

UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CÂMPUS DE BOTUCATU

**PRODUÇÃO DE FITASES E PROTEASES FÚNGICAS,
APLICAÇÃO EM RAÇÕES E VALORES DIGESTÍVEIS PELA
TILÁPIA-DO-NILO**

PAULA KERN NOVELLI

Trabalho de Tese apresentado
ao Programa de Pós-graduação
em Zootecnia como parte das
exigências para obtenção do
título de Doutor em Zootecnia

BOTUCATU - SP
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"The important thing is not to stop questioning. Curiosity has its own reason for existing.

Never lose a holy curiosity."

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein (1879-1955)

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CAPÍTULO I

CONSIDERAÇÕES INICIAIS

1. Introdução

O presente trabalho objetivou a produção de fitases e proteases, enzimas utilizadas como aditivos em ração animal, produzidas por Fermentação em Estado Sólido (FES) utilizando diferentes resíduos agroindustriais. Foram selecionadas várias espécies fúngicas para análise quanto à produção ou não de fitases e proteases e verificação das atividades enzimáticas encontradas. Ademais, os estudos das características bioquímicas das enzimas produzidas e a alta atividade encontrada, viabilizaram sua adição às rações formuladas para dieta da tilápias-do-Nilo.

Nos últimos anos, processos biotecnológicos inovadores têm explorado a FES como uma tecnologia promissora (Castro, 2015). A utilização de fungos filamentosos em substrato sólido é outra vantagem alcançada, uma vez que o meio mimetiza a condição natural de crescimento do micro-organismo, viabilizando ainda uma maior produção. Além disso, a utilização de resíduos ou co-produtos da agroindústria pode diminuir custos, além de ser sustentável e ecologicamente correta.

Dentre os metabólitos produzidos durante a fermentação microbiana, as enzimas apresentam grande potencial de aplicação. Neste sentido, o uso de enzimas na alimentação animal é prática comum nos dias atuais e a utilização de enzimas exógenas de micro-organismos tem mostrado bons resultados para o aumento da eficiência de utilização dos nutrientes (Pariza e Cook, 2010). Neste contexto, o aumento na produtividade requer o uso de rações que atendam as exigências nutricionais de cada espécie.

Embora o uso de enzimas digestivas exógenas de micro-organismos na alimentação animal ser prática comum nos dias atuais e ter mostrado bons resultados para o aumento da eficiência de utilização dos nutrientes, o custo de produção, ainda, pode ser limitante na expansão de seu uso em larga escala (Graminha et al., 2008). Assim, as enzimas produzidas neste trabalho viabilizaram sua produção em maior escala. Posteriormente, foram adicionadas às dietas para tilápias-do-Nilo possibilitando a redução de custo e melhorando o valor nutricional dos ingredientes da ração.

2. Fermentação em Estado Sólido (FES)

A fermentação tradicional já vem sendo utilizada a milhares de anos para produção de alimentos e bebidas, como por exemplo, vinho, cerveja, queijos e pães, sendo que atualmente estes processos evoluíram para a indústria química, farmacêutica e agricultura, incluindo tratamento de resíduos. A fermentação compreende um conjunto de reações enzimáticas controladas, pelas quais uma molécula orgânica é degradada em compostos mais simples, liberando energia. A glicose é uma das substâncias mais empregadas pelos micro-organismos como ponto de partida na fermentação. Resultam da atividade de micro-organismos, como leveduras, bactérias e fungos filamentosos e consequente produção de metabólitos e compostos bioativos. Neste sentido, a fermentação utilizada a milhares de anos em processos biotecnológicos e na indústria alimentícia, aproveita as biomoléculas produzidas neste processo pelos micro-organismos. É importante lembrar que a fermentação dentro do conceito de FES é utilizada no sentido mais amplo de processos microbianos controlados e não implica somente na utilização da glicose nas vias metabólicas fermentativas durante o cultivo microbiano (Mitchell et al., 2006).

Os processos fermentativos podem ser em meio líquido ou sólido. A fermentação líquida ou submersa (FSm) foi durante muitos anos a mais utilizada na indústria biotecnológica e são reações que ocorrem em meio fermentativo líquido com nutrientes solúveis, sendo que cada parte do reator contém, ao mesmo tempo, a mesma quantidade de micro-organismos, nutrientes e metabólitos. Por outro lado, a Fermentação em Estado Sólido (FES) é o processo fermentativo que ocorre na ausência de fase aquosa livre entre as partículas (Lonsane et al., 1985), sendo que a matriz sólida utilizada no processo pode ser, tanto a fonte de nutriente, quanto simplesmente um suporte impregnado com nutrientes adequados ao desenvolvimento do micro-organismo (Pandey, 2003). Neste tipo de fermentação pode-se usar como substratos resíduos agrícolas e agroindustriais, como farelos e cascas de arroz, trigo, milho, laranja e outros, que apresentam baixo valor agregado, são ricos em nutrientes e apresentam restrita disponibilidade de água que ajuda a eliminar seletivamente contaminantes, especialmente bactérias e leveduras. Alguns estudos já têm ressaltado a promissora produção de enzimas por FES com diferentes resíduos agroindustriais como farelo de soja, trigo e arroz, cascas de laranja, maçã e banana (Fleuri et al., 2013; Karatas et al.,

2013; Monton et al., 2013; Chutmanop et al., 2008). A baixa atividade da água no meio de cultivo sólido influencia nos aspectos fisiológicos dos micro-organismos, tais como seu crescimento vegetativo, esporulação, germinação de esporos e produção de enzimas (Graminha et al., 2008) A obtenção do produto final por FES é simplificada e a quantidade de efluentes resultantes deste tipo de fermentação é minimizada (Lima et al., 2001). Assim, a fermentação sólida permite redução do valor do produto final e, ainda, pode ser considerada ambientalmente correta, pois utiliza matérias-primas de baixo custo rejeitadas nas agroindústrias para seu processo de produção.

As células microbianas cultivadas por processos fermentativos são fontes promissoras para a produção enzimática, pois podem produzir maior quantidade em menor tempo; sua natureza diversa permite a produção de biomoléculas e enzimas variadas; podem ser manipuladas geneticamente, o que permite a obtenção de linhagens melhoradas quanto à produção e qualidade da enzima produzida; e permitem um processo em condições controladas. Assim, a FES pode produzir várias biomoléculas, entre elas as enzimas, de maneira eficiente e com baixo custo. Análises comparativas entre os processos de fermentação submersa e FES demonstram as várias vantagens deste último, como simplicidade, baixo custo e abundância dos meios de cultura, redução de contaminações pela baixa atividade de água, baixa demanda de energia, (Sandhya et al., 2005; Biesebeke et al., 2002). Contudo, ainda muitas questões devem ser elucidadas como, por exemplo: por que algumas espécies fúngicas apresentam diferente comportamento em fermentação sólida quando comparadas à fermentação submersa; por que algumas enzimas produzidas em FES têm diferentes características físico-químicas em relação às produzidas em meio submerso; e por que muitas vezes a FES produz maiores quantidades de enzimas e metabólitos secundários (Barrios-González, 2012). Os diferentes resíduos modificam a expressão metabólica dos fungos, produzindo enzimas com diferentes características e que podem ser aplicadas de várias formas na indústria biotecnológica. A combinação da célula microbiana e diferentes substratos passa por processos metabólicos e de biotransformação que podem gerar diversos produtos celulares. A seleção do micro-organismo, substrato e a caracterização bioquímica das enzimas produzidas são importantes para avaliar seu potencial biotecnológico e direcionar as possíveis aplicações para processos industriais (Castro e Sato, 2013).

No cenário brasileiro, com sua economia fortemente voltada à agricultura, o potencial tecnológico para a reutilização eficiente do resíduo agroindustrial, assim como a adição de valor ao “lixo” agroindustrial produzido em grande quantidade, os processos biotecnológicos, especialmente a FES, têm contribuído para o desenvolvimento de produtos de alto valor agregado como, enzimas, ácidos orgânicos, sabores e aromas, pigmentos, polissacarídeos e hormônios (Soccol e Vandenberghe, 2003), os quais podem ser aplicados, reduzir custos e viabilizar o uso das enzimas para a alimentação humana e animal.

3. Enzimas

Enzimas são proteínas que apresentam atividade catalítica, ou seja, aceleram as reações químicas diminuindo a energia de ativação da reação. São macromoléculas formadas por aminoácidos ligados covalentemente por ligações peptídicas. A estrutura primária das proteínas corresponde à sequência desses aminoácidos e a interação entre aminoácidos adjacentes leva a arranjos espaciais, denominadas estruturas secundárias e terciárias. Neste último, há a configuração do sítio catalítico da enzima ou sua atividade biológica e especificidade (Sant’Anna Jr., 2001). Sua especificidade refere-se à capacidade de realizar somente uma reação bioquímica junto a um substrato, garantindo a formação de um produto sem a produção de subprodutos na maioria das vezes. As enzimas possuem várias aplicações industriais determinadas por sua especificidade, atividade, estabilidade, disponibilidade e custo.

As enzimas são responsáveis pelas reações bioquímicas envolvidas nos processos biológicos dos sistemas vivos. Algumas são haloproteínas constituídas somente por unidades de aminoácidos, e outras são heteroproteínas que possuem uma parte proteica e mais um cofator, necessário para a atividade catalítica, que pode ser uma coenzima (vitamina) ou um íon metálico. A atividade de uma enzima é determinada por sua concentração e de seu substrato, cofatores, presença ou não de inibidores, pH, temperatura e tempo de reação, sendo que em condições ótimas elas podem catalisar reações com velocidades que chegam a ser 10^{17} vezes mais elevadas (Tortora et al., 2012).

A Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB – União Internacional de Bioquímica e Biologia Molecular) divide as

enzimas em seis principais grupos: oxidorreduções (1), transferases (2), hidrolases (3), liases (4), isomerases (5) e ligases (6) nas quais estão incluídas subclasses de acordo com o tipo de reação catalisada. Nesse sistema cada enzima recebe seu nome recomendado, geralmente formado pelo nome do substrato catalisado e sufixo ase. Cada enzima também recebe seu nome sistemático constituído por 4 dígitos, sendo que cada número descreve a enzima de maneira progressivamente mais detalhada. O primeiro número refere-se à classe, ou tipo de reação geral catalisada. O segundo especifica a sub-classe, o terceiro sub-subclasse e o quarto diferencia a sub-subclasse. É importante ressaltar que o código se baseia na reação catalisada em condições naturais, já que a enzima que realiza a catálise de determinada reação em seu meio de origem pode catalisar outras reações em condições controladas. As mais comuns usadas na indústria biotecnológica são as carboidrolases (hidrólise dos dissacarídeos, oligossacarídeos e polissacarídeos), proteases (hidrólise das ligações peptídicas das proteínas e peptídeos) e lipases (hidrólise de gorduras e triglicerídeos) (IUBMB, 1993).

As enzimas também podem ser classificadas em constitutivas e indutivas, sendo as primeiras sintetizadas independentemente do meio-ambiente celular e estão presentes sempre e em quantidade constante nas células. Já as enzimas indutivas são aquelas cuja síntese é aumentada pela presença de meio indutor, os quais são normalmente iguais ou derivados do substrato fornecido. Alguns indutores conhecidos são amido, dextrina, maltose, lactose, sacarose, monopalmitato, lactose e colesterol (Rhodes, 1969).

Atualmente, o número de enzimas identificadas que constam na lista da Comissão Internacional de Enzimas (E.C.) é de cerca de 3000. Porém, somente cerca de 60 enzimas têm aplicação industrial e são utilizadas em quantidades significativas, sendo que 75% correspondem às hidrolases (Brasil, 2015). O mercado está distribuído principalmente em enzimas utilizadas em detergentes (37%), têxteis (12%), processamento do amido (11%), panificação (8%) e ração animal (6%) (Figura 1).

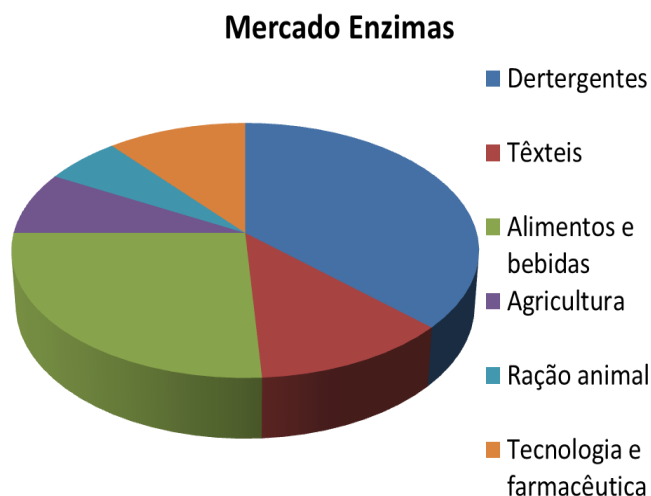


Figura 1: Distribuição do mercado brasileiro de enzimas (Brasil, 2015).

Neste sentido, reações enzimáticas e de biotransformação catalisadas por enzimas fúngicas extracelulares são usadas na indústria, agricultura, tecnologia de alimentos, medicina entre outros setores. A vantagem do uso de enzimas fúngicas está na variedade das reações bioquímicas que catalisam e na sua alta especificidade. Há séculos a humanidade usa as leveduras e fungos filamentosos em diversos processos biotecnológicos como produção de pão, queijos e bebidas alcoólicas. Os fungos filamentosos têm sido usados há anos como fontes de produção de diversos metabólitos e enzimas, sendo que muitos fungos secretam naturalmente proteínas e têm sido explorados comercialmente como fábricas desses biocatalisadores (Paiva e Sá-Pereira, 2008). Os fungos filamentosos de maior interesse industrial incluem espécies de *Aspergillus* sp., como *A. awamori*, *A. niger*, *A. oryzae* e *A. nidulans* (Van der Homborgh et al., 1997). Além disso, várias espécies do gênero *Aspergillus* são consideradas não tóxicas, sendo avaliado como micro-organismo seguro, segundo o Food and Drug Administration (FDA), recebendo a denominação Generally Recognized as Safe (GRAS), podendo ser utilizada na alimentação humana e animal (Morita et al., 2010; Vishwanatha et al., 2009; Gotou et al., 2009).

3.1. Fitase

O uso de alimentos de origem vegetal como farelos de soja, trigo e milho, glúten de milho, entre outros coprodutos da agroindústria, em rações para peixes é alternativa

para a substituição da farinha de peixe e alimentos de origem animal. Contudo, estes alimentos, geralmente, contêm grande parte do seu fósforo na forma de ácido fítico, forma esta indisponível aos animais monogástricos. Ao mesmo tempo, o fitato age como antinutriente ligando-se às proteínas, aminoácidos, lipídeos e minerais, interagindo com enzimas digestivas e reduzindo suas atividades, o que influencia na digestão e prejudica o aproveitamento dos nutrientes da dieta (Figura 2) (Bohn et al., 2007; Nagashima et al., 1999).

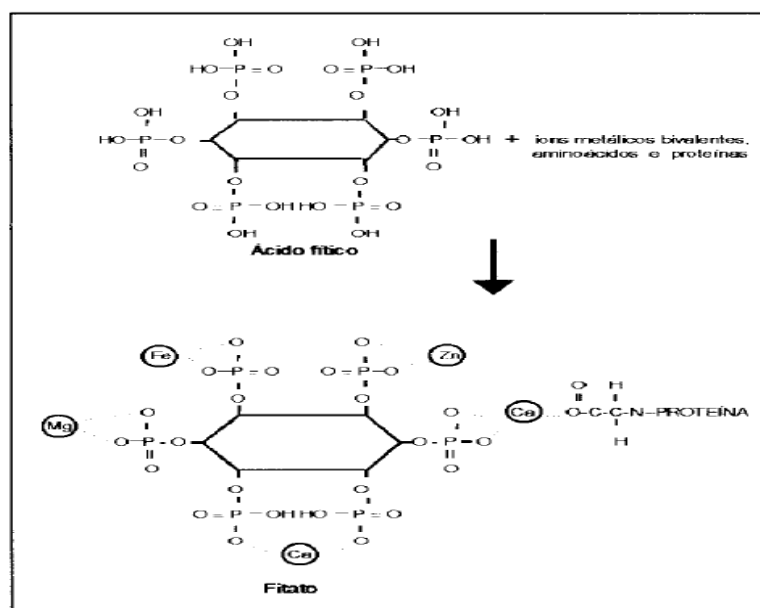


Figura 2: Formação do fitato e complexação de proteínas e minerais (Fireman e Fireman, 1998).

