induced by ultraviolet light (unpublished data) denominated as *Aspergillus welwitschiae* UELAs 15.262/35, was also used in this study. These samples were part of Microoganisms Collection of Londrina State University.

2.2. SELECTION OF *ASPERGILLUS WELWITSCHIAE* STRAINS *AMYLASES* PRODUCING

The *A. welwitschiae* strains were inoculated punctually in Petri dishes, containing Czapeck-Dox agar medium (20 g/L soluble starch; $NaNO_3$ 1 g/L; K_2HPO_4 1 g/L; $MgSO_4.7H_2O$ 1 g/L, $FeSO_4$ 0.01 g/L, Agar 15 g/L and pH 4.5) for selection of the potential *amylases* production. The dishes were incubated at 28 ± 2 ºC for 5 days and stained with iodine (Kl 1 g/100 mL; I_2 0,5 g/100 mL), then the dishes were incubated again for 10 days under the same conditions. The potential *amylases* production was evaluated by the Enzymatic Index (EI) expressed by the relationship between the diameter of the halo of degradation + colony growth and the diameter of the growth of the colony [16].

The 24 *A. welwitschiae* strains were also evaluated for the potential for *amylases* in liquid Czapeck-Dox medium. Suspensions of 10^5 conidia/mL from each strain were prepared and they were inoculated in 5 mL of Czapeck-Dox medium, at 28 ± 2 ºC for 4 days. After the fermentation period, the Crude Enzymatic Extract (CEE) was collected and the production potential of *amylases* was indirectly evaluated by quantifying reducing sugars by 3.5 - dinitrosalicylic acid (DNS), according to [23]. All the experiments were performed in experimental triplicate, the mean and standard deviation were performed with the help of the R software.

The *A. welwitschiae* strains that showed higher EI and production of reducing sugars were selected for further evaluations.

2.3. EVALUATION OF ABIOTIC PARAMETERS FOR THE PRODUCTION OF *AMYLASES* BY *ASPERGILLUS WELWITSCHIAE* STRAINS

The *A. welwitschiae* strains selected, were subjected to evaluation of abiotic parameters such as temperature and pH for *amylases* production according to the Rotational Central Compound Design (RCCD). The evaluated temperatures and pHs were 28 °C, 30 °C, 35 °C, 40 °C and 42 °C and 4.2, 5.0, 7.0, 9.0 and 9.8, respectively, in liquid Czapeck-Dox medium for 4 days. All experiments were performed in experimental triplicate, the mean, standard deviation, DCCR were performed with the aid of the R software.

2.4. *AMYLASES* PRODUCTION BY *A. WELWITSCHIAE* UNDER SUBMERGED FERMENTATION

A. welwitschiae strains selected under better abiotic parameters for *amylases* production were subjected to *amylases* production kinetics, under Submerged Fermentation, in Czapeck-Dox medium. Together with the selected *A. welwitschiae* strains we introduced under the same abiotic conditions the UELAs 15.262/35 mutant strain for the analysis of *amylases* production kinetics. Each day a portion (a flask with the repetition) of the kinetics was removed to determine the amylolytic activity, until the eighth day.

2.5. DETERMINATION OF AMYLOLYTIC ACTIVITY

After each fermentation period, the cultivation was interrupted by filtration (Whatman paper n ° 1) to obtain the Crude Enzymatic Extract (CEE). The activity of *amylases* was determined according to [40].

The determination of the amylolytic activity was conducted by means of the enzymatic reaction, consisting of 200 µL of citrate-phosphate buffer 0.05M, pH 6.0 (citric acid 1,05 g/100 mL; NaH_2PO_4 1,38 g/100 mL), 300 µL of soluble starch (1%) and 100 µL of the Crude Enzymatic Extract. The reactional mixture was incubated for 30 minutes at 40 °C. To interrupt the enzymatic reaction, 1.5 mL of DNS was added to each sample, followed by incubation at 100 °C for 5 minutes, and subsequent addition of 17.9 mL of distilled water. The amount of reducing sugars was evaluated at A_{550nm} (Biochrom Libra S22). Under such conditions, one unit (U) of *amylases* activity is defined as the amount of enzyme that releases 1 µmoL of reducing sugars per mL of sample per minute.

