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EXPLORANDO O POTENCIAL DAS PROTEÍNAS DE VÍSCERAS DE FRANGO: UMA ABORDAGEM DIRECIONADA À OBTENÇÃO DE PRODUTOS COM PROPRIEDADES ANTIOXIDANTES E ENZIMAS

EXPLORING THE POTENTIAL OF CHICKEN VISCERA PROTEINS: A TARGETED APPROACH TO OBTAINING PRODUCTS WITH ANTIOXIDANT PROPERTIES AND ENZYMES

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Orientador: Prof. Dr. Ruann Janser Soares de Castro

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RESUMO

A produção de carne de frango vem aumentando gradativamente ao longo dos anos, o que acarreta maior geração de resíduos. As vísceras do animal são uma parcela importante, comumente destinadas à compostagem, incineração ou transformadas em farinha para consumo animal. Todavia, a busca por alternativas de reaproveitamento do material para obtenção de produtos com maior valor agregado vem ganhando espaço no meio industrial. Para tanto, o objetivo deste trabalho foi avaliar o potencial da farinha de vísceras de frango como meio para obtenção de produtos com propriedades antioxidantes e proteases. Inicialmente, foi realizado um processo de hidrólise enzimática utilizando o concentrado proteico da farinha de vísceras de frango e as proteases comerciais Flavourzyme e Alcalase. Nesta etapa, foram avaliados os efeitos das condições do processo (tempo de hidrólise e concentração da mistura de enzimas) sobre a formação de peptídeos com atividade antioxidante, e ainda uma avaliação inicial do aumento de escala do processo. Os resultados mostraram que, embora as variáveis independentes não tenham apresentado efeitos significativos sobre as respostas (positivo ou negativo) (p > 0,10), a modificação destes fatores resultou em aumento significativo da atividade antioxidante, sendo os melhores resultados obtidos com atividade inicial de protease de 170,5 U/mL e 120 min de hidrólise. A hidrólise enzimática resultou em aumentos de até 300% na atividade antioxidante quando comparada à amostra não hidrolisada. Nos estudos de aumento de escala foi possível manter a atividade antioxidante dos hidrolisados ao partir de um volume inicial de trabalho de 50 mL (Erlenmeyer) para um volume de 3L em biorreator. O segundo bloco de experimentos deste trabalho incluiu a aplicação da farinha de vísceras de frango como substrato para produção de proteases e obtenção de produtos antioxidantes pelo micro-organismo *Aspergillus oryzae* IOC3999 em fermentação em estado sólido. As variáveis de processo umidade inicial do meio de cultivo, temperatura e quantidade de inóculo foram avaliados. Adicionalmente, as proteases produzidas também foram caracterizadas bioquimicamente. Para a fermentação, as variáveis independentes apresentaram efeito estatisticamente significativo (p < 0,10) para a maioria das respostas. A condição de cultivo conduzida com 40% de umidade inicial do meio de cultivo, temperatura de incubação de 30°C e inóculo inicial de $5{,}05 \times 10^6$ esporos/g, apresentou os melhores resultados para atividade antioxidante e produção de proteases nos dois tempos de fermentação avaliados (24 e 48 h). As proteases apresentaram atividade ótima na faixa de pH entre 5,0 e 6,0 e na temperatura de 50°C. Os parâmetros termodinâmicos indicaram que o processo de inativação térmica da protease não é espontâneo (ΔG^*d > 88,78 kJ/mol), aumenta com o aumento da temperatura (ΔH^*d 27,01-

26,88 kJ/mol) além de apresentar um indicativo de redução da desordem no sistema ($\Delta S^*d <$ −197,74 kJ/mol), provavelmente relacionado à aglomeração de enzimas parcialmente desnaturadas. Em conclusão, os resultados obtidos permitiram demonstrar o grande potencial de exploração de resíduos da indústria do frango, em especial as vísceras para obtenção de produtos antioxidantes e enzimas utilizando dois tipos de processos biotecnológicos (hidrólise enzimática e fermentação).

Palavras-chave: *proteases, farinha de vísceras de frango, peptídeos bioativos, atividade antioxidante, fermentação em estado sólido, termodinâmica enzimática.*

ABSTRACT

The production of chicken meat has been increasing gradually over the years, which leads to greater generation of waste. The animal viscera are an important part, commonly destined for composting, incineration or transformed into flour for animal consumption. However, the search for alternatives to reuse the material to obtain products with greater added value has been gaining ground in the industrial environment. Therefore, the objective of this work was to evaluate the potential of chicken viscera meal to obtain products with antioxidant properties and proteases. Initially, an enzymatic hydrolysis process was carried out using the protein concentrate of chicken viscera meal and the commercial proteases Flavourzyme and Alcalase. In this step, the effects of the process conditions (hydrolysis time and concentration of the enzyme mixture) on the formation of peptides with antioxidant activity were evaluated, as well as an initial evaluation of the scale-up of the process. The results showed that, although the independent variables did not present significant effects in all response variables (positive or negative) ($p > 0.10$), the modification of these variables resulted in a significant increase in antioxidant activity, in which the best results were obtained using initial protease activity of 170.5 U/mL and 120 min of hydrolysis. Enzymatic hydrolysis resulted in increases of up to 300% in antioxidant activity when compared to the unhydrolyzed sample. In the scale-up studies, it was possible to maintain the same antioxidant activity of the hydrolysates from an initial working volume of 50 mL (Erlenmeyer) to a volume of 3L in a bioreactor. The second block of experiments of this work included the application of chicken viscera flour as a substrate to produce proteases and obtaining antioxidant products by the microorganism *Aspergillus oryzae* IOC3999 in solid state fermentation. The process variables initial moisture of the culture medium, temperature and amount of inoculum were evaluated. Additionally, the proteases produced were also biochemically characterized. For fermentation, the independent variables showed a statistically significant effect ($p < 0.10$) for most responses. The cultivation condition performed with 40% initial moisture of the culture medium, incubation temperature of 30°C and initial inoculum of 5.05×10^6 spores/g, showed the best results for antioxidant activity and production of proteases in the two times of fermentation evaluated (24 and 48 h). The proteases showed optimal activity in the pH range between 5.0 and 6.0 and at a temperature of 50°C. The thermodynamic parameters indicated that the process of thermal protease inactivation is not spontaneous ($\Delta G^*d > 88.78$ kJ/mol), it increases with increasing temperature (ΔH^*d 27.01-26.88 kJ/mol), in addition to presenting an indication of reduced disorder in the system ($\Delta S^*d < -197.74$ kJ/mol), probably related to the agglomeration of partially denatured enzymes. In

conclusion, the results showed the great potential of exploitation of residues from the chicken industry, especially the viscera, to obtain antioxidant products and enzymes using two types of biotechnological processes (enzymatic hydrolysis and fermentation).

Keywords: *proteases, chicken viscera meal, bioactive peptides, antioxidant activity, solid-state fermentation, enzymes thermodynamics.*

SUMÁRIO

1. INTRODUÇÃO

O Brasil é o maior exportador de carne de frango do mundo, exportando aproximadamente 30% a mais que o segundo colocado, os Estados Unidos. Somente em 2019, o país produziu 13,3 milhões de toneladas do produto, representando um aumento de 3,03% em relação ao ano anterior (ABPA, 2020). Porém, a grande produção é responsável pela geração de uma grande fração de resíduos de frango, tanto sólidos quanto líquidos, o que representa de 40-60% do peso do animal (THORESEN et al., 2020).

Os resíduos sólidos são constituídos basicamente de vísceras, músculos, gordura, sangue, ossos e penas, que por sua composição representam um material potencialmente nocivo ao meio ambiente, por causarem contaminação de solos e pela geração de gases do efeito estufa (FERREIRA et al., 2018). Como forma de controlar os possíveis danos ao ambiente e à saúde humana, o tratamento desses resíduos ocorre por meio da queima, incineração, compostagem e produção de ração animal, sendo a compostagem e a produção de ração animal, as alternativas mais viáveis (DEMATTÊ et al., 2016; WARE; POWER, 2017).

Devido à baixa rentabilidade dos tratamentos convencionais, diversos países vêm investindo em projetos e legislações que viabilizem a utilização desses resíduos para a aplicação em recursos rentáveis como a produção de produtos com maior valor agregado. Pela composição rica em proteínas, diversos estudos vêm relatando a utilização de técnicas de extração química de proteínas utilizando métodos clássicos como a precipitação com solventes orgânicos (etanol, isopropanol) e sais, seguidos de processos de hidrólises (ácida e alcalina) (THORESEN et al., 2020). Apesar do baixo custo, os métodos de hidrólise química apresentam desvantagens, como a degradação de aminoácidos essenciais, certa toxicidade, controle mais difícil do processo e condições de execução mais extremas em termos de pH e temperatura (BEN HAMAD BOUHAMED; KECHAOU, 2017).

No caso da hidrólise por vias biotecnológicas, que inclui a hidrólise enzimática e a fermentação, há vantagens que estão relacionadas principalmente com os aspectos ambientalmente amigáveis do processo, que vêm sendo cada vez mais requisitados em processos industriais. Para a hidrólise enzimática, utilizando proteases comerciais, o maior benefício é a possibilidade de a reação ser conduzida em condições moderadas (temperatura e pH), além de manter o valor nutricional da fonte, e formar peptídeos com atividade biológica e/ou com funções tecnológicas mais atrativas como capacidade espumante, emulsificante, maior solubilidade, dentre outras (HONG; MIN; JO, 2019).

Quanto ao processo de hidrólise proteica envolvendo a fermentação microbiana, utiliza-se micro-organismos capazes de produzir enzimas proteolíticas que realizam a clivagem das ligações peptídicas de proteínas presentes no substrato durante o seu crescimento. Vantagens adicionais do processo fermentativo em relação a outros métodos incluem a possibilidade de produção simultânea de outros metabólitos (enzimas e ácidos orgânicos, por exemplo) que podem agregar maior valor ao produto final, além da incorporação de biomassa que pode conferir maior teor proteico ao produto fermentado assim como possíveis efeitos probióticos (NASRI, 2017; OLUKOMAIYA et al., 2019; OLIVEIRA et al., 2020).

Diversas ações fisiológicas são associadas aos peptídeos bioativos que contêm geralmente entre 2 e 20 resíduos de aminoácidos, com massa molecular de até 6000 Da. Algumas das principais ações biológicas de peptídeos incluem atividade antimicrobiana, antioxidante, anti-hipertensiva, antidiabética, além de funções imunomoduladoras. A bioatividade está relacionada com a composição aminoácidos nesses peptídeos e sua massa molecular, resultando em características específicas responsáveis pelas propriedades benéficas (HONG; MIN; JO, 2019).

Enquanto componentes da estrutura nativa da proteína, os peptídeos não apresentam bioatividade e somente após a clivagem das ligações peptídicas são capazes de realizar tais funções. Além disto, a geração de peptídeos com essas propriedades pode ser influenciada pelas condições do processo, bem como do mecanismo de ação da protease envolvida (ARTE et al., 2019; LACOU; LÉONIL; GAGNAIRE, 2016).

Neste sentido, o trabalho teve como objetivo explorar a farinha de vísceras de frango, que é um resíduo/subproduto do processamento de frango de corte, como substrato para obtenção de peptídeos com propriedades antioxidantes, por meio da hidrólise enzimática utilizando proteases comerciais. Adicionalmente, o processo de fermentação em estado sólido utilizando o micro-organismo *A. oryzae* IOC3999 também foi explorado com o objetivo de obter produtos com propriedades antioxidantes e proteases, que em seguida foram caracterizadas bioquimicamente.

CAPÍTULO I

Resíduos da agroindústria do frango e suas potenciais aplicações para obtenção de bioprodutos por processos fermentativos e enzimáticos

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1. REVISÃO BIBLIOGRÁFICA

1.1. Produção e comercialização da carne de frango no mundo

A produção de carne de frango no mundo vem aumentando gradativamente ao longo dos anos, decorrência direta do aumento do seu consumo, que está associado à busca por produtos mais saudáveis que forneçam alto teor de proteínas, porém com reduzida quantidade de gordura. O aumento no consumo também está relacionado com fatores sociais, como o menor preço e a alta praticidade, sendo um produto de rápido preparo e grande diversidade de formas de consumo (SKUNCA et al., 2018).

Dados fornecidos pela OECD mostram que o consumo de carne de frango aumentou em aproximadamente 54% ao longo de 30 anos, entre 1990 e 2020, e em 2021 atingiu seu maior valor, com média mundial de 14,9 kg/per capita/ano sendo a carne mais consumida no mundo, seguida pela carne de porco e bovina, com 10,8 e 6,4 kg/per capita/ano, respectivamente (OECD-FAO, 2021).

Para suprir o alto consumo, os principais produtores da carne de frango em ranking mundial são, EUA, China, Brasil e Rússia; somados, eles foram os responsáveis pela produção de quase 100 mil toneladas de carne de frango só no ano de 2020. Embora a principal destinação seja para o consumo interno, alguns países como os EUA e principalmente o Brasil destinam uma boa fatia ao mercado estrangeiro; o Brasil, por exemplo, destina mais de 30% da produção ao comércio exterior, sendo uma importante fonte de rentabilidade para o país, além de demonstrar o seu grande potencial produtivo (ABPA, 2021). O lugar de destaque do Brasil na produção de frango de corte deve-se principalmente à sua extensão territorial, alta capacidade de produção de grãos, melhoramento genético e tecnologias desenvolvidas para criação e abate de aves (POHLMANN et al., 2020; SPURIO et al., 2016).

Todavia, a alta demanda por este produto corrobora com a busca por formas de suprir as necessidades proteicas da população em consonância com práticas mais ecológicas, uma vez que o processo de produção de carne de frango, no que concerne à criação até o abate do animal, representa menor consumo de recursos naturais em comparação às carnes suína e bovina, a segunda e a terceira carnes mais consumidas no mundo, respectivamente (SWAIN et al., 2018)

Enquanto a produção de carne de frango necessita de aproximadamente 4300 m^3 de água para cada tonelada de carne produzida, para a carne bovina, esse valor pode chegar a até 15400 m^3, e 6000 m^3 para a carne de porco. Além disso, a criação de frangos de abate é realizada em granjas com alta densidade populacional, enquanto que suínos e bovinos necessitam de maior extensão territorial (MEKONNEN; HOEKSTRA, 2010; OONINCX; DE BOER, 2012). Outro

ponto importante é a geração de gases do efeito estufa. Suínos e bovinos geram somados cerca de 140 kg CO_2-eq/kg de produto, enquanto que os frangos produzem quantidades 20 vezes menores (SWAIN et al., 2018).

Ainda assim, a maior preocupação com o aumento da produção e do consumo de carnes de frango, é o consequente aumento da geração de resíduos, tanto sólidos quanto líquidos. O processo produtivo da carne de frango pode ser representado, de forma geral, por um fluxograma simples (Figura 1) (FERREIRA et al., 2018).

Figura 1. Fluxograma e geração de resíduos do processamento da carne de frango (Fonte: Adaptado de FERREIRA et al., 2018). Autorização de uso disponível no Anexo.

1.1.1. Destinação dos resíduos do processamento da carne de frango

Como exposto no fluxograma (Figura 1), são gerados resíduos durante todo o processamento da carne de frango. O consumo de água e a geração de efluentes são um dos mais graves problemas ambientais gerados por este setor, sendo necessário um tratamento adequado das águas residuárias que possui alta carga orgânica, sólidos suspensos, lipídeos, compostos nitrogenados e fosfato oriundos do sangue, penas, vísceras, urina e fezes, tornando o descarte inadequado um grande perigo ambiental (AZIZ et al., 2018; RAJAB et al., 2017).

A quantidade de efluente gerada varia entre 8 e 15 L por abate de ave, e esse efluente é bombeado até as estações de tratamento (ETE) onde é separado em duas fases: 1) o líquido propriamente dito, que deve ser destinado ao processo de crescimento microbiano aeróbico, anaeróbico ou a junção dos processos, e 2) os sólidos suspensos que podem passar pelo processo de evaporação e centrifugação, resultando em lodo centrifugado e óleo residual (FAGNANI et al., 2019; RAJAB et al., 2017).

O lodo centrifugado apresenta alto potencial de utilização como fonte de energia, podendo sofrer combustão direta e ser utilizado como substituto da madeira na geração de vapor em caldeiras. Outra destinação renovável para este material é a compostagem, porém apresenta uma série de empecilhos, como os gastos de transporte do material, o período de estabilização do composto orgânico que pode levar de 90 a 180 dias, além da baixa demanda (FAGNANI et al., 2019).

Em relação aos resíduos sólidos, a maior proporção é de estrume, penas, ossos, vísceras não comestíveis e frações gordurosas. O estrume, que corresponde às fezes e à urina do animal, é um dos resíduos sólidos mais preocupantes durante o descarte, já que devido ao seu alto conteúdo de fósforo, enxofre e nitrogênio representa um grande perigo de contaminação de águas, ar e solo. Já as penas apresentam mais de 90% de proteína rica em cisteína, arginina e treonina, da mesma forma que as vísceras que apresentam uma composição rica em proteínas e lipídeos (SINGH et al., 2018).

Dentre os tratamentos/destinações convencionais mais utilizados para os resíduos da indústria do frango estão a incineração, a queima, a compostagem, a alimentação animal, a aplicação como fertilizante e como fonte de energia. Ainda assim, esses métodos podem apresentar certas desvantagens; a incineração, por exemplo, é um método muito caro e precisa de controle na emissão de fumaça, bem como a poluição atmosférica pela queima; a compostagem, por sua vez, ocasiona em problemas de mau cheiro e emissão de gases do efeito estufa (SINGH et al., 2018).

Por outro lado, a exploração dos resíduos para a produção de farinhas e incorporação em rações animais se tornou uma opção rentável e sustentável que vem sendo explorada por empresas para a destinação correta desses resíduos. Estima-se que no Brasil sejam produzidas 905 mil de toneladas de farinhas por ano. Dados ainda mostram que a utilização desses resíduos na composição de rações animais pode reduzir o custo do produto de 4 a 8% quando comparado à ração produzida com fontes vegetais tradicionais (FERREIRA et al., 2018). No entanto, a

utilização de farinhas dos resíduos sólidos do processamento da carne de frango na alimentação animal pode ser limitada pela digestibilidade do material (CARDOSO et al., 2020).

No mais, a aplicação como fertilizante não atende à grande quantidade gerada enquanto que a geração de bioenergia apesar de possuir um grande potencial, pode apresentar problemas de baixa eficiência (FERREIRA et al., 2018).

Sendo assim, formas alternativas de reaproveitamento desses resíduos vêm sendo estudadas no sentido de agregar mais valor aos produtos e gerar lucros às empresas. Com base na composição com alto teor de proteínas, as quais podem representar até 67% em relação à matéria seca do resíduo, a hidrólise enzimática para aumento de digestibilidade e geração de biomoléculas com potencial bioativo se apresenta como uma das alternativas mais vantajosas (THORESEN et al., 2020; WARE; POWER, 2017; ROSTAGNO et al., 2017).

Por exemplo, quando se trata da utilização de resíduos como matéria prima para a ração animal, a principal preocupação é em relação à qualidade da proteína, e neste caso, hidrolisados proteicos de resíduos de frango podem ser importantes fontes de aminoácidos essenciais. Soares et al., (2020) reportaram que hidrolisados de frango apresentaram um total de 720,5 g/kg em matéria seca de proteína bruta, sendo encontradas quantidades adequadas para os 9 aminoácidos essenciais: histidina (17,9 g/kg), isoleucina (28,9 g/kg), leucina, (53,7 g/kg), lisina (47,3 g/kg), metionina (15,0 g/kg), fenilalanina (28,2 g/kg), treonina (29,8 g/kg), valina (32,6 g/kg) e triptofano (4,9 g/kg), e também para o aminoácido não essencial arginina (45,7 g/kg).

No entanto, é importante frisar que apenas a qualidade nutricional das proteínas não é suficiente para viabilizar a utilização do resíduo como ração animal, já que uma boa digestibilidade e consequentemente biodisponibilidade, também deve estar atrelada. Neste sentido, os hidrolisados podem ser fontes importantes de proteínas com digestibilidade melhorada.

Isto pode ser observado por meio dos resultados obtidos por Cardoso et al. (2020), que não apenas demonstraram a melhoria na eficiência da produção de animais utilizando resíduos hidrolisados de frango, como representaram uma possível solução econômica e ambiental, uma vez que a substituição parcial da dieta de referência de Tilápia do Nilo por hidrolisados proteicos de resíduos de frango resultaram em menor custo. Os autores observaram que a substituição parcial da dieta de referência por hidrolisados proteicos de frango resultaram em aumento de digestibilidade proteica de 88,6% (dieta de referência) para 90,84% (hidrolisados de resíduos de frango), garantindo um aumento de 6% no fornecimento energético, além de aumento de digestibilidade para diversos aminoácidos.

Por outro lado, a importância de maximizar o reaproveitamento de proteínas se dá pelo problema mundial de crescimento populacional. Países subdesenvolvidos já sofrem com a desnutrição proteico-energética, problema este que pode se agravar e atingir até mesmo países desenvolvidos, pois esperasse que até 2050 a demanda por proteína de origem animal deva dobrar (FAO, 2014).

Neste sentido, o redirecionamento de resíduos industriais ricos em proteínas da alimentação animal para a alimentação humana vem sendo cada vez mais estudada, mas esbarra em problemas como: alergenicidade, toxicidade, sabor estranho e fatores antinutricionais. As proteínas recuperadas podem ser destinadas à alimentação humana, ou como coadjuvantes tecnológicos de diversas formas, como ingredientes para fortificação de alimentos e suplementos dietéticos, ingredientes com propriedades tecnofuncionais (capacidades emulsificante, de formação de espuma e de gelificação, por exemplo), ou ainda como material de biopolímeros e na biorrefinaria (POJIĆ; MIŠAN; TIWARI, 2018).