Neste sentido a fitase catalisa a hidrólise dos grupos fosfato e ácido fítico a fosfato inorgânico, aumentando a biodisponibilidade do fósforo para animais monogástricos. Segundo Lei e Porres (2003), sua classificação é baseada na posição do primeiro fosfato a ser hidrolisado, nomeadas de 3-fitase (E. C. 3.1.3.8) e 6-fitase (E. C. 3.1.3.26) (Figura 3).

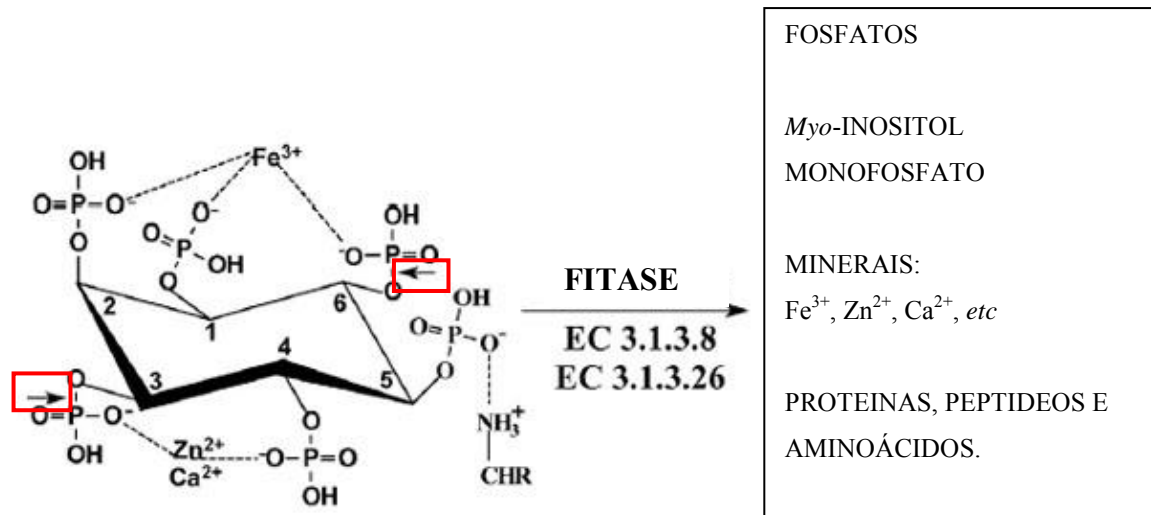


Figura 3: Degradação do ácido fítico pela 3-fitase liberando o fosfato iniciando pela ligação éster do carbono C3, e pela 6-fitase liberando o fosfato inicialmente do carbono C6. As setas indicam onde as ligações são inicialmente quebradas. (Agranoff, 2009).

O mercado mundial da enzima fitase como um aditivo alimentar é estimado em US\$500 milhões, sendo a China o maior produtor (Schauer e Borriss, 2004). A primeira fitase comercial produzida derivada do fungo *Aspergillus niger* com capacidade de liberar o fosfato ligado ao fitato e reduzir a sua excreção, foi introduzida no mercado em 1991 (Selle e Ravindram, 2006). A suplementação da enzima fitase na ração auxilia na nutrição dos animais (Kies et al., 2001; Lei e Stahl, 2000). Várias pesquisas, com a tilápia-do-Nilo, foram desenvolvidas objetivando determinar a ação da fitase na disponibilidade de nutrientes das dietas. Segundo Furuya et al. (2006) a adição de fitase tem potencial para aumentar o desempenho dos peixes e a disponibilidade do fósforo e da proteína, reduzindo a excreção de P e N para o meio aquático. Gonçalves et al. (2005) estudaram a suplementação de níveis de fitase em dietas purificadas e marcadas com Cr_2O_3 e concluíram que a enzima aumentou a disponibilidade dos minerais Mg, Cu, Zn e Mn em alimentos de origem vegetal e, que o nível recomendado de suplementação depende do alimento. Do mesmo modo, Bock et al. (2006) demonstraram que a suplementação de fitase em rações para a tilápia-do-Nilo melhora a digestibilidade da proteína, energia bruta e aumenta a disponibilidade do cálcio, fósforo, zinco, manganês e magnésio. Aumentando a disponibilidade do fósforo para o animal, a fitase pode ainda diminuir a eutrofização nos ambientes aquáticos, reduzindo a poluição.

Contudo, dados sobre as condições de hidrólise da fitase no trato das espécies de peixes são limitados e doses ótimas para a adição da enzima que possam substituir o

fósforo inorgânico precisam ser mais bem avaliadas em dietas para organismos aquáticos. O uso da fitase na alimentação de peixes cresce conforme a necessidade de dietas menos onerosas e mais efetivas e de acordo com o aumento da preocupação quanto à conservação do meio ambiente (Cao et al., 2007).

3.2. Protease

As proteases (E. C. 3.4.21.12) catalisam a reação de hidrólise das ligações peptídicas das proteínas e, ainda, podem apresentar atividade sobre ligações éster e amida nas reações de síntese. Como todas as enzimas, as proteases apresentam certo grau de especificidade quanto ao substrato, em geral, relacionado aos aminoácidos envolvidos na ligação peptídica a ser hidrolisada (Santos e Koblitz, 2008). As proteases constituem importante grupo de enzimas produzidas comercialmente (Uyar e Baysal, 2004), têm aplicação em diferentes indústrias e vêm sendo utilizadas como aditivos na ração animal. Estas enzimas estão amplamente distribuídas na natureza e podem ser obtidas a partir de diversas fontes como plantas, animais e micro-organismos. As proteases de origem microbiana são predominantemente extracelulares, o que diminui a necessidade das complexas etapas de recuperação da enzima a partir do meio fermentativo (Muthulakshmi et al., 2011). Ao mesmo tempo, a expressão e secreção de diferentes proteases por micro-organismos podem ser reguladas pelo tipo de fermentação e substrato utilizado para a sua produção.

Dentre as aplicações de protease, processos que envolvem a hidrólise de proteínas vêm sendo estudados. Neste sentido, a utilização destas enzimas para melhorar a disponibilidade de nutrientes de alimentos proteicos contendo fatores antinutricionais e de baixa digestibilidade são comuns na indústria de ração animal. Elevados teores de ingredientes inibidores de proteases são encontrados, principalmente, no farelo de soja. Estudos realizados com ingredientes vegetais e a complementação enzimática na dieta de frangos, coelhos e peixes têm demonstrado resultados promissores (Toral et al., 2002). A suplementação com enzimas exógenas na ração de animais monogástricos visa a remoção de fatores antinutricionais dos alimentos de origem vegetal, o aumento da digestibilidade das dietas, a potencialização da ação das enzimas endógenas e a redução da poluição ambiental (Garcia et al., 2000).

Guimarães et al. (2009) determinaram que um complexo enzimático contendo protease para tilápia-do-Nilo melhorou a digestibilidade da proteína, do extrato etéreo, do carboidrato e da energia das rações. Estudos com peixes carnívoros também demonstraram que o uso de proteases exógenas melhora a digestibilidade aparente dos nutrientes e que influencia nos parâmetros corpóreos dos peixes (Soares et al., 2008; Silva et al., 2007). Sajjadi e Carter (2004) comprovaram a melhora no desempenho de salmão (*Salmo salar*) e Ng et al. (2002) observaram a diminuição dos efeitos antinutricionais e aumento no valor nutritivo de farelo de palmeira para tilápia vermelha (*Oreochromis sp.*) quando houve adição de enzimas exógenas na formulação das dietas.

Do mesmo modo, grande número de trabalhos vêm demonstrando a utilização de diferentes proteases visando à liberação de peptídeos com distintas atividades biológicas como, antiadipogênica (Merija et al., 2010; Tsou et al., 2010), antimicrobiana (Boman, 2003; Adje et al., 2011), anti-hipertensiva (Adje et al., 2011; Alemán et al., 2011), antioxidante e anti-inflamatória (Zang et al., 2009; Oseguera-Toledo et al., 2011; Ahn et al., 2012) e anticancerígena (Alemán et al., 2011). Sendo assim, a ampla diversidade de proteases, assim como a existência de fontes proteicas com composição variada de aminoácidos, torna possível a obtenção de peptídeos com funções biológicas distintas e/ou até mesmo multifuncionais, assim como as condições de processo (Castro, 2015).

4. Proteína vegetal na alimentação de peixes

Apesar da longa história ao redor do mundo, a aquicultura só apresentou impacto como importante fonte de proteína animal nas últimas quatro décadas, quando se percebeu que os desembarques de peixes oriundos da captura extrativista estavam em declínio, indicando que os volumes capturados seriam insuficientes para suprir a demanda por este tipo de proteína. Como consequência, houve aumento da procura por animais aquáticos oriundos da aquicultura, sendo que a produção de peixes cresceu como atividade agroindustrial. A partir daí foi imperativa a necessidade de se desenvolver a indústria comercial de dietas para organismos aquáticos (Sussel et al., 2014). No início as rações utilizadas eram para aves e suínos, porém notou-se que além da inadequada forma de apresentação para fornecimento aos peixes, estas apresentavam baixa eficiência alimentar e grande lixiviação na água. Somente após o final da década de 1980 é que começaram a formular rações específicas para peixes e a peletização

passou a ser alternativa para o fornecimento de ração mais adequada aos peixes. Peletização é a aglomeração de pequenas partículas por meio de processo mecânico que combina umidade, calor e baixa pressão. Mais tarde percebeu-se que a extrusão, processo mecânico que combina alta pressão e alta temperatura, proveria a ração mais digestível aos peixes e com menor impacto ao ambiente. Embora a extrusão resulte em aumento no custo do produto em relação à dieta peletizada, este é compensado pela melhora na eficiência alimentar e menor eutrofização da água, melhorando sua qualidade e possibilitando o crescimento mais rápido dos peixes, com melhor aproveitamento dos nutrientes e reduzindo, assim, o custo do alimento por unidade de peixe produzida (Kubitza, 1999).

Neste contexto, o aumento na produtividade requer o uso de rações que atendam as exigências nutricionais da espécie, pois o alimento natural não é capaz de atendê-las em condições intensivas de cultivo. As rações comerciais possuem de 25 a 40% de proteína bruta, o que implica em elevada participação de ingredientes proteicos, e isso corresponde a mais de 50% de seu custo total de produção (Furuya et al., 2005). O Brasil possui grande disponibilidade de matéria prima de origem vegetal tanto quanto animal para a produção de rações para organismos aquáticos, sendo assim, estudos são necessários para formulação do alimento ideal para esses últimos com melhor utilização dos insumos disponíveis e menor custo de produção. A substituição da proteína animal pela proteína vegetal em rações para os organismos aquáticos pode diminuir custos e adequa-se aos atuais padrões sanitários e ambientais.

Muitos estudos realizados para determinar a exigência em proteína para a aquicultura podem apresentar valores superestimados, uma vez que parte dos aminoácidos da dieta pode ser catabolizada na forma de esqueleto carbônico e utilizada como fonte de energia pelos peixes, aumentando o custo da alimentação e, ao mesmo tempo, a excreção de amônia com maior potencial poluente (Wood, 1993; Wilson, 1989; Lovell, 1989, apud Gonçalves et al., 2009). Por outro lado, em muitas espécies o metabolismo do glicogênio é mais lento e a glicose para suporte metabólico é obtida dos aminoácidos da dieta.

Para as espécies tropicais, poucas são as informações dos valores digestíveis da proteína e da energia da maioria dos alimentos nacionais em função do manejo alimentar aplicado. Segundo Ustaoglu Tiril & Alagil (2009) não há diferenças nos

valores de digestibilidade da proteína pela truta arco-íris quando se aumenta a frequência de alimentação. Somando-se à isso, a menor eficiência alimentar também pode estar relacionada à taxa de alimentação. Windell et al. (1978) observaram que a truta arco-íris quando arraçadas com taxas de alimentação entre 0,4 e 0,6% do peso corporal apresentaram coeficientes de digestibilidade aparente para proteína bruta superiores aos apresentados pelos peixes arraçados com 1,6 % do peso corporal ao dia.

Assim como o tipo de insumo utilizado pode afetar a digestibilidade e o destino metabólico da proteína nos peixes, o maior nível de proteína na dieta não significa que esta está sendo destinada ao crescimento muscular e sim, pode estar sendo destinada a outras vias metabólicas. A deficiência no suprimento de proteína na dieta pode determinar o retardo no crescimento e perda de peso, contudo quando fornecida em excesso, a proteína é catabolizada e usada como energia, aumentando o depósito de nitrogênio no ambiente, com conseqüente poluição (Ahuja et al., 2004).

Gonçalves et al. (2009) concluíram que é possível a utilização de níveis inferiores a 34% de proteína digestível (PD) na ração com base no conceito de proteína ideal para juvenis de tilápia e determinaram o melhor índice de eficiência econômica para peixes alimentados com ração com 30% de proteína digestível (PD) e 3.000 kcal/kg de energia digestível (ED), ou seja, relação ED:PD de 10 kcal g⁻¹. Entre os principais fatores que influenciam a exigência proteica na alimentação dos peixes estão a qualidade da proteína, o teor de energia não proteica (carboidratos e lipídeos) e a relação energia/proteína bruta (Brandt, 1991). O excesso de energia não proteica como resultado da formulação com alta relação energia digestível/proteína bruta (ED:PB) pode inibir a ingestão voluntária antes do consumo suficiente da dieta, o que prejudica a utilização de outros nutrientes.

A digestibilidade de um ingrediente da ração depende, principalmente, da sua composição química aliada à capacidade digestiva da espécie (McGoogan e Reigh, 1996), sendo que quanto maior a disponibilidade para absorção de um nutriente, menor a quantidade excretada pelo peixe. Alimentos de origem vegetal vêm sendo utilizados com maior frequência na alimentação de peixes, pois possuem baixo custo e maior disponibilidade no mercado. Contudo, apresentam proteína deficiente em um ou mais aminoácidos essenciais, contém menor valor energético que os alimentos de origem animal e podem conter vários antinutricionais.

Os tipos de proteína na ração interferem, ademais, nos custos de produção. Avaliar o efeito da adição de enzimas microbianas de custo relativo baixo, comparada com os diferentes alimentos disponíveis da ração e aproveitamento dos nutrientes podem diminuir ainda mais estes custos.

Observa-se que diversos são os fatores que afetam a digestibilidade e o metabolismo da proteína e nutrientes nos animais aquáticos, entretanto, a partir de rações balanceadas e utilização de aditivos adequados e de baixo custo, torna-se possível a obtenção de melhores respostas de produção, maximização dos lucros e, principalmente, minimização do impacto ambiental que alguns alimentos podem proporcionar.

4.1. Farelo de soja

O farelo de soja é excelente fonte de proteína e aminoácidos para tilápias, com boa disponibilidade no mercado nacional, contudo tem a metionina como aminoácido limitante (Furuya et al., 2001). Possui boa digestibilidade dos aminoácidos, teor de proteína entre 45 e 49% (Pezzato et al., 2002; Gonçalves et al., 2009) e digestibilidade aparente da proteína de 92,4% (Guimarães et al., 2008). Boscolo et al. (2002) também demonstraram que a tilápia-do-Nilo utiliza a energia e proteína do farelo de soja eficientemente. Contudo, o farelo de soja possui alguns fatores antinutricionais como inibidores da protease, a lecitina, o ácido fítico e interfere na disponibilidade das vitaminas A e D.