2.6.1. Partial Purification of the Crude Enzymatic Extract of *A. welwitschiae* strains

In a flask, was added a total of 680 mL of each crude enzyme extract obtained by the selected *A. welwitschiae* strain and the mutant strain UELAs 15.262/35, which were precipitated with 80% (1:1 v/v) ammonium sulfate saturated solution, under shaking for 2 hours at 4 °C. The resulting precipitate was collected at 9,000 rpm, by 15 minutes at 4 °C. The precipitate was solubilized with 12 mL citrate-phosphate buffer (0.05 M, pH 6.0). The solubilized precipitate was dialyzed for 24 hours, with three changes of distilled water.

2.6.2. Characterization of *amylases* of the Crude Enzymatic Extract Partially Purified of *A. welwitschiae* strains

The Crude Enzymatic Extract Partially Purified (CEEPP), was evaluated for the effect of pH on amylolytic activity. The CEEPP, was incubated for 30 minutes, 40 °C in 0.05 M citrate-phosphate buffer at pHs 5.5, 6.0, 6.5, 7.0 and tris/HCl buffer at pHs 7.5 and 8.0. The *amylases* activity was then determined as described to [40].

Likewise, the effect of temperature on amylolytic activity was performed using 100 µL of CEEPP. The temperature range verified was 30 °C, 35 ° C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C for 30 minutes. The pH selected in item 2.6.2. was used. The amylolytic activity was determined according to [40].

Under conditions of pH and temperature favorable to greater *amylases* activity, the thermal stability of CEEPP was analyzed. Thus, the amylolytic reaction containing 700 µL of CEEPP occurred for different time periods (0, 20, 40, 60 and 100 minutes). For each period of reaction, the amylolytic activity was analyzed according to [40].

For the evaluation of the effects of metallic ions, 500 µL of EBEPP were incubated together with different solutions of metallic salts (1:1 v/v), of cations: Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+} at 5 mM at 30°C for 1 hour. Then, aliquots of 100 µL were collected and amylolitic activity was evaluated according to [40].

3. RESULTS

3.1. Selection and evaluation of abiotic parameters of *Aspergillus welwitschiae* strains for *amylases* producing

The 24 *A. welwitschiae* strains showed potential for *amylases* production (Table 1). The UELAs 15.262 strain were able to produce *amylases*, both in solid and liquid Czapeck-Dox medium (Table 1). Due to the higher production in both situations this strain was selected for the other evaluations.

Table 1: Selection of *amylases* production potential by *A. welwitschiae* strains.

***A. welwitschiae* strains**	**Enzymatic Index (EI)**	**Amount of reducing sugar (mg/mL)**	***A. welwitschiae* strains**	**Enzymatic Index (EI)**	**Amount of reducing sugar (mg/mL)**
UELAs 15.262	**1.61**	**7.50**	UELAs 26.365	1.39	0.84
UELAs 20.290	1.50	1.59	UELAs 32.459	1.39	1.01
UELAs 1.05	1.47	0.69	UELAs 29.432	1.39	1.24
UELAs 11.225	1.45	0.68	UELAs 11.228	1.38	1.06
UELAs 7.182	1.43	0.90	UELAs 28.422	1.38	0.81
UELAs 12.247	1.42	0.70	UELAs 27.405	1.37	0.65
UELAs 25.348	1.41	1.22	UELAs 28.430	1.37	1.44
UELAs 28.425	1.41	0.62	UELAs 7.200	1.36	0.85
UELAs 24.232	1.41	0.52	UELAs 6.144	1.36	0.97
UELAs 28.411	1.40	0.86	UELAs 12.233	1.35	1.11
UELAs 34.262	1.40	1.69	UELAs 2.14	1.34	1.16
UELAs 6.136	1.40	0.74	UELAs 27.237	1.28	0.68

The strain in bold was selected due it has the highest EI value and quantity of reducing sugars generated. Average Enzyme Index obtained in experimental triplicates, and the dishes were incubated at 28 ° C for 4 days. The amount of reducing sugar generated was evaluated in liquid Czapeck-Dox medium, incubated at 28 °C for 4 days with 10^5 conidia/mL

The abiotic parameters for greater production of *amylases* by UELAs 15.262, were 40 °C and pH 5.0, (Table 2), obtained by the RCCD. However, lower temperatures and neutral pH are favorable for the future use of this strain in an industrial environment. Due to this fact, the abiotic parameters of 35 °C, pH 7.0 for the production of *amylases* by *A. welwitschiae* UELAs 15.262 was selected for Submerged Fermentation.