Nesse contexto, os estudos que buscam métodos de extração proteica e que possibilitem a redução desses problemas encontram um terreno fértil para desenvolvimento. As técnicas de extração úmidas, dentre elas a extração assistida por enzimas, ou mais especificadamente a hidrólise enzimática de proteínas utilizando as proteases está dentre essas possibilidades (POJIĆ; MIŠAN; TIWARI, 2018).

1.2. Hidrólise de proteínas

A hidrólise proteica consiste na quebra de ligações peptídicas das proteínas originando os hidrolisados contendo frações de peptídeos e aminoácidos livres. Esses hidrolisados proteicos possuem uma ampla gama de aplicações e propriedades, como suplementos alimentares (MORA et al., 2019), aditivos em produtos cosméticos (AGUIRRE-CRUZ et al., 2020), propriedades farmacológicas (SHAIBANI et al., 2019), e ainda como ingredientes em alimentos conferindo características sensoriais e funcionais aos produtos (ANZANI; ÁLVAREZ; MULLEN, 2020). Os métodos de hidrólise proteica mais comuns incluem os processos físico-químicos, fermentativos e enzimáticos (ASHAOLU, 2020).

1.2.1. Hidrólise química

A hidrólise química consiste na utilização de soluções ácidas ou básicas que são eliminadas ao final do processo. A hidrólise ácida envolve a aplicação de altas temperaturas, superiores a 100°C durante um período que varia de 20 a 96 h. Desta forma, a taxa na qual as ligações peptídicas são quebradas é de suma importância para a eficiência do processo, sendo

o ácido clorídrico (HCl) um dos agentes mais utilizados, pois permite uma clivagem rápida das ligações peptídicas quando comparado a outros ácidos. Ao final do processo, o ácido é eliminado por evaporação (ASHAOLU, 2020).

Em relação à hidrólise utilizando soluções básicas, os agentes hidróxido de potássio (KOH) e hidróxido de sódio (NaOH) são os mais utilizados. A reação ocorre a 180ºC e muitas vezes é definida como saponificação, já que as bases não somente realizam a decomposição de proteínas em peptídeos e aminoácidos, mas também formam sabão a partir da quebra de macromoléculas lipídicas. Alguns sais de sódio, potássio e cálcio podem ser utilizados para solubilizar as proteínas após o aquecimento a 130ºC (ASHAOLU, 2020).

Apesar de a utilização de agentes ácidos e básicos fortes apresentar uma alternativa prática e econômica para as indústrias, há perdas nutricionais por degradação de aminoácidos essenciais. A asparagina e a glutamina sofrem reações de desaminação sendo convertidos em ácido aspártico e ácido glutâmico, enquanto a serina e a treonina têm o seu conteúdo reduzido de 5 a 15% no processo (MUSTĂȚEA; UNGUREANU; IORGA, 2019).

A cisteína também pode ser destruída no processo de hidrólise ácida, enquanto a metionina pode sofrer oxidação tornando-se sulfóxido de metionina e sulfona de metionina; em ambos os casos, a perda dos aminoácidos pode ser parcialmente evitada pela remoção de oxigênio no meio. No mais, resíduos de tirosina podem sofrer halogenação enquanto que resíduos de lisina podem participar da reação de Maillard (MUSTĂȚEA; UNGUREANU; IORGA, 2019).

Atrelados aos riscos ambientais, essa prática torna-se inapropriada e cada vez mais vem perdendo espaço para os outros métodos de hidrólise. Além disso, as clivagens das ligações peptídicas realizadas por esses reagentes são de difícil controle, gerando hidrolisados com composições química e funcionalidades variadas; podem ainda apresentar maior conteúdo de cinzas pela necessidade de neutralização da reação e formação de sais, o que limita as possíveis aplicações dos hidrolisados proteicos (NASRI, 2017; OVISSIPOUR et al., 2012)

1.2.2. Hidrólise enzimática

A hidrólise por processos bioquímicos compreende a fermentação microbiana e a ação enzimática. Em relação ao método enzimático, diversas vantagens em relação aos métodos químicos podem ser apontadas, como: condução em condições de temperatura e pH mais brandas, alto rendimento, maior velocidade de reação, fácil controle, alta especificidade, além de maior possibilidade de manutenção do valor nutricional do substrato (WUBSHET et al.,

2019). Adicionalmente, processos enzimáticos apresentam maior reprodutibilidade quando se trata da produção de peptídeos bioativos (NASRI, 2017).

O processo de hidrólise enzimática de proteínas pode ser conduzido em biorreatores, onde a fonte proteica nativa é homogeneizada e suspensa em uma quantidade apropriada de água ou solução tampão e em seguida é adicionada a protease de interesse. O controle das variáveis de processo, como temperatura, pH e agitação, é um dos grandes atrativos desse método, uma vez que o torna prático (SELEME, 2019).

O tempo de hidrólise também é uma importante variável pois está diretamente relacionado com o grau de hidrólise das proteínas, o que consequentemente influencia no tamanho e na composição de aminoácidos dos peptídeos gerados, afetando assim também as suas funções biológicas (MARCINIAK et al., 2018). Após o tempo considerado adequado para hidrólise, ocorre a inativação da protease utilizada por tratamento térmico, sendo muitas vezes considerada a única desvantagem desse processo (SELEME, 2019). Por fim, os hidrolisados proteicos são recuperados e submetidos às etapas posteriores como secagem e armazenamento (WUBSHET et al., 2019).

De toda forma, considerando a complexidade de matrizes alimentares, subprodutos e resíduos, uma condição ótima de hidrólise enzimática pode ser um desafio a se determinar devido às barreiras de contato entre enzima/substrato. Em razão disto, diversos estudos vêm sendo desenvolvidos para determinar condições ótimas de trabalho para uma aplicação viável do processo industrialmente. Para tanto, algumas estratégias auxiliares e de pré-tratamento do material vêm sendo estudadas como o processo de ultrassom, micro-ondas, campo elétrico pulsante de alta voltagem e alta pressão hidrostática (HABINSHUTI; MU; ZHANG, 2020; HALL; LICEAGA, 2020; MARCINIAK et al., 2018).

Além das variáveis de processo, a escolha das enzimas utilizadas deve ser cuidadosamente avaliada. As proteases comerciais geralmente são produzidas por micro-organismos do gênero *Aspergillus* sp. e *Bacillus* sp., bactérias láticas ou ainda extraídas de vegetais e animais. Alguns exemplos incluem a Alcalase, Neutrase, papaína, Flavourzyme e Proteinase K (MERZ et al., 2015; SHU et al., 2015).

As proteases são biocatalisadores responsáveis por inúmeras reações biológicas nos mais diversos sistemas orgânicos, como plantas, animais e micro-organismos. Elas são enzimas capazes de realizar a clivagem da ligação peptídica em uma proteína e por este motivo são responsáveis pelo controle da ativação, síntese e "*turnover*" de proteínas para regular os processos fisiológicos (RAZZAQ et al., 2019).

Mas sua importância não para por aí, devido à sua alta especificidade e velocidade de reação, as proteases são largamente utilizadas industrialmente em processos, nos quais, pela modificação de proteínas, a liberação de peptídeos e aminoácidos livres, fornece uma série de benefícios tecnológicos e biológicos. Essa grande relevância é expressa de forma incontestável pelos números de mercado, onde as proteases correspondem a 60% do mercado mundial de enzimas, sendo aplicadas na indústria de detergentes (VOJCIC et al., 2015), de alimentos (DA SILVA; DINI; GOMES, 2017), farmacêutica (WITHANA et al., 2016), têxtil (SINGH et al., 2016) e biotecnológica (KULLMANN, 2018).

Devido à grande variedade de proteases, foi estabelecido pelo comitê de nomenclatura internacional de classificação de enzimas (EC) uma série de classes e subclasses para estes biocatalisadores com base em suas características bioquímicas de atuação. Uma de suas classificações é dada pela posição na qual executa sua atividade enzimática, podendo ser no interior (endoproteases) ou nas extremidades das proteínas (exoproteases). As exoproteases, por sua vez, podem atuar tanto na região N-terminal quanto na C-terminal das proteínas, e por esse motivo são denominadas aminopeptidases e carboxipeptidases, respectivamente (DE SOUZA et al., 2015).

Quanto ao pH de atuação, as proteases podem ser classificadas em: ácidas (pH 2,0 a 6,0), as neutras (pH 6,0 a 8,0) e as alcalinas (pH 8,0 a 13,0). Com base na natureza do sítio ativo, as proteases podem ainda ser classificadas em 5 principais grupos: serino-proteases, cisteíno-proteases, proteases aspárticas, metalo-proteases e treonino-proteases (SHARMA et al., 2017).

As proteases podem ser obtidas de diversas fontes, seja pela extração de tecidos vegetais e animais ou pela produção e secreção por parte dos micro-organismos. Algumas das principais proteases oriundas de tecidos vegetais incluem a bromelina (abacaxi), a papaína (mamão) e a ficina (figo), sendo largamente utilizadas na indústria de alimentos principalmente na fabricação de produtos para amaciamento de carne (FENG et al., 2017; MORELLON-STERLING et al., 2020).

Quanto às proteases de origem animal, as de maior destaque são as responsáveis pela digestão alimentar encontradas principalmente no trato gastrointestinal dos animais, como a quimosina, pepsina, quimotripsina, tripsina pancreática e a renina (SLAMANI et al., 2018).

Entretanto, a utilização de proteases de origem animal e vegetal pode apresentar alguns fatores limitantes, como a baixa produtividade, fatores sazonais e por não atender a certas características necessárias para todas as aplicações industriais desejadas. Por este motivo, as

pesquisas buscaram formas de produção de proteases utilizando micro-organismos, as quais resultaram em altos rendimentos, menor consumo de tempo, menor necessidade de espaço, além de permitir a utilização de ferramentas de manipulação genética, o que propicia a produção de proteases com características desejadas (RAZZAQ et al., 2019).

A produção de proteases de origem microbiana se dá por processos fermentativos, sendo as duas principais a Fermentação em Estado Sólido (FES), que será comentada em um tópico a parte, e a Fermentação Submersa (FSm), sendo o controle das condições de cultivo de ambas as fermentações de grande importância para o processo (SOCCOL et al., 2017).

Dentre as condições de cultivo a se controlar, destacam-se a composição do substrato que pode induzir a produção de determinada enzima: substratos com alto conteúdo de proteína, por exemplo, podem induzir a produção de proteases, considerando que haja uma relação carbono e nitrogênio (C:N) suficiente para sustentar o crescimento adequado do micro-organismo. No mais, fatores físicos como temperatura, pH, umidade, oxigênio dissolvido e agitação, além do tempo de incubação devem ser avaliados pois influenciam fortemente no rendimento e característica das enzimas (SOUZA et al., 2017).

A Tabela 1 resume os dados de alguns trabalhos que relatam a produção de proteases por micro-organismos e suas características bioquímicas.

Tabela 1. Proteases produzidas por diferentes micro-organismos, classe catalítica e condições ótimas de pH e temperatura.

Micro-organismo	Classe catalítica	pH ótimo	Temperatura ótima	Referência bibliográfica
***Bacillus safensis* RH12**	Serino-protease	9,0	60°C	(REKIK et al., 2019)
***Bacillus licheniformis* K7A**	Serino-protease	10,0	70°C	(HADJIDJ et al., 2018)
***Bacillus halodurans* SW-X**	Serino-protease	10,0	70°C	(KAEWSALUD et al., 2021)
***Bacillus amyloliquefaciens* S13**	Serino-protease	8,0	60°C	(HAMICHE et al., 2019)
Lactobacillus curvatus	Metalo-protease	6,0	40°C	(SUN et al., 2019)
Rhizomucor miehei	Aspártico-protease	5,5	55°C	(SUN et al., 2018)
Aspergillus oryzae	Serino-protease	9,0	40°C	(GAO et al., 2019)
***Penicillium chrysogenum* X5**	Serino-protease	10,0	80°C	(OMRANE BENMRAD et al., 2018)
***Aspergillus tamarii* Kita UCP1279**	Serino-protease	9,0	50°C	(AMARAL et al., 2020)
***Aspergillus heteromorphus* URM0269**	Serino-protease	8,0	50°C	(FERNANDES et al., 2020)
***Aspergillus oryzae* Y1**	Serino-protease	7,0	55°C	(AO et al., 2018)
***Aspergillus terreus* 7461**	Serino-protease	6,5	50°C	(DE LIMA et al., 2021)
Aspergillus foetidus	Aspártico-protease	5,0	55°C	(SOUZA et al., 2017)
***Candida kefyr* 41PSB**	Metalo-protease	7,0	105°C	(YAVUZ et al., 2017)
***Bacillus cereus* AG1**	Metalo-protease	10,0	50°C	(PATEL; GUPTE; GUPTE, 2018)
***Bacillus luteus* H11**	Serino-protease	10,5	45°C	(KALWASIŃSKA et al., 2018)
***Aspergillus oryzae* CH93**	Metalo-protease	8,0	50°C	(SALIHI; ASOODEH; ALIABADIAN, 2017)
***Aspergillus flavus* MTCC 9952**	Serino-protease	11,0	40°C	(YADAV et al., 2015)
Aspergillus terréus	Serino-protease	7,0	45°C	(BIAGGIO et al., 2016)
Aspergillus avenaceus URM6706	Serino-protease	7,0	50°C	(DA SILVA et al., 2021)

Uma protease comercial muito utilizada industrialmente é a Flavourzyme que consiste em uma preparação contendo atividades de endo/exopeptidases derivadas do *Aspergillus oryzae* e é muito utilizada na indústria para hidrólise de proteínas, possui pH ótimo em torno de 7,0 e temperatura ótima de 50ºC (NOVOZYMES, 2001). O nome "Flavourzyme" é uma referência à produção de agentes aromatizantes a partir da hidrólise das proteínas, tanto vegetal (WANG et al., 2016), quanto animal (ZHANG et al., 2017). No entanto, diversos estudos reportaram a ação desta enzima para produção de peptídeos bioativos, como antioxidantes e antimicrobianos (BORRAJO et al., 2020), inibição da enzima conversora de angiotensina (BAO et al., 2016), dentre outras.

Outra importante protease comercial é a Alcalase. Produzida a partir de *Bacillus licheniformis* é uma serino endopeptidase com faixa de atuação ótima de pH entre 7,0 e 9,0 e de temperatura entre 30 e 65ºC (NOVOZYMES, 2016). Assim como para a Flavourzyme, diversos estudos demonstraram a melhoria na qualidade tecnológica de proteínas hidrolisadas pela Alcalase, como aumento da solubilidade, da capacidade emulsificante e de formação de espuma, assim como maior capacidade de retenção de água (HALIM; SARBON, 2020). Adicionalmente, também foi reportada a liberação de peptídeos bioativos, com atividade antioxidante (HU et al., 2018; AGUILAR; GRANATO CASON; DE CASTRO, 2019), anticancerígena (KARAMI et al., 2019) e inibidora da enzima conversora de angiotensina (BAO et al., 2016; DE MATOS et al., 2022).

1.2.3. Hidrólise de proteínas durante processos fermentativos

A hidrólise proteica envolvendo processos fermentativos caracteriza-se pela utilização de micro-organismos capazes de produzir enzimas proteolíticas que realizam a clivagem das ligações peptídicas de proteínas presentes no substrato durante o seu crescimento, liberando frações de peptídeos e aminoácidos livres (NASRI, 2017). Muitas vezes, a geração de peptídeos bioativos é uma consequência da utilização da fermentação para outros fins, assim, pode-se entender a fermentação como um processo indireto de hidrólise proteica. Na produção de leites fermentados, por exemplo, o objetivo primário do processo não é hidrolisar proteínas e obter peptídeos bioativos. No entanto, o produto fermentado possui uma infinidade de peptídeos provenientes de proteínas hidrolisadas por ação de proteases produzidas pelas bactérias ácido láticas durante a obtenção do produto. Além disso, a fermentação pode conferir características sensoriais diferenciadas (CHEN et al., 2016), e diminuir a alergenicidade de proteínas em determinados produtos (LI et al., 2019).

Geralmente, os processos fermentativos, como a fermentação submersa e a fermentação em estado sólido, utilizam como micro-organismos bactérias láticas, leveduras e fungos do gênero *Aspergillus* sp. classificados como seguros para geração de produtos destinados ao consumo humano. Da mesma forma que em outros processos biotecnológicos, as condições de cultivo como temperatura, pH, umidade, aeração, substrato, dentre outros, devem ser criteriosamente avaliados quando o objetivo é utilizar a fermentação como processo para produção de peptídeos com propriedades biológicas (CHAI; VOO; CHEN, 2020).

Taha et al., (2017) detectaram a produção de peptídeos bioativos com propriedades antioxidantes e antibacterianas durante o processo fermentativo para produção de iogurte utilizando leite de búfala como substrato. Sanjukta e Rai, (2016) estudaram um processo fermentativo utilizando soja como substrato e diferentes fungos filamentosos e bactérias e relataram a produção de peptídeos bioativos com ação inibidora da enzima conversora de angiotensina (ECA), além de apresentarem propriedades antioxidante, antitumoral, antidiabética e antimicrobiana.

1.3. Fermentação em Estado Sólido (FES)

Enquanto a fermentação submersa (FSm) é o processo biotecnológico mais convencional e utiliza meios de cultivo na forma líquida, a fermentação em estado sólido é um processo biotecnológico de crescimento microbiano em um substrato sólido com quantidade adequada de umidade (CERDA et al., 2019; HANSEN et al., 2015). Sendo assim, a FES simula o habitat natural dos micro-organismos, propiciando melhor desempenho para sua adaptação e crescimento, o que pode resultar na produção de biomoléculas com alto rendimento.

A matriz sólida pode conter todos os nutrientes essenciais para o crescimento eficiente do micro-organismo, ou pode servir de suporte infundido com solução de nutrientes (KARIMI et al., 2021). Em ambos os casos, os micro-organismos são inoculados sobre uma malha de espessura relativamente fina de substrato, para que sejam capazes de penetrar e ter acesso aos nutrientes durante todo o período de fermentação. Neste sentido, resíduos sólidos da indústria de alimentos se tornam excelentes substratos para utilização na FES devido à sua composição rica em macro e micronutrientes essenciais (SADH; DUHAN; DUHAN, 2018).

Ainda que a FSm apresente diversas vantagens, como maior facilidade na aplicação de métodos de purificação e refino das biomoléculas e melhor controle das condições de processo, o que possibilita a implementação de processos industriais em biorreatores de grande escala, a a larga geração de efluentes ao final do processo torna-se um aspecto negativo por representar um potencial problema para o meio ambiente (BEHERA et al., 2019).

Em contrapartida, a FES pode ser considerada um processo com menor impacto ambiental, pois é uma técnica de menor custo, menor utilização de energia e produz muito pouco efluente. Além disso, o tempo de fermentação é mais curto, produz enzimas em maior concentração e as condições de cultivo limitam a contaminação por bactérias. Neste caso, essa técnica é mais direcionada para o crescimento de fungos, em especial os fungos filamentosos, pois são capazes de se distribuir por toda a superfície do substrato (LEITE et al., 2021; VERDUZCO-OLIVA; GUTIERREZ-URIBE, 2020).

Todavia, a FES possui suas desvantagens, as quais incluem: dificuldades na manutenção da aeração, quantificação da biomassa formada e realização de estudos da cinética de crescimento, além do controle adequado das variáveis de processo, como temperatura, transferência de massa e calor, controle do pH e a não uniformidade da massa celular e do substrato, fatores estes que dificultam a aplicação dessa técnica em escala industrial (LEITE et al., 2021; VANDENBERGHE et al., 2021). Cada uma dessas variáveis apresenta um papel fundamental para o crescimento do micro-organismo e produção eficiente da biomolécula de interesse.

A temperatura e o pH, por exemplo, têm papel fundamental durante o crescimento do micro-organismo. Embora haja uma temperatura adequada para o seu desenvolvimento, durante a atividade metabólica há geração de calor que pode elevar a temperatura no meio e afetar negativamente a germinação de esporos, o crescimento celular, a produção de metabólitos e a esporulação; desta forma, o calor deve ser rapidamente dissipado o que só pode ser atingido com aeração adequada (CHILAKAMARRY et al., 2022). No caso do pH, o próprio substrato apresenta um valor específico de pH, resultante da presença de ácidos orgânicos e outros nutrientes. O consumo destes elementos e a produção de outros metabólitos durante o crescimento microbiano resulta em variação do pH, que afeta diretamente a ação de enzimas extracelulares e consequentemente o crescimento celular. Neste sentido, é necessário a utilização de soluções tamponantes e sais de amônio, ureia, dentre outros, que ajudam a eliminar a necessidade de controle de pH durante a fermentação (CHILAKAMARRY et al., 2022).

Em relação à água, embora presente em pouca quantidade, esta desempenha papel fundamental para difusão de nutrientes pelo substrato e absorção pelo micro-organismo, além de manter a estabilidade e a função de estruturas biológicas, como proteínas, carboidratos e nucleotídeos (KUMAR et al., 2021). Quando a umidade no substrato é muito baixa, a solubilidade dos nutrientes é limitada o que dificulta a absorção destes pelo micro-organismo, enquanto uma umidade muito alta limita a difusão de ar pelo substrato devido ao preenchimento

dos espaços vazios por espessas camadas de água, além de propiciar um aumento no risco de contaminação. Além disso, valores de atividade água entre 0,6-0,7 são suficientes para o crescimento de fungos filamentosos, pois favorecem a germinação de esporos e o crescimento micelial (KUMAR et al., 2021).

Embora a composição química do substrato seja o principal indicador para sua aplicação na FES, características físicas atreladas ao tamanho de partícula, como cristalinidade, área acessível e superficial e porosidade também devem ser levadas em consideração no momento da escolha (SRIVASTAVA et al., 2019). Partículas pequenas, por exemplo, são preferíveis em relação às partículas grandes, pois resultam em maior área superficial, garantem melhor transferência de calor e trocas gasosas, bem como uma distribuição uniforme de umidade ao longo de uma fina película superficial. A porosidade adequada ainda permite a transferência de nutrientes por caminhos com menos barreiras (SRIVASTAVA et al., 2019). Ainda assim, deve se tomar cuidado, pois partículas excessivamente pequenas podem levar a compactações que resultam na formação de aglomerados de substrato e até mesmo de grande concentração de massa celular alocada em uma determinada região, dificultando a penetração e a distribuição uniforme de gases e do próprio micro-organismo pelo meio de cultivo. Meios de cultivo com partículas de tamanhos variados no intervalo entre 0,25 e 7,5 mm são considerado ideais para esse tipo de processo (ŠELO et al., 2021; SRIVASTAVA et al., 2019).