Segundo Kaushik et al. (1995), a substituição da farinha de peixe pela proteína da soja na alimentação de trutas (*Oncorhynchus mykiss*) não afetou o crescimento e a utilização de nutrientes. Meurer et al. (2008) recomendou a inclusão de até 42% de farelo de soja na ração para tilápia-do-Nilo na fase de reversão sexual. Do mesmo modo, Souza et al. (2004) verificaram que o farelo de soja é fonte proteica adequada para a substituição da farinha de peixe ao nível de 50% para a tilápia-do-Nilo. De acordo com Aguiar et al. (2005), a lisina é necessária para o desenvolvimento muscular de tilápias-do-Nilo e importante para a proliferação das células musculares desta espécie, sendo o farelo de soja boa fonte deste aminoácido e o ingrediente de origem vegetal que apresentou melhor disponibilidade aparente para este aminoácido segundo Guimarães et al. (2008).

Bouraoui et al. (2011) concluíram que a substituição combinada da farinha e óleo de peixe por farinha de soja e glúten de milho para a dourada do mar (*Sparus aurata*) permitiram crescimento e utilização dos nutrientes da dieta satisfatórios, com poucas mudanças no metabolismo lipídico, contudo, futuras investigações são necessárias para o melhor entendimento da utilização dos ingredientes de origem vegetal pelos peixes.

4.2. Glúten de milho

Dentre os alimentos proteicos de origem vegetal, pode-se destacar também o glúten de milho-60, que apresenta bons valores de digestibilidade aparente pela tilápia-do-Nilo (Pezzato et al., 2002). O glúten de milho é obtido após a remoção da maior parte do amido, gérmen e porções fibrosas por tratamento enzimático do endosperma (Butolo, 2002). Possui bom teor de metionina, porém é deficiente em lisina, arginina e triptofano (Gonçalves et al., 2009). O teor de proteína bruta está em torno de 62% para o glúten de milho-60 e seu coeficiente de digestibilidade aparente é 91,4% (Guimarães et al., 2008), com baixo teor de fibra e rico em vitamina B e E (Regost et al., 1999).

Pongmaneerat e Watanabe (1991) encontraram 93,7% a 95,5% de digestibilidade aparente da proteína do glúten de milho para carpa comum (*Cyprinus carpio*). Robaina et al. (1997) substituíram de 20 a 40% da proteína da farinha de peixe pela proteína do glúten de milho e não encontraram diferença no ganho de peso para a dourada do mar. Estudos confirmam a possibilidade da adição de até 40% de glúten de milho em dietas para várias espécies de peixes sem comprometimento do desempenho ou da eficiência e utilização dos nutrientes (Robaina et al., 1997; Rodehutschord et al., 1995).

Segundo Gamboa-Delgado et al. (2013) a substituição de 50 a 60% de proteína animal por soja e glúten em rações para camarões mantiveram as taxas de crescimento e sobrevivência. Estudos anteriores concluíram que a proteína do glúten de milho pode substituir até 42% (19,82% de inclusão na ração) da proteína do farelo de soja em rações para alevinos de tilápias-do-Nilo (Hisano et al., 2003), assim melhorar ainda mais sua disponibilidade por meio da adição de enzimas de baixo custo pode ser alternativa viável para melhorar a performance na produção aquícola.

5. Metabolismo e valor nutricional

O conjunto de enzimas relacionadas ao metabolismo de açúcares, gorduras e proteínas é bastante distinto, ou comporta-se de maneira diferente para as diferentes espécies e respostas às mudanças na dieta podem ser variáveis para as inúmeras espécies de peixes. Existem vários relatos correlacionando as enzimas do metabolismo energético e de proteínas e o estado nutricional dos peixes (Kim et al., 1992; Metón et al., 1999; Melo et al., 2006; Peres e Oliva-Teles, 2007;). Componentes destes três grupos de nutrientes são passíveis de serem usados como combustível para as vias metabólicas, tais reações na presença de oxigênio irão liberar CO₂ e água com a produção de energia.

As proteínas, assim como todos os compostos constituintes de um organismo, carboidratos e lipídeos estão em constante processo de degradação e síntese. Dentro deste contexto os nutrientes devem ser oferecidos de forma que os organismos consigam preservar a vida e se desenvolver. Os nutrientes necessários em quantidades substanciais são chamados macronutrientes como, proteínas, carboidratos e lipídeos, sendo a proteína o mais oneroso destes macronutrientes. Portanto, entender o metabolismo dos peixes, gerenciando a oferta dos carboidratos e lipídeos como fonte de energia, poupando assim as proteínas, resulta em vantagens no custo de produção e traz menos risco a natureza.

Assim, as proteínas são macromoléculas que contém carbono, hidrogênio, oxigênio, nitrogênio e, em alguns casos, enxofre; e são importantes constituintes da estrutura animal e essenciais para seu desenvolvimento. As unidades estruturais fundamentais da proteína são os aminoácidos que são unidos por ligações peptídicas. Todos os aminoácidos possuem um terminal carboxila e outro amino ligados ao carbono central. Os aminoácidos essenciais para os peixes são: metionina, treonina, fenilalanina, lisina, valina, leucina, histidina, triptofano e isoleucina (Halver e Hardy, 2002). As proteínas não têm seus valores nutricionais baseados somente na sua composição de aminoácidos, mas sim em como estes aminoácidos estão disponíveis à ação das enzimas. Neste sentido, a adição de enzimas exógenas pode auxiliar no aproveitamento metabólico dos nutrientes pelo animal (Kies et al., 2001).

Os peixes são capazes de usar e metabolizar mais facilmente as proteínas em vez dos carboidratos. As proteínas da dieta têm normalmente duas vias metabólicas, o

catabolismo para a produção de energia e o anabolismo para a síntese de novas proteínas. O crescimento relacionado à ingestão de proteínas pode ser determinado da relação entre a síntese e a degradação desta proteína, contudo a síntese proteica tem alto custo energético para o metabolismo, podendo corresponder a 42% do total de energia consumida pelo organismo. Outro aspecto a ser considerado quanto ao metabolismo das proteínas é o limite no qual os peixes não são mais capazes de usá-las para crescimento, estocando-as e usando como fonte de energia, produzindo, conseqüentemente, altos índices de amônia na água pelo processo metabólico de hidrólise chamado desaminação (Jobling, 1994). Deve-se considerar ainda que o desbalanço nutricional pode causar redução na ingestão e retardo no crescimento.

Antes dos macronutrientes chegarem às vias metabólicas o alimento deve sofrer digestão química. Alguns fatores podem influenciar esta atividade química e enzimática como idade, fisiologia, clima, genética, saúde; sendo assim estudos são necessários para unir o conhecimento em nutrição e biotecnologia com os mecanismos fisiológicos e metabólicos dos peixes.

Do mesmo modo, micronutrientes, como minerais e vitaminas, são de vital importância para o desempenho metabólico animal, sendo que a ausência de algum destes pode levar à vários distúrbios metabólicos. Neste sentido, o fósforo é mineral de extrema importância na alimentação animal, sendo o segundo mineral de importância na estrutura óssea (Steffens, 1987). O fósforo também se encontra nos ácidos nucleicos, fosfolipídios e numa série de enzimas, uma vez que participa de importantes processos metabólicos (Berne et al., 2000).

O íon fosfato é de grande importância nos sistemas biológicos, é componente integrante dos compostos envolvidos no metabolismo energético, desde a glicose-1-fosfato até o fosfoenolpiruvato. Faz parte da estrutura dos compostos de alta energia como o ATP e a creatinina fosfato, de coenzimas como o NAD^+ , NADP^+ e a tiamina-pirofosfato, e de lipídeos como a fosfatidilcolina. Atua ainda como cofator de várias enzimas. As reservas de fosfato nos tecidos moles, como a massa muscular, são grandes e, a transferência entre essas reservas e o líquido extracelular, constitui fator importante na regulação do fosfato plasmático (Berne et al., 2000).

Os peixes utilizam em seu metabolismo, preferencialmente o fósforo proveniente da dieta. Parte deste mineral pode ser absorvida da água, embora em quantidades

insuficientes para o seu crescimento, sendo que a assimilação de fósforo do alimento pode ser 200 vezes maior do que o absorvido da água (Hepher, 1993). Assim, sua suplementação em rações para peixes se faz necessária quando se utilizam alimentos de origem vegetal, nos quais o fósforo se apresenta em grande parte indisponível na forma de fitato (Miranda, 2000).

Neste sentido, o valor nutricional da dieta está relacionado à digestibilidade dos ingredientes, sua absorção e disponibilidade para o metabolismo (Moraes e Almeida, 2011). Muitos estudos já relataram o valor nutricional de alimentos, Pezzato et al. (2004) determinaram a digestibilidade aparente e valor nutricional de alguns alimentos alternativos pela tilápia-do-Nilo e que podem ser utilizados para formulação de rações para peixes tropicais, encontrando bons valores de coeficiente de digestibilidade aparente para fontes de origem vegetal como silagem de milho, raspa de mandioca e levedura de álcool. Anteriormente, Bureau et al. (1999), iniciou pesquisas sobre a digestibilidade e valor nutricional de alguns alimentos de origem animal utilizados na alimentação dos peixes. Guimarães et al. (2008) relatou a digestibilidade aparente da matéria seca, proteínas, energia e de aminoácidos de ingredientes de origem vegetal como milho, farelo de trigo, casca e farelo de arroz e sorgo, assim como Magalhães et al. (2015), mais recentemente estudou a digestibilidade de novos resíduos agroindustriais que podem ser aproveitados na ração de organismos aquáticos.

O crescimento da aplicação de enzimas na agroindústria é influenciado pelo aumento na demanda dessas biomoléculas associadas à formulação de rações animais. O mercado de enzimas em alimentos compostos para animais tem aumentado bastante devido ao custo cada vez maior das matérias-primas e a busca por ingredientes alternativos (Sá-Pereira et al., 2008). As enzimas também são empregadas para reduzir a contaminação ambiental com nutrientes nas fezes, como fósforo, nitrogênio, cobre e zinco (Ahuja et al., 2004).

Assim, estudos sobre alternativas para a melhoria de aproveitamento de rações para a produção animal e a aquicultura são necessários. O uso de novas fontes de ingredientes vem sendo avaliado há alguns anos, cabe agora à biotecnologia estudar maneiras que possam melhorar a disponibilidade destes novos ingredientes e maximizar a produção. A produção de enzimas exógenas com menor custo, por Fermentação em Estado Sólido, pode viabilizar o uso de enzimas em grande escala para aplicação na

ração animal. Contudo a capacidade de tornar os alimentos melhor disponíveis para o metabolismo animal pode melhorar o desempenho e produtividade na indústria agropecuária. Neste contexto, esta tese compreende os estudos da produção das enzimas fitase e protease por Fermentação em Estado Sólido (FES), caracterização bioquímica e adição à rações para tilápia-do-Nilo, a fim de analisar a digestibilidade de ingredientes de origem vegetal nas dietas, e está dividida nos seguintes capítulos.

O Capítulo – II, intitulado “**Screening of fungi phytases produced by solid state fermentation with different agricultural by-products as substrate**” redigido de acordo com as normas publicação em **Journal of Applied Microbiology**.

Capítulo – III, “**Novel inexpensive fungi proteases: production by solid state fermentation and characterization**” enviado para revista **Food Chemistry**.

Capítulo – IV, “**Production of *Aspergillus niger* 40018 enzymes with different agroindustrial co-products as substrates**” enviado para a revista **Applied Biochemistry and Biotechnology**.

Capítulo – V, “**Digestible values for Nile tilapia using fungal phytases and proteases produced by solid state fermentation**” redigido de acordo com as normas para ser publicado em **Aquaculture Nutrition**.

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CAPÍTULO II

**SCREENING OF FUNGI PHYTASES PRODUCED BY SOLID STATE
FERMENTATION WITH DIFFERENT AGRICULTURAL BY-PRODUCTS AS
SUBSTRATE**

Abstract: This study was carried out for screening the phytase production using agroindustrial residues as substrate for solid state fermentation (SSF) and several fungal strains. High phytase production was observed for most of the microorganisms studied, as well as very different biochemical characteristics, including activities at specific pH values. The phytases showed very distinct behavior of optimum and stability pH, optimal values of temperature and stability. *A. niger* showed the higher values of enzyme activity. Lyophilized enzyme produced by *A. niger* reached 8,575 U/g and yield of 826,096.50 U/Kg of substrate when fermented in wheat bran. Therefore, the substrate as well as the microorganism strain can affect the biochemical character of the enzyme produced. The high phytase activity and very distinct characteristics established, plus the low cost of substrates, make these fungal phytases potential alternatives for biotechnological industry.

Keywords: Fungal phytase, agroindustrial residues, solid state fermentation, biochemical characteristics

1. Introduction

Phytases catalyse the sequential hydrolysis of myo-inositol P6 (phytic acid) to a series of lower phosphoric esters that release the organic phosphorous. These enzymes are classified based on the first phosphorous to be hydrolyzed, named 3-phytase (E. C. 3.1.3.8) and 6-phytase (E. C. 3.1.3.26). Moreover, since global phosphate reserves are not renewable, phytate-P is an alternative and economical phosphorous source that can be effectively converted to available phosphorous by this enzyme (Cao et al., 2007).

Phytases can be obtained from several sources, including plants, animals, and microorganisms and the annual world market for phytase as a feed additive can reach up to US\$500 million (Schauer and Borriss, 2004). Even though a wide variety of microbial phytases are available, use of these enzymes on an industrial scale is still limited by their high production costs and the fact that their activity is often limited to a restricted range of biochemical characteristics. There are several reports on phytase production by submerged fermentation (SmF) and solid state fermentation (SSF) and many authors have compared its productivity values in different fermentation systems trying to explain how the fermentation system affects fungi physiology (Ebune et al., 1995; Papagianni et al., 2000; Krishna and Nokes, 2001; Roopesh et al., 2006).

Although most microbial enzymes are produced by submerged fermentation, solid state fermentation (SSF) using agroindustrial residues allows the production of these biomolecules at a lower cost and is environmentally friendly (Fleuri et al., 2013; Salihi et al., 2012). SSF is also a cost-effective process as it uses agroindustrial wastes, like seeds, peels, husks, bark, and bran to produce valuable bioactive molecules. Another important advantage of SSF is the higher growth rate exhibited by fungi on solid substrate as compared to submerged fermentation; the morphology of filamentous fungi allows them to colonize the substrate surface and matrix in search of nutrients, consequently secreting higher levels of metabolites and enzymes (Barrios-González, 2012).

In this study we evaluated the production of several phytases by GRAS fungal strains using agroindustrial waste, wheat bran and soybean bran as substrates; we also determined optimum and stability pH and temperature for these enzymes, as this

characteristics can indicate biocatalyst performance and lead for specific applications of the enzyme.

2. Materials and methods

2.1. Microorganisms

Fungal samples from lyophilized strains of *Aspergillus niger* (INCQS 40018), *Aspergillus flavipes* (INCQS 40024), *Aspergillus brasiliensis* (INCQS 40036), *Aspergillus oryzae* (INCQS 40068), and *Penicillium roquefortii* (INCQS 40074) from Osvaldo Cruz Institute (FIOCRUZ) were inoculated in Potato Dextrose Agar (PDA) and incubated at 30 °C until satisfactory growth. They were maintained at 4 °C in PDA under a sterile layer of vaseline and sub-cultured every three months. These strains together with 5 more fungi strains from the Bioprocess Laboratory of Biosciences Institute (UNESP) were used for the enzyme production. Strains selected were not yet classified, coded as: 13, 58, 77, 159, and 162.

Fungi selected for this study are considered nontoxic and safe microorganisms by the FDA, designated GRAS, and therefore can be used for human and animal nutrition.