Table 2: Abiotic parameters for *amylases* production by *A. welwitschiae* UELAs 15.262.

***A. welwitschiae* strains**	**Temperature (°C)**	**pH**	**Amount of reducing sugar (mg/mL)**
UELAs 15.262	35	4.2	6.0
	40	**5.0**	**8.5**
	30	5.0	6.4
	35	9.8	1.7
	40	9.0	1.9
	30	9.0	1.3
	35	**7.0**	**7.8**
	42	7.0	5.7
	28	7.0	6.7

The analysis was performed in experimental triplicate, using 4 mL of Czapek-Dox medium, incubated for 4 days, 10^5 conidia/mL.

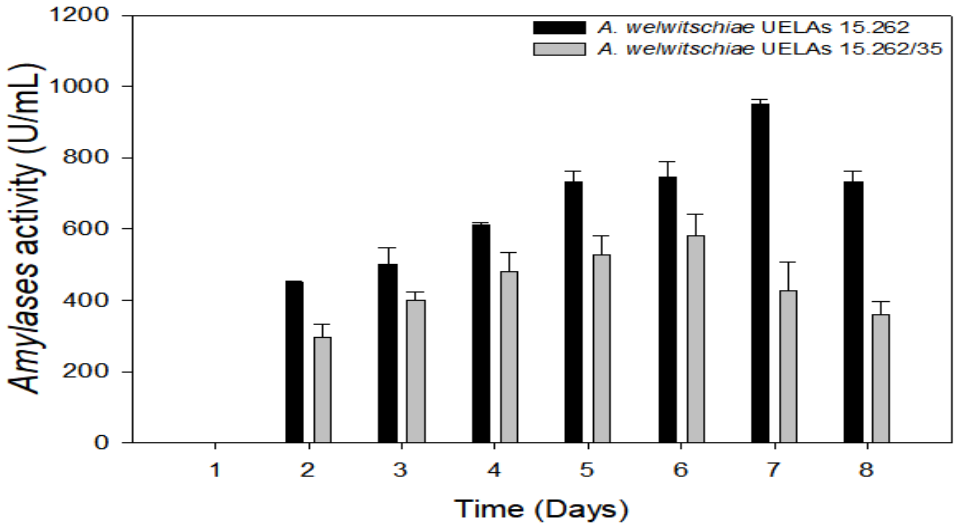
3.2. KINETICS OF *AMYLASES* PRODUCTION BY *A. WELWITSCHIAE* IN SUBMERGED FERMENTATION

Using the previously selected abiotic parameters and the UELAs 15.262 strain, we verified the kinetics of *amylases* production. At this stage under the same conditions, we introduced the *A. welwitschiae* UELAs 15.262/35 mutant strain, due to this strain presents potential for the production of some metabolites, and citric acid (unpublished data).

There was an increase in the production of *amylases* by *A. welwitschiae* UELAs 15.262 throughout the fermentation process, with a higher production (951 U/mL) of these enzymes on the seventh day. As for the UELAs 15.262/35 strain, there was also an increase in the production of *amylases*, however, only until the sixth day, with

highlight to a greater production of these enzymes on the sixth day (580 U/mL), according to Fig. 1.

Figure 1: Kinetics of *amylases* production by *Aspergillus welwitschiae* UELAs 15.262 and *Aspergillus welwitschiae* UELAs 15.262/35 in Submerged Fermentation



Submerged Fermentation using 4mL of Czapeck-Dox culture medium, 10^5 conidia/mL inoculum, incubated at 35 °C, pH 7.0.