A aeração é outra variável importante a se controlar, pois deve ser suficiente para manter o ambiente em aerobiose, eliminar o dióxido de carbono gerado, e regular a temperatura e a umidade no meio (FARINAS, 2015).

Ainda que o inóculo inicial não seja uma variável problemática para se controlar, é válido salientar a importância em avaliar a concentração adequada de micro-organismo a ser inoculada, bem como o tipo de inoculação. Grandes concentrações de inóculo podem levar a um esgotamento precoce dos nutrientes, antes mesmo que o micro-organismo sintetize os produtos de interesse em quantidades desejadas. Enquanto baixas concentrações podem não ser suficientes para iniciar o seu crescimento (YOON et al., 2014). O método de inoculação, a ser utilizado irá depender tanto da natureza dos fungos envolvidos, como a finalidade dos estudos. Os métodos de preparação de inóculo normalmente aplicados para FES incluem suspensão de esporos, disco de micélios, suspensão de micélios e substratos pré-inoculados (YOON et al., 2014).

Apesar de a FSm representar cerca de 90% dos processos industriais, a FES vem sendo bastante estudada na produção de diversos bioprodutos como antibióticos, toxinas bacterianas, drogas imunológicas, alcaloides, enzimas, biocombustíveis, e na obtenção de produtos alimentícios (ARORA; RANI; GHOSH, 2018). A Tabela 2 traz um retrato da importância da aplicação da FES para produção de diversas biomoléculas e reaproveitamento de resíduos agroindustriais.

Tabela 2. Fermentação em Estado Sólido para produção de diversas biomoléculas, utilizando micro-organismos e substratos diferentes.

Micro-organismo	Substrato	Produto	Referência bibliográfica
Aspergillus oryzae	Casca de soja, resíduos de farinha de trigo	α-amilase	(MELNICHUK et al., 2020)
Trichoderma reesei CCT2768	Carnaúba	Celulases, xilanases	(DA SILVA et al., 2018)
Aspergillus niger, *A. fumigatus*	Biomassa de sorgo	Hemicelulases, celulases	(DIAS et al., 2017)
Aspergillus niger	Resíduos de vinícola, azeite e cervejaria	Xilanase, celulase, β-glicosidase, compostos antioxidantes	(LEITE; BELO; SALGADO, 2021)
Rhizopus oryzae MUCL28168	Sorgo, café	n-desmetilases	(PEÑA-LUCIO et al., 2020)
Aspergillus niger	Farelo de trigo, farelo de soja, farelo de algodão e casca de laranja	Lipase, CMCase, α-amilase, β-glicosidase	(OHARA et al., 2018)
Yarrowia lipolytica	Esponja de luffa	y-decalactonas	(TRY et al., 2018)
Kluyveromyces marxianus	Bagaço de cana, melaço de beterraba	Ésteres aromáticos	(MARTÍNEZ et al., 2018)
Trichoderma harzianum IOC4042	Bagaço de cana	6-pentil-α-pirona	(DA PENHA; ROCHA-LEÃO; LEITE, 2019)
Monascus purpureus CH01	Bagaço de batata	Pigmento amarelo, vermelho e roxo	(CHEN et al., 2021)
Monascus purpureus FTC5356	Folha de palmeira	Pigmento vermelho	(SAID; HAMID, 2019)
Aspergillus niger	Concha de camarão	Proteases	(OOI; RASIT; ABDULLAH, 2021)
Aspergillus tamarii Kita UCP1279	Casca de limão, farelo de aveia, farelo de soja, farelo de trigo	Proteases	(AMARAL et al., 2020)
Bacillus subtilis MTCC5480	Farelo de glúten de milho	Peptídeos bioativos	(JIANG et al., 2020)
Bacillus subtilis lwo	Grão de bico	Protease, peptídeos bioativos	(LI; WANG, 2021)
Lactobacillus reuteri K777, *Lb. plantarum* K779	Tremoço, trigo, quinoa	Peptídeos bioativos, compostos fenólicos	(AYYASH et al., 2019)
Aspergillus terreus, A. *oryzae*	Biomassa lignocelulósica	Ácido fumárico, ácido itacônico	(JIMÉNEZ-QUERO et al., 2020)
Aspergillus niger, *Rhizopus oryzae*	Resíduos de frutas e vegetais	ácido succínico	(DESSIE et al., 2018)
Penicillium roqueforti ATCC10110	Resíduos de farelo de cacau	Lipases	(ARAUJO et al., 2021)

Devida à essa grande importância, diversos estudos vêm apresentando formas de realizar a produção em larga escala de biomoléculas utilizando a FES. Dentre as tecnologias desenvolvidas, os biorreatores estáticos e agitados são os que merecem maior destaque (VANDENBERGHE et al., 2021).

Biorreatores de bandeja são bastante utilizados na cultura asiática para a fermentação de alimentos como o arroz. Consistem basicamente em uma bandeja perfurada construída com madeira ou aço inoxidável onde o substrato é disposto em uma fina camada, entre 5 e 15 cm (Figura 2). As bandejas são armazenadas em câmaras que possibilitem o controle de temperatura e umidade; a aeração ocorre de forma natural por convecção. Como já é de se imaginar, o principal problema envolvido nessa prática é a necessidade de grandes áreas, além do maior risco de contaminação (SOCCOL et al., 2017).

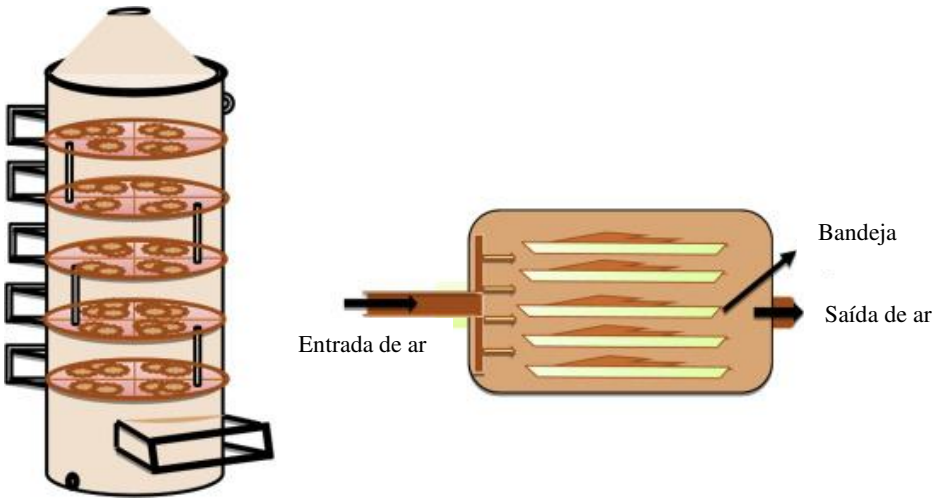

Figura 2. Representação do biorreator de bandeja (Fonte: KRISHANIA et al., 2018). Autorização de uso disponível no Anexo.

Os biorreatores de leito compactado (Figura 3) são uma versão da técnica já utilizada industrialmente. No caso da FES, o leito é formado pelo substrato misturado ao micro-organismo e preso em cilindros ou tambores de metal encamisados para controle da temperatura, uma das principais vantagens desse método é a possibilidade de forçar o ar a atravessar o sistema, repondo o oxigênio, com consequente controle da umidade e mitigação do acúmulo de CO_2 e calor. Entretanto, alguns desafios ainda precisam ser superados, como a compactação do leito, a queda de pressão, o acúmulo de calor e a heterogeneidade no meio (ARORA; RANI; GHOSH, 2018).

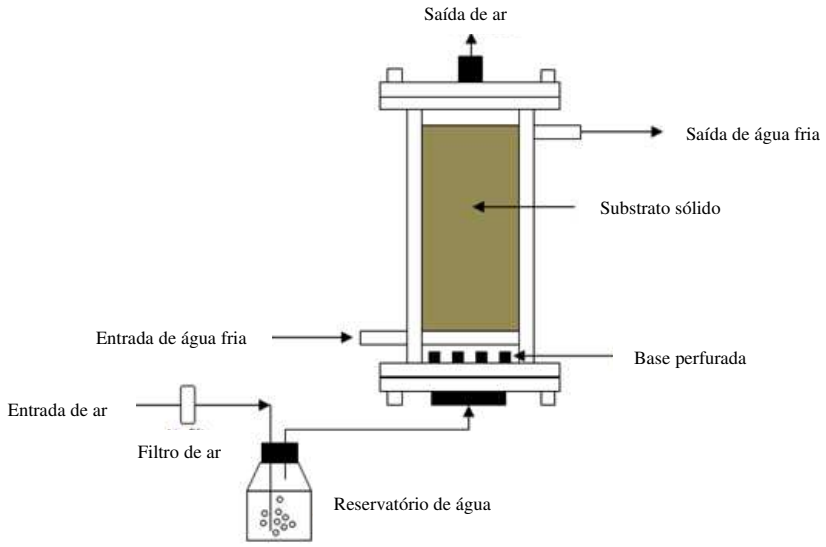

Figura 3. Representação do biorreator de leito compactado (Fonte: MANAN; WEBB, 2017). Autorização de uso disponível no Anexo.

Biorreatores de leito fluidizado (Figura 4), as partículas ficam suspensas livremente em um espaço pela ação de um fluido, o que garante maior transferência de calor, difusão de oxigênio e umidade (NALINI et al., 2021).

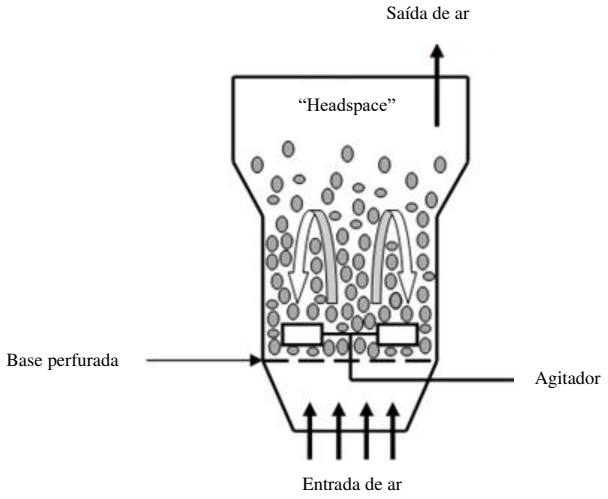

Figura 4. Representação do biorreator de leito fluidizado (Fonte: MANAN; WEBB, 2017). Autorização de uso disponível no Anexo.

Em relação aos biorreatores agitados, o objetivo é a melhoria na homogeneidade do substrato, bem como melhorar a transferência de massa e oxigênio. O controle de temperatura pode ser realizado por meio do encamisamento do equipamento, ou na passagem do ar aquecido. Estes biorreatores podem trabalhar tanto de forma intermitente com ciclos de

agitação, como de forma contínua, onde a principal preocupação é a intensidade da agitação, para não haver ruptura micelial do fungo (VANDENBERGHE et al., 2021).

Exemplos de biorreatores com essa funcionalidade incluem: o tambor de rotação horizontal (Figura 5) e o Koji (comercializado pela Fujiwara no Japão). O primeiro consiste de um tambor cilíndrico que pode conter hastes no seu interior e onde a mistura é realizada de forma inclinada garantido maior suavidade e uniformidade. Enquanto que no Koji, o substrato é empilhado em um disco rotativo com uma camada de espessura máxima de 50 cm, com rotação no próprio eixo (HEMANSI et al., 2019; VANDENBERGHE et al., 2021).

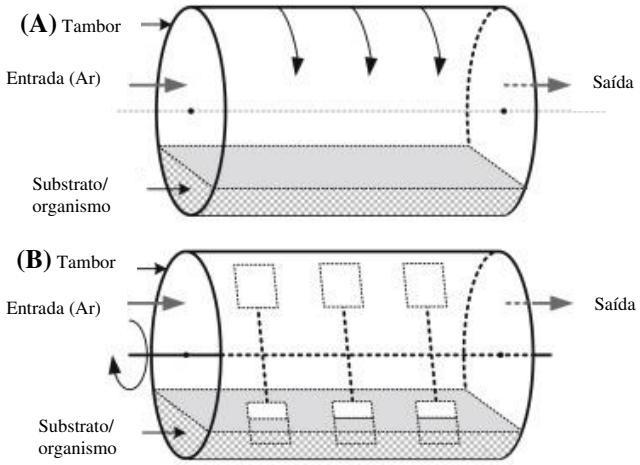

Figura 5. Representação do biorreator de tambor agitado (A) e do biorreator com hastes de agitação (B) (Fonte: GE; VASCO-CORREA; LI, 2017). Autorização de uso (Anexo).

1.4. Importância comercial de fungos filamentosos do gênero *Aspergillus*

Fungos filamentosos do gênero *Aspergillus* incluem uma grande diversidade de espécies, tendo sido identificadas mais de 350 espécies. São fungos, em sua maioria, saprófitos e crescem em uma grande quantidade de substratos, principalmente no solo e em diversos tipos de matéria orgânica, além de possuírem a capacidade de colonizar plantas e animais (DE VRIES et al., 2017).

A grande importância desse gênero comercialmente é devido à sua ampla e diversa produção de biomoléculas, as quais têm sido amplamente utilizadas em vários setores, como na indústria de alimentos, química e farmacêutica. Outro ponto relevante é que a maior parte dos bioprodutos sintetizados por fungos do gênero *Aspergillus* é considerada GRAS pela *Food and Drug Administration* (FDA) (PARK et al., 2017).

Algumas das principais biomoléculas produzidas pelo gênero *Aspergillus* são: enzimas (CHIMBEKUJWO; JA'AFARU; ADEYEMO, 2020; XIANG et al., 2021), pigmentos (SARAVANAN et al., 2020), ácidos orgânicos (OZDAL; KURBANOGLU, 2019) e antibióticos/terapêuticos (AL-FAKIH; ALMAQTRI, 2019; IMAMURA; TSUYAMA; HIRATA, 2011).

Industrialmente, diferentes espécies de fungos do gênero *Aspergillus* vêm sendo utilizadas na produção de biomoléculas há mais de um século. Essa utilização foi iniciada após a identificação da produção de ácido cítrico por *Aspergillus niger* em meios de cultura de baixo custo à base de açúcar (NTANA et al., 2020). Hoje, uma série de outros ácidos orgânicos, como ácido málico, glucônico e itacônico também estão sendo produzidos utilizando espécies desse micro-organismo (CAIRNS; BARTHEL; MEYER, 2021). Outro exemplo é a produção de lovastatina, um medicamento utilizado no controle de colesterol que é hoje produzido por meio de processos fermentativos com o *Aspergillus terreus* (HUANG et al., 2021).

Assim como o *Aspergillus niger*, o *Aspergillus oryzae* é uma espécie com ampla exploração industrial. Tradicionalmente, essa espécie é responsável pela manufatura do Koji pelos japoneses, uma massa de grão de soja e de trigo umidificados com água e fermentada pelo *A. oryzae* a 30°C durante 5 dias. Esse que foi um dos primeiros processos em larga escala da fermentação em estado sólido (NOUT, 2007).

A importância da produção do Koji é a liberação de diversas enzimas, incluindo amilases, proteases, celulases, invertases e lipases. Posteriormente, essa massa rica em enzimas hidrolíticas é utilizada na produção de alimentos fermentados típicos da região como molho de soja, missô e saquê (DABA; MOSTAFA; ELKHATEEB, 2021).

A grande produção de enzimas exógenas pelo *A. oryzae* e o seu status de GRAS, levou esse micro-organismo a um lugar de destaque, sendo uma das principais linhagens utilizadas para produção e posterior comercialização dessas biomoléculas por parte das indústrias de biotecnologia. Como é o caso da AB Enzymes, BASF, Christian Hansen, DuPont e Novozymes, que são algumas das empresas que utilizam o *A. oryzae* para produção de enzimas e outras biomoléculas (NTANA et al., 2020).

1.5. Peptídeos bioativos

Peptídeos bioativos são sequências específicas de moléculas proteicas, formados a partir da quebra das ligações peptídicas entre aminoácidos (SÁNCHEZ; VÁZQUEZ, 2017). Os peptídeos bioativos são assim definidos por possuírem atividades biológicas associadas que podem promover benefícios à saúde excedendo o papel único e exclusivamente nutricional.

Quando estão ligados à proteína nativa, não apresentam bioatividade, sendo necessária a realização da clivagem das ligações peptídicas por meio dos processos de hidrólise para a liberação dos mesmos (KARAMI; AKBARI-ADERGANI, 2019).

Diversos peptídeos de diferentes tamanhos e configurações podem ser originados a partir da clivagem das ligações peptídicas da proteína nativa. Já foram relatados mais de 3900 diferentes peptídeos bioativos (MINKIEWICZ; IWANIAK; DAREWICZ, 2019). A composição dos aminoácidos e a forma como estão dispostos no peptídeo é o que determina sua ação benéfica à saúde, onde, dentre as principais funções fisiológicas estão: ação antimicrobiana, anti-inflamatória, antioxidante, antitrombótica, anti-hipertensiva, opioide, imunomoduladora e quelante de metais (SÁNCHEZ; VÁZQUEZ, 2017). Os peptídeos bioativos geralmente têm entre 2 e 20 resíduos de aminoácidos e massa molecular de até 6000 Da (MAESTRI; MARMIROLI; MARMIROLI, 2016).

Além da sequência e da composição em aminoácidos, massa molecular e distribuição de cargas, a funcionalidade dos peptídeos bioativos também está associada a outros fatores como a realização de pré-tratamentos dos substratos, a protease aplicada e as condições do processo de hidrólise, como pH, temperatura e tempo de reação (KARAMI; AKBARI-ADERGANI, 2019).

A obtenção de peptídeos bioativos pode se dar a partir de diversas fontes proteicas tanto animal quanto vegetal, como carnes (XING et al., 2019), leite (ABD EL-SALAM; EL-SHIBINY, 2017), peixes (YATHISHA et al., 2019), arroz, trigo (ESFANDI; WALTERS; TSOPMO, 2019), insetos (YOON et al., 2019), e até mesmo algas marinhas (ADMASSU et al., 2018). No entanto, a utilização de resíduos agroindustriais vem ganhando cada vez mais credibilidade e espaço, pelo apelo sustentável e por atribuir maior rentabilidade a fontes de baixo custo (LAPEÑA et al., 2018).

Apesar das diversas funções fisiológicas benéficas à saúde proporcionadas pelos peptídeos bioativos, a ação biológica vai depender de sua biodisponibilidade e bioacessibilidade pelo organismo. Desta forma, para que os peptídeos bioativos exerçam sua função fisiológica, sua integridade estrutural deve ser mantida enquanto são transportados para os locais de ação. Assim, essas moléculas devem resistir à degradação proveniente das proteases endógenas (SUN et al., 2020). Em todo caso, a biodisponibilidade de peptídeos bioativos consumidos por via oral na circulação sanguínea de animais e humanos já foi relatada em quantidades nano e picomolares (XU et al., 2019).

Além da função nutricional e fisiológica, os hidrolisados proteicos, fontes de peptídeos bioativos, também conferem importantes propriedades tecnofuncionais aos alimentos, apresentando melhores características como elevada solubilidade e capacidade de retenção de água, propriedades emulsificantes, espumantes e gelificantes (HALIM; YUSOF; SARBON, 2016).

1.5.1. Peptídeos antioxidantes

Concentrações moderadas de espécies reativas de oxigênio (ROS - *reactive oxygen species*) estão normalmente presentes em organismos, pois desempenham funções biológicas essenciais como, modulação do ciclo de vida celular, transdução de sinal intracelular e sinalizações de expressão gênica (WANG et al., 2018). No decorrer das atividades fisiológicas diárias, a geração e eliminação dessas moléculas, como radicais hidroxila, ânions superóxidos, peróxido de hidrogênio, dentre outros, ocorre naturalmente (NWACHUKWU; ALUKO, 2019).

Para combater esses agentes oxidantes, as células possuem diversos agentes de defesa, os antioxidantes, que podem estar naturalmente presentes no organismo como as enzimas catalase e a superóxido dismutase ou podem ser consumidos pela alimentação como os antioxidantes naturais, compostos fenólicos e vitaminas A, C e E (LIOU; STORZ, 2010).

No ser humano, quando a geração de ROS ocorre em concentrações que excedem a capacidade de neutralização pelo organismo, seja pela dieta alimentar, uso de químicos (ex. tabaco) ou assimilação de fontes externas, como a radiação por luz UV, pode ocorrer a geração de sérios danos à membrana lipídica, proteínas e no DNA, causando distúrbios de saúde, como câncer e doenças neurodegenerativas e inflamatórias (BERGAMINI et al., 2005; WONG et al., 2020).

Em outro caso, os ROS ainda são agentes preocupantes na produção de alimentos e cosméticos, pois causam a formação de *off-flavors*. Neste caso, alguns produtos sintéticos podem ser utilizados, pois apresentam forte ação antioxidante, como é o caso do hidroxianisol butilado (BHA) e hidroxitolueno butilado (BHT); todavia, o uso desses compostos químicos começou a ser restrito por conta de indícios associados à indução de danos ao DNA e potencial toxicidade (SILA; BOUGATEF, 2016; WU et al., 2015).

Neste sentido, a suplementação da dieta utilizando antioxidantes naturais vem há tempos recebendo grande atenção e o desenvolvimento de tecnologias e busca por novas fontes de obtenção desses componentes está sendo cada vez mais inserido no campo cientifico e industrial (PRASAD; GUPTA; TYAGI, 2017). Os peptídeos bioativos, por exemplo, estão entre as classes de compostos com grande relevância nesse campo, em especial, àqueles obtidos de

fontes proteicas subutilizadas como resíduos e subprodutos da indústria alimentícia (GÖRGÜÇ; GENÇDAĞ; YILMAZ, 2020; MORA; REIG; TOLDRÁ, 2014).