2.2. Enzyme Production

The proteases were obtained by SSF using two different substrates: 1) wheat bran and 2) soybean bran. The culture medium was composed of 10 g of substrate with ratio of 1:1, substrate and water. Erlenmeyer flasks (250 mL) containing the culture medium were sterilized for 15 minutes at 121 °C under 1 atm of pressure, then subsequently inoculated with 2 mL of fungi suspension (10^7 spores/mL), followed by incubation at 30 °C for 120 hours (Fleuri et al., 2014). After incubation, 50 mL of distilled water was added to each flask. The culture medium was mixed manually using a glass rod, then left to stand for 1 hour with occasional stirring. Enzyme extracts were collected in single flasks by filtration through cheesecloth. The crude extract enzyme activities were determined as described below.

2.3. Phytase activity

The phytase activity was determined using *p*-nitrophenyl phosphate as substrate, 1000 μ L of 5 mmol L⁻¹ *p*-nitrophenyl phosphate were added to 500 μ L of acetate buffer

pH 5.0 (0.8 mol L^{-1}) and 500 μL of crude enzyme extract. The system was incubated for 10 minutes at 37 °C. The reaction was stopped with 2 mL of sodium hydroxide (0.1 mol L^{-1}). Phytase activity unit was the quantity of enzyme necessary to release one μmol of *p*-nitrophenol per reaction minute at 410 nm. The control sample was determined with denaturated crude enzyme extract (Stockmann et al., 2003).

2.4. Enzymatic kinetics

Maximum enzyme activity kinetics of selected fungi strains in wheat bran and soybean bran were performed at different fermentation times (from 72 to 168 hours) by SSF under the conditions established above in the enzyme production.

2.5. Biochemical characterization

For biochemical characterization, the optimal activity and stability of enzymes at different pH and temperature were tested using enzymes produced by SSF in both substrates at the best fermentation time according production kinetics above.

Effect of temperature on activity and stability

The optimum temperature was determined as described for each enzyme activity (pH 5.0) at different temperatures, as follows: 20, 30, 37, 40, 50, 60, 70, 80, and 90 °C.

The temperature stability was determined by incubating the crude enzyme extract at different temperatures for 1 hour, followed by determining the residual activity as described in the section above for phytase activity.

Effect of pH on activity and stability

Optimum pH was determined as described for each enzyme activity (37 °C) using buffer solutions at different pH values as follows: 0.1 mol L^{-1} acetate buffer pH 4.0 and 5.0; 0.1 mol L^{-1} sodium phosphate buffer pH 6.0 and 7.0; and 0.1 mol L^{-1} borax-boric acid buffer pH 8.0 and 9.0.

The pH stability was determined by incubating the enzyme in buffer solutions at different pH values at 30 °C for 24 hours, followed by determining the residual activity as described in the section on phytase activity.

3. Results and discussion

Phytase production by solid state fermentation (SSF) was assessed with both soybean and wheat bran as substrate for 10 fungi strains. On the soybean bran substrate, enzyme activity was not assessed for *Aspergillus oryzae*, *Penicillium roquefortii*, 58, 77, 159, and 162, as these strains did not show satisfactory growth on this substrate. Likewise, the fungus coded 13 did not have satisfactory growth in wheat bran.

The initial analysis at pH 5.0 (0.8 mol L⁻¹) and 37 °C showed increasing levels of enzymatic activity (in U/mL) by SSF for 159 (0.19), 162 (0.23), *A. flavipes* (1.09), 77 (1.73), *A. brasiliensis* (3.01), 58 (3.85), *A. niger* (5.02) and *A. oryzae* (7.16) when the substrate was wheat bran. *Penicillium roquefortii* did not show enzyme activity when fermented in wheat bran. For soybean bran, increasing enzymatic activity levels were observed for strains 13 (0.80), *A. flavipes* (2.31), *A. brasiliensis* (3.30) and *A. niger* (4.23).

Enzymatic production kinetics of all strains was evaluated. Our results showed that there was no consistent pattern of phytase production over the course of fermentation time for the different fungi, with the highest levels of phytase activity associated with different fermentation times for different strains. For a same strain, changing the substrate also led to differences in phytase activity during fermentation (Figure 1). If on the one hand the observed differences in activity reflect underlying adaptations in the molecular and physiological machinery of each strain, on the other knowledge of the specific biological mechanisms associated with this process is not required to achieve optimal levels of biomolecule production by SSF. However, this information will also be of key importance to design efficient methods for strain improvement for SSF and create new technologies and new enzymes, as two different peaks of activity for the same strain and substrates may lead to different biomolecules. Barrios-González (2012) stated that specific SSF environmental marks induce higher transcription of the specific transcription factor of the fungus as well as the biosynthetic genes, and achieve higher production of enzymes.

Fermentation times associated with highest enzymatic activity were elected for further biochemical characterization. We did not pursue further biochemical characterization of *A. flavipes* fermented in soybean bran and fungi 159, 162 and *P. roquefortii* as these strains were very unstable when fermented, showing inconsistent

enzyme activities. In those cases where similar activity was observed for more than one fermentation time, the shortest time was elected for analysis. The optimum temperatures for the fungi studied followed a normal pattern for enzymatic activity, which increased until it reached an optimum temperature of 37 °C, subsequently decreasing at higher temperatures, possibly indicating protein denaturation (Figure 2a). *A. niger* and 58 fermented in wheat bran showed two values for optimum temperature, 37 and 70 °C, and 37 and 50°C, respectively, what can demonstrate the presence of isozymes. The pattern for stability temperature of the phytases studied was very distinct, demonstrating for some fungi stability at high temperatures like 80 °C (Figure 2b). Similar results were found for fungal phytases of *Aspergillus niger* although with maximum stability activity at 60 °C (Bhavsar et al., 2013). Same authors stated that phytases in solid fermentation are less thermostable as compared to submerged fermentation. Differently than majority of the plant phytases that are irreversibly inactivated at temperatures above 70 °C (Cao et al., 2007), some of our phytases produced retained significant activity at high temperatures (Figure 2b). Crude thermozyms, namely those enzymes that support high temperatures, can be useful in those food processes where high temperatures are required. They also reduce the risk of microbial contamination, reduce viscosity, and improve substrate solubility since they are able to act at higher temperatures.

Fungi phytases showed acid optimum pH (5.0). Even though, fungi with enzyme activity below 200 U/mL had basic optimum pH (9,0) (Figure 2c). Initial methodology for phytase activity used acetate buffer 0.8 mol L⁻¹. However, buffers with 0.1mol L⁻¹ were used for tests of biochemical characteristics with different pH and, it was observed higher enzyme activity when the crude extract was added to those buffers of lower concentration. So, concentration also interfered on the enzyme activity for the studied fungus, showing levels of phytase activity extremely higher than those found on previous studies for biochemical characterization (Greiner and Konietzny, 2006; Rani and Ghosh, 2011; Morales et al., 2011). Besides *A. niger* fermented in wheat bran and *A. brasiliensis* fermented in both substrates, only subtle differences in phytase activity were observed for optimum and stability pH (Figure 2c and 2d). Phytase stability of enzyme produced by *A. niger* (wheat bran), *A. brasiliensis* (soybean bran) and *A. brasiliensis* (wheat bran) decreased after pH 5.0, and reached almost zero at pH 6.0 (Figure 2d). The optimum and stability pH profile of phytases determine their ability to

act efficiently in several environments, especially through the digestive tract of animals, where this enzyme is mostly applicable. Fungal phytases degrade phytate in stomach at low pH (Bhavsar et al., 2013), consequently the enzymes produced in this study are suitable to fit this criteria. The observed diversity in the biochemical behavior of the enzymes produced by different strains and substrates should translate into a variety of possible applications.

Additionally, the production of these enzymes is substrate specific, consistent with our observation that their production was different for wheat bran and soybean bran. *A. niger* phytase showed different behavior when fermented in wheat bran and soybean bran (Figure 2c and 2d). The other fungi evaluated here were stable at pH values greater than 6 (Figure 2d), but with lower enzyme activity. According Greiner and Konietzny (2006), stability of most plant phytases decrease at pH values above 8.0 and below 3.0, unlike then the microbial phytases in this study. These differences highlight the contribution of type of substrate for the characteristics of the enzyme produced. The observation of high phytase activity at acid pH values it is an opportunity of using these enzymes as animal nutrition additives and improve feed availability, especially through the acid digestion in the stomach.

A. niger and *A. brasiliensis* were selected for upscale phytase production given the high activity of the former and pH characteristic of the latter. Lyophilized *A. niger* reached 8,575 U/g of enzyme activity and yield of 826,096.50 U/Kg of substrate, values higher than those previously reported by Vats and Banerjee (2005) on submerged fermentation; Rodríguez-Fernández et al. (2012) in orange peel; Gupta et al. (2014) in wheat bran; Singh et al. (2015) in sugarcane bagasse; and similar to those achieved by Cao et al. (2007) and Bhavsar et al. (2013). Yet, yield of lyophilized phytase from *A. brasiliensis* (860,576 U/Kg of substrate) was higher than lyophilized phytase from *A. niger*. In light of these results, we estimate that phytase production by these fungi using SSF and different substrates can be low cost and used for several applications.

4. Conclusion

The high yield of phytases produced by SSF and the diverse nature of the biochemical characteristics found for the fungal strains analyzed in this study, including those with high activity under specific conditions, highlight their promising use for a wide range of industrial applications, besides the utilization of different agroindustrial residues as substrates can be environmentally friendly and reduce cost of production.

Acknowledgements

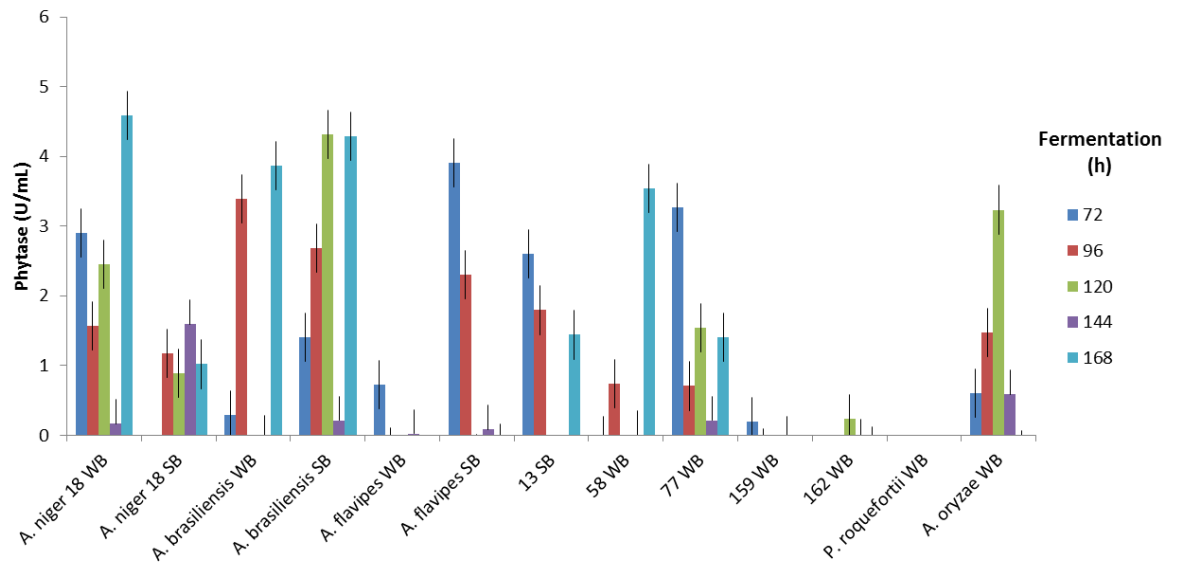
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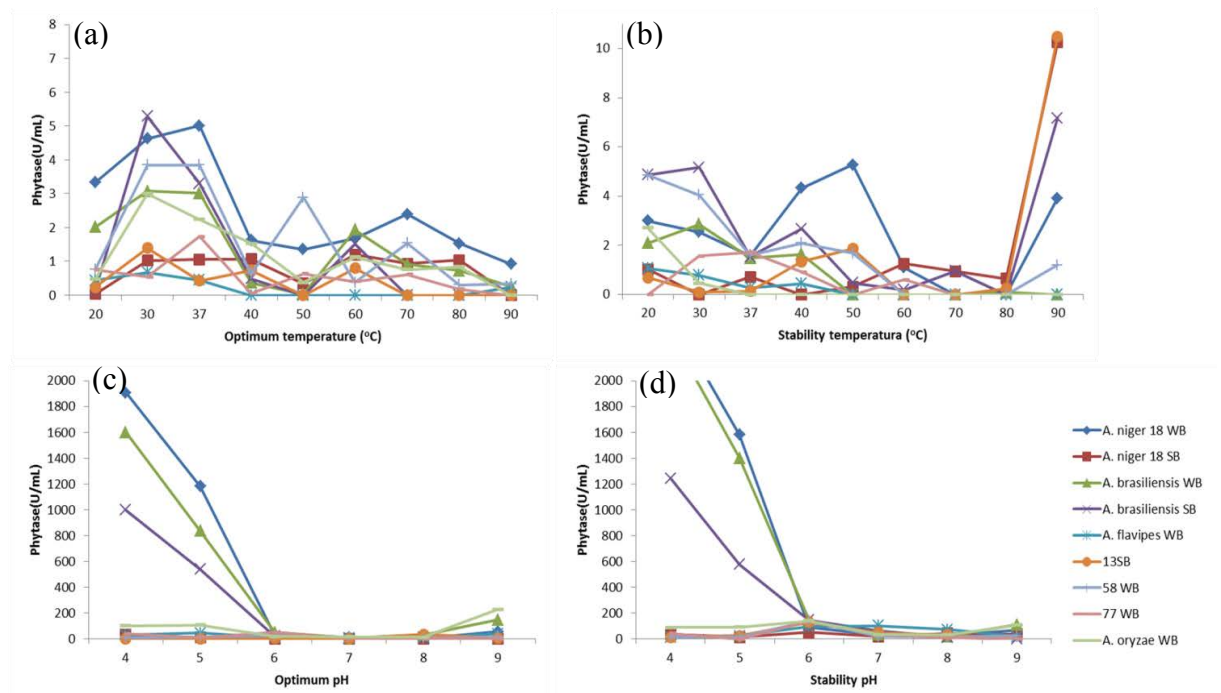
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5. Figures



*Standard error by procmixed SAS statistics 0.3235

Figure 1. Kinetics of phytase production by different fungal strains in wheat bran (WB) and soybean bran (SB).



* Standard error for biochemical characteristics measured by procmixed SAS statistics was between 0.3880 to 20.59 U/mL.

Figure 2. Influence of temperature (a – optimum, and b – stability) and pH (c – optimum, and d – stability) on phytase activity produced by solid state fermentation with wheat bran (WB) and soybean bran (SB) as substrate.

CAPÍTULO III

NOVEL INEXPENSIVE FUNGI PROTEASES: PRODUCTION BY SOLID STATE FERMENTATION AND CHARACTERIZATION

Abstract: A comparative study was carried out for proteases production using agroindustrial residues as substrate for solid state fermentation (SSF) of several fungal strains. High protease production was observed for most of the microorganisms studied, as well as very different biochemical characteristics, including activities at specific temperatures and a wide range of pH values. The proteases showed very distinct behavior of optimum pH, optimal values of temperature and stability at 50 °C. *A. oryzae* showed stability at all pH values studied. *P. roquefortii* and *A. flavipes* presented optimum activity at temperatures of 50 and 90 °C, respectively. Lyophilized protease from *A. oryzae* reached 1,251.60 U/g and yield of 155,010.66 U/Kg of substrate. Therefore, the substrate as well as the microorganism strain can affect the biochemical character of the enzyme produced. The high protease activity and stability established plus the low cost of substrates, make these fungal proteases potential alternatives for the biotechnological industry.