3.3. CHARACTERIZATION OF *AMYLASES* FROM CRUDE ENZYMATIC EXTRACT PARTIALLY PURIFIED OF *A. WELWITSCHIAE*

The amylolytic activity of the Crude Extract Enzymatic Partially Purified of *A. welwitschiae*, was characterized as to pH, temperature, thermal stability and metallic ions effect.

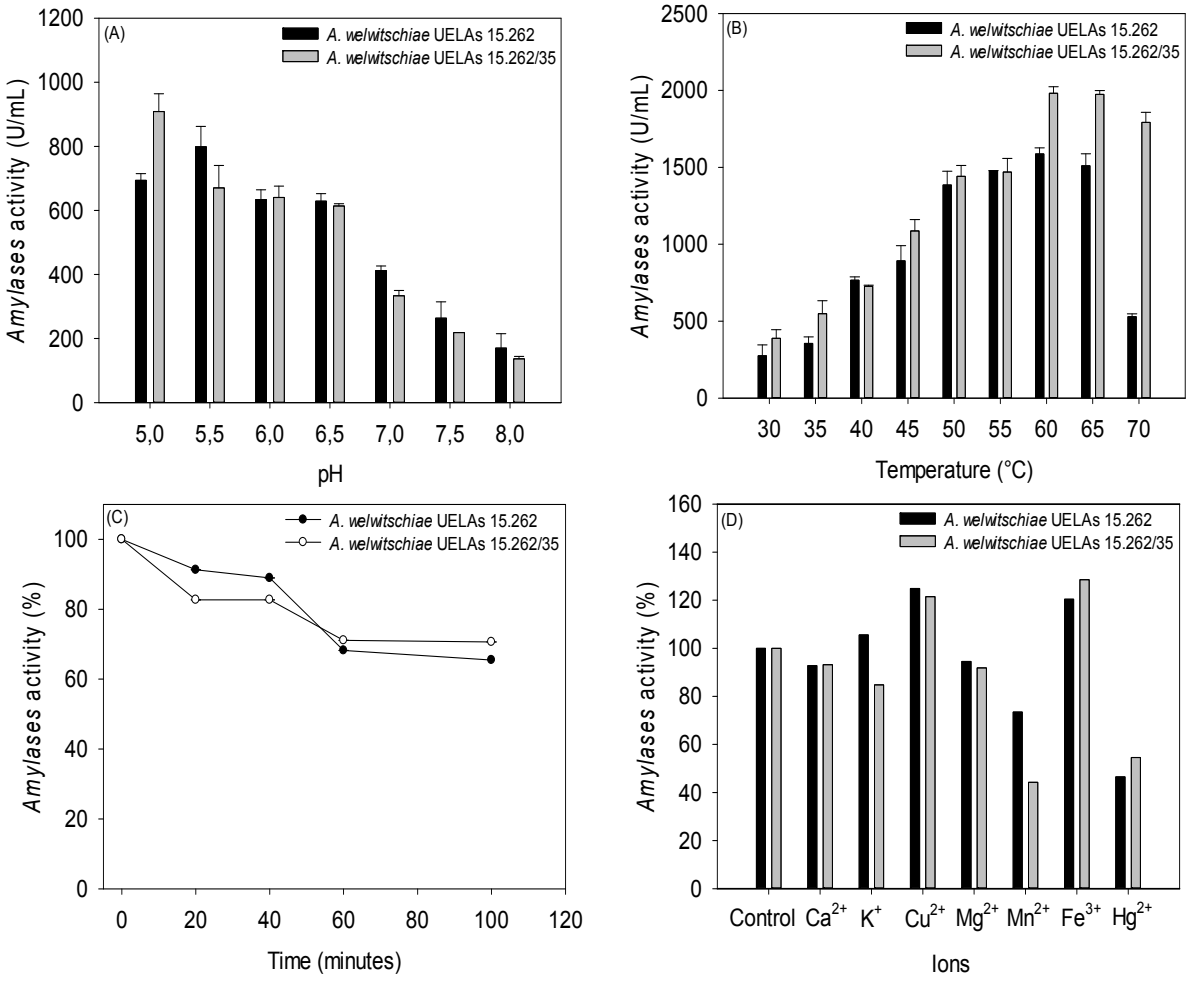
The highest *amylases* activity of *A. welwitschiae* UELAs 15.262 was obtained with pH 5.5 (798.7 U/mL), while for UELAs 15.262/35 it was with pH 5.0 (908.7 U/mL), as shown in Fig.2 (A).