Certos peptídeos são capazes de atuar como agentes antioxidantes devido à presença de resíduos de aminoácidos que podem realizar a doação de elétrons ou íons hidrogênio. Aminoácidos aromáticos, como a fenilalanina, podem doar um par de elétrons e ainda assim manter sua estabilidade através do mecanismo de ressonância. Aminoácidos que possuem um anel indol e pirrolidina, como no caso do triptofano e da prolina, podem realizar a doação de elétrons através de seus grupos hidroxilas. De forma semelhante, o anel imidazol da histidina também pode exercer essa função, além de apresentar a capacidade de quelar íons metálicos (ZAKY et al., 2020; ZOU et al., 2016).

Estudos também têm demonstrado que peptídeos contendo aminoácidos hidrofóbicos, como prolina, valina e leucina na posição N-terminal geralmente apresentam maior atividade antioxidante. Esse maior potencial está associado ao caráter hidrofóbico do peptídeo, o que confere ao mesmo maior presença na interface água-lipídeo, e consequentemente, maior acesso aos radicais livres lipídicos (SILA; BOUGATEF, 2016; WEN et al., 2020).

Peptídeos com resíduos de aminoácidos ácidos e básicos, como os ácidos glutâmico e aspártico ou arginina e lisina (básicos), também pode desempenhar grande capacidade antioxidante, já que pela presença excessiva de cargas, podem realizar a doação de elétrons, sequestro de radicais livres e redução de íon metálicos (LIU et al., 2016). Os aminoácidos cisteína e metionina também estão envolvidos na eliminação de radicais livres, através da formação de pontes dissulfeto (KIM; WEISS; LEVINE, 2014).

1.6. Potencial de aplicação de resíduos proteicos hidrolisados na alimentação animal

Ainda que a produção de peptídeos bioativos com distinto valor agregado, esteja cada vez mais elucidada em estudos científicos, poucos são os casos em que a exploração da bioatividade desses peptídeos atingem a malha industrial e se tornam produtos comerciais (LI-CHAN, 2015; NASRI et al., 2022). Um dos melhores exemplos da forma prática na qual esse produto pode ser comercializado é na destinação como ração animal, que envolve uma série de benefícios.

O aumento da eficiência na produção de alimentos (carne, leite e ovos) é um dos maiores desafios da agropecuária, já que esse aumento está associado com a nutrição ideal destes animais, definidas pela maior absorção intestinal dos nutrientes presentes na ração (HOU et al., 2017; SALEMDEEB et al., 2017). O aproveitamento de resíduos provenientes de atividades industriais, em especial relacionadas ao beneficiamento de alimentos tanto de origem vegetal

quanto animal, representa um olhar importante para inserção destes como ingredientes nutricionalmente atrativos para a alimentação animal.

Nesse contexto, é importante indicar certas desvantagens inerentes aos resíduos, as quais podem limitar o seu uso. Os resíduos vegetais, por exemplo, possuem como principais entraves a composição não balanceada de aminoácidos essenciais, a presença de fatores antinutricionais e a baixa palatabilidade. Ademais, mudanças na composição química a cada safra fazem com que seja necessária a constante reformulação das rações (RIMOLDI et al., 2020; SIDDIK; HOWIESON; FOTEDAR, 2019).

Quando comparados aos resíduos de origem vegetal, os subprodutos/resíduos de origem animal são geralmente superiores para alimentação animal, fator que se deve pela maior qualidade e proporção de aminoácidos proteinogênicos (como glicina, prolina, ácido glutâmico, leucina, lisina e arginina) (LI; WU, 2020). É importante ressaltar que não só a quantidade e a presença de aminoácidos são suficientes para aplicação desse tipo de substrato. A baixa digestibilidade de algumas fontes de proteínas, muitas vezes pode representar uma barreira para a incorporação destes resíduos em rações, como ocorre com as vísceras e com as penas de frango. O que fazer neste caso?

O aumento da digestibilidade das proteínas dos resíduos animais pode ser aprimorada pela aplicação da hidrólise enzimática, sendo esta uma maneira eficaz de converter esse substrato em ingredientes proteicos com qualidade superior, garantindo um aumento da presença de peptídeos que contribuem com a melhoria da saúde intestinal, crescimento e desempenho da produção (CHAKLADER et al., 2020). Hou et al., (2017) descreveram alguns mecanismos responsáveis por esta melhoria, como:

- Maior taxa de absorção de pequenos peptídeos em relação a uma quantidade equivalente de aminoácidos livres;
- A taxa de catabolismo de peptídeos pequenos pelas bactérias do intestino delgado é menor que uma quantidade equivalente de aminoácidos livres;
- A composição dos aminoácidos livres que entram na veia porta é mais equilibrada com o transporte intestinal de pequenos peptídeos do que a dos aminoácidos livres;
- Aumento do fornecimento de aminoácidos proteinogênicos, o que pode melhorar as reações antioxidantes e síntese de proteínas musculares;
- Presença de alguns peptídeos específicos, como os bioativos, que podem melhorar a morfologia, motilidade, função gastrointestinal (como, secreção e reações anti-inflamatórias) e status endócrino a favor do anabolismo.

Economicamente, a utilização de resíduos para alimentação animal por meio da incorporação como ingrediente em rações apresenta uma grande vantagem, já que as rações representam os maiores custos na criação de animais, sendo a parte proteica responsável por 40-70% dos custos (ALVES et al., 2019).

Estudos demonstraram que formulações contento hidrolisados proteicos de peixe não afetaram as variáveis relacionadas à produção do peixe-gato africano, como taxa de crescimento e peso final, e mantiveram a digestibilidade da ração comumente utilizada. No entanto, os hidrolisados exibiram ação adicional, promovendo o aumento da produção de imunoglobulinas nos animais (SWANEPOEL; GOOSEN, 2018). Já Opheim et al., (2016) observaram um aumento na eficiência de crescimento de frangos ao incorporar hidrolisados proteicos de salmão na dieta inicial dos animais. Aloysius et al., (2018) observaram uma redução dos parâmetros inflamatórios associados à obesidade, em camundongos alimentados com hidrolisados de frango.

Outras pesquisas ainda demonstraram que a incorporação de hidrolisados de proteínas de diversas fontes foram responsáveis pela melhoria no crescimento e desenvolvimento de várias espécies de peixes. Como é o caso da melhoria no crescimento do robalo asiático com dieta suplementada (1-4g/100g) de hidrolisados de vísceras de atum, descrito por Chotikachinda et al., (2013) e do pregado (*Scophthalmus maximus*) com hidrolisados de cabeças de pollok (3,7g/100g) em pesquisa realizada por Zheng et al., (2013).

A principal limitação da utilização de hidrolisados proteicos seja para a alimentação animal ou mesmo humana é devido a problemas de palatabilidade causadas pela formação de peptídeos hidrofóbicos que causam amargor. Esse problema pode ser contornado utilizando condições e enzimas com especificidades que evitem a formação de peptídeos com essa caraterística, ou mesmo realizando a incorporação de agentes de sabor (DALIRI; LEE; OH, 2018; SCHLOTHAUER et al., 2006).

1.7. Considerações finais

Resíduos da indústria do frango são materiais ricos em proteínas e lipídeos. O seu reaproveitamento por meio da transformação em farinhas, na alimentação animal e na produção de biocombustíveis tem sido importante para redução de custos e de possíveis impactos ambientais. Todavia, os processos biotecnológicos de transformação podem otimizar a eficiência dessas práticas e aumentar as possibilidades de reutilização deste material. Tanto a hidrólise enzimática de proteínas, quanto os processos de fermentação, já estão bem estabelecidos como práticas eficientes para obtenção de biomoléculas, desde que as variáveis

do processo sejam devidamente controladas. Desta forma, concentrar esforços no desenvolvimento de novos processos que viabilizem e ampliem as possibilidades de uso de resíduos da agroindústria do frango apresenta-se como uma alternativa muito interessante. Dentre as principais tendências, destacam-se: i) a utilização de hidrolisados proteicos como ingredientes na preparação de alimentos, dadas as suas melhores características biológicas e funcionais, fato este que poderá reduzir a necessidade por proteínas convencionais; ii) a purificação e a identificação dos peptídeos bioativos gerados durante a hidrólise ou fermentação, no sentido de direcioná-los para aplicações específicas e iii) aplicação na alimentação animal, com estudos mais aprofundados relacionando o impacto da substituição das proteínas tradicionais por hidrolisados de resíduos de frango sobre a saúde e a produtividade dos animais.

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CAPÍTULO II

Unraveling the biological potential of chicken viscera proteins: a study based on their enzymatic hydrolysis to obtain antioxidant peptides

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Abstract

The production of chicken meat has been growing over the years, with a proportional increase in waste generation. Since it is a residue rich in protein, enzymatic hydrolysis through proteases can provide technological and biological improvements in this substrate offering new opportunities for exploration by the industries. Therefore, this work aimed to study the enzymatic hydrolysis of proteins from chicken viscera and to characterize the hydrolysates in terms of their antioxidant properties, solubility and molecular weight distribution. Additionally, hydrolysate production was scaled-up from 125 mL flasks with 50 mL of protein solution (100 mg/mL) to 3 L using a 6 L bioreactor. The enzymatic hydrolysis of chicken viscera proteins using a binary mixture of proteases resulted in an increase of up to 245% for ABTS, 353% for DPPH, 69% for FRAP and 145.95% for total reducing capacity (TRC). During scale-up, the antioxidant activity of protein hydrolysates did not show significant differences ($p < 0.05$), demonstrating that the proposed process is promising. The hydrolysates showed molecular weights between 6 and 14 kDa. Enzymatic hydrolysis proved to be an efficient strategy to add value to solid wastes from the poultry industry.

Keywords: *proteases, chicken viscera meal, bioactive peptides, antioxidant activity*

1. Introduction

The constant population growth has demonstrated concerns about the increase in demand for food production, with animal protein sources being a major protagonist of these discussions. It is estimated that over 20 years, until 2018, meat consumption increased by 58%, with 54% being responsible for the growth of the world population. Among the most consumed meats in the world, beef, pork and chicken stand out [1].

Driven by nutritional aspects, the need for greater convenience and lower prices, chicken meat is currently the most consumed in the world. The Organization for Economic Co-operation and Development (OECD) data showed that the per capita consumption of chicken meat was 14.9 kg/capita/year, followed by pork with 10.7 kg/capita/year in 2020 [2]. Fortunately, the production of this meat is largely motivated by the lower need for natural resources such as water and land area compared to beef, for example, in addition to generating lower levels of greenhouse gases [3,4].

To supply the high productive demand for chicken meat in the world, the main producers and exporters of this product are the USA, China, Brazil, and Russia. Data from the United States Department of Agriculture (USDA) in partnership with the Brazilian Association of Agricultural Production (ABPA) showed that the USA was responsible for the production of 20.239 thousand tons of chicken meat in 2020. On the other hand, Brazil deserves to be highlighted for its 13.845 thousand tons of chicken meat produced in the same year, and it is the largest exporter of this product in the world [5].

As a result of the growth in chicken meat production, there is also an increase in the generation of waste, which, if not treated or disposed of properly, can cause several environmental problems [6]. However, due to the high protein content, the potential for reusing this waste is indisputable, and it is common for them to become byproducts transformed into flour and destined for animal feed [7].

Even so, the incorporation of these residues in animal feed is quite limited, since the productive efficiency of animal husbandry depends on the adequate nutritional balance of the ration, which can only be achieved through an effective digestibility of macronutrients in particular. Chicken meat residues can have a digestibility up to 20% lower than that of conventional sources, such as soy, when consumed by some animals [8,9].

Among such processing strategies, enzymatic hydrolysis through proteases stands out, as it is able to cleave larger proteins in peptides, which in general have greater digestibility, in addition to ensuring significant improvements in antioxidant activity that are beneficial to the

animal's metabolism. It is a process with effective control of variables, and a wide range of temperature and pH work conditions without the need to resort to extreme conditions [8,10–12].

Furthermore, protein hydrolysates with antioxidant properties can be used as ingredients in food formulations as an agent for the preservation or promotion of health. As a technological adjuvant, protein hydrolysates have better technological properties, such as solubility, emulsifying capacity and foaming capacity, than native protein [13,14].

In this context, this work studied the enzymatic hydrolysis of proteins from chicken viscera to obtain hydrolysates with improved antioxidant properties. The hydrolysates production was also scaled-up from 50 mL to 3 L by using a bioreactor.

2. Materials and methods

2.1. Commercial reagents and Enzymes

The reagents azocasein, trichloroacetic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 4, 6-tris(2 -pyridyl)-s-triazine (TPTZ), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, bovine serum albumin, the Folin-Ciocalteu reagent, and enzyme preparations Flavourzyme™ 500 L (from *Aspergillus oryzae*) and Alcalase™ 2.4 L (from *Bacillus licheniformis*) were purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were purchased commercially available grade.

2.2. Determination of Protease Activity

The proteolytic activity was determined according to the method proposed by Charney and Tomarelli [15] with slight modifications proposed by De Castro and Sato [16]. The reaction was carried out in 2 mL microtubes containing 0.5 mL of enzyme solution and 0.5 mL of azocasein substrate (0.5% m/v) prepared in sodium phosphate buffer (100 mmol/L, pH 7.0), and incubated at 50ºC for 40 min. The reaction was stopped by adding 0.5 mL of trichloroacetic acid (10% m/v). The reaction blank was prepared similarly to the enzymatic reactions; however, the paralyzing agent was added before the enzymatic solution. Then the tubes were centrifuged at 17,000 x g for 15 min at 25ºC. The colorimetric reaction was performed by mixing a 1.0 mL aliquot of the supernatant of the reaction mixture with 1.0 mL of the neutralizing agent KOH (5 mol/L). One unit of enzyme activity (U) was defined as the amount of enzyme needed to increase the absorbance by 0.01 at 428 nm relative to the blank.

2.3. Production of protein concentrate from chicken viscera (PCCV)

The chicken viscera meal was kindly provided by the company Ad'oro S/A (Sao Paulo, Brazil). According to information from the manufacturer, chicken viscera meal has the following proximate composition: 1.94% moisture, 6.33% carbohydrates, 9.64% lipids, 17.15% ash and 64.94% protein.

For the production of protein concentrate, the chicken viscera meal was subjected to successive washings with ethanol (95%) in the proportion (1:3 m/v). The mixture was kept under constant agitation for 1h at 35ºC and then centrifuged at 17,000 x g at 25ºC for 15 min. The supernatant was discarded, and the precipitated material was subjected to a second wash with ethanol under the same conditions described above. The final precipitate was distributed in trays and kept in an oven with forced air circulation at 50ºC for 24 h to remove residual ethanol. The protein content of the defatted material was estimated by the Lowry method [17]. The PCCV was stored in vacuum packaging and kept under refrigeration until used for enzymatic hydrolysis.

2.4. Effect of process variables on the enzymatic hydrolysis of PCCV

The commercial protease preparations Alcalase™ 2.4L and Flavourzyme™ 500L were used for the production of PCCV hydrolysates.

Samples with 50 mL of protein solutions (100 mg/mL) added to the binary mixture of proteases in equal proportions were incubated under agitation of 100 rpm at 50°C, as previously determined by our research group [11]. After hydrolysis, the solutions were subjected to heat treatment (100°C for 20 min) to inactivate the enzymes. The samples were centrifuged at 17,000 x g at 5°C for 20 min and the supernatants containing the bioactive peptides were collected, frozen and freeze-dried for further analysis.

To determine the most suitable process conditions for enzymatic hydrolysis, a Central Composite Rotational Design (CCRD) was used, in which the amount of enzyme added to the reaction medium (units per mL of reaction – U/mL) (from 29.5 to 170.5 U/mL) and hydrolysis time (from 35.4 to 204.6 min) were evaluated (Table 1). The antioxidant activity of the hydrolysates was used as response to CCRD.

Table 1. CCRD matrix used to verify the effects of protease concentration (U/mL) and hydrolysis time (min) on the enzymatic hydrolysis of PPCV.

Assay	Independent variables	
	Enzyme concentration (U/mL) – X_1	**Hydrolysis time (min) – X_2**
1	-1.00 (50)	-1.00 (60)
2	1.00 (150)	-1.00 (60)
3	-1.00 (50)	1.00 (180)
4	1.00 (150)	1.00 (180)
5	-1.41 (29.5)	0.00 (120)
6	1.41 (170.5)	0.00 (120)
7	0.00 (100)	-1.41 (35.4)
8	0.00 (100)	1.41 (204.6)
9	0.00 (100)	0.00 (120)
10	0.00 (100)	0.00 (120)
11	0.00 (100)	0.00 (120)

The multiple determination coefficient (R^2) and Fisher's test (variance analysis – ANOVA) were used to verify the statistical adequacy of the proposed coded models. The software Statistica 13.3 from TIBCO Software Inc. Company (Palo Alto, California, USA) was used on the experimental design, to analyze the data and generate the models.

Defined the most suitable condition in the CCRD, a hydrolysis kinetics was performed to obtain the protein hydrolysates and to investigate some kinetic parameters of enzymatic inactivation. The results obtained in this step were used as a basis for the scale-up studies.

2.5. Modulation of enzyme activity over time using kinetic parameters

The stability of the binary mixture of commercial proteases as a function of time was evaluated during the enzymatic hydrolysis process. Aliquots were collected at predefined times to determine the residual protease activity throughout the reaction. The residual activity values were used to estimate some kinetic parameters of enzymatic inactivation.

The value of the inactivation constant (k_d) for the proteases, expressed as exponential decay, was determined by plotting ln (A/A_0) versus time using the experimental data as follows:

$$A = A_0 \times e^{-kd \times t} \text{ (Eq. 1).}$$

where t is time, A_0 is the initial enzyme activity and A is the residual enzyme activity for a given time t.

The half-life ($t_{1/2}$) of proteases, defined as the time required for enzymes to reduce their initial activity by 50%, was estimated as shown below:

$$t_{1/2} = \ln (0.5) / k_d \text{ (Eq. 2).}$$

The decimal reduction time (D value), defined as the time required to reduce the initial enzyme activity by 90% at a specific temperature, was calculated as described below:

$$D = 2.303 / k_d \text{ (Eq. 3).}$$

2.6. Determination of antioxidant properties

2.6.1. ABTS radical inhibition

The antioxidant activity measured by the ABTS method was performed according to the method proposed by Al-Duais et al. [18]. The working solution was prepared at the time of analysis and in the absence of light, consisting of the dilution of the concentrated ABTS radical solution (prepared at least 16 h in advance), with absorbance at 734 nm adjusted to 0.70 ± 0.02. In each well of the microplate, 20 µL of hydrolysate solutions (1 mg/mL), blank (distilled water) or standard (Trolox) was added to 220 µL of the ABTS radical solution. After incubation for 6 min in the absence of light, the absorbance of the reaction mixtures was measured at 734 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland). The antioxidant activity was expressed in µmol of Trolox equivalents per g of protein hydrolysate (µmol TE/g).

2.6.2. DPPH radical inhibition

The antioxidant activity determined by DPPH free radical scavenging was performed following the methodology described by Al-Duais et al. [18]. The analysis consisted of the addition of 66 µL of the hydrolysate solutions (5 mg/mL), blank (distilled water) or standard (Trolox) to 134 µL of ethanolic DPPH solution (150 µmol/L) in each well of the microplate. After incubation for 45 min in the dark, the absorbance was measured at 517 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland) and the antioxidant activity was expressed in µmol TE/g.

2.6.3. Iron ion reducing power – FRAP

The reducing power of iron was evaluated according to the methodology proposed by Benzie and Strain [19] and described by Matos, Novelli and De Castro [20] The FRAP reagent was prepared by mixing 2.5 mL of the TPTZ solution (10 mmol/L in HCl); 25 mL of phosphate buffer (0.3 mol/L, pH 3.6) and 2.5 mL of $FeCl_3 \cdot 6H_2O$ (20 mmol/L). Aliquots of 25 µL of the solutions containing the hydrolysates (1 mg/mL), blank or Trolox (5-150 µmol/L) were added to 175 µL of FRAP reagent. The reaction mixture was incubated in a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland) at 37°C for 30 min. The absorbance was monitored at 593 nm and the results were expressed in µmol TE/g.

2.6.4. Total Reducing Capacity - Folin-Ciocalteu

The determination of the total reducing capacity of the samples was evaluated by the Folin-Ciocalteu method [21] with minor modifications. Aliquots of 25 µL of the protein hydrolysate solutions (1 mg/mL), standard or blank, were mixed with 25 µL of 50% (v/v) Folin-Ciocalteu solution and 200 µL of 5% (m/v) sodium carbonate solution (Na_2CO_3) and kept for 20 min at 40°C in the absence of light. Then, the reading was performed on a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland) to determine the absorbance at 760 nm. The calibration curve was prepared with a standard solution of gallic acid (0 to 0.1 mg/mL) and the results were expressed in mg of gallic acid equivalent (GAE) per g of protein hydrolysate (mg GAE/g).

2.7. Determination of TCA-soluble protein content

The TCA-soluble protein content was used as an indirect measure of the degree of protein hydrolysis according to the method described by Castro and Sato [22]. A total of 1.0 mL aliquots of solutions containing the hydrolysates or control (nonhydrolyzed protein) at 10 mg/mL were mixed with 1.0 mL of TCA solution (0.44 mol/L). The mixture was incubated for 30 min at 25ºC and then centrifuged at 17,000 x g for 15 min. The protein content of the supernatant and the sample without precipitation with TCA was quantified by the Lowry method [17] using bovine serum albumin as a standard. The ratio between the soluble protein content in TCA (supernatant) and the total protein content in the samples (without TCA) was determined and expressed as a percentage.