Keywords: Fungal protease, agroindustrial residues, solid state fermentation, biochemical characteristics

1. Introduction

Proteases (EC 3.4.23.18) are one of most important and abundant enzymes produced by the biotechnology industry, consisting of more than 25% of biomolecules produced for industrial application and 60% of the whole enzyme market (Kumar, Sahai & Bisaria, 2012; Ramakrishna, Rajasekhar & Reddy, 2010). Proteases contribute to several chemical and biochemical reactions that take place in the food, beverage, pharmaceutical, cosmetics, and other industries, hydrolyzing peptide bonds in proteins and polypeptides (Uyar & Baysal, 2004). In the animal food industry, where protein-based food is often characterized by the presence of several anti-nutritional peptides and low nutritional availability, proteases can be particularly helpful by improving protein digestibility, as well as reducing environmental pollution (Pariza & Cook, 2010).

Proteases can be obtained from several sources, including plants, animals, and microorganisms (Akhtar et al., 2013). Even though a wide variety of microbial proteases are available, use of these enzymes on an industrial scale is still limited by their high production costs and the fact that their activity is often limited to a restricted range of biochemical characteristics. Therefore, studies involving other microbial enzyme sources are necessary.

Most microbial enzymes are produced by submerged fermentation, however, solid state fermentation (SSF) using agroindustrial residues allows the production of these biomolecules at a lower cost and is environmentally friendly (Fleuri et al., 2013; Salihu, Alama, Karim & Salleh, 2012). SSF is also a cost-effective process as it uses agroindustrial wastes, like seeds, peels, husks, bark, and bran to produce valuable bioactive molecules.

Another important advantage of SSF is the higher growth rate exhibited by fungi on solid substrate as compared to submerged fermentation; the morphology of filamentous fungi allows them to colonize the substrate surface and matrix in search of nutrients, consequently secreting higher levels of metabolites and enzymes (Barrios-González, 2012). Filamentous fungi of industrial interest include several species of *Aspergillus* sp. (Van Der Hombergh, Van de Vondervoortb, Fraissinet-Tachetb & Visserb, 1997), as they are capable of substrate adaptation and produce several metabolites with high biological activities. The genus *Aspergillus* is also considered nontoxic, recognized as a safe microorganism by the Food and Drug Administration

(FDA), denominated Generally Recognized as Safe (GRAS), and used for human and animal nutrition (Gotou, Shinoda, Mizuno & Yamamoto, 2009; Morita et al., 2010; Vishwanatha, Rao & Singh, 2009).

In this study we evaluated the production of inexpensive proteases from several GRAS fungal strains using agroindustrial waste, wheat bran and soybean bran. Because the biochemical characterization of enzymes indicates biocatalyst performance and can predict the successful use of the enzyme for specific applications (Couto & Sanromán, 2006; Okino-Delgado & Fleuri, 2014), we also determined optimum and stability pH and temperature for these proteases.

2. Materials and methods

2.1. Microorganisms

Fungal samples from lyophilized strains of *Aspergillus niger* (INCQS 40018), *Aspergillus niger* (INCQS 40065), *Aspergillus flavipes* (INCQS 40024), *Aspergillus brasiliensis* (INCQS 40036), *Aspergillus oryzae* (INCQS 40068), and *Penicillium roquefortii* (INCQS 40074) from Osvaldo Cruz Institute (FIOCRUZ) were inoculated in Potato Dextrose Agar (PDA) and incubated at 30 °C until satisfactory growth. They were maintained at 4 °C in PDA under a sterile layer of Vaseline and sub-cultured every three months. These strains together with 10 more fungi strains from the Bioprocess Laboratory of Biosciences Institute (UNESP) were used for the enzyme production initial study. Strains selected were: two new strains of *Aspergillus niger* (01 and 40015), and eight strains not classified, coded as: 13, 51, 58, 68, 77, 129, 159, and 162.

2.2. Enzyme Production

The proteases were obtained by SSF using two different substrates: 1) wheat bran and 2) soybean bran. The culture medium was composed of 10 g of substrate with a ratio of 1: 1, substrate and water. Erlenmeyer flasks (250 mL) containing the culture medium were sterilized for 15 minutes at 121 °C under 1 atm of pressure, then subsequently inoculated with 2 mL of fungi suspension (10^7 spores/mL), followed by incubation at 30 °C for 120 hours (Fleuri et al., 2014). After incubation, 50 mL of distilled water was added to each flask. The culture medium was mixed manually using a glass rod, then left to stand for 1 hour with occasional stirring. Enzyme extracts were

collected in single flasks by filtration through cheesecloth. The filtrate was used as crude enzyme extract and protease activity were determined as described below.

2.3. Protease activity

The protease activity was determined using azocasein as substrate, with modifications (Charney & Tomarelli, 1947). The reaction media were 0.5 mL of azocasein 0.5% (m/v) in sodium phosphate buffer pH 7.0 (0.1 mol L^{-1}) and 0.5 mL of crude enzyme extract, which were incubated for 40 minutes at $37 \text{ }^{\circ}\text{C}$. The reaction was stopped with 0.5 mL trichloroacetic acid (TCA) 10% (m/v) and centrifuged at $4,677 \times g$ for 15 minutes at $15 \text{ }^{\circ}\text{C}$; 1 mL of potassium hydroxide (5 mol L^{-1}) was added to 1 mL of the filtrate. The protease activity unit was defined as the quantity of enzyme necessary to increase 0.1 of absorbance at 428 nm in the assay conditions.

2.4. Enzymatic kinetics

Maximum enzyme activity kinetics of selected fungi strains in wheat bran and soybean bran were performed at different fermentation times (from 72 to 168 hours) by SSF under the conditions established above in the enzyme production.

2.5. Biochemical characterization

For biochemical characterization, the optimal activity and stability of enzymes at different pH and temperature were tested using enzymes produced by SSF in both substrates at the best fermentation time according production kinetics above.

Effect of temperature on activity and stability

The optimum temperature was determined as described for each enzyme activity (pH 7.0 to protease) at different temperatures, as follows: 20, 30, 37, 40, 50, 60, 70, 80, and $90 \text{ }^{\circ}\text{C}$.

The temperature stability was determined by incubating the crude enzyme extract at different temperatures for 1 hour, followed by determining the residual activity as described in the section above for protease activity.

Effect of pH on activity and stability

Optimum pH was determined as described for each enzyme activity ($37 \text{ }^{\circ}\text{C}$) using buffer solutions at different pH values as follows: 0.1 mol L^{-1} acetate buffer pH 4.0 and 5.0; 0.1 mol L^{-1} sodium phosphate buffer pH 6.0 and 7.0; and 0.1 mol L^{-1} borax-boric acid buffer pH 8.0 and 9.0.

The pH stability was determined by incubating the enzyme in buffer solutions at different pH values at 30 °C for 24 hours, followed by determining the residual activity as described in the section on protease activity.

3. Results and discussion

Protease production by solid state fermentation (SSF) was assessed with both soybean and wheat bran as substrate for 16 fungi strains. On the soybean bran substrate, enzyme activity was not assessed for *Aspergillus oryzae*, *Penicillium roquefortii*, 58, 77, 159, and 162, as these strains did not show satisfactory growth on this substrate.

The initial analysis at pH 7.0 and 37 °C showed increasing levels of enzymatic activity (in U/mL) by SSF for *A. oryzae* (5.10), 159 (11.88), *A. brasiliensis* (11.89), 77 (13.74), *Penicillium roquefortii* (25.19), 58 (27.10), and *A. flavipes* (27.78) when the substrate was wheat bran. For soybean bran, increasing enzymatic activity levels were observed for strains 13 (8.24), *A. brasiliensis* (10.13), and *A. flavipes* (21.99). Although *A. niger* 40018 (fermented with both substrates) and 162 showed satisfactory growth, protease activity was low for these strains in preliminary tests. For *A. niger* 40065, *A. niger* 01, *A. niger* 40015, 51, 68, and 129 protease activity was not detected.

With the exception of those strains that did not have satisfactory growth when fermented in soybean bran, enzymatic production kinetics of all other strains was evaluated. Our results showed that there was no consistent pattern of protease production over the course of fermentation for the different fungi, with the highest levels of protease activity associated with different fermentation times for different strains. For a same strain, changing the substrate also led to differences in protease activity during fermentation (Figure 1). If on the one hand the observed differences in activity reflect underlying adaptations in the molecular and physiological machinery of each strain, on the other knowledge of the specific biological mechanisms associated with this process is not required to achieve optimal levels of biomolecule production by SSF.

Fermentation times associated with highest enzymatic activity were elected for further biochemical characterization. We did not pursue further biochemical characterization of proteases from *A. flavipes* as this strain was very unstable when fermented in soybean bran. In those cases where similar activity was observed for more

than one fermentation time, the shortest time was elected for analysis. The optimum and stability temperatures for the fungi studied followed a normal pattern for proteolytic activity, which increased until it reached an optimum temperature of 50 °C, subsequently decreasing at higher temperatures, possibly indicating protein denaturation (Figure 2a–b). Similar results were found for fungal proteases of *Myceliophthora* sp. (Zanphorlin et al., 2011), *Colletotrichum gloesporioides* (Dunaevsky, Matveeva, Beliakova, Domash & Belozersky, 2007), *Trichoderma reesei* QM9414 (Dienes et al., 2007), and *Fusarium culmorum* (Pekkarinen, Jones & Niku-Paavola, 2002). *A. flavipes* and *P. roquefortii* showed two peaks of optimal protease activity at 50 °C and 90 °C (Figure 2a), indicating the presence of isozymes.

Fungal proteases of *A. oryzae*, *A. flavipes*, *Penicillium roquefortii*, 13, 159, and 162 showed optimum activity at alkaline pHs, whereas *A. niger* 40018 and *A. brasiliensis* fermented in wheat bran had optimum activity at acid pHs. *A. brasiliensis* fermented with soybean bran and 58 had optimum activity at pH 7. Only subtle differences in protease activity were observed for *A. niger* 40018 fermented in soybean bran and 77 fermented in wheat bran (Figure 2c). The observed diversity in the biochemical behavior of the enzymes produced by different strains and substrates should translate into a variety of possible applications. Alkaline proteases are characterized not only by a high activity at basic pH, but also high stability at high temperatures and low water activity (Takami, Akiba & Horikishi, 1990). Additionally, the production of these enzymes is substrate specific, consistent with our observation that their production was different using wheat bran and soybean bran. *A. oryzae* protease showed high stability at a wide range of pHs in this study (4.0–9.0), a range wider than that previously observed for this same strain under fermentation conditions similarly involving SSF of wheat bran and soybean bran (Castro & Sato, 2014). The other fungi evaluated here were stable at pH values greater than 6 (Figure 2d). *A. niger* 40018 was stable at pH 6.0 and 8.0 using soybean bran as substrate, with a high activity of 51.35 U/mL and 115.6 U/mL, respectively (Figure 2d), indicating the presence of isozymes. Otherwise, optimal initial pH and temperature for acid protease from *Aspergillus niger* fermented in rice stillage were determined to be 4.0 and 30 °C, respectively (Yang & Lin, 1998); and lower activity with rice combined with C- and N-sources when compared to our fermentation exclusively in soybean bran. These

differences highlight the contribution of type of substrate for the characteristics of the enzyme produced. The observation of high protease activity at alkaline pH values indicates the possibility of using these enzymes as animal nutrition additives and improving feed availability, especially through the intestinal alkaline digestion.

A. oryzae and *A. flavipes* were selected for upscale protease production given the high activity of the former and optimum activity of the latter at 90 °C. Lyophilized protease from *A. oryzae* reached 1,251.60 U/g of enzyme activity and yield of 155,010.66 U/Kg of substrate, values higher than those previously reported (Castro & Sato, 2014; Sandhya, Sumantha, Szakacs & Pandey, 2005) and similar to those achieved by Belmessikh, Boukhalfa, Mechakra-Maza, Gheribi-Aoulmi & Amrane (2013). Yet, yield of lyophilized protease from *A. oryzae* was 8.7 times higher than lyophilized protease from *A. flavipes* (17,752.46 U/Kg of substrate). In light of these results, we estimate that protease production by *A. oryzae* using SSF and wheat bran as substrate can be up to 90% less expensive than commercial enzyme produced by this same strain through submerged fermentation.

4. Conclusion

The high levels of protease production by SSF and the diverse nature of the biochemical characteristics found for the fungal strains analyzed in this study, including those with high activity under specific conditions, highlight their promising use for a wide range of industrial applications at low cost using different agroindustrial residues as substrates.

Acknowledgements

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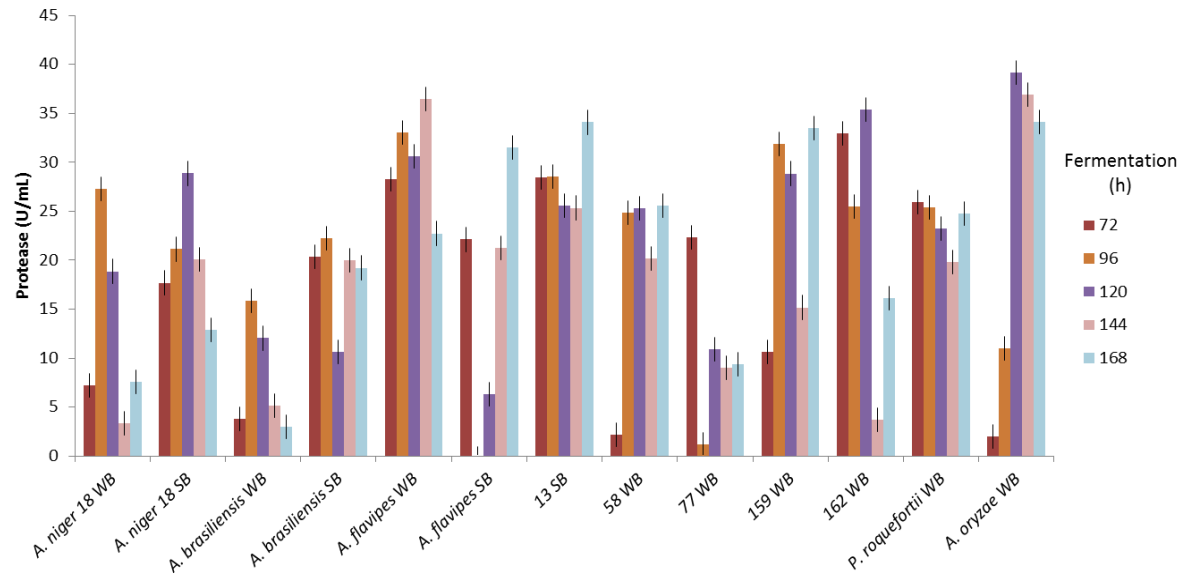
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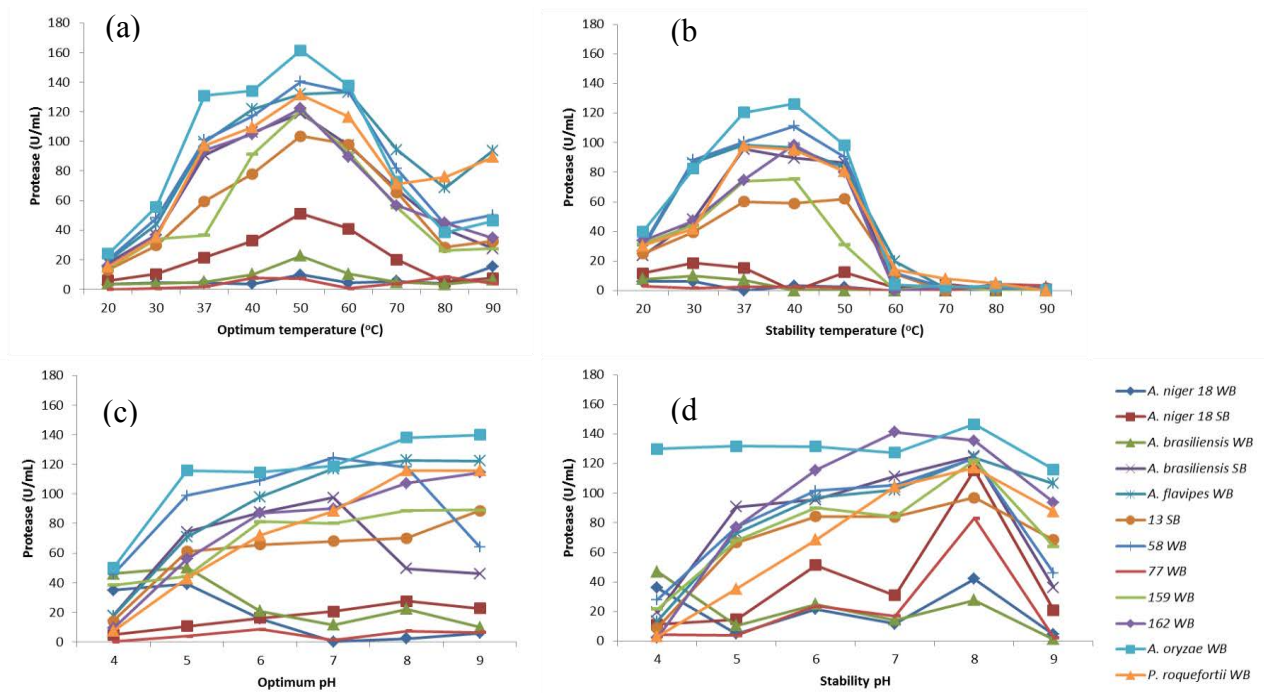
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5. Figures



*Standard error by procmixed SAS statistics 1,1223.