As for the influence of temperature on the amylolytic activity of both *A. welwitschiae* strains, an increase in *amylases* activity was observed with an increase in temperature until 65 °C, when we maintain the best pH obtained previously. The highest *amylases* activities were obtained at 55 °C (1,477 U/mL), 60 °C (1,587 U/mL) and 65 °C (1,510 U/mL) by *A. welwitschiae* UELAs 15.262. Furthermore, for *A. welwitschiae* UELAs 15.262/35, the highest *amylases* activities were obtained at 60 °C (1,982 U/mL) and 65°C (1,973 U/mL). At 70 °C there was 66% reduction in *amylases* activity from UELAs 15.262, in contrast to only 10% reduction in *amylases* activity by UELAs 15.262/35 (Fig. 2 (B)).

Under the conditions previously established, there was a decrease in *amylases* activity over the time. The *amylases* activity of UELAs 15.262 strains was more stable in the first 40 minutes, when compared to *amylases* of UELAs 15.262/35 strain. However, *amylases* activity from the UELAs 15.262/35 strain showed greater thermal stability between 60 to 100 minutes, as shown in Fig.2 (C).

The Cu^{2+} and Fe^{3+} ions provided greater increasing on *amylases* activity for both UELAs 15.262 and UELAs 15.262/35 strains (Fig.2 (D)). The other metallic ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}) were not efficient as cofactors.

Figure 2: Characterization of *amylases* activity from crude enzymatic extract partially purified of *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains. (A) Influence of pH on *amylases* activity; (B) Influence of temperature on *amylases* activity; (C) Influence of thermal stability on amylase activity; (D) Influence of metallic ions on *amylases* activity



(A)Influence of pH on amylolytic activity of CEEPP of UELAs 15.262 and UELAs 15.262/35, at 40 °C. (B) Influence of temperature on the amylolytic activity of the CEEPP of UELAs 15.262, pH 5.5 and UELAs 15.262/35, pH 5.0. (C) CEEPP thermal stability of UELAs 15.262 and UELAs 15.262/35 at 60 °C. (D) Influence of metal ions on amylolytic activity of CEEPP of UELAs 15.262 and UELAs 15.262/35, obtained at 60 °C, pH 5.5 and 5.0, respectively.

4. DISCUSSION

The production of *amylases* by microorganisms is dependent of the genetic background of strains and/or species, as well as of abiotic factors during the fermentation process, such as temperature, pH, type of substrate among others [4, 29, 38, 46]. The selection of strains with genetic characteristics that favor the production of *amylases* has been of great importance. Strains of *Aspergillus* are described as good *amylases* producers and among the species with the greatest potential for *amylases* production, are the species of *Aspergillus* section *Nigri* [12, 32, 49]. *A. niger* has

been the most studied species of *Aspergillus* section *Nigri* regarding the production of *amylases* [17, 54, 55]. However, in this study we have detected that *A. welwitschiae* is a potential producer of *amylases*.

In addition, *A. niger* and *A. welwitschiae* harbor potentially productive strains of Ochratoxin A and Fumonisin B2 [1, 20, 25, 50]. This fact highlights the importance of using strains capable of producing *amylases*, which are safe in terms of the inability to produce Ochratoxin A and Fumonisin B2. In this sense, all strains in this study were evaluated as not producing both mycotoxins [50] and, all strains produced *amylases*.

The UELAs 15.262 strain stood out in terms of production of these enzymes. The screening of *Aspergillus* strains regarding the potential for amylase production has also been reported in other studies, which evaluated the potential of production of amylases for EI, from species of genus *Aspergillus* [30, 43, 56].