2.8. Bioreactor scaling-up test

The enzymatic hydrolysis of PCCV was evaluated for scale-up using the conditions defined as the most appropriate in the CCRD. Scale-up was conducted using working volumes of 100 and 250 mL using Erlenmeyer flasks. Then, the reactions were carried out in a bioreactor (Bioflo-IIc, New Brunswick Co., USA) with capacities of 3 and 6 L and working volumes of 1 and 3 L, respectively. For bioreactors, the process was carried out at 40°C to respect the working range of the equipment, with agitation of 100 rpm for the 3 L bioreactor. The agitation for the 6 L bioreactor was calculated from Eq. 4, to maintain the fluid dynamics of the process [23]. After hydrolysis, samples were quickly collected, heat treated at 100°C for 20 min to inactivate the proteases and centrifuged at 17,000 x g for 20 min at 5ºC. Supernatants containing the bioactive peptides were collected, frozen and freeze-dried for further analysis.

$$Re_{3l}=Re_{6l} \rightarrow \left(\frac{A \,.\, Di^2 \,.\, \rho}{\mu} \right)_{3l} = \left(\frac{A \,.\, Di^2 \,.\, \rho}{\mu} \right)_{6l} \qquad \text{(Eq. 4)}$$

where: A is the agitation speed (rpm); Di is the impeller diameter; ρ is the density; and μ is the viscosity.

2.9. Statistical analysis

The results were statistically analyzed by Analysis of Variance (ANOVA) followed by Tukey's test using the software Minitab 19 from Minitab LLC (State College, Pennsylvania, USA). Values were expressed as the arithmetic mean (n = 3) and considered statistically different when p values were less than 0.05.

3. Results and discussion

3.1. Effects of process parameters on enzymatic hydrolysis of PCCV and on antioxidant properties of hydrolysates

The results for the antioxidant activity of the PCCV hydrolysates as well as its variation in relation to the control sample for each assay of the experimental design can be seen in Table 2. The highest values obtained for the antioxidant activity in each method were as follows: assay 6 for ABTS (1808.21 µmol TE/g) and DPPH (89.70 µmol TE/g) radical scavenging, assay 8 for FRAP (353.54 µmol TE/g) and assay 4 for TRC (41.68 mg GAE/g), which led to increases of 245, 353, 69 and 145%, respectively, compared to the control samples. The limited variability of the central points (assays 9–11) indicated good reproducibility of the experimental data (Table 2).

Table 2. Results obtained for the antioxidant properties of the control assay (nonhydrolyzed protein) and hydrolysates under conditions of experimental design CCRD 2^2 quantified by different methods and variation in antioxidant activity compared to the control.

	Antioxidant activity				**Variation of antioxidant activity (%)**			
Assay	**ABTS (µmol TE/g)**	**DPPH (µmol TE/g)**	**FRAP (µmol TE/g)**	**TRC (mg GAE/g)**	**ABTS**	**DPPH**	**FRAP**	**TRC**
Control	523.68 ± 68.46^{g}	19.76 ± 3.40^{f}	208.85 ± 18.21^{bc}	16.95 ± 1.70^{g}	-	-	-	-
1	1446.22 ± 47.04^{ef}	75.15 ± 1.75^{bcd}	192.18 ± 43.22^{c}	30.82 ± 1.08^{e}	176	280	-8	82
2	1744.62 ± 82.41^{ab}	79.28 ± 0.26^{b}	261.95 ± 44.02^{ab}	27.39 ± 0.98^{f}	233	301	25	62
3	1741.78 ± 66.62^{abc}	87.87 ± 4.41^{a}	222.71 ± 23.24^{bc}	25.78 ± 0.40^{f}	233	345	7	52
4	1580.24 ± 111.75^{cde}	69.44 ± 4.71^{cde}	229.79 ± 27.86^{bc}	41.68 ± 0.95^{a}	202	251	10	146
5	1507.89 ± 28.26^{de}	69.73 ± 3.03^{de}	331.64 ± 43.35^{a}	36.92 ± 0.27^{bcd}	188	253	59	118
6	1808.31 ± 62.45^{a}	89.70 ± 2.26^{a}	322.42 ± 4.43^{a}	37.42 ± 0.46^{bcd}	245	354	54	121
7	1664.21 ± 15.62^{abcd}	67.53 ± 2.32^{e}	195.13 ± 18.81^{c}	39.88 ± 0.90^{ab}	218	242	-7	135
8	1270.45 ± 67.41^{f}	72.64 ± 7.43^{bc}	353.54 ± 39.71^{a}	39.66 ± 1.09^{abc}	143	267	69	134
9	1615.82 ± 46.37^{bcde}	65.68 ± 3.03^{e}	317.85 ± 30.52^{a}	36.68 ± 1.00^{cd}	208	232	52	116
10	1559.13 ± 77.93^{de}	67.87 ± 4.20^{e}	320.35 ± 8.70^{a}	37.27 ± 0.80^{bcd}	198	243	53	120
11	1562.93 ± 22.05^{de}	64.60 ± 0.76^{e}	342.81 ± 29.64^{a}	34.65 ± 1.75^{d}	198	227	64	104

The results are presented as the mean (n = 3) ± SD and when followed by different letters in the same column there is a statistically significant difference at the 5% level of significance by Tukey's test.

In general, the best results were achieved using a greater enzyme activity and longer reaction time, although these values did not significantly differ ($p > 0.05$) from other assay conditions with lower enzyme activity and shorter reaction times. This reflected directly on the analysis of effects and the significance of factors on antioxidant activity, in which the enzyme activity and hydrolysis time did not show significant effects ($p > 0.10$) (Table 3).

Table 3. Estimates for the coefficients obtained from the CCRD for determination of the most adequate conditions of enzyme activity and hydrolysis time to obtain PCCV hydrolysates with antioxidant activity.

Response: Antioxidant activity (ABTS) (µmol TE/g)					
Factors	**Coefficients**	**Standard error**	***t*-value (5)**	***p*-value**	R^2
Intersection	1579.29	73.08	21.61	< 0.001	
x_1 (L)	70.22	44.75	1.57	0.184	
x_1 (Q)	55.77	53.26	1.05	0.347	0.65
x_2 (L)	-53.22	44.75	-1.19	0.297	
x_2 (Q)	-39.61	53.26	-0.74	0.494	
$x_1 \times x_2$	-114.96	63.29	-1.82	0.131	
Response: Antioxidant activity (DPPH) (µmol TE/g)					
Factors	**Coefficients**	**Standard error**	***t*-value (5)**	***p*-value**	R^2
Intersection	66.05	4.10	16.09	< 0.001	
x_1 (L)	1.74	2.51	0.69	0.520	
x_1 (Q)	7.59	2.99	2.54	0.052*	0.66
x_2 (L)	1.26	2.51	0.50	0.636	
x_2 (Q)	2.78	2.99	0.93	0.396	
$x_1 \times x_2$	-5.64	3.55	-1.59	0.173	
Response: Antioxidant activity (FRAP) (µmol TE/g)					
Factors	**Coefficients**	**Standard error**	***t*-value (5)**	***p*-value**	R^2
Intersection	327.00	35.32	9.26	< 0.001	
x_1 (L)	7.98	21.63	0.37	0.727	
x_1 (Q)	-18.49	25.74	-0.72	0.505	0.51
x_2 (L)	27.80	21.63	1.28	0.255	
x_2 (Q)	-44.84	25.74	-1.74	0.142	
$x_1 \times x_2$	-15.67	30.59	-0.51	0.630	

Table 3 (continuation). Estimates for the coefficients obtained from the CCRD for determination of the most adequate conditions of enzyme activity and hydrolysis time to obtain PCCV hydrolysates with antioxidant activity.

Response: Antioxidant activity (TRC) (mg GAE/g)					
Factors	**Coefficients**	**Standard Error**	***t*-value (5)**	***p*-value**	R^2
Intersection	36.20	2.96	12.21	< 0.001	
x_1 (L)	1.65	1.81	0.91	0.405	
x_1 (Q)	-1.27	2.16	-0.59	0.581	0.51
x_2 (L)	1.12	1.81	0.62	0.564	
x_2 (Q)	0.02	2.16	0.01	0.992	
$x_1 \times x_2$	4.83	2.57	1.88	0.118	

* *p*-value < 0.10 represent variables that have a statistically significant effect for a 90% confidence level.

Since the estimated effects were not significant ($p > 0.10$), the mathematical models (equations) and their respective contour plots were not generated.

The efficiency in cleavage of proteins in peptide fractions with acidic, basic and aromatic ring residues, or in just exposing their hydrophobic residues, which are largely responsible for the antioxidant activity of hydrolysates, seems to be achieved even in a short reaction time and with low enzyme concentration [24].

Although higher enzyme concentrations and hydrolysis time resulted in a higher degree of hydrolysis, estimated by the result of TCA-soluble protein (Table 4), it did not generate a response correlated with antioxidant activity, except for antioxidant activity by the FRAP method which demonstrated a significant correlation ($p < 0.05$) with the change in TCA-soluble protein (R^2 of 0.75, data not shown).

The FRAP method quantifies the reduction power of the iron ion, Fe^{3+} to Fe^{2+}, a process that occurs in acidic medium (pH 3.6) influencing the loads of amino acid residues, mainly acids and bases, modifying its reduction potential and possibly justifying the correlation with the degree of hydrolysis [24].

Table 4. TCA-soluble proteins of PCCV hydrolysates in each assay from CCRD.

Assay	TCA-soluble protein (%)
1	43.14 ± 0.43^{f}
2	50.97 ± 0.39^{e}
3	54.71 ± 0.91^{d}
4	62.77 ± 0.48^{b}
5	60.41 ± 0.31^{c}
6	64.93 ± 0.13^{a}
7	55.52 ± 0.13^{d}
8	64.93 ± 0.26^{a}
9	61.45 ± 0.23^{bc}
10	60.33 ± 0.46^{c}
11	60.38 ± 0.85^{c}

The results are presented as mean (n = 3) ± SD and when followed by different letters in the same column there is a statistically significant difference at the 5% level of significance by Tukey's test.

TCA-soluble protein is frequently used to quantify the level of proteolysis of hydrolysates as it has a positive correlation with the degree of hydrolysis (DH). This parameter requires special attention, since the size of the peptide is directly related to its biological and functional properties. [25]. However, it is common to observe that in certain studies, increasing the degree of hydrolysis increases antioxidant activity, but in some cases the effect may be reversed. [26,27].

Although the size of the peptide influences its antioxidant activity by leaving free residues that allow the donation of electrons/metal ion reduction, the generation of peptides with amino acids that present these residues is linked to the specificity of the proteases involved in the process [25].

Alcalase® 2.4L from *B. licheniformis*, for example, is an endoprotease with high specificity for the cleavage of aromatic amino acids, while Flavourzyme® 500L from the fungus *A. oryzae* is a mixture of endo and exoproteases with low specificity, thus causing a high degree of hydrolysis in the protein [20]. The mixture of proteases can result in a large number of peptide compositions that may or may not have antioxidant activity, hence the reason for the lack of correlation with the degree of hydrolysis.

Such observation was also carried out by Hall, Johnson and Liceaga [28], where, in the hydrolysis of crickets (*Gryllodes sigillatus*) using Alcalase in the proportion of 3% E:S and with a time of 20 min a degree of hydrolysis of 52% was quantified, while the same process but with a time of 80 min showed a degree of hydrolysis of 85%, even so, no significant difference was observed in the antioxidant activity between the samples to the ABTS method.

Hu et al. [27] also reported results that reinforce the concept that a greater degree of hydrolysis does not necessarily increase the antioxidant activity. The authors observed that peptides from pecan meal hydrolysates with Alcalase in the ratio 1:20 (w:w) for 180 min having molecular weight between 5-10 kDa had lower antioxidant activity, estimated by the ABTS and FRAP methods, when compared to peptides with molecular weight > 10 kDa.

The antioxidant property in hydrolysates from organic waste is a strong ally for the destination of this product as an ingredient in food [27, 28] and in animal feed, since they reduce the effects of the body's excess oxidative reactions [31].

The optimization of process conditions is a tool widely used in studies of enzymatic hydrolysis of proteins, because understanding the effect of variables on the hydrolysis process is the key to maximizing the yield of the product, and enabling its industrial implementation [32].

Similar to this work, Nikhita and Sachindra (2021) [32] performed the enzymatic hydrolysis of chicken blood using the Alcalase enzyme, studying the effect of time, enzyme concentration and temperature, and found that neither time nor concentration of enzyme had a significant effect ($p > 0.05$) on antioxidant activity by DPPH, although the enzyme concentration had a significant effect ($p > 0.05$) on the degree of hydrolysis, temperature was significant in both cases.

Even so, the authors observed an increase in the range of 30% in the inhibition of the DPPH radical between the lowest result (20°C, 30 min and 0.5% w/v of enzyme) and the highest result (60°C, 30 min and 1.5% w/v of enzyme).

However, Bhaskar et al., (2008) [33] verified a different behavior, where, in the hydrolysis of proteins of Catla (*Catla catla*) the effect of the concentration of the commercial enzyme Alcalase (0.5, 1.0 and 1.5 (%)v/w), and time (45, 105 and 165 min), showed a statistically significant positive effect on the response variable degree of hydrolysis ($p < 0.05$).

Based on all these observations, the most adequate conditions selected to perform the following experiments were according to assay 6, 170.5 U/ml enzymes concentration and 120 min of hydrolysis, since on average it presented the best results for antioxidant activity.

3.2. Kinetics of hydrolysis of PCCV

The enzymatic hydrolysis kinetics of PCCV were performed to evaluate the antioxidant activity of the hydrolysates after 30, 60, 90, 120 and 150 min, in addition to verifying the residual protease activity over time.

It was observed that the increase in reaction time and protein hydrolysis estimated by the TCA-soluble proteins did not result in a proportional increase in the antioxidant activity, with the highest values detected after 120 min of hydrolysis for all methods applied, and this condition was selected for the next step. (Table 5).

In the study of the protease activity over time, a high stability of the binary mixture of Alcalase and Flavourzyme was observed, even after 150 min of the process, there was still more than 80% residual activity (Table 5). The inactivation constant value of the enzyme mixture was estimated to be 0.0014 min^{-1}, with a $t_{1/2}$ of 495.10 min and a *D*-value of 1645 min.
Aguilar et al. [34] suggested that the mixture of enzymes results in an increase in the half-life of action of these enzymes, as they form an enzyme complex with greater resistance to thermal inactivation.

Temperature plays a special role in the enzymatic hydrolysis of proteins, on the one hand it contributes to the increase in the catalytic activity of the protease and the partial denaturation of proteins, exposing their hydrophobic residues and increasing the susceptibility to hydrolysis; on the other hand the constant supply of energy in the system can cause the breaking of important bonds in the structure of the enzyme and in its catalytic site, which eventually leads to the loss of enzymatic activity as well as its irreversible denaturation [35–37].

In the case of time, the increase is associated with the increase in the degree of hydrolysis, where at the beginning, the reaction is faster due to the lower competitiveness between the protein and the peptides; however, at a given moment, there will be a low availability of substrate or the enzyme may reach saturation of its catalytic sites [36,38]. These phenomena may have been responsible for maintaining the same degree of hydrolysis (TCA-soluble protein) between 120 and 150 min, as indicated by Tukey's test ($p < 0.05$) (Table 5).

In addition, small peptides and free amino acids can electrostatically interact with the enzyme, destabilizing its structure and causing loss of activity. Or even interacting with each other, forming complexes that reduce the interaction between the residues that promote the donation of electrons with the free radical [38], this may explain the reduction of antioxidant activity in 150 min of hydrolysis.

Table 5. Results of the antioxidant activity for hydrolysates obtained during the kinetics of enzymatic hydrolysis of chicken viscera protein concentrate using a binary mixture of enzymes.

Assay	Antioxidant activity				TCA-soluble protein (%)	Residual protease activity (%)
	ABTS (µmol TE/g)	DPPH (µmol TE/g)	FRAP (µmol TE/g)	TRC (mg GAE/g)		
30 min	1288.89 ± 19.20^{c}	265.92 ± 10.96^{b}	107.81 ± 20.83^{c}	37.39 ± 0.89^{c}	63.85 ± 1.07^{d}	99.97 ± 1.84
60 min	1207.26 ± 8.74^{d}	235.79 ± 6.50^{c}	238.46 ± 9.70^{b}	21.49 ± 1.08^{d}	66.77 ± 0.72^{c}	92.24 ± 0.52
90 min	1145.94 ± 4.36^{e}	307.03 ± 8.89^{a}	251.75 ± 5.15^{b}	21.52 ± 1.51^{d}	70.25 ± 0.57^{b}	87.61 ± 0.66
120 min	1680.98 ± 9.96^{a}	310.34 ± 7.09^{a}	353.15 ± 17.97^{a}	86.16 ± 2.99^{a}	72.62 ± 0.69^{a}	83.49 ± 0.76
150 min	1372.86 ± 22.79^{b}	271.30 ± 16.69^{b}	342.07 ± 16.97^{a}	49.24 ± 1.11^{b}	73.28 ± 0.55^{a}	80.54 ± 0.95

The results are presented as mean (n = 3) ± SD and when followed by different letters in the same column there is a statistically significant difference at the 5% level of significance by Tukey's test.

3.3. Scale-up of the PCCV enzymatic hydrolysis process

One of the major barriers to industrially implementing enzymatic hydrolysis processes is their scaling to large volumes, which may not correspond to the results obtained on a laboratory scale. Therefore, the ideal is that maintaining the process conditions and fluid dynamics, it is possible to obtain the same results regardless of the process volume [23].

Initially, an increase in scale was carried out in Erlenmeyer flasks, starting from 125 mL (working volume of 50 mL), the standard volume of the experiments, to 250 and 500 mL (working volumes of 100 mL and 250 mL, respectively); considering similar geometric characteristics of the glassware, both tests were performed under the same hydrolysis conditions. Following the enzymatic hydrolysis in the bioreactor, 3 L (working volume 1 L) started from the standard condition with agitation at 100 rpm, while the subsequent increase to 6 L (working volume 3 L) ensured the same fluid dynamics in the process. The agitation was set at 60 rpm, according to Eq. 4.

For the scale-up, taking into account the greater geometric modification of the equipment, and respecting the fluid dynamics of the process, the results matched. This made it possible to obtain PCCV hydrolysates with antioxidant properties on a semi-pilot scale similar to those produced in Erlenmeyer flasks (Table 6).

Table 6. Antioxidant activity of PCCV hydrolysates obtained during the scale up study.

Assay	Antioxidant activity			
	ABTS (µmol TE/g)	**DPPH (µmol TE/g)**	**FRAP (µmol TE/g)**	**CRT (mg GAE/g)**
50 mL	964.61 ± 5.24^{ab}	153.62 ± 15.08^{a}	188.08 ± 11.49^{a}	56.06 ± 6.60^{a}
100 mL	963.41 ± 27.54^{ab}	154.79 ± 21.34^{a}	207.05 ± 7.69^{a}	54.21 ± 3.29^{a}
250 mL	972.71 ± 5.68^{a}	158.37 ± 18.67^{a}	206.85 ± 9.39^{a}	55.79 ± 2.07^{a}
1 L	914.49 ± 30.14^{b}	142.03 ± 13.30^{a}	187.48 ± 9.87^{a}	54.22 ± 5.64^{a}
3 L	953.26 ± 22.04^{ab}	153.76 ± 15.09^{a}	207.84 ± 7.09^{a}	53.51 ± 3.32^{a}
Integral material*	936.23 ± 14.57^{ab}	148.29 ± 11.02^{a}	196.42 ± 10.45^{a}	55.77 ± 5.91^{a}

*Integral material, not defatted and hydrolyzed under the same conditions as the defatted protein concentrate and 50 mL volume.

Some authors report behaviors similar to those obtained in the present work, as is the case of Grimaldos and Zapata [39], where in the scale-up in a 1 to 5 L bioreactor of the enzymatic hydrolysis process of bovine plasma using the Alcalase enzyme (60.4°C, E/S 10% w/w, pH 9.0),they obtained the same results of antioxidant activity.

However, not always just maintaining the fluid dynamics of the process can lead to the same results as at the laboratory scale, as seen from the results of Sierra-Lopera and Zapata-Montoya [40] in the scale-up in a 0.5 to 7.5 L bioreactor of the process of enzymatic hydrolysis of red tilapia scales, using the Alcalase enzyme (58.5°C, E/O 0.098 g enzyme/g protein, pH 8.05), it was necessary to increase the agitation from 250 rpm (calculated by eq. 4) to 350 rpm (arbitrary value) to achieve the same antioxidant activity results. Justifying the importance of the study to ensure greater use of the process industrially.

An additional test was carried out to verify whether the removal of lipids from the substrate before hydrolysis would have any effect on the antioxidant properties of the hydrolysates. The results showed that (Table 6) this step is optional as it had no significant effect ($p > 0.05$) on the response of interest. Failure to carry out this step can reduce process costs and increase the nutritional value of the product. However, it should be noted that the presence of lipids may be a factor that reduces the stability of the product during storage, as it is more susceptible to oxidation reactions, which may (or may not) be minimized by the presence of antioxidant peptides.

3.4. Electrophoretic profile of hydrolysates

Samples of PCCV hydrolysates A1 (Erlenmeyer flask containing 100 mL of protein solution) and A2 (3 L of protein solution in bioreactor) showed similar peptide electrophoretic profiles on SDS-PAGE, with two main bands of 14 kDa and 6 kDa being observed (Figure 1). The A3 hydrolysate sample obtained with chicken viscera meal not defatted showed electrophoretic bands of 14 kDa, 6 kDa and 3.5 kDa.