Figure 1. Kinetics of protease production by different fungal strains in wheat bran (WB) and soybean bran (SB).



* Standard error for biochemical characteristics measured by SAS statistics was between 1.41 to 4.62 U/mL.

Figure 2. Influence of temperature (a – optimum, and b – stability) and pH (c – optimum, and d – stability) on protease activity produced by solid state fermentation with wheat bran (WB) and soybean bran (SB) as substrate.

CAPÍTULO IV

PRODUCTION OF *Aspergillus niger* 40018 ENZYMES WITH DIFFERENT AGROINDUSTRIAL CO-PRODUCTS AS SUBSTRATES

Abstract: Utilization of agricultural co-products as substrate for solid state fermentation (SSF) was studied, aimed the achievement of phytases and proteases from *Aspergillus niger* with different biological characteristics. Activity for phytase and protease substantially increased when the different biochemical characteristics were considered in the study. Optimum pH and stability of the phytase with wheat bran as substrate was between 4.0 - 5.0 and optimum temperature of activity was 37°C. Phytase fermented in soybean bran showed constant values at all pHs studied, for optimal and stability. Phytase with both substrates showed stable activity for temperatures higher than 80°C. Protease showed very distinct behavior of optimum pH, acid for wheat bran and basic for soybean bran, respectively and optimal values of temperature and stability at 50°C. Protease produced with wheat bran and soybean bran was stable in the range of pH 7.0 - 9.0, however with higher production yield in wheat bran. As a result, the substrate, as well as, the microorganism species can affect the biochemical character of the enzyme produced.

Keywords: phytase, protease, *Aspergillus niger*, isozymes, Solid fermentation.

1. Introduction

Phytase catalyzes the hydrolyses of inorganic phosphorous from phytate and phytic acid, increasing its availability for animal nutrition. Moreover, phytate can act as an anti-nutritional factor for animal feeding, since it binds to proteins, amino acids, lipids and minerals, interacting with digestives enzymes and reducing its activity, and influencing nutritional availability. Phytase classification is based on the first phosphorous to be hydrolyzed, named 3-phytase (E. C. 3.1.3.8) and 6-phytase (E. C. 3.1.3.26). Many researches showed the benefit of phytase supplementation on animal nutrition [1,2]. At the same time, adding phytase to animal ration can reduce phosphorous and nitrogen release to environment [3], reducing environmental problems.

Proteases (EC 3.4.23.18) hydrolyze peptide bounds, so protein became more digestible for the animal. These enzymes constitute important group of enzymes commercially produced [4], they have different industrial applications and they are being used as animal feed additive recently. Nowadays, proteases constitute more than 25% of biomolecules produced for industrial usage. Protein based food with several anti-nutritional factors and low nutritional availability are common in the animal feed industry. High levels of proteases inhibitors are found, mainly in soybean bran, which is one of the most important protein ingredients in animal fed. Studies with vegetable ingredients and enzyme addition in diets for animals showed promising results [5,6,7]. Additionally, proteases can also help with reduction of environmental pollution. Moreover, soybean bran fermentation with the right microorganism and physic-chemical environment can be a solution for protease production and also enhancement of soybean nutritional values.

Even with a wide variety of microbial phytases and proteases, use of these enzymes on industrial scale is still limited due to high production costs, low activity, and limited biochemical characteristics, which facilitates searching of other microbial enzyme sources. The bioconversion of agro industrial residues for enzymes production, as well as other value-added products occupies prominent position, mainly due to the possibility of obtaining biocatalysts at lower cost using environmentally friendly techniques [8].

In this scenario, filamentous fungi are versatile for enzymes production by solid state fermentation (SSF). One of the most important advantages of SSF is a consequence of the different physiology shown by fungi and other microorganisms on a solid substrate [9]. This type of fermentation can use agricultural and agro industrial waste as substrates, which present low value, are nutrient rich, and have restricted water availability that helps to select contaminants, especially bacteria and yeasts. The achievement of the final product by SSF is easier and the amount of waste is minimized [10,11]. *Aspergillus niger* is considered nontoxic and safe microorganism by the Food and Drug Administration (FDA), designated Generally Recognized as Safe (GRAS), therefore it can be used for human and animal nutrition [12,13,14]. Thus, the present study focused on phytase and protease production and characterization with two substrates using one strain of *Aspergillus niger*.

2. Materials and methods

2.1. Microorganisms

Fungal samples from lyophilized strains of *Aspergillus niger* (INCQS 40018), from Osvaldo Cruz Institute (FIOCRUZ) were inoculated in Potato Dextrose Agar (PDA) and incubated at 30°C until satisfactory growth. They were maintained at 4°C in PDA under sterile layer of vaseline and sub-cultured every three months. This strain was elected between 16 different fungus previously studied and it was considered the best enzyme producer at experimental conditions.

2.2. Enzymes Production

The phytases and proteases from *A. niger* 40018 were obtained by SSF using two different substrates: 1) wheat bran and 2) soybean bran [15]. The culture medium was composed of 10 g of substrate with 100% (m/v). Erlenmeyer flasks (250 mL) containing the culture medium were sterilized for 15 minutes at 121°C under 1 atm of pressure, then subsequently inoculated with 2 mL of fungi suspension (10^7 spores/mL), followed by incubation at 30°C for 120 hours. After

incubation, 50 mL of distilled water was added to each flask. The culture medium was mixed manually using a glass rod, then left stand for 1 hour with occasional stirring. Enzyme extracts were collected in single flasks by filtration through cheesecloth. The crude extract enzymes activities were determined as described below.

2.3. Phytase activity

The phytase activity was determined using p-nitrophenyl phosphate as substrate, 1000 μL of 5 $\text{mmol}\cdot\text{L}^{-1}$ p-nitrophenyl phosphate were added to 500 μL of acetate buffer pH 5.0 (0.8 $\text{mol}\cdot\text{L}^{-1}$) and 500 μL of crude enzyme extract. The system was incubated for 10 minutes at 37°C. The reaction was stopped with 2 mL of sodium hydroxide (0.1 $\text{mol}\cdot\text{L}^{-1}$). The phytase activity unit was the quantity of enzyme necessary to release one μmol of p-nitrophenol per reaction minute at 410 nm. The control sample was measured with denaturized crude enzyme extract [16].

2.4. Protease activity

The protease activity was determined using azocasein as substrate, with modifications [17]. The reaction media was 0.5 mL of azocasein 0.5% (m/v) in sodium phosphate buffer pH 7.0 (0.1 $\text{mol}\cdot\text{L}^{-1}$) and 0.5 mL of crude enzyme extract, they were incubated for 40 minutes at 37°C. The reaction was stopped with 0.5 mL TCA 10% (m/v) and centrifuged at 6,000 rpm for 15 minutes at 15°C; 1 mL of potassium hydroxide (5 $\text{mol}\cdot\text{L}^{-1}$) was added to 1 mL of the supernatant. The protease activity unit was defined as the quantity of enzyme necessary to increase 0.1 of absorbance at 428 nm in the assay conditions.

2.5. Enzymatic kinetics

Maximum enzymes activity kinetics of *A. niger* 40018 in wheat bran and soybean bran were performed at different fermentation times (from 72 to 168 hours) by SSF under the conditions established above in the enzyme production.

2.6. Characterization

The optimal activity and stability of enzymes at different pH and temperature were tested using enzymes produced by SSF in both substrates at the best fermentation time according production kinetics above.

Effect of temperature on activity and stability

The optimum temperature was determined as described for each enzyme activity (pH 5.0 to phytase and pH 7.0 to protease) at different temperatures, as follows: 20, 30, 37, 40, 50, 60, 70, 80, and 90°C.

The temperature stability was determined by incubating the crude enzyme extract at different temperatures for 1 hour, followed by determining the residual activity as described in section above for phytase and protease activity.

Effect of pH on activity and stability

Optimum pH was determined as described for each enzyme activity (37°C) using buffer solutions at different pH values as follows: 0.1 mol L⁻¹ acetate buffer pH 4.0 and 5.0; 0.1 mol L⁻¹ sodium phosphate buffer pH 6.0 and 7.0; and 0.1 mol L⁻¹ borax-boric acid buffer pH 8.0 and 9.0.

The pH stability was determined by incubating the enzyme in buffer solutions at different pH values at 30°C for 24 hours, followed by determining the residual activity as described in section phytase and protease activity.

3. Results and Discussion

Initial phytase activity from *A. niger* 40018 obtained by solid-state fermentation (SSF) on wheat bran and soybean bran were 4.05 and 4.23 U/mL, respectively. The protease activity was 4.03 and 0.95 U/mL for wheat bran and soybean bran, respectively. Phytase produced by *Aspergillus niger* shown activity from 0.16 to 8.09 U/mL, with increase of activity when wheat bran was added as substrate [18]. Other study using crude protease extract from *Aspergillus oryzae* produced by solid state fermentation presented activity of 15.478 U/mL, however they used wheat bran as substrate enhanced with calcium chloride [19]. This study

achieved phytase higher production with 168 and 144 hours of fermentation, for wheat bran and soybean bran, respectively. Protease kinetics showed more activity at 96 hours using wheat bran as substrate and 120 hours for soybean bran (Figure 1). Likewise, kinetics production of isozymes from *Aspergillus niger* establish improvement activity at 96 and 120 hours [20].

The optimum temperature for the phytase of *A. niger* studied was 37°C (Figure 2a), therefore, for both substrates, especially soybean bran, the enzyme activity was stable at higher temperatures of 90°C (3.92 and 10.24 U/mL, for wheat bran and soybean bran, respectively), which allows the utilization of this enzyme in situations where the high temperature can be a limitation in the process (Figure 2b). Although the stability of phytase decreases for temperatures higher than 60°C, the presence of enzyme activity at 90°C in the stability studies demonstrates the peculiar characteristics of these biomolecules. Plants and microbial phytases [21] are optimum and stable at pH 4.5 to 6.0 and at temperatures lower than 60°C. However, extremely variations on pH and high temperature can reduce phytase activity and it may cause enzyme denaturation. In this study, phytase activity produced in wheat bran was higher at pH 4.0 (1,908 U/mL) when compared to soybean bran (33.18 U/mL) (Figure 2c). Furthermore, the stability of phytase activity from *A. niger* 40018, in wheat bran and soybean bran as substrate decreased at pH higher than 5.0, and it was nearly zero at pH 6.0 (Figure 2d). Several studies showed values of optimum pH and temperature for phytase between 1.5 - 5.0 and 45°C, respectively [20,22,23]. Phytase activity for *Aspergillus niger* ranged from 50 to 103 U/mg with optimum pH of 5.0 - 5.5 and temperature of 55 - 58°C [24]. However the initial values found were lower, when biochemical characteristics, especially pH, were tested with buffer with lower concentration, the phytase activity for *A. niger* 40018 were much higher than those found on literature. Initial methodology for phytase activity used acetate buffer 0.8 mol L⁻¹. However, buffers with 0.1 mol L⁻¹ were used for tests of biochemical characteristics with different pH and, it was observed higher enzyme activity when the crude extract was added to those buffers of lower concentration. So, concentration also interfered on the enzyme activity for the studied fungus, showing levels of phytase activity extremely higher than those found on previous

studies for biochemical characterization [23,25]. Phytase activity improved from 4.05 up to 2,506.55 U/mL with changes in concentration and pH. Studies regarding concentration, effect of ions and solvents should be a next step in our study of biochemical characteristics for *A. niger* 40018 enzymes.

The optimal temperature for protease produced by *A. niger* 40018 followed a normal pattern for proteolytic activity, raising until optimum temperature of 50°C and decreasing at high temperatures. Similar results were found for fungal proteases of *Myceliophthora* sp. [26], *Colletotrichum gloesporioides* [28], *Trichoderma reesei* QM9414 [28] e *Fusarium culmorum* [29]. Yet, the optimum activity for protease produced using soybean bran as substrate was higher than the activity found for wheat bran, 51.10 and 9.95 U/mL at 50°C, respectively (Figure 3a). Stability temperature for protease followed similar curve as for optimum temperature for the enzymes produced by SSF, as the enzyme activity decreased at temperatures higher than 60°C. The proteases were stable at two temperature, 30 and 40°C; and 30 and 50°C, for wheat bran and soybean bran, respectively (Figure 3b), indicating possible isozymes. Alkaline proteases are characteristic because their high activity at basic pH, specificity for the substrate, resistance to high temperatures and the ability to resist low water activity [30], likewise SSF in our study showed high protease activity at those conditions. *A. niger* 40018 fermented in wheat bran produced protease with decrease activity when incubated at alkaline pH, in opposition, protease produced using soybean bran as substrate presented optimum pH as 8.0 (Figure 3c), what demonstrate very distinct characteristics for the enzyme produced by the same microorganism when different substrates were used for solid fermentation. Values found for optimum pH and temperature for the microbial protease of *Myceliophthora* sp. in others studies were of 9.0 and 40 - 45°C, respectively [27]. *A. niger* 40018 showed high stability at pH 8.0 for soybean bran and wheat bran, with activity of 115.6 and 42.1 U/mL, respectively (Figure 3d). Biochemical characteristics of protease from *Aspergillus oryzae* produced by SSF using agro industrial waste, including wheat bran and soybean bran, were optimum pH 5.0 – 5.5, stability pH 4.5 – 6.0, optimum temperature 55 – 60°C and stability temperature 35 – 45°C; with maximum enzyme activity of 29.27 and 8.20 IU for wheat bran and soybean bran, respectively [31]. Otherwise, acid protease

from *Aspergillus niger* fermented in rice stillage were optimal initial pH 4.0 and temperature 30°C [32]. Protease activity by *A. niger* 40018 in this study were higher, up to 115.6 U/mL, and with very distinct biochemical characteristics.

4. Conclusion

The high phytase and protease activity yield detected, the easy extraction added the abundance and low cost of substrates, and peculiar biochemical characterization, made this fungal enzymes potential alternatives to commercial exogenous additive for industrial usage. Change of substrate for solid fermentation can produce biomolecules, maybe isozymes, with extremely different characteristics that can be applied for several biotechnological applications in animal food industry. *Aspergillus niger* 40018 fermented in wheat bran produced phytases with very high activity. As for protease production, wheat bran and soybean bran produced enzymes with high activity and very distinctive characteristics. This study can also lead to others using different types of substrate, especially agroindustrial waste, to produce cheaper and promising biomolecules that can be used to improve animal husbandry.