Besides the genetic background of the strains, the abiotic factors interfere the production of *amylases* [9, 44]. Temperature and pH are crucial factors that have provided the production of *amylases*. The production of *amylases* by UELAs 15.262 strain was favored at slightly acidic pH (pH 5.0) and temperature between 35 °C and 40 °C. These data are according to reports of *amylases* production by species of *A. niger*, pH 6.0, 28 °C [48], pH 4.0, 30 ± 2 °C [41], and *Aspergillus* sp., pH 5.5, 30 °C [11].

In addition, the *A. welwitschiae* mutant UELAs 15.262/35 strain stood out by the ability to improve the amylase production in fewer days and in most favorable conditions to industrial applications. This strain was obtained by random mutation induced by ultraviolet light (unpublished data). It is known that the use of mutant strains has contributed to increased production of metabolites, such as *amylases* [3, 36, 54]. In this sense, the kinetics of *amylases* production by selected strains under Submerged Fermentation, showed higher *amylases* production with seven and six days of fermentation by UELAs 15.262 (951 U/mL) and UELAs 15.262/35 (580 U/mL), respectively. Others studies also reported the highest *amylases* production by *Aspergillus* strains in periods of time longer than four days of Submerged Fermentation (502 U/mL - 6 days – 30 °C – 180 rpm [45]; 1,780 U/mL - 4 days – 40 °C – pH 4,5 – under shaking [42]), under standardized conditions. In contrast, *Aspergillus* strains under Submerged Fermentation for up to four days, the highest *amylases* production obtained was from 0.88 U/mL to 350 U/mL (33.52 U/mL – 35 °C – 4 days [6], 70.29 EU/mL – 30 °C – 88 hours – 200 rpm [17], 230 U/mL – 35 °C – 3 days – 200 rpm [26], 0,48 U/mL – 28 °C - 2 days – 200 rpm [35], in standardized conditions. Also, like *A. welwitschiae* UELAs 15.262/35, *Aspergillus* mutant strains have shown satisfactory *amylases* activity as in the reports by [36] that obtained 171.4 U/mL in 48 hours, in conditions of 30 °C, pH 6.5, by [3], obtained 687 U/mL of *amylases* activity in 72 hours, 30 °C, pH 5.0 and the group by [54] in conditions of 30 °C, 200 rpm, obtained 2,189 U/mL in 48 hours.

Whereas *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 showed considerable production of *amylases*, the characterization of Crude Enzymatic Extract Partially Purified (CEEPP) was relevant. The highest *amylases* activity from CEEPP of UELAs 15.262 strain was obtained at pH 5.5, 50 °C to 65 °C, provided a 2-fold increase in *amylases* activity (800 U/mL - pH 5.5 to 1,600 U/mL - pH 5.5, 65 °C). Similarly, the *amylases* activity from CEEPP of UELAs 15.262/35 strain obtained at pH 5.0, 60 °C to 65 °C, increased 2.2-fold the activity of these enzymes (900 U/mL - pH 5.0 to 2,000 U/mL - pH 5.0, 65 °C). Most of the *amylases* produced by *Aspergillus* species have better enzymatic activity with pH around 4.5 to 6.0, between 50 °C and 65 °C [5, 7, 24, 28]. Higher or lower pH and temperature variations cause a drastic drop-in enzymatic activity [2, 53].

The thermal stability of an enzyme is one of the main requirements for its application. The reduction of 35%

and 30% in the activity of *amylases* by UELAs 15.262 and UELAs 15.262/35 strains, respectively, at the end of 100 minutes highlights the importance of the *amylases* produced by *A. welwitschiae* in future applications. In contrast, studies using *Aspergillus* strains for the production of *amylases* obtained between 40% to 70% reduction in *amylases* activity, in a period between 50 to 180 minutes, and between 50 °C to 60 °C [35, 51, 53].

The addition of Cu^{+2} and Fe^{+3} ions to CEEPP provided an increase of 25% and 20% (2,410 U/mL and 2,324 U/mL) for *amylases* activity of UELAs 15.262 strain, and 21% and 29% (2,976 U/mL and 3,150 U/mL) for UELAs 15.262/35 strain, respectively. Selim [34], evaluating *amylases* from *A. niger,* obtained an increase in amylolytic activity of 53% using the Cu^{2+} ion and 38 % with Fe^{3+} ion. Xian and Feng [57], using the ions of Cu^{2+} and Fe^{3+} (5mM) showed an increase of 14% and 23%, respectively, in the enzymatic activity of *amylases* produced by the species *A. tritici* WZ99.