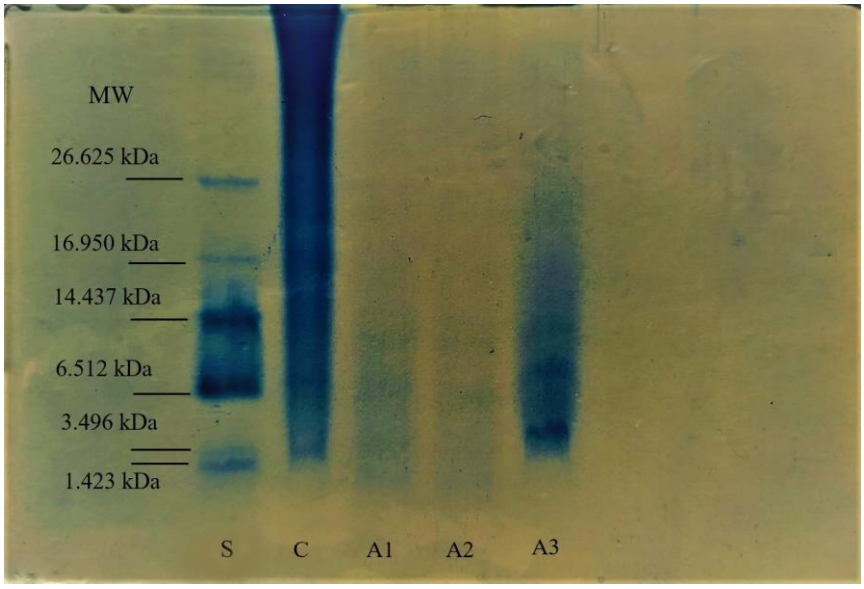

Figure 1. Molecular mass distribution (MW) by SDS-PAGE of the hydrolysates obtained from the protein concentrate of chicken viscera from the peptide standard with MW between 1.4 and 26.6kDa. Where P - Standard; C - Control; A1 – 100 mL volume hydrolysates; A2 – 3 L volume hydrolysates; A3 – Integral material hydrolysates.

It is clear that hydrolysis using the mixture of Alcalase and Flavourzyme enzymes in fact generates a greater amount of peptides with molecular masses between 6 kDa and 14 kDa which corresponds to the same masses of peptides on the 100 mL and 3 L scales. Demonstrating its reproducibility and that even with the PCCV not degreased, the results are the same, making the process cheaper.

4. Conclusion

The use of proteins from chicken viscera as substrates for enzymatic hydrolysis met the proposal to obtain hydrolysates with improved antioxidant properties. The enzyme concentration and hydrolysis time variables in the studied levels from the CCRD design had no significant effects on the antioxidant activity of PCCV hydrolysates. Despite this, an individual analysis of each assay allowed the identification of conditions that resulted in hydrolysates with better antioxidant properties, as follows: 170.5 U/mL as enzyme activity and 120 min as hydrolysis time. Furthermore, this work provided preliminary information for a scale-up of the proposed enzymatic hydrolysis for PCCV and obtaining hydrolysates with antioxidant properties.

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CAPÍTULO III

Chicken viscera meal as substrate for the simultaneous production of antioxidant compounds and proteases by *Aspergillus oryzae*

Revista: Process Biochemistry

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Abstract

Alternative destinations for the use of chicken waste, such as substrates for solid-state fermentation (SSF), can contribute to the development of new processes and obtaining molecules with high added value. For this, an experimental design was applied to evaluate the effect of some process variables including moisture, temperature and inoculum size on the production of antioxidant peptides and proteases by *A. oryzae* IOC3999 through SSF of chicken viscera meal. As a result, it was possible to verify that process conditions strongly influenced protease production and antioxidant activity of the fermented products, with moisture being the factor with the most significant effect ($p < 0.10$). A global analysis of the results indicated that the most adequate conditions for SSF were: 40% initial moisture, 30°C as the incubation temperature, 5.05×10^6 spores/g as the inoculum size and 48 h fermentation as the fermentation time. Under this condition, the antioxidant activities for the ABTS, DPPH and FRAP methods were 376.16, 153.29 and 300.47 (µmol TE/g), respectively, and the protease production reached 428.22 U/g. The proteases were biochemically characterized and showed maximal activity at pH values ranging from 5.0 to 6.0 and a temperature of 50°C. The thermodynamic parameters indicated that the process of thermal protease inactivation is not spontaneous ($\Delta G^*d > 88.78$ kJ/mol), it increases with increasing temperature (ΔH^*d 27.01-26.88 kJ/mol), in addition to presenting an indication of reduced disorder in the system ($\Delta S^*d < -197.74$ kJ/mol), probably related to the agglomeration of partially denatured enzymes.

Keywords: *proteases*, *bioactive peptides*, *solid-state fermentation*, *biochemical characterization*, *enzyme thermodynamics.*

1. Introduction

During the production of chicken meat, it is estimated that 40-60% of liquid and solid waste is generated in relation to the animal's weight [1]. Among solid wastes, chicken viscera represent a significant fraction, which is usually reused in the form of flour for animal feed or destined for composting [2]. However, such destinations are unable to fully reuse the large amount of waste produced [3,4].

In view of this, there are several opportunities for the application of agro-industrial wastes as substrates for the development of new processes. The chicken viscera, for example, are an excellent option for this purpose, since its composition rich in macronutrients, especially proteins, can be exploited in different types of applications, such as ingredients for animal feed [5], obtaining bioactive peptides [6] or as substrates for fermentation [7]. Among the most used technologies, is Solid-State Fermentation (SSF), has been suggested as a potential waste recycling process in which microorganisms convert solid wastes as substrates into value-added products [8–10].

In this fermentation process, the addition of water to the medium is sufficient to keep the substrate solid with the water adsorbed on its surface, unlike submerged fermentation, which take place in an aqueous medium. For this reason, SSF is considered an environmentally friendly method, due to the lower generation of effluents and the use of the substrate in an integral way, in addition to limiting the contamination of most bacteria [9].

Filamentous fungi, such as the genus *Aspergillus* spp., are the class of microorganisms that best make use of SSF, as they are able to grow radially across the surface of the substrate in low moisture conditions, in addition to their rapid growth and the presence of a wide variety of genes that produce different biomolecules [11,12]. The production of specific biomolecules, such as enzymes, for example, can be induced by the substrate composition and process conditions such as temperature, pH, moisture, and C:N ratio, among other factors [13]. Thus, several studies have been carried out on the production of different enzymes [14–16], organic acids [17,18], pigments [19], and other biomolecules [20,21] by different strains of *Aspergillus* spp. through SSF.

The production of proteases under SSF is also one of the most studied technologies, since proteases have great commercial relevance, accounting for approximately 60% of the world enzyme market [22]. This class of enzymes is widely used in the enzymatic hydrolysis of proteins to produce hydrolysates with better technofunctional properties and containing bioactive peptides [23]. When the production of proteolytic enzymes on a protein substrate

occurs, the bioactive peptides are naturally released during the growth of the microorganism [24–26]. Peptides that exert biological functions are known as bioactive peptides, and among the various functions they can exert, antioxidant activity stands out, since these peptides are able to perform electron donation and metal reduction ions [27].

In this context, the main objective of this work was to use chicken viscera meal as a substrate for the simultaneous production of proteases and antioxidant compounds under SSF by *Aspergillus oryzae* IOC 3999. Additionally, studies on the biochemical properties of the proteases produced were performed.

2. Materials and methods

2.1. Reagents

The reagents azocasein, trichloroacetic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, bovine serum albumin and Folin-Ciocalteu reagent were purchased from Sigma Aldrich (Steinheim, Germany). The other reagents (salts and solvents) used were of analytical grade.

2.2. Determination of protease Activity

The determination of protease activity was performed according to the method proposed by Charney and Tomarelli [28] adapted by Castro and Sato [29]. The reaction was carried out in 2 mL microtubes containing 0.5 mL of enzyme solution and 0.5 mL of azocasein substrate (0.5% m/v) prepared in sodium phosphate buffer (100 mmol/L, pH 7.0), and incubated at 50ºC for 40 min. The reaction was stopped by adding 0.5 mL of trichloroacetic acid (10% m/v). The reaction blank was carried out similarly to the enzymatic reactions, however the paralyzing agent was added before the enzymatic solution. Then, the tubes were centrifuged at 17,000 *g* for 15 min at 25ºC. The colorimetric reaction occurred by mixing a 1.0 mL aliquot of the supernatant of the reaction mixture with 1.0 mL of the neutralizing agent KOH (5 mol/L). One unit of enzyme activity (U) was defined as the amount of enzyme required to increase the absorbance by 0.01 at 428 nm in relation to the blank.

2.3. Substrate

The chicken viscera meal was kindly provided by the company Ad'oro S/A (São Paulo, Brazil). According to the manufacturer's information, the flour prepared from chicken viscera

had the following composition: 1.94% moisture, 6.33% carbohydrates, 9.64% ether extract, 17.15% ash and 64.94% proteins.

2.4. Microorganism and pre-inoculum

The strain used in this study was *Aspergillus oryzae* IOC 3999 from Oswaldo Cruz Foundation – Fiocruz (Rio de Janeiro, Brazil), and maintained in the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, University of Campinas. The strain was periodically subcultured and maintained on potato dextrose agar slants. To produce the pre-inoculum, the microorganism was inoculated into a medium composed of 10 g wheat bran and 5 mL of solution containing 1.7% (w/v) $NaHPO_4$ and 2.0% (w/v) $(NH_4)_2SO_4$ and incubated at 30°C for 3-4 days. To prepare the inoculum for fermentation, the fungal spores were dispersed into sterile Tween 80 solution (0.3%) and the number of spores per milliliter in the suspension was determined using a Neubauer cell counting chamber [30].

2.5. Solid-State Fermentation (SSF) for protease production and obtaining the crude enzymatic extract

Protease and antioxidant compound production were studied under SSF using chicken viscera meal as a substrate in 250 mL Erlenmeyer flasks containing 20 g medium previously sterilized at 121°C for 15 min. The effects of the cultivation parameters were investigated using a central composite rotatable design (CCRD) in which the independent variables initial moisture of the culture medium, incubation temperature and amount of inoculum were evaluated at different levels and combinations (Table 1). The protease and antioxidant activities were tested at 24 h and 48 h of fermentation. The extracts were obtained by the addition of 50 mL distilled water followed by maceration, maintenance at rest for 1 h and filtration through gauze tissue. The solution free of any solid material was used as crude extract for the determinations of protease and antioxidant activities.

Table 1. The CCRD matrix used to verify the effects of independent variables (initial moisture, temperature of incubation and inoculum) on the production of antioxidant molecules and protease by *A. oryzae* IOC 3999 using chicken viscera meal as a solid substrate at 24 and 48h fermentation.

Runs	Independent variables		
	X_1	X_2	X_3
	Initial Moisture (%)	**Temperature (°C)**	**Amount of inoculum (spores/g)**
1	-1.00 (44.05)	-1.00 (27)	-1.00 (2.00×10^6)
2	1.00 (55.95)	-1.00 (27)	-1.00 (2.00×10^6)
3	-1.00 (44.05)	1.00 (33)	-1.00 (2.00×10^6)
4	1.00 (55.95)	1.00 (33)	-1.00 (2.00×10^6)
5	-1.00 (44.05)	-1.00 (27)	1.00 (8.00×10^6)
6	1.00 (55.95)	-1.00 (27)	1.00 (8.00×10^6)
7	-1.00 (44.05)	1.00 (33)	1.00 (8.00×10^6)
8	1.00 (55.95)	1.00 (33)	1.00 (8.00×10^6)
9	-1.68 (40.00)	0.00 (30)	0.00 (5.05×10^6)
10	1.68 (60.00)	0.00 (30)	0.00 (5.05×10^6)
11	0.00 (50.00)	-1.68 (25)	0.00 (5.05×10^6)
12	0.00 (50.00)	1.68 (35)	0.00 (5.05×10^6)
13	0.00 (50.00)	0.00 (30)	-1.68 (1.00×10^5)
14	0.00 (50.00)	0.00 (30)	1.68 (1.00×10^7)
15	0.00 (50.00)	0.00 (30)	0.00 (5.05×10^6)
16	0.00 (50.00)	0.00 (30)	0.00 (5.05×10^6)
17	0.00 (50.00)	0.00 (30)	0.00 (5.05×10^6)

The experiments were randomized to maximize the variability in the observed responses caused by extraneous factors. A second-order model equation was used for this model, represented by Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \qquad (1)$$

where Y represents the response estimated by the model, i and j correspond to the variation of 1 to the number of variables (n), β_0 is the mean, β_i and β_{ij} represent the linear and quadratic coefficients, respectively and x_i and x_j are the coded independent variables. The multiple determination coefficient (R^2) and Fisher's test (analysis of variance-ANOVA) were used to verify the statistical adequacy of the proposed models using the software Statistica version 13.3 from TIBCO Software Inc (Palo Alto, California, USA).

2.6. Determination of antioxidant properties

2.6.1. ABTS radical inhibition

The antioxidant activity measured by the ABTS method was performed on a microplate according to the methodology proposed by Al-Duais et al. [31]. The working solution was prepared at the time of analysis and in the absence of light, consisting of the dilution of the concentrated ABTS radical solution (prepared at least 16 h in advance), with absorbance at 734 nm adjusted to 0.70 ± 0.02. In each well of the microplate, 20 µL of crude extract or standard (Trolox) was added (0 to 400 µmol/L), and 220 µL of the ABTS radical solution was added. After 6 min in the absence of light, the absorbance of the reaction mixtures was measured at 734 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland). The antioxidant activity was expressed in µmol of Trolox equivalents per g of protein in the crude extract (µmol TE/g).

2.6.2. DPPH radical inhibition

The antioxidant activity determined by DPPH free radical scavenging in the microplate was performed following the methodology described by Al-Duais et al. [31]. The analysis consisted of adding 66 µL of the crude extract, Trolox (0-125 µmol/L), and 134 µL of ethanolic DPPH solution (150 µmol/L) to each well of the microplate. After 45 min in the dark, the absorbance was measured at 517 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland), and the antioxidant activity was expressed in µmol TE/g.

2.6.3. Ferric Reducing Antioxidant Power – FRAP

The reducing power of iron was evaluated according to the methodology proposed by Benzie and Strain [32] with modifications proposed by Matos, Novelli and De Castro [33]. The FRAP reagent was prepared by mixing 2.5 mL of the TPTZ solution (10 mmol/L in HCl), 25 mL of phosphate buffer (0.3 mol/L, pH 3.6) and 2.5 mL of $FeCl_3 \cdot 6H_2O$ (20 mmol/L). Then, 25 µL aliquots of the crude extract, blank (distilled water) or Trolox (5-150 µmol/L) were added to 175 µL of FRAP reagent. The reaction mixture was incubated in a microplate reader (Multiskan GO, Thermo Fisher Scientific, Finland) at 37°C for 30 min. Absorbance was monitored at 593 nm, and the results were expressed in µmol TE/g.

2.7. Effects of pH and temperature on the activity and stability of the protease determined using an experimental design

The optimum values of pH and temperature for activity and stability were determined using a CCRD with three replicates at the central point and four axial points (a total of 11 runs) (Table 4). Stability assays were carried out by incubating the enzyme solutions for 1 h at different pH and temperature conditions, followed by determination of residual protease activity at optimal conditions previously determined. Final results were expressed as U/g for optimal activity and as a percentage for stability.

The statistical analysis was performed in the same way described in topic 2.6 using the software Statistica version 13.3 from TIBCO Software Inc (Palo Alto, California, USA).

2.8. Kinetic and thermodynamic parameters of enzymatic inactivation of the protease produced by *A. oryzae* IOC 3999

The kinetic and thermodynamic parameters of enzyme inactivation were investigated as described by De Castro et al. [34] following the described first order kinetic equations:

$$\ln\Psi = -k_d t \quad (2)$$

where Ψ is the residual enzyme activity coefficient, defined as the ratio of the enzyme concentration (E) at a given time, in relation to the initial concentration of exposure at a given temperature ($\Psi = E/E_0$), k_d is the thermal inactivation constant and t is time.

The k_d was estimated at different temperatures (40-55°C) from the slopes of the lines obtained by plotting the experimental data of $\ln\Psi$ over time.

Knowing that the inactivation constant depends on temperature according to the Arrhenius equation:

$$k_d = Ae^{-E^*d/RT} \quad (3)$$

The $\ln k_d$ values were plotted against 1/T (T – temperature) and the activation energy of irreversible enzymatic inactivation (denaturation) (E^*_d) was estimated from the slope of the resulting line.

From E^*_d, the other thermodynamic parameters enthalpy of denaturation (ΔH^*_d), Gibbs free energy (ΔG^*_d) and entropy (ΔS^*_d) were calculated according to the following equations:

$$\Delta H^*_d = E^*_d - RT \quad (4)$$

$$\Delta G^*_d = -RT\ln(k_d h/k_b T) \quad (5)$$

$$\Delta S^*_d = (\Delta H^*_d - \Delta G^*_d)/T \quad (6)$$

where h is the Planck constant (6.626×10^{-34} Js) and k_b is the Boltzmann constant (1.381×10^{-23} J/K).

The half-life of proteases, defined as the time required for enzymes to reduce their initial activity by 50%, was still calculated and estimated as shown below:

$$t_{1/2} = \ln (0.5)/k_d \quad (7).$$

The decimal reduction time (D value), defined as the time required to reduce the initial enzyme activity by 90% at a specific temperature, was calculated as described below:

$$D = 2.303/k_d \quad (8).$$

2.9. Statistical analysis

The results were statistically analyzed by Analysis of Variance (ANOVA) followed by Tukey's test using the software Minitab 19 from Minitab LLC (State College, Pennsylvania, USA). Values were expressed as the arithmetic mean (n = 3) and considered statistically different when *p* values were less than 0.05.

3. Results and discussion

3.1. Optimization of the production of proteases and antioxidant peptides

The results for the antioxidant activity and protease production detected for the extracts obtained after fermentation of chicken viscera meal by *A. oryzae* IOC3999 at 24 and 48 h for each assay of the experimental design are shown in Table 2. The highest values obtained for the antioxidant activity in each method were: run 6 for ABTS (401.08 µmol TE/g) at 48 h, run 8 for DPPH (208.69 µmol TE/g) radical scavenging at 24 h and assay 9 for FRAP (300.47 µmol TE/g), which led to increases of 119, 195, and 231%, respectively, compared to the control samples. The highest protease production was detected at 48 h fermentation in run 1 and reached 501.39 U/g (Table 2).

Table 2. Results for antioxidant activity and protease production after 24 and 48h fermentation as a function of the different culture conditions of *A. oryzae* IOC 3999 in chicken viscera meal using CCRD.

	Antioxidant activity (µmol TE/g)						Protease activity (U/g)	
	ABTS		DPPH		FRAP			
Control	183.09 ± 7.41		70.74 ± 3.01		90.60 ± 2.58		*Not detected*	
Runs	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**	**24h**	**48h**
1	254.50 ± 4.80^{f}	349.51 ± 3.16^{cd}	166.33 ± 1.80^{efgh}	157.92 ± 2.73^{a}	142.69 ± 1.71^{hi}	295.51 ± 9.13^{a}	66.55 ± 2.21^{f}	501.39 ± 7.97^{a}
2	240.26 ± 7.39^{fgh}	203.08 ± 2.74^{j}	162.95 ± 1.77^{fgh}	104.30 ± 2.44^{e}	185.48 ± 1.44^{cd}	196.90 ± 11.89^{b}	26.02 ± 2.24^{h}	244.48 ± 5.16^{h}
3	304.66 ± 6.14^{bcd}	401.08 ± 3.34^{a}	184.79 ± 6.19^{bc}	104.01 ± 4.05^{e}	166.01 ± 3.06^{f}	163.82 ± 3.91^{d}	104.27 ± 1.91^{b}	456.55 ± 14.26^{b}
4	247.87 ± 8.65^{fg}	242.40 ± 3.77^{i}	157.40 ± 6.47^{gh}	73.40 ± 2.31^{i}	143.40 ± 2.13^{hi}	80.27 ± 5.34^{gh}	51.23 ± 1.52^{g}	200.56 ± 3.92^{i}
5	301.94 ± 6.27^{bcd}	324.89 ± 3.99^{f}	190.97 ± 4.63^{b}	129.19 ± 1.92^{d}	282.69 ± 4.04^{b}	194.89 ± 5.48^{b}	98.78 ± 5.37^{bc}	254.77 ± 4.82^{h}
6	285.27 ± 7.57^{e}	255.91 ± 4.88^{h}	155.98 ± 5.66^{h}	59.20 ± 4.48^{j}	135.97 ± 4.12^{ij}	63.04 ± 4.77^{i}	71.69 ± 1.24^{f}	179.87 ± 5.07^{i}
7	313.09 ± 5.15^{b}	343.90 ± 4.77^{de}	120.47 ± 1.42^{i}	98.24 ± 2.86^{ef}	139.43 ± 2.90^{i}	90.90 ± 2.87^{g}	112.82 ± 2.97^{a}	191.88 ± 7.21^{i}
8	308.80 ± 6.77^{bc}	369.85 ± 3.70^{b}	208.69 ± 6.18^{a}	98.51 ± 6.48^{ef}	184.40 ± 3.65^{cd}	108.71 ± 6.20^{f}	52.68 ± 2.34^{g}	63.18 ± 8.08^{l}
9	334.99 ± 2.24^{a}	376.16 ± 3.34^{b}	192.71 ± 4.74^{b}	153.29 ± 2.07^{ab}	291.54 ± 4.93^{a}	300.47 ± 7.79^{a}	97.68 ± 2.69^{bc}	428.22 ± 6.59^{c}
10	252.96 ± 1.10^{f}	261.89 ± 2.76^{h}	156.07 ± 3.65^{h}	81.99 ± 3.66^{h}	136.38 ± 2.63^{i}	70.00 ± 2.39^{hi}	22.16 ± 0.23^{h}	106.94 ± 6.27^{k}
11	235.03 ± 4.64^{gh}	239.48 ± 1.49^{i}	167.97 ± 4.69^{defg}	89.73 ± 0.59^{g}	142.84 ± 1.23^{hi}	60.59 ± 3.06^{i}	53.95 ± 0.46^{g}	230.51 ± 8.94^{h}
12	306.26 ± 4.83^{bc}	294.05 ± 3.00^{g}	178.50 ± 1.81^{cd}	98.27 ± 1.77^{ef}	191.49 ± 3.70^{c}	145.06 ± 5.00^{e}	101.54 ± 3.78^{b}	357.86 ± 12.72^{de}
13	227.36 ± 4.22^{h}	159.08 ± 7.60^{k}	166.01 ± 0.93^{fgh}	93.55 ± 2.01^{fg}	148.99 ± 3.86^{gh}	57.80 ± 1.95^{i}	5.30 ± 0.17^{i}	134.10 ± 0.90^{j}
14	288.12 ± 8.22^{de}	377.68 ± 0.78^{b}	156.99 ± 3.79^{gh}	93.34 ± 2.11^{fg}	127.69 ± 1.66^{j}	121.70 ± 6.45^{f}	88.29 ± 1.62^{de}	290.68 ± 6.42^{g}
15	314.13 ± 3.58^{b}	357.24 ± 6.57^{c}	172.42 ± 5.39^{def}	149.90 ± 1.27^{b}	178.72 ± 1.21^{de}	182.65 ± 2.38^{bc}	93.47 ± 3.57^{cd}	340.01 ± 21.19^{ef}
16	302.44 ± 12.78^{bcd}	336.78 ± 2.27^{e}	177.31 ± 4.49^{cde}	154.34 ± 2.07^{ab}	155.41 ± 5.59^{g}	176.90 ± 3.05^{cd}	84.90 ± 2.61^{e}	330.63 ± 7.05^{f}
17	294.95 ± 6.24^{cde}	334.16 ± 5.64^{ef}	167.75 ± 3.00^{defg}	139.38 ± 2.56^{c}	173.08 ± 2.74^{ef}	190.63 ± 3.93^{bc}	85.31 ± 1.39^{e}	372.98 ± 0.91^{d}

The results are presented as mean (n = 3) ± SD and when followed by different letters in the same column there is a statistically significant difference at the 5% level of significance by Tukey's test

Most of the mathematical models for the extracts obtained at 24 h fermentation showed R^2 greater than 0.80, values of F-calculated higher than F-tabulated with statistical significance ($p \leq 0.10$) according to ANOVA (Table 3). On the other hand, the variation of responses as a function of the significance of the independent variables for the extracts obtained in 48 h of fermentation resulted in nonsignificant models ($p > 0.10$), with the exception of the antioxidant activity measured by the DPPH method. For responses in which the statistical parameters were not satisfactory, the mathematical models (equations) were not generated.