Acknowledgements

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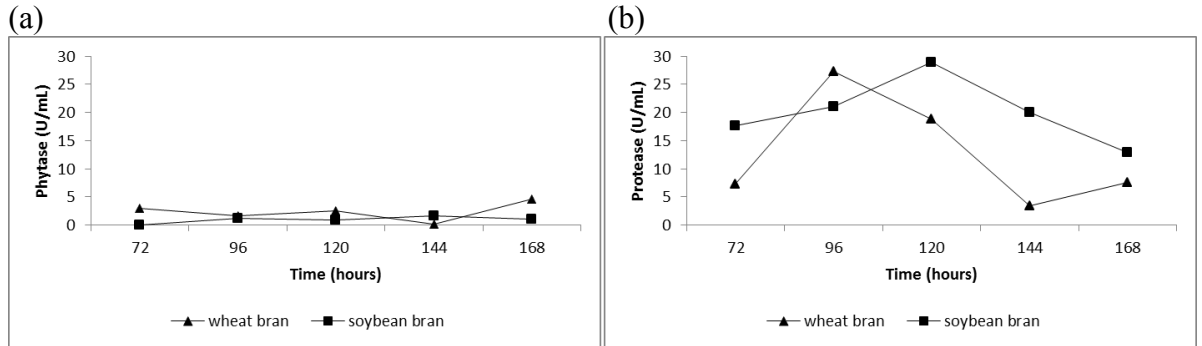
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5. Figures



Figures 1. Kinetics of phytase (a) and protease (b) produced by *A. niger* 40018 fermented in wheat bran and soybean bran.

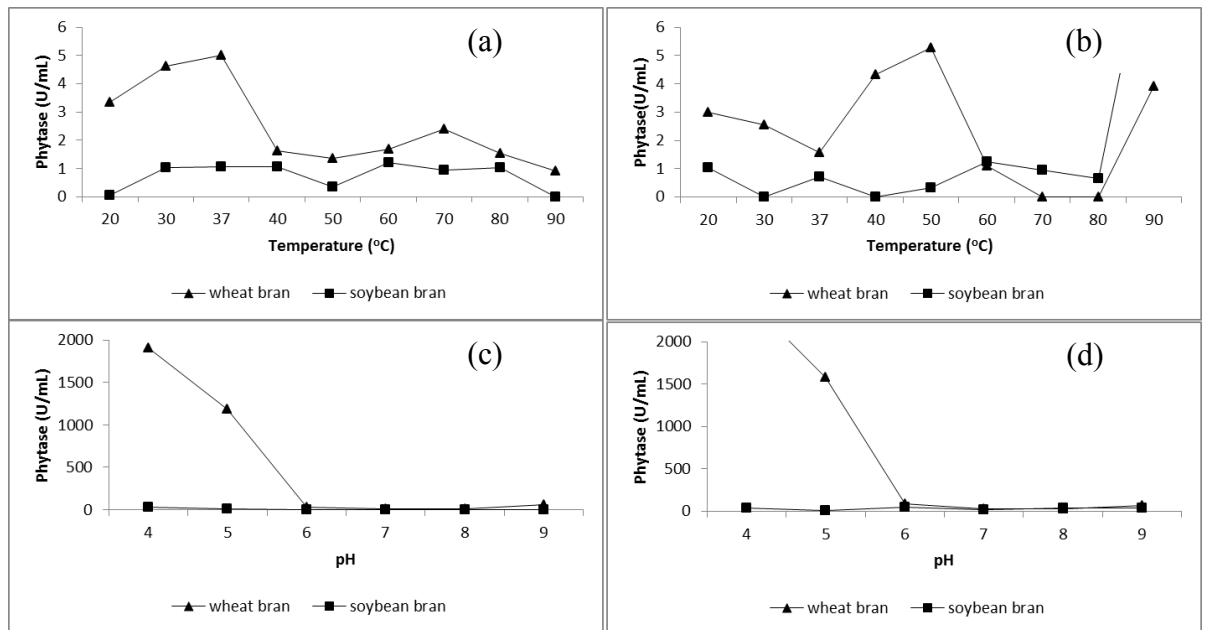


Figure 2. Influence of temperature (a – optimum, and b – stability) and pH (c – optimum, and d – stability) on phytase activity produced by solid state fermentation with wheat bran and soybean bran as substrate using *A. niger* 40018.

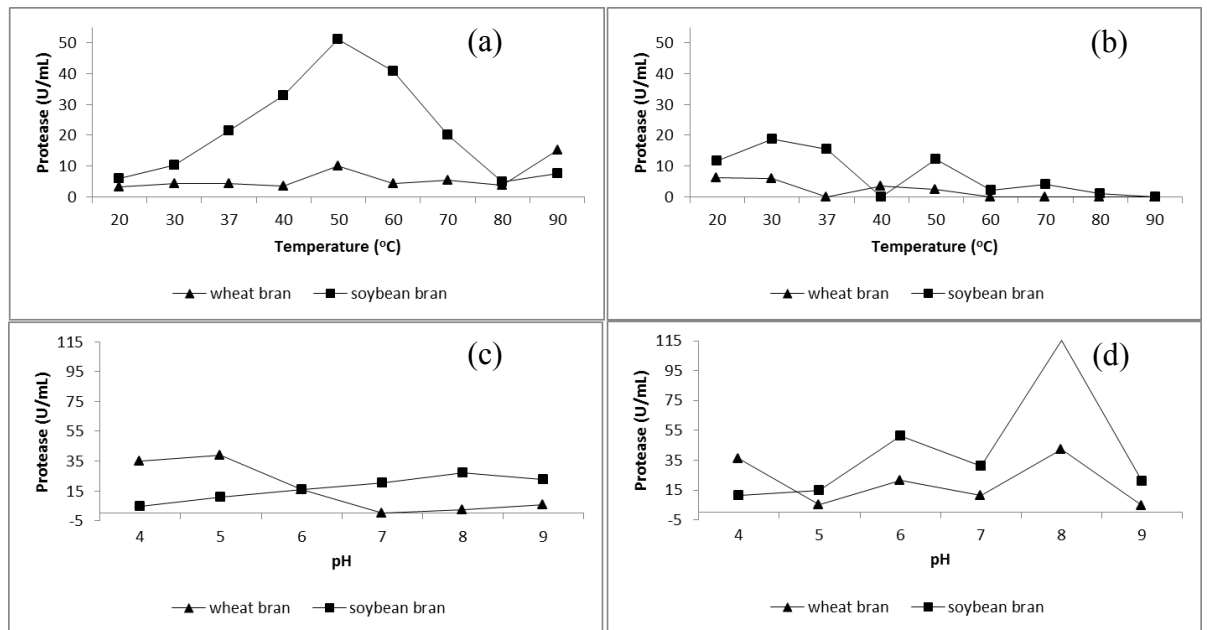


Figure 3. Influence of temperature (a – optimum, and b – stability) and pH (c – optimum, and d – stability) on protease activity produced by solid state fermentation with wheat bran and soybean bran as substrate using *A. niger* 40018.

CAPÍTULO V

**DIGESTIBLE VALUES FOR NILE TILAPIA (*Oreochromis niloticus*)
USING FUNGAL PHYTASES AND PROTEASES PRODUCED BY
SOLID STATE FERMENTATION**

Abstract: The use of enzymes in animal feed is common practice nowadays and the use of exogenous microbial enzymes has shown good results for improving the efficiency of nutrients utilization. The ingredients used for fish feed formulation aim to provide nutrients to meet their requirements, and affect the availability in the animal intestinal tract. In this sense, the goal of this work was to add low cost fungal phytases and proteases produced by Solid State Fermentation using agroindustrial co-products to improve nutritional availability of plant based diets for Nile tilapia. Concentrated enzyme extract of phytase, protease and both enzymes was added to diets with soybean meal or corn gluten as test ingredients for further analysis of nutrients digestibility. Phytase supplementation increased dry matter, protein, energy, lipids and phosphorous utilization for soybean meal ($P < 0.05$). Protease affects energy, lipids, phosphorous and magnesium availability for soybean meal ($P < 0.05$). As for corn gluten, the enzymes did not show improvement for nutrients and minerals utilization. When the enzymes were combined in the diets they did not show improved as they were used alone. These low cost enzymes are an alternative as additives to improve soybean meal digestibility in fish plant based diets.

Keywords: Phytase, Protease, Solid State Fermentation (SSF), *Oreochromis niloticus*, Nutritional requirements, Phytate-protein, minerals complexing.

1. Introduction

The intensification of fish production requests improved production technology and low cost and low pollution feeding systems. Due to high cost and limited availability of animal protein sources (Naylor et al., 2000), the replacement of fish meal by plant protein is encouraging (Fontainhas-Fernandes et al., 1999; Mabahinzireki et al., 2001). However, plant protein ingredients contain anti-nutritional factors that reduce the availability of nutrients and minerals, increasing waste output (Higgs et al., 1995; Hardy, 1996). Phytic acid is the major anti-nutritional factor, which is the phosphorous storage compound in most plant protein sources (Cheryan, 1980). Phytate (IP6) is a salt of *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, which is present in several portions in plant seeds. Most of phosphorous bound to phytate (P-IP6) is excreted by fish into the water due to its low availability, what can make phosphorous less available for fish utilization on its metabolism. In addition, this molecule bounds to metal ions and also to cationic groups in proteins and aminoacids. It has been demonstrate that IP6 can inhibit pepsin and other proteases activity *in vitro* by the formation of binary protein-IP6 complexes (Vaintraub & Bulmaga, 1991; Morales et al., 2011).

Phytases (E. C. 3.1.3.8 and E. C. 3. 1. 3. 26) are a special class of phosphatases that catalyse the sequential hydrolysis of IP6 to less phosphorylated *myo*-inositol derivatives and inorganic phosphate. One of the main commercial phytases used in animal nutrition is produced from *Escherichia coli* and has two pH optima at 2.5 and 4.5 (Elkhalil et al., 2007). However, some latest studies demonstrate the utilization of new fungal phytases in fish diets with promising results (Liebert & Portz, 2005; Dalsgaard et al., 2009). It has been demonstrated that the addition of phytase to plant

ingredients used in fish nutrition improves phosphorous availability and also prevents binding of IP6 to protein, this resulting in increased nutrients utilization (Storebakken et al., 1998). It has been also suggested that a number of factors such as pH, nature of the protein source and the presence of digestive proteases may determine the effect of phytase on protein and minerals bioavailability within fish digestive tract (Morales et al., 2011). Details of the specific effects of such factors on the action of phytase efficacy have not been elucidated yet and more research is needed to obtain a better insight into the mechanisms underlying phytase–protein interactions and subsequent availability of proteins and amino acids after digestion in fish. Moreover, the right phytase, regarding especially pH and temperature, used as nutritional additive can have different effects in nutrients digestibility.

Proteases (EC 3.4.23.18) hydrolyze peptide bounds, so protein became more digestible for the animal. These enzymes constitute important group of enzymes commercially produced (Uyar & Baysal, 2004) they have different industrial applications and they are being used as animal feed additive recently. Nowadays, proteases constitute more than 25% of biomolecules produced for industrial usage. Protein based food with several anti-nutritional factors and low nutritional availability are common in the animal feed industry. High levels of proteases inhibitors are found, mainly in soybean meal, which is one of the most important protein ingredients in animal fed. Studies with vegetable ingredients and enzyme addition in diets for animals showed promising results (Pariza & Cook, 2010; Denstadli et al., 2011; Morales et al., 2013). Additionally, proteases can also help with reduction of environmental pollution (Guimarães et al., 2009).

The aim of this study was to determine the effect of supplemental low-cost homemade fungal phytase and protease on protein, energy and minerals availability by juvenile Nile-tilapia fed plant based protein diets.

2. Materials and methods

2.1. Diets

Two diets were prepared with the test ingredients (soybean meal and corn gluten) added to a reference diet formulated as fish nutritional requirements in a proportion of 30% (m/m), as proposed by Pezzato et al. (2004). Table 1 shows the calculated formulation and analysis of the experimental diets.

Phytase from *Aspergillus niger* produced by Solid State Fermentation (SSF) at the Bioprocess Laboratory, Institute of Biosciences São Paulo State University was added to the diet in a ratio of 2,000 U kg⁻¹, as recommended by Portz et al., (2003) and, for protease produced by SSF with *Aspergillus oryzae*, 1,500 U kg⁻¹ was added, as recommended by Mahmoud et al., (2014). Enzymes were sprayed over the diets and the soybean oil was added at the end of the process. The determination of phytase activity in the experimental diets was performed using p-nitrophenyl phosphate as substrate, an aliquot of 1000 µL of 5 mmol L⁻¹ p-nitrophenyl phosphate were added to 500 µL of acetate buffer pH 5.0 (0.1 mol L⁻¹) and 100 mg of the experimental diet. The system was incubated for 10 minutes at 37 °C. The reaction was stopped with 2 mL of sodium hydroxide (0.1 mol L⁻¹). The phytase activity unit was the quantity of enzyme necessary to release one µmol of p-nitrophenol per reaction minute at 410 nm. The protease activity was determined using azocasein as substrate. The reaction media was 0.5 mL of azocasein 0.5% (m/v) and 0.5 mL of diet solution 1:5 (m/v) in borax-boric acid buffer

pH 9.0 (0.1 mol L⁻¹), they were incubated for 40 minutes at 37 °C. The reaction was stopped with 0.5 mL TCA 10% (m/v) and centrifuged at 6,000 rpm for 15 minutes at 15°C; 1 mL of potassium hydroxide (5 mol.L⁻¹) was added to 1 mL of the supernatant. The protease activity unit was defined as the quantity of enzyme necessary to increase 0.1 of absorbance at 428 nm in the assay conditions.

2.2. Culture conditions, fish and feeding

The experiment was evaluated by the Ethic Commission for Animal Utilization of the Veterinary and Animal Science College, University of São Paulo State “Julio de Mesquita Filho” under protocol number 20/2013-CEU.

Nine 250 L aquaria were used for feeding procedure and, five conic 310 L aquaria were used to collect faeces. Settlement column was used to collect faeces. Both systems were connected to a biological filter, and water temperature was controlled by an electronic thermostat. The water was circulated at 6.59 L min⁻¹ through the aquaria and temperature was maintained at 27.0 ± 0.5 °C.

Fish with 150 g average weight were stocked at a density of 10 fish in each experimental unit (circular net cages inside the feeding aquaria) at São Paulo State University Aquaculture Research Nutrition Laboratory. These fish were fed a growth ration produced in our laboratory until they reached the weight to begin the experiment. Then, 90 fish were selected by weight and distributed in nine net cages.

Diets were randomly assigned to aquaria and fish fed 7 days prior to the beginning of faecal collection (acclimatization period). For the first faecal collection the first five groups of fish were transferred to collecting faeces aquaria and on the consecutive day the remaining four groups were transferred and faeces collected. This

procedure was carried out until representative volume of faeces for a replication (each group of fish) chemical analysis was collected. Test diets were then reassigned to the net cages for each following round. On the collecting day, fish were fed twice in the morning, and every hour in the afternoon. The acclimatization and faecal collection process were repeated three times to obtain triplicate measurements per treatment (test diets). The cages were transferred to faeces collection aquaria at 18:00 h, where they remained until the morning of the following day. Then, the cages were returned to the respective feeding aquaria. Fish were fed in an independent system from faeces collection system in order to avoid the presence of feed in the faecal samples, as reported by Pezzato et al. (2002). Faeces were oven-dried at 55 °C, ground and stored at -20.0 °C until chemical analysis.