5. CONCLUSION

Thus, this is the first report of the association of selected strains with the best abiotic factors provided satisfactory *amylases* production and consequent *amylases* activity by *A. welwitschiae* strains. In the characterization of the Crude Enzyme Extract Partially Purified, the wild strain UELAs 15.262 showed better enzymatic activity in the abiotic combination of pH 5.5, 60 °C, in contrast, the mutant strain UELAs 15.262/35 using the abiotic combination of pH 5.0, 60 °C, showed a 20% increase in enzymatic activity, when compared with the strain UELAs 15.262. The stability of both strains showed only a 35% (UELAs 15.262) and 30% (UELAs 15.262/35) reduction in amylolytic activities over 100 min of incubation at 60 °C. The effect of Cu^{2+} and Fe^{3+} metal ions contributed to an increase in enzyme activity in both strains. The improved activity at acidic pHs and elevated temperatures contribute to both strains being good candidates for various industrial applications.

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6 CONSIDERAÇÕES FINAIS

Neste estudo, através da triagem, a linhagem UELAs 15.262 apresentou o maior potencial produtor de enzimas amilolíticas, tanto no meio sólido quando líquido. Na Fermentação Submersa a cepa mutante UELAs 15.262/35 apresentou atividades enzimáticas inferiores, quando comparada a cepa selvagem UELAS 15.262.

Na caracterização do Extrato Bruto Enzimático Parcialmente Purificado, a linhagem selvagem UELAs 15.262 apresentou melhor atividade enzimática na combinação abiótica de pH 5,5, 60 °C, em contrapartida, a cepa mutante UELAs 15.262/35 utilizando a combinação abiótica de pH 5,0, 60 °C, apresentou um aumento de 20% na atividade enzimática, quando comparada com a cepa UELAs 15.262. A estabilidade de ambas as linhagens mostrou uma redução de apenas 35% (UELAs 15.262) e 30% (UELAs 15.262/35) nas atividades amilolíticas, ao longo de 100 minutos de incubação a 60 °C. O efeito dos íons metálicos Cu^{2+} e Fe^{3+} contribuíram para um aumento na atividade enzimática em ambas as linhagens. A melhor atividade em pHs ácidos e temperaturas elevadas, contribuem para ambas as linhagens serem boas candidatas para diversas aplicações industriais.

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ANEXOS

ANEXO 1

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- Book

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- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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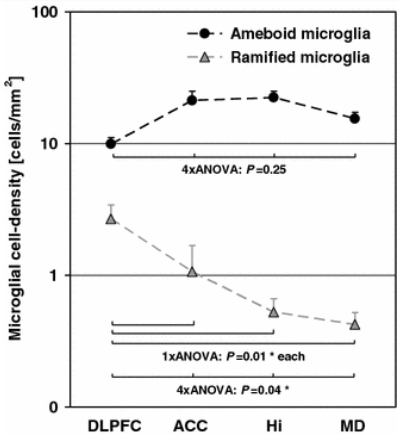
Line



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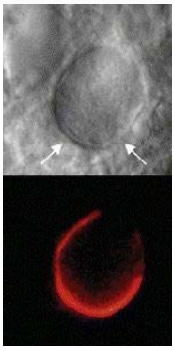

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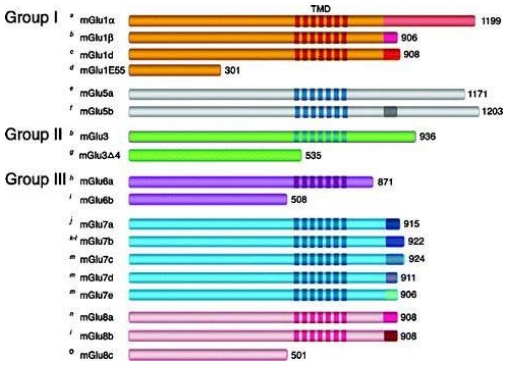

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Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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