Table 3. Analysis of variance (ANOVA) including models, R^2 and probability values for the final reduced models for antioxidant and protease activities of the crude extract obtained after fermentation of chicken viscera meal by *A. oryzae* IOC3999 at 24 and 48 h.

Response: antioxidant activity ABTS at 24 h fermentation						
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-calculated/ *F*tabulated	R^2	*p*-value
Regression	15660.07	5	3132.01	6.23	0.87	0.0001
Residual	2256.52	11	205.14			
Total	17916.59	16				
Regression model: $Y = 301.52 - 16.85X_1 + 15.52X_2 + 19.35X_3 - 9.19(X_2)^2 - 13.75(X_3)^2$						
Response: antioxidant activity DPPH at 48 h fermentation						
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-calculated/ *F*tabulated	R^2	*p*-value
Regression	16215.98	6	2702.66	6.73	0.89	0.0001
Residual	1630.6	10	163.06			
Total	17846.58	16				
Regression model: $Y = 147.60 - 20.05X_1 + 11.66X_1X_2 + 11.65X_2X_3 - 9.76(X_1)^2 - 18.11(X_2)^2 - 18.31(X_3)^2$						
Response: Protease production at 24 h fermentation						
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-calculated/ *F*tabulated	R^2	*p*-value
Regression	13390.76	4	3347.69	5.70	0.83	0.0002
Residual	2840.14	12	236.68			
Total	16230.90	16				
Regression model: $Y = 79.50 - 22.54X_1 + 10.10X_2 + 16.66X_3 - 9.87(X_3)^2$						

The variations in antioxidant and protease activities of the extracts were also depicted using contour plots (Figure 1). In general, the interpretation of contour plots confirmed the significant effects of independent variables on the assessed responses, in which, lower levels of initial moisture of the culture medium and incubation temperatures and amount of inoculum in the central point region favored the production of antioxidant compounds and proteases by *A. oryzae* IOC 3999 during the fermentation process.

The moisture initial of the medium in SSF is one of the main variables to be considered, since the objective of the method is that the water present in the medium is enough to carry out the diffusion of nutrients, absorption by the microorganism and maintain the stability and the function of biological components but does not result in excessive amounts. High levels of initial humidity can cause filling of empty spaces, compaction and consequently limitation of the air passage of [13].

The statistically significant ($p \leq 0.05$) and negative effect of initial moisture was also observed by Amaral et al., [35] in the production of proteases by *A. tamarii* Kita UCP1279 using soybean meal with an initial moisture content ranging from 30 to 50%. On the other hand, Benabda et al., [36] found higher protease production by *Rhizopus oryzae* at higher percentages of initial moisture when it was cultivated in bread bran.

In relation to temperature, the increase in this parameter means an increase in free energy in the system, which contributes to the acceleration of metabolic reactions, which justifies the positive effect of this variable. Even so, the exaggerated increase in this variable can generate heat sources that can compromise the growth and development of the microorganism [10]. This behavior was observed by Balachandran et al., [37] during SSF of wheat bran by *Bacillus halodurans*, in which protease production was impaired at temperatures above 40°C.

It is expected that the greater the amount of inoculum, the faster the microbial growth will occur, and in fact this occurs up to a certain limit. However, a very high initial inoculum can result in early nutrient depletion without adequate yield for enzyme production [38].

The antioxidant activity of the fermented extract produced under SSF of chicken viscera meal by *A. oryzae* IOC3999 is probably mostly provided by the formation of bioactive peptides, given the rich protein composition of the substrate and high production of proteases. However, other secondary metabolites generated during the growth of the microorganism may contribute to this activity.

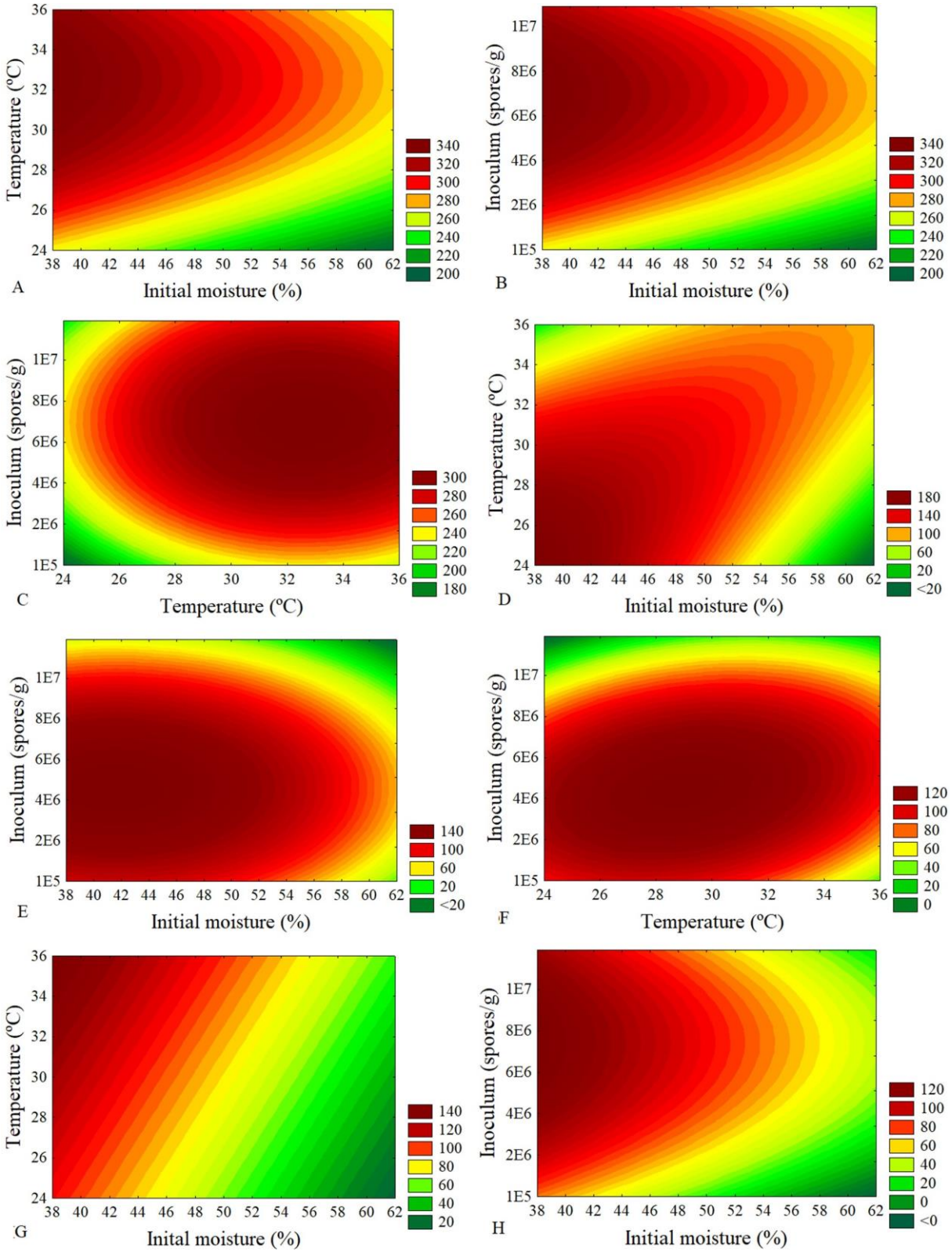

Figure 1. Contour plots for antioxidant activity ABTS 24h fermentation (A)(B)(C), DPPH 48h fermentation (D)(E)(F) and protease production 24h fermentation (G)(H) in SSF by *A. oryzae* IOC 3999 using chicken viscera meal as a solid substrate as a function of the independent variables (initial moisture, temperature and amount of inoculum) used in the CCRD.

During microbial growth, a series of secondary metabolites can be produced, such as organic acids, pigments and bioactive compounds that can have antioxidant action. In addition to compounds conjugated with sugars, fatty acids and proteins are released during fermentation by the action of carbohydrases, lipases and proteases [39,40].

Punia et al., [41] demonstrated that after the fourth day of rice flour fermentation by *A. oryzae,* there was a considerable increase in the concentration of gallic acid and ascorbic acid produced by the fungus, which consequently increased the antioxidant activity of the fermented extract.

In another case, Yin et al., [42] observed a significant increase ($p < 0.05$) in the total content of phenolics with high antioxidant activity after fermentation of wheat bran using *A. niger, A. oryzae* and *A. awamori*. This increase was associated to release of ferulic, chlorogenic and syringic acids by the action of *A. niger*, *A. oryzae* and *A. awamori*. According to the authors, these acids were esterified or covalently linked to the polysaccharides present in wheat, and they were released by the action of carbohydrases produced by these microorganisms.

Based on these comments, from a global analysis of the results, it was possible to select the most adequate conditions for SSF that offered a balance between the responses favoring the production of the compounds of interest, as follows: 40% initial moisture, 30°C as the incubation temperature, 5.05×10^6 spores/g as the inoculum size at 48 h as the fermentation time. Under these conditions, the antioxidant activities for the ABTS, DPPH and FRAP methods were 376.16, 153.29 and 300.47 (µmol TE/g), respectively, and the protease production reached 428.22 U/g.

3.2. Biochemical characterization of the protease produced by *A. oryzae* IOC 3999 under SSF

Biochemically characterizing enzymes provides crucial information for their destination in industrial processes. Temperature and pH are two of the most important operational conditions for enzymatic action, as they directly influence the conformation of the catalytic site of the enzyme, leading to an increase or reduction in the reaction rate and even causing irreversible denaturation of the enzyme [43].

The results of the CCRD experimental design in relation to the study of the effect of pH and temperature on the optimum activity and stability of the protease produced by *A. oryzae* IOC 3999 under solid state fermentation, using chicken viscera meal as a substrate, are shown in Table 4.

Table 4. CCRD matrix used to verify the effects of pH and temperature (°C) on the activity and stability of the protease produced by *A. oryzae* IOC 3999 under solid-state fermentation of chicken viscera meal.

CCRD			Protease activity	
Run	Independent variables		Optimum (U/g)	Stability (%)
	X_1	X_2		
	pH	Temperature (°C)		
1	-1.00 (3.73)	-1.00 (39.64)	52.38 ± 6.47^{g}	38.57 ± 1.23^{b}
2	1.00 (7.27)	-1.00 (39.64)	453.19 ± 8.76^{b}	97.30 ± 1.06^{a}
3	-1.00 (3.73)	1.00 (60.64)	36.44 ± 3.94^{gh}	6.65 ± 0.70^{f}
4	1.00 (7.27)	1.00 (60.64)	104.19 ± 4.52^{e}	7.38 ± 0.70^{f}
5	-1.41 (3.00)	0.00 (50.00)	19.36 ± 3.94^{h}	12.89 ± 0.70^{e}
6	1.41 (8.00)	0.00 (50.00)	144.61 ± 3.94^{d}	17.05 ± 1.96^{d}
7	0.00 (5.50)	-1.41 (35.00)	410.49 ± 4.93^{c}	100.00 ± 0.53^{a}
8	0.00 (5.50)	1.41 (65.00)	80.85 ± 3.56^{f}	2.18 ± 0.70^{g}
9	0.00 (5.50)	0.00 (50.00)	601.79 ± 6.90^{a}	28.59 ± 0.93^{c}
10	0.00 (5.50)	0.00 (50.00)	602.92 ± 8.88^{a}	29.11 ± 1.15^{c}
11	0.00 (5.50)	0.00 (50.00)	601.79 ± 6.47^{a}	29.63 ± 0.70^{c}

The results are presented as mean (n = 3) ± SD and when followed by different letters in the same column there is a statistically significant difference at the 5% level of significance by Tukey's test.

For the optimum reaction activity, the central point condition (pH 5.5 and 50°C) showed the highest values of protease activity, around 600 U/g. In addition, there was no statistically significant difference ($p < 0.05$) between these values, indicating good repeatability of the analysis.

Regarding to the stability of the protease under different pH and temperature conditions, the behavior was different from its optimum condition. Although, the protease showed maximum performance at 50°C, its stability was not as high under this condition. Compared to the run in which the proteases were more stable (run 7 - pH 5.5 and 35°C), the central point condition (runs 9 -11, pH 5.5 and 50°C) resulted in retention of around 28 - 29% of its relative activity after 1 h of incubation.

The statistical treatment of the data showed that the multiple determination coefficients obtained (R^2) were 0.98 for the response optimum activity and 0.90 for the stability, indicating models that were highly explanatory of the responses obtained. Additionally, ANOVA (Table 5) resulted in values of F calculated higher than F tabulated, indicative of the good adjustment and predictive capacity of the coded models.

Table 5. Analysis of variance (ANOVA) including models, R^2 and probability values for the final reduced models for the activity and stability of the protease produced by *A. oryzae* IOC 3999 using chicken viscera meal as a solid substrate.

Response: Protease activity - Optimal						
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-calculated/ *F*tabulated	R^2	*p*-value
Regression	730443.40	5	146088.68	12.15	0.98	0.0007
Residual	11908.90	5	2381.78			
Total	742352.30	10				
Regression model: $Y = 602.17 + 161.42X_1 - 207.78X_2 - 166.53X_1X_2 - 521.32(X_1)^2 - 357.63(X_2)^2$						
Response: Protease activity - Stability						
Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*-calculated/ *F*tabulated	R^2	*p*-value
Regression	10488.59	3	3496.20	2.77	0.90	0.0002
Residual	1167.02	7	233.40			
Total	11655.61	10				
Regression model: $Y = 23.50 - 65.04X_2 - 29.00X_1X_2 + 27.71(X_2)^2$						

The variations in protease activity and stability were also depicted using contour plots (Figure 2). The interpretation of contour plots confirmed the significant effects of independent variables on the assessed responses, in which, pH values ranging from 5.0 to 6.0 and temperatures between 45 and 55°C resulted in maximum protease activity (Figure 2A), while the lower temperatures preserved more enzymatic activity in the stability study when the pH values were close to neutrality (Figure 2B).

Similar values were observed by Da Silva et al. [44] with the protease produced by *A. avenaceus* URM6706 that showed maximum activity at 50°C and pH at 7.0. In contrast, Fernandes et al. [45] also observed an optimal temperature at 50°C, but with an optimal pH more alkaline (8.0) for the protease produced by *A. heteromorphus* URM0269.

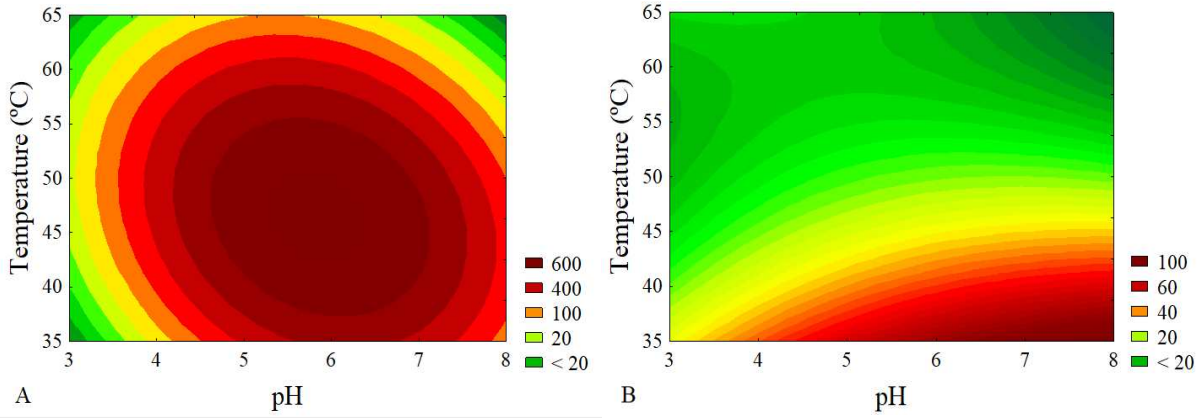

Figure 2. Contour plots for the optimum activity (A) and stability (B) of the protease produced by *A. oryzae* IOC 3999 using chicken viscera meal as solid substrate as function of the independent variables (pH and temperature) used in the CCRD.

Kaewsalud et al., [46] conducted a study on the production of keratinase by *Bacillus halodurans* SW-X using chicken feather residues as substrate and observed that although the enzyme showed optimal activity at 70°C, after 1 h incubation at this temperature, the residual enzymatic activity was below 10%, similar to that observed in our study .

This behavior can be expected, since the increase in temperature exerts two phenomena simultaneously on the enzymatic activity: i) the increase in temperature increases the free energy in the system, decreasing the minimum energy necessary for the reaction to occur, that is, it increases the reaction rate and ii) the increase in temperature promotes protein denaturation, which may be irreversible [47].

3.3. Kinetic and thermodynamic parameters for protease inactivation

One of the most limitations for the use of enzymes in industry is the lack of knowledge about their inactivation during the process [48]. In this sense, knowledge of the kinetic and thermodynamic parameters of the decay of enzymatic activity can provide more precise information for the development of equipment and processes that maximize the efficiency of their use [44].

The protease produced by *A. oryzae* IOC 3999 under SSF using chicken viscera meal followed first-order Arrhenius kinetics with satisfactory correlation for the inactivation constant (k_d) ($0.95 \leq R^2 \leq 0.99$) at temperature ranging from 40 to 55 °C from the slopes of the straight lines of semi-log plots of $\ln\psi$ vs. Time (min) (Figure 3).

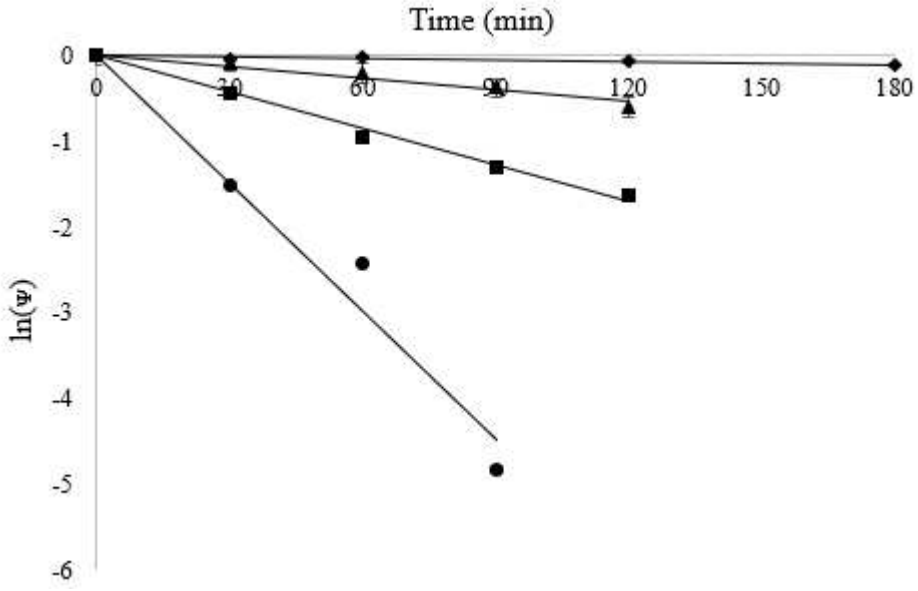

Figure 3. Semi-log plots of thermal denaturation of protease from *A. oryzae* as a function of different temperatures: 40 (◆), 45 (▲), 50 (■) and 55 (●).

From the inactivation constant, it is possible to calculate the $t_{1/2}$ and the D-value for the protease enzymatic kinetics. The half-life time ($t_{1/2}$) is the time at which the enzyme loses half of its initial enzymatic activity, while the D-value is the time at which it loses 90% of its initial activity. Therefore, higher values of these parameters are desirable for industrial applications, as they indicate greater thermal resistance of the enzyme [49].