Chemical analysis of feedstuffs, diets and faeces were determined according to AOAC (1995) protocols. Chromium oxide content of diets and faeces were determined according to Bremer-Neto et al. (2005) and gross energy content was determined in an adiabatic calorimetric bomb (Parr Instrument Company, Moline-IL, EUA). Calcium, magnesium, manganese and phosphorous were mineralized with nitro-perchloric acid solution for further analyses. Phosphorous was determined by phospho-vanado-molybdate colorimetric method according Moraes et al. (2009); calcium, magnesium and manganese were determined using a SHIMADZU AA-6800 atomic absorption spectrometer equipped with a background absorption correction with a deuterium lamp and self-reverse (SR) system, according Neves et al. (2009).

2.3. Apparent digestibility coefficients (ADC) calculation

Nose (1960) equation was used to assess the apparent digestibility coefficients (ADC, %) of dry matter protein, energy lipid and availability of phosphorous, calcium and magnesium of the experimental diets, as:

$$ADC = 100 - [100 [\%Cr_2O_3r / Cr_2O_3f] \times [\%Nf / \%Nr]]$$

Where:

ADC = Apparent digestibility coefficient (%);

$\%Cr_2O_3r$ = diet chromium oxide III percentage;

$\%Cr_2O_3f$ = faeces chromium oxide III percentage;

$\%Nf$ = crude energy or percentage of dry matter, protein, lipids or minerals in the faeces;

$\%Nr$ = crude energy or percentage of dry matter, protein, lipids or minerals in the diet.

The apparent digestibility coefficient of dry matter, energy, protein and minerals availability of the ingredients when added enzymes were calculated based on Bureau (2006).

$$ADC_{\text{test ingredient}} = ADC_{\text{test diet}} + (ADC_{\text{test diet}} - ADC_{\text{ref diet}}) \times ((0.7 \times N_{\text{ref diet}}) / (0.3 \times N_{\text{test ingredient}}))$$

2.4. Experimental design

Nine compound diets were evaluated, eight containing the two plant test ingredients (soybean meal and corn gluten) and the addition or not of the supplemental enzymes (phytase, protease, both and no enzyme), and one reference practical diet, with three replicates for each combination. The digestibility was determined indirectly using 0.1% (m/m) of chromium oxide III as an inert marker. Analysis of variance test by SAS statistics was used and, when there was difference between averages, it was applied the Duncan test with 5% significance.

3. Results and discussion

There were significant differences in the apparent digestibility coefficient (ADC) of dry matter, protein, energy and lipids when phytase was added to soybean meal as a plant based ingredient (Table 2). The digestibility varied significantly with or without phytase utilization, and was significantly lower when soybean meal was used as an ingredient test alone. The availability of phosphorous was also affected by fungal phytase addition (Table 3). A significant effect of phytase supplementation on protein and energy utilization rate was observed by Liebert & Portz (2005), and similar level of phytase addition was reported by Furuya et al. (2001), as an additive for maximum growth performance for Nile tilapia. Most microbial phytase act efficiently under conditions present in the stomach (acid pH), however, not all phytase have the same pH profile (Morales et al., 2011). In this study, *Aspergillus niger* phytase showed optimum pH at 5. Also, as Nile tilapia stomach pH is acid (Hepher, 1993), phytase activity increase in low pH and protein digestion occurs in the stomach; this condition may help to improve protein digestibility.

Vielma et al. (2004) reported positive effects of supplemented phytase on protein digestibility in trout; however, protein utilization was not significantly increased. Some recent papers show enhanced amino acid availability due to supplemental phytase in land animals (Sebastian et al., 1997; Martin et al., 1998) and fish (Guimarães et al., 2008; Gonçalves et al., 2007). Plant protein utilization has been reported to increase (Storebakken et al., 1998; Vielma et al., 1998; Sugiura et al., 2001) or to remain unchanged (Lanari et al., 1998; Yan and Reigh, 2002) by enzymes utilization.

As expected, the supplementation effect of microbial phytase and/or protease on phosphorous, magnesium, calcium and manganese utilization was significant when added to soybean meal (Table 3). Apparent phosphorous availability and bone mineralization are considered as the most sensitive conditions for assessing the influence of phytase on phosphorous utilization (Cao et al., 2007). Currently, the capacity of phytase to increase total phosphorous availability in fish has been demonstrated. Sugiura et al. (2001) observed that rainbow trout fed diet with 50% soybean meal pretreated with 200 U phytase/kg, the ADC of phosphorous reached 93%. Sajjadi and Carter (2004) reported that phosphorous availability after phytase supplementation was significant higher for Atlantic salmon (*Salmon salar*) when compared to control diets without phytases; also, Ai et al. (2007) suggested that supplementation of 500 IU phytase per kg diet was able to improve phosphorous, calcium and zinc utilization, but not improve protein digestibility of Japanese seabass (*Lateolabrax japonicus*). Additionally, the positive effect of phytase on ADC of phosphorous has also been observed in tilapias fed soybean meal based diets (Liebert & Portz, 2005).

Moreover, the digestibility of energy and lipid were increased with addition of fungal protease to soybean meal (Table 2). The availability of phosphorous also ranged from 36.39 to 82.63%, with no enzyme and protease addition in soybean meal, respectively (Table 3). The enzyme activity is linked to the animal feeding habits (Sabapathy & Teo, 1993), this may modify the enzyme addition effect on the ingredient, as it can be more efficient for some fish species than others. Likewise, exogenous protease added to diet of *Cichla* sp., a carnivorous fish, affected body characteristics and growth performance (Soares et al., 2008), however, this authors did not studied the effect of protease on nutrients digestibility. Ng et al. (2002) observed reduction of anti-nutrients effect and increase of nutritional value of palm bran for Nile tilapia; and the use of an enzyme complex, including protease, for Nile tilapia diets improved the digestibility of protein, fat and gross energy digestibility (Guimarães et al., 2009). The exogenous protease could be responsible for increase and complement the enzyme-substrate machinery, improving even carbohydrates and lipids digestion. And more, similarly to phytase, protease can degrade some proteins that act as anti-nutritional factors and improve nutrients digestibility.

Otherwise, there were no differences for corn gluten treatments with enzymes addition (Table 2 and 3). Corn gluten nutritional composition, as amino acids, minerals or other components, may have caused an inhibition on the activity site of the enzyme produced and, for this reason, enzyme addition did not work for this ingredient. Even more, when the enzymes were combined in the treatments, they did not show improvement on ingredients digestibility. This may show that the fungal protease produced can degrade the phytase and increase the negative effect on gluten digestibility and its anti-nutrients. In this sense, the stability of phytase during stomach digestion in

the presence of pepsin may be a limiting factor for its efficiency and more studies simulating the fish stomach conditions and interaction with ingredients might be needed. On the other hand, calcium availability improved with protease addition in the diet for both plant ingredients. Calcium chemical characteristic may be responsible for that improvement, as it is a metal ion that can also bound to phytate and anti-nutritional factors. Many studies reported increase in protein digestibility with microbial phytase supplementation in plant based diets using soybean bran (Liebert and Portz, 2005; Vielma et al., 2004; Cheng and Hardy, 2003), on the other hand, corn gluten needs further considerations. Likewise, Gonçalves et al. (2007) identified that phosphorous availability varies according the vegetable ingredient used and definite that mineral availability for corn gluten diets with phytase addition was improved only with high levels of enzyme addition, also due to its natural phytase occurrence or the amount of phytate present in the corn gluten. Furthermore, high levels of plant ingredients in the diet result in high content of insoluble carbohydrates, as well as elevated levels of anti-nutritional ingredients like phytates (Cain and Garling, 1995; Francis et al., 2001; Riche et al., 2001). According to Liener (1975) and Tacon (1993), oilseed meals contain many thermolabile anti-nutrients and most importantly enzyme inhibitors. Whether these or other anti-nutrients factors were totally inactivated was not determined in this study. Also, it is important to remember that the enzymes are substrate specific and the corn gluten has different composition than soybean meal, so this might affect in the nutrients utilization as well.

The lipids digestibility was over estimated when both enzymes were added to the ingredients; this may indicate the presence of other enzymes, like lipases in the fungal enzyme complex produced, since the homemade enzymes were used as a crude

extract and they were not purified. Furthermore, because of lipase presence, lipids release in the faeces can be more than what should be expected. Supplementation of phytase may also improve the bioavailability of protein and might lead to additional improvements in growth and energy deposition or lipids excretion. The reduction of phytate–protein complexes in the gut and increased nutrient availability could be an explanation for this observation. In vitro studies of Singh and Krikorian (1982) demonstrated negative effects of phytates on protein utilization.

In addition, the optimum supplemental doses of the enzymes for each ingredient may need further research. Also, the interaction between enzymes and sources need more attention to encounter an ideal way to maximize enzyme efficacy. Further investigations are necessary to clarify the influence of supplemental fungal enzymes on nutrients and minerals utilization in fish.

4. Conclusion

In conclusion, both microbial enzymes demonstrated to be efficient for increasing nutritional utilization of soybean meal for Nile tilapia. Thereby, this additive may reduce environmental pollution by phosphorous and other minerals excreted, as they increase mineral availability; and more, these low cost enzymes are alternative additives to improve soybean meal digestibility for a cost-effective and environmentally friendly fish diet.

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5. Tables

Table 1: Formulation (g kg⁻¹) and proximate composition (% dry matter) of the experimental diets with two plant protein based ingredients and enzyme addition.

<i>Diets</i>	Reference	SB ¹	CG ¹	SBF ¹	SBP ¹	SBFP ¹	CGF ¹	CGP ¹	CGFP ¹
<i>Ingredients</i>									
Soybean	561.61	692.95	392.95	692.95	692.95	692.95	392.95	392.95	392.95
Corn	367.69	257.27	257.27	257.02	256.27	256.02	257.02	256.27	256.02
Corn gluten			300.00				300.00	300.00	300.00
Soybean oil	14.20	9.94	9.94	9.94	9.94	9.94	9.94	9.94	9.94
L-Lysine	1.35	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94
DL-Methionine	3.49	2.44	2.44	2.44	2.44	2.44	2.44	2.44	2.44
Threonine	3.08	2.16	2.16	2.16	2.16	2.16	2.16	2.16	2.16
Dicalcium phosphate	39.58	27.71	27.71	27.71	27.71	27.71	27.71	27.71	27.71
Premix ²	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Vit C ³	0.50	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
BHT ⁴	0.20	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
NaCl	1.00	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Coline	1.30	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91
Cr ₂ O ₃ ⁵	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Phytase				0.25		0.25	0.25		0.25
Protease					1.00	1.00		1.00	1.00
<i>Analyzed composition</i>									
Dry matter	93.0	94.2	94.3	92.5	93.6	93.4	93.0	93.6	92.9
Protein	34.8	40.6	46.0	40.4	40.4	38.9	45.4	45.2	45.4
Lipids	3.7	3.2	2.2	3.2	3.5	2.8	1.3	2.2	1.8
Energy (kJ g ⁻¹)	18.8	19.2	20.7	19.0	18.9	19.0	20.7	20.5	20.5
Ash	6.4	5.8	5.1	5.4	5.8	6.6	5.7	5.7	5.5
Cr ₂ O ₃ ⁵	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phosphorous	1.2	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0
Magnesium	0.45	0.41	0.29	0.38	0.37	0.44	0.27	0.29	0.31
Calcium	1.25	0.98	0.87	0.94	1.07	1.08	0.82	0.98	0.85
Manganese	0.008	0.004	0.006	0.005	0.008	0.008	0.006	0.008	0.006

¹SB- : Soybean meal test diet without enzyme; CG- : Corn gluten test diet without enzyme; SBF: Soybean meal diet with phytase; SBP: Soybean meal diet with protease; SBFP: Soybean meal diet with phytase and protease; CGF: corn gluten diet with phytase; CGP: Corn gluten diet with protease; CGFP: Corn gluten diet with phytase and protease.

²Vitamin and mineral supplement (levels per kg of product): vitamin A = 1,200,000 IU; vitamin D3 = 200,000 IU; vitamin E = 12,000 mg; vitamin K3 = 2,400 mg; vitamin B1 = 4,800 mg; vitamin B2 = 4,800 mg; vitamin B6 = 4,000 mg; vitamin B12 = 4,800 mg; folic acid = 1,200 mg; calcium pantothenate = 12,000 mg; vitamin C = 48,000 mg; biotin = 48 mg; choline = 65,000 mg; nicotinic acid = 24,000 mg; Mn = 4,000 mg; Zn = 6,000 mg; I = 20 mg; Co = 2 mg; Cu = 4 mg e Se = 20 mg; ³Vitamin C Rovimix@ Stay-35, DMS Nutritional Products, Switzerland; ⁴Butyl-hydroxytoluene; ⁵Chromium oxyde.

Table 2: Apparent digestibility coefficients (ADC) of dry matter, protein, energy, lipids of plant ingredients, soybean meal and corn gluten, with fungal enzymes addition.

<i>Treatment</i>	<i>Soybean meal</i>				<i>Corn gluten</i>			
	<i>no enzyme</i>	<i>phytase</i>	<i>protease</i>	<i>Phytase+ protease</i>	<i>no enzyme</i>	<i>phytase</i>	<i>protease</i>	<i>Phytase+ protease</i>
dry matter	72.62 c	93.48 ab	94.39 ab	92.37 ab	94.43 ab	80.22 bc	84.57 abc	97.92 a
protein	93.92 bcd	98.47 a	97.36 ab	97.51 ab	95.17 abc	91.47 d	91.96 cd	96.43 ab
energy	78.39 b	94.87 a	94.87 a	93.33 a	95.60 a	85.75 ab	88.52 ab	96.97 a
lipid	95.53 ab	104.83 a	105.02 a	110.83 a	77.23 bc	33.61 e	64.73 cd	54.81 d

*Values with different letters within a row indicate significant difference ($P < 0.05$).

Table 3: Phosphorous (P), Magnesium (Mg), Calcium (Ca) and Manganese (Mn) availability of plant based ingredients, soybean meal and corn gluten, and enzymes addition.

<i>Treatment</i>	<i>Soybean meal</i>				<i>Corn gluten</i>			
	<i>no enzyme</i>	<i>phytase</i>	<i>protease</i>	<i>Phytase+ protease</i>	<i>no enzyme</i>	<i>phytase</i>	<i>protease</i>	<i>Phytase+ protease</i>
Phosphorous	36.39 c	80.22ab	82.63 a	75.03 ab	66.55 abc	48.08 bc	54.41 abc	68.70 abc
Magnesium	66.09 c	79.45 b	78.51 bc	92.05 a	73.71 bc	49.74 d	66.22 c	76.78 bc
Calcium	18.03 cd	38.91 bc	73.28 a	62.50 ab	5.29 d	15.32 cd	56.23 ab	52.92 ab
Manganese	-281.78e	37.17 d	70.86 b	94.41 a	47.00 cd	46.51 cd	65.68 bc	51.55 bcd

*Values with different letters within a row indicate significant difference ($P < 0.05$).

CAPÍTULO VI

CONSIDERAÇÕES FINAIS

1. Implicações

A produção enzimática por fermentação em estado sólido (FES) utilizando resíduos da agroindústria é alternativa para produção a baixo custo de enzimas de alto valor comercial. Podem, ainda, ser obtidas enzimas e biomoléculas com características físico-químicas muito distintas e com diferentes e promissoras aplicações para indústria biotecnológica.

O uso de enzimas fúngicas produzidas por FES na alimentação de tilápias-do-Nilo em dietas vegetais, pode melhorar a biodisponibilidade do fósforo e magnésio e, ainda, a digestibilidade de proteínas, lipídeos e energia. Igualmente, por melhorarem a disponibilidade dos minerais, podem ser promissores aditivos nutricionais com finalidade de amenizar a poluição das águas, fator importante e premissa básica para implantação das modernas aquiculturas.

Contudo, a utilização de ingredientes vegetais alternativos e mais, de nutrientes adequados e balanceados é necessária para que os peixes atinjam desempenho satisfatório e parâmetros metabólicos normais. Assim sendo, os estudos relacionados e a otimização da produção enzimática devem ser continuados e correlacionados à viabilidade econômica.