The inactivation constant increased exponentially with increasing temperature, while the $t_{1/2}$ and D-values decreased proportionally. At 40°C, the protease had a $t_{1/2}$ of 1155.24 min and a D-value of 3838.33 min, while at 55°C, the $t_{1/2}$ was 13.95 min and the D-value was 46.34 min, which represented a reduction of more than 80 times when the different temperatures were compared (Table 6).

Table 6. Kinetic and thermodynamic parameters of the irreversible thermal inactivation of protease from *A. oryzae* IOC 3999 at different temperatures.

Kinetic parameters				
Temperature (°C)	**k_d (min^{-1})**	**$t_{1/2}$ (min)**	**D-value (min)**	**R^2**
40	0.0006	1155.24	3838.33	0.95
45	0.0045	154.03	511.78	0.98
50	0.0142	48.81	162.18	0.99
55	0.0497	13.95	46.34	0.99
Thermodynamic parameters				
Temperature (°C)	**ΔH^*_d (kJ/mol)**	**ΔG^*_d (kJ/mol)**		**ΔS^*_d (J/molK)**
40	27.01	96.09		-220.70
45	26.97	92.34		-208.85
50	26.92	90.74		-203.90
55	26.88	88.78		-197.74

This means that the thermal denaturation of the protease becomes more pronounced with increasing temperature, because the high vibration of the molecules at these temperatures promotes the breaking of strong electrostatic bonds, such as hydrogen bonds that stabilize the enzyme [48].

In other works, the protease produced by *A. avenaceus* URM6706 in SmF of soybean flour presented $t_{1/2}$ and D-values ranging between 277.2-19.97 min and 921.2-66.36 min, respectively, in temperatures ranging from 50 to 70°C [44]. According to the studies of De Castro et al., [34], the protease produced by *A. niger* LBA02 in soybean meal under SSF showed $t_{1/2}$ between 1386.29-14.97 min, and D-values ranging from 4605.20 to 49.73 min in a temperature range between 40 and 60°C, similar to those observed in our work.

The thermal denaturation energy (E^*_d) of *A. oryzae* IOC3999 protease was also calculated from the slope coefficient of the graph line $\ln(k_d)$ vs 1/T for each temperature (40-55°C) (Figure 4) and the value was 29.61 kJ/ mol ($R^2 = 0.98$). Once the energy barrier to thermal inactivation is overcome, the enzyme is denatured and cannot refold to its native form. Thus, the higher this value is, the greater the thermostability of the enzyme [50].

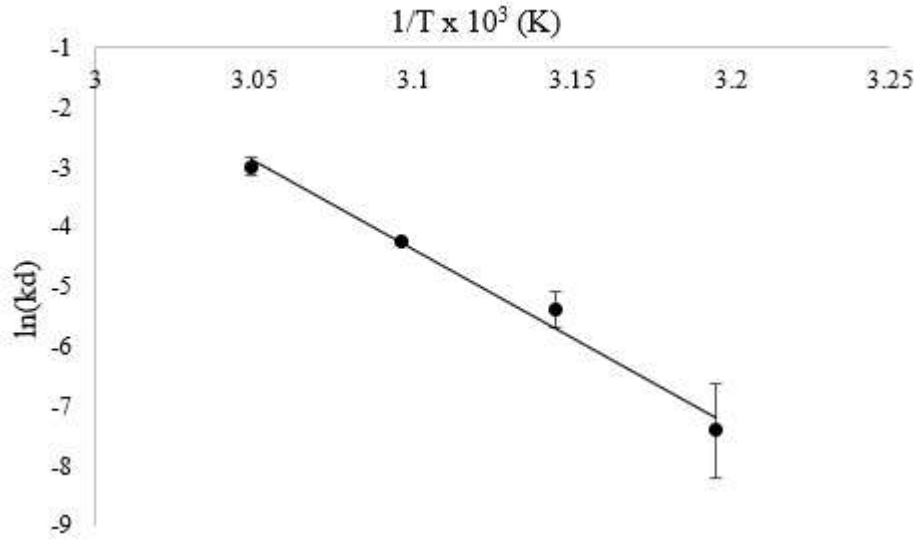

Figure 4. Arrhenius plot to estimate the thermodynamic parameters of thermal inactivation of protease from *A. oryzae* IOC 3999.

In comparison to the work carried out by Da Silva et al., [50], the E^*_d for the protease produced by *A. oryzae* IOC 3999 (29.61 kJ/mol) was similar to the purified protease produced by *A. tamarii* URM4634 (28.8 kJ/mol) in wheat bran by SSF (5g substrate, 40% moisture, 30 °C and pH 7.0), but it was lower when compared to unpurified protease (49.7 kJ/mol).

E^*_d is directly related to the enthalpy of protease denaturation (ΔH^*_d). This thermodynamic parameter expresses the total value of energy required for denaturation, and likewise, the higher this value is, the greater thermostable the enzyme is at a specific temperature [51].

Furthermore, the energy required for thermal inactivation of the enzyme is expected to decrease with increasing temperature. This occurs because the increase in thermal energy in the system causes structural destabilization of the enzyme, leading to the breaking of non-covalent bonds. It has been reported that the energy required for breaking hydrophobic -CH_2 bonds is 5.4 kJ/mol [52]. For the protease produced by *A. oryzae* IOC3999, the highest value of ΔH^*_d (27.01 kJ/mol) was observed for the temperature of 40°C, at 55°C this value dropped to 26.88 kJ/mol (Table 7).

These values are similar to those observed for the pre-purified protease produced by *A. heteromorphus* URM0269 under SSF using wheat bran as substrate, which presented ΔH^*_d between 27.7 and 27.4 kJ/mol at temperatures ranging from 10 to 40°C [45].

The thermal denaturation entropy (ΔS^*_d) is another thermodynamic parameter that measures the size of the disturbance/disorder in the system as a result of the enzyme thermal inactivation process [53]. The higher this value is, the greater the disorder of the system, which can be explained by the large number of protein molecules in the transition state for denaturation [54]. In this work, the protease showed a high negative value for ΔS^*_d, between -220.70 and -197.74 J/molK (40-55°C, respectively), as expected, the entropy increases with increasing temperature (Table 6).

The negative value of entropy indicates that the reduction of enzymatic activity may be a result of the agglomeration of proteins from their partial denaturation, which reduces the disorder in the system [34].

Singh and Chhatpar [55] reported a similar behavior for the protease produced by *Streptomyces* sp. A6 under SmF, which showed ΔS^*_d ranging between -126.95 and -138.82 J/molK at temperatures varying from 30 to 70°C. Otherwise, Souza et al., [56] observed a high disorder in the system during the denaturation of the protease produced by *A. foetidus* under SmF, which showed ΔS^*_d values between 599.59 and 610.49 J/molK in a temperature range of 55-70°C.

Gibbs energy provides more accurate information about the protease enzymatic inactivation process, because it takes into account the contribution of ΔH^*_d and ΔS^*_d [54]. Positive ΔG^*_d values ($\Delta G^*_d > 0$) indicate that the protease thermal inactivation process is not a spontaneous process, requiring the insertion of an external energy source to occur. Therefore, the higher the value of ΔG^*_d is, the greater the thermostability of the enzyme [57].

The ΔG^*_d of the protease produced by *A. oryzae* IOC3999 ranged from 96.09 to 88.78kJ/mol (Table 6) (40-55°C). The decrease demonstrated a reduction in the thermostability of the protease with increasing temperature, but the positive value indicates that the process did not occur spontaneously. Similar results have been reported for a number of proteases, such as the protease produced by *A. fumigatus* that had ΔG^*_d between 89.2 and 91.4 kJ/mol (50-80°C) [58] and the protease produced by *Bacillus stearothermophilus* that showed ΔG^*_d between 91.71 and 90.84 kJ/mol (50-60°C) [59].

4. Conclusion

The SSF using *A. oryzae* IOC 3999 was an interesting process to increase the antioxidant properties from chicken viscera meal, in addition to having allowed the simultaneous production of proteases. From the optimization study using an experimental design, the following parameters were defined as the most suitable to obtain a fermented product with improved antioxidant activity and higher levels of protease: 40% initial moisture, 30°C as the incubation temperature, 5.05×10^6 spores/g as the inoculum size after fermentation and 48 h as the fermentation time. Through the biochemical characterization of the enzyme, the optimal conditions for its performance in terms of activity and thermal stability were determined.

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Discussão geral

A farinha de vísceras de frango demonstrou ser um ótimo substrato para obtenção de biomoléculas com alto valor agregado utilizando processos biotecnológicos. Na hidrólise enzimática do concentrado proteico das vísceras de frango utilizando uma mistura de proteases comerciais, Flavourzyme e Alcalase, foi possível identificar um aumento significativo ($p < 0,05$) na formação de peptídeos com atividade antioxidante em relação à amostra não hidrolisada (controle). Os maiores valores obtidos para a atividade antioxidante em cada método foram: ensaio 6 para eliminação de radicais ABTS (1808,21 µmol TE/g) e DPPH (89,70 µmol TE/g), ensaio 8 para FRAP (353,54 µmol TE/g) e ensaio 4 para CRT (41,68 mg GAE/g), o que levou a aumentos de 245, 353, 69 e 145%, respectivamente, em relação às amostras controle.

Apesar das variáveis de processo tempo de hidrólise (min) e quantidade da mistura de enzimas (U/mL) não terem apresentado efeitos estatisticamente significativos ($p > 0,10$) para o planejamento experimental, ficou claro que a variação no nível dessas variáveis foi responsável pelo aumento do grau de hidrólise, quantificada pelo método indireto da proteína solúvel em TCA, e pelo aumento das propriedades antioxidantes dos peptídeos formados. Diante destas observações, o ensaio 6 com a concentração de enzimas de 170,5 U/mL e 120 min de tempo de hidrólise, apresentou, em média, os melhores resultados para atividade antioxidante. Assim, esta condição foi selecionada para execução das demais etapas.

Durante o estudo de cinética da hidrólise enzimática, o tempo de 120 min foi confirmado como o ideal para obtenção de hidrolisados com maior atividade antioxidante. Além disso, a mistura de proteases apresentou alta resistência à desnaturação térmica ao longo do processo, mantendo 80% da atividade inicial mesmo após 150 min de hidrólise a 50°C e pH 7,0.

A escalabilidade do processo também foi avaliada, partindo de Erlenmeyers com 50, 100 e 250 mL (volume de trabalho) até biorreatores de 1 e 3L (volume de trabalho). Como resultado, os hidrolisados produzidos em todas as escalas de trabalho apresentaram valores semelhantes de propriedades antioxidantes. A análise eletroforética ainda revelou que os hidrolisados apresentaram maior fração de peptídeos com massa molecular entre 6 e 14kDa.

Utilizando um segundo processo, neste caso, a fermentação em estado sólido, a farinha de vísceras de frango foi utilizada como substrato para o cultivo do micro-

organismo *Aspergillus oryzae* IOC3999 a fim de avaliar a produção de proteases e de biomoléculas com propriedades antioxidantes. Os maiores valores para a atividade antioxidante em cada método foram: ABTS (401,08 µmol TE/g), DPPH (208,69 µmol TE/g) e FRAP (300,47 µmol TE/g), o que levou a aumentos de 119, 195 e 231%, respectivamente, em relação às amostras controle. Já a maior produção de protease foi detectada em 48h de fermentação e atingiu 501,39 U/g.

O delineamento composto central rotacional (DCCR) demonstrou que as variáveis: umidade inicial (%), temperatura (°C) e quantidade de inóculo (esporos/g) desempenharam papel fundamental na produção de proteases e nas propriedades antioxidantes do produto fermentado, onde para as respostas que apresentaram efeitos significativos ($p < 0,10$), o R^2 foi superior a 0,80. Os modelos e as curvas de contorno geradas deixaram claro que os níveis mais baixos de umidade inicial, bem como a temperatura e quantidade de inóculo próximos ao ponto central resultaram na melhoria da produção de proteases e das propriedades antioxidantes dos produtos fermentados. Desta forma, as condições de cultivo 40% de umidade inicial, 30°C temperatura de incubação e $5,05 \times 10^6$ esporos/g como quantidade de inóculo em 48h de tempo de fermentação foram selecionadas como as mais adequadas para maximização das respostas de interesse.

O controle da umidade em processos de fermentação em estado sólido deve ser bastante cuidadoso, porque embora deva existir uma quantidade suficiente para realizar a difusão de nutrientes e a absorção pelo micro-organismo, altos níveis de umidade inicial podem causar preenchimento de espaços vazios, compactação e consequentemente limitação da passagem de ar (KUMAR et al., 2021).

No caso da temperatura e da quantidade de inóculo, o aumento dessas variáveis contribui positivamente com o desempenho do crescimento e na produção de metabólitos pelo micro-organismo, mas até certo ponto. O aumento exagerado da temperatura pode levar a danos estruturais em proteínas e na ação de enzimas, enquanto o excesso de inóculo pode levar à exaustão precoce dos nutrientes (CHILAKAMARRY et al., 2022; YOON et al., 2014).

Nos estudos de caracterização bioquímica da protease produzida pelo *A. oryzae* IOC3999 em FES da farinha de vísceras de frango, o efeito do pH e da temperatura sobre a atividade ótima e estabilidade da enzima foi avaliado por meio de um DCCR. Em ambos os casos, os níveis das variáveis estudadas causaram efeitos significativos ($p < 0,05$) sobre a ação das proteases e resultaram em modelos matemáticos com R^2 acima de 0,90.

Para a atividade ótima, o ponto central (pH de 5,5 e temperatura de 50°C) resultou nos maiores valores de atividade enzimática, em torno de 600 U/g. Para a estabilidade, o ensaio que resultou em máxima retenção da atividade enzimática foi o ensaio 7 (pH 5,5 e 35°C) enquanto com nas condições do ponto central, onde a atividade ótima foi detectada, a enzima perdeu aproximadamente 70 % da sua atividade inicial. A partir das curvas de contorno geradas, foi possível identificar que a faixa ótima de atuação da protease ficou entre o pH 5,0 e 6,0 e temperaturas entre 45 e 55°C. Por outro lado, a maior estabilidade da protease foi identificada em temperaturas abaixo de 40°C e pH próximo à neutralidade.

O comportamento da inativação térmica da protease também foi avaliado por meio da determinação de parâmetros cinéticos e termodinâmicos entre as temperaturas de 40 e 55°C. A 40°C, a protease apresentou $t_{1/2}$ de 1155,24 min e um valor D de 3838,33 min, enquanto a 55°C o $t_{1/2}$ foi de 13,95 min e um valor D de 46,34 min, o que representa uma redução de mais de 80 vezes quando as temperaturas são comparadas.

Isso significa que a desnaturação térmica da protease se torna mais pronunciada com o aumento da temperatura, pois a alta vibração das moléculas nessas temperaturas promove a desestabilização de certas interações, como pontes de hidrogênio, forças de van der Waals e interações hidrofóbicas, as quais são responsáveis pela manutenção da estrutura da enzima e consequentemente por sua estabilidade.

A energia de desnaturação térmica (E^*_d) da protease também foi calculada e atingiu 29,61 kJ/mol. Uma vez que esse valor representa a energia mínima necessária para que o processo de inativação térmica se inicie, quanto maior este valor, mais termoestável é a enzima (DA SILVA et al., 2018).

Em relação às variáveis termodinâmicas, a entalpia de desnaturação (ΔH^*_d) representa a energia necessária para a inativação da protease, e os valores ficaram entre 27,01 e 26,88 kJ/mol, entre as temperaturas de 40 e 55°C, respectivamente. Para a entropia de desnaturação térmica, que quantifica o grau de desordem no sistema, os valores foram negativos e ficaram entre -220,70 e -197,74 J/molK (40-55°C). O valor negativo pode significar que a desnaturação das proteases promove um estado de aglomeração entre as proteínas, o que reduz a desordem no sistema (DE CASTRO et al., 2015).

A energia de Gibbs (ΔG^*_d) leva em consideração a contribuição da entalpia e da entropia e por este motivo é uma variável mais precisa como informação do processo de inativação térmica da protease. Valores positivos de ΔG^*_d significam que o processo não ocorre de forma espontânea e quanto maior o valor, mais termoestável é a protease

(OLIVEIRA et al., 2020). Para a protease em questão, a ΔG^*_d ficou entre 96,09 e 88,78 kJ/mol nas temperaturas de 40 e 55°C, respectivamente.

Conclusão geral

Diante dos resultados obtidos, foi possível demonstrar o potencial de aproveitamento de resíduos da agroindústria do frango utilizando processos biotecnológicos. A hidrólise enzimática com proteases comerciais do concentrado proteico de vísceras de frango foi capaz de fornecer hidrolisados com alto grau de hidrólise e que apresentaram peptídeos de massa molecular entre 6 e 14 kDa com atividade antioxidante até 300% maior do que a amostra não hidrolisada. Além disso, a escalabilidade do processo mostrou-se promissora, indicando que o aumento na escala pode ser conduzido, com os devidos ajustes, sem que haja modificação nas propriedades antioxidantes dos hidrolisados. No processo de fermentação em estado sólido, a farinha de vísceras de frango foi um excelente substrato para o crescimento do fungo filamentoso *A. oryzae* IOC3999, onde foi possível identificar o efeito das variáveis de processo para a maximização da produção de proteases e de biomoléculas com atividade antioxidante. As condições que melhor contribuíram com o aumento dos resultados foram: 40% de umidade inicial, 30°C temperatura de incubação e 5.05×10^6 esporos/g como quantidade de inóculo em 48h de tempo de fermentação, levando a aumentos na atividade antioxidante em relação ao material não fermentado de, 105% para o método ABTS, 115% DPPH e 231% FRAP, para a produção de protease o valor foi de 428,22 U/g. As proteases produzidas também foram caracterizadas bioquimicamente, apresentando condições ótimas de atuação na faixa de pH entre 5,0 e 6,0 e de temperaturas entre 40 e 50°C. Em relação à estabilidade, a enzima apresentou alta estabilidade em temperaturas abaixo de 40°C e valores de pH em torno de 7,0. Os parâmetros cinéticos e termodinâmicos de inativação térmica das proteases forneceram informações adicionais sobre as proteases produzidas por *A. oryzae*, contribuindo para sua aplicação em processos futuros.

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Design aspects of solid state fermentation as applied to microbial bioprocessing

Abstract

Solid state fermentation (SSF) refers to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected micro organisms, especially fungi, are naturally adapted. SSF has been used in the world for a long time. This technology is commonly known in the East, for traditional manufacture of fermented foods, and in the west, for mould-ripened cheese. It can be defined as a system, in which the growth of selected microorganism(s) occurs on solid materials with low moisture contents and has been identified as a potentially important methodology and technique in biotechnology. Nowadays, SSF is an economically viable, practically acceptable technology for large-scale bioconversion and biodegradation processes. Development of sustainable SSF and bioprocess technology is an emerging, multidisciplinary field with possible application to the production of enzymes, chemicals, bioethanol and pharmaceuticals. SSF offers many advantages over conventional submerged fermentation (SMF) such as, simple and inexpensive substrates, elimination of the need for solubilisation of nutrient from within solid substrates, elimination of the need for rigorous control of many parameters during fermentation, product yields are mostly higher, lower energy requirements, produce less waste water, no foam generation and relatively easy recovery of end products. SSF provides flexibility in terms of the raw materials to be used and their capability to produce various value-added products.

Keywords: solid state fermentation, submerged fermentation, biological factors, physico-chemical factors, bioreactors, mass transfer phenomena, polyurethane foam

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Introduction

Solid state fermentation (SSF) has been practiced for centuries in the Orient and Asian region in both large and small scale applications in food processing and production of traditional fermented foods. Products such as fermented soybean (tempe, soy sauce, annatto, miso, etc) and fermented rice (tapai, koji, red fermented rice, brewing of the Japanese rice wine (sake)) among others are based on SSF. Some of these products have been in existence for over a thousand years, and their production was probably based on trial and error methods. Bread making is one of the oldest techniques known to man, and archaeological discoveries indicate that ancient Egyptians were making bread using a fermentation process even as early as 2600 BC.[1] Fermented foodstuffs serve as an important component in the daily diet of a large majority of the families in that region as a source of protein and vitamins. However, the low level of traditional technology in the preparation of these products, the various sanitary requirements, the lack of built-in safeguards against undesirable microbial growth and toxins and other closely related constraints pose challenging problems to scientific and technological workers. There is therefore a perceived need for more basic understanding, in-depth studies and scientific as well as technological research on the various aspects of SSF. The process of SSF has been practiced since ancient times and most of the processes are still practiced without any major modifications. The techniques applied are very simple, principally needing only raw materials, simple pre-treatment of the substrate to be fermented and microorganisms as an inoculum. The fermentation process proceeds at room temperature. Table 1 shows the development of SSF from the past to the present and future. SSF technology has contributed many products for humans since the beginning of human civilisation.[2]

In recent years, the application and development of SSF technology has greatly expanded especially in Western countries because of its perceived advantages in the production of various secondary metabolites and novel foods.[3] Detailed studies carried out in laboratories have helped to understand and to confirm the chemical and microbiological changes during fermentation. Some of the originally small scale, traditional SSF processes have become large-scale industries; it should therefore be worthwhile to study those factors that are conducive for large-scale production. In the future, SSF may become more important as a technology to process alternative, bio-based feedstocks that will replace the declining petroleum resources. There will be efforts and ever-increasing pressure to move towards biorefineries for the production of industrial biochemicals.[4] The large-scale cultivation of microorganisms will be an integral part of such biorefineries. This can be achieved since SSF technology offers the advantage and potential to minimise the addition of water and thus optimise process economics in biorefineries. On top of that, in order to meet the requirements of SSF as a potential technology, it is necessary to produce effective large-scale SSF bioreactors with optimised performance.

Definition of solid state fermentation

SSF has been defined in many ways. Many researchers in the field have introduced their own ways to define SSF. For example, Pandey et al.[5] defined SSF as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can also be used as carbon and energy source. Mitchell et al.[6] described SSF as any process in which substrates in a solid particulate state are utilised, while Viniegra-Gonzàlez[7] defined SSF as a microbial process