FERNANDA LOPES MOTTA

HUMIC ACID PRODUCTION BY FERMENTATION OF WASTE FROM PALM OIL PROCESSING USING *TRICHODERMA* STRAINS

PRODUÇÃO DE ÁCIDOS HÚMICOS POR FERMENTAÇÃO DE RESÍDUOS DO PROCESSAMENTO DE ÓLEO DE PALMA UTILIZANDO CEPAS DE TRICHODERMA

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UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia Química

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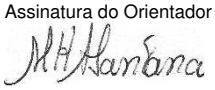
Thesis presented to the School of Chemical Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Chemical Engineering.

Tese apresentada à Faculdade de Engenharia Química da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Engenharia Química.

Orientadora: Profª. Drª. Maria Helena Andrade Santana

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE
DEFENDIDA PELA ALUNA FERNANDA LOPES MOTTA
E ORIENTADA PELA PROFª. DRª. MARIA HELENA ANDRADE SANTANA

Assinatura do Orientador



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RESUMO

Ácidos húmicos (AH) são macromoléculas orgânicas de alta complexidade estrutural. Na agricultura, seus efeitos diretos na produtividade e na qualidade de diversos cultivos têm sido explorados desde 1940 e na última década houve um grande crescimento no seu emprego na medicina e na biologia. Embora suas inúmeras aplicações tenham atraído a atenção de muitos pesquisadores, os AH comerciais são ainda extraídos da turfa e do carvão mineral, fontes não renováveis de carbono, indicando a necessidade do desenvolvimento de um processo biotecnológico para sua produção. Empty-fruit-bunches (EFB) são resíduos do processamento do óleo de palma subutilizados ou descartados. Além disso, EFB são fibras constituídas de celulose, hemicelulose e lignina, cuja degradação pode ser alcançada por meio da ação de micro-organismos com alta capacidade de degradação de material lignocelulósico, como o *Trichoderma*. Neste contexto, este trabalho visou produção de AH a partir da fermentação de EFB com cepas de *Trichoderma*. Os resultados obtidos demonstraram que aveia é um substrato adequado para produção de esporos de *Trichoderma* em profundidade para sua aplicação como inóculo das fermentações submersas (FS) de EFB para produção de AH. A investigação dos precursores dos AH dentre os polímeros constituintes do EFB indicaram um efeito sinergético destes polímeros. Quanto à substituição do extrato de leveduras por peptonas vegetais nos meios de cultivo, para ambas fontes de nitrogênio os resultados encontrados para produção de biomassa e de AH foram muito similares. Através da otimização da produção dos AH por FS utilizando ferramentas de planejamento experimental foram obtidos 428,4±17,5 mg/L destes ácidos, permitindo um aumento de 7 vezes em relação às condições inicialmente utilizadas neste trabalho. A comparação da performance de espécies de *Trichoderma* na produção de AH por FS indicou a superioridade do *T. reesei* na produção destes ácidos. Por fim, a caracterização do processo de produção dos AH por fermentação em estado sólido (FES) utilizando colunas de Raimbault, onde foram obtidos 88,1 ±2,9 mg/L destes ácidos, confirmou a relação entre a esporulação do fungo e a produção dos AH, assim como a hipótese do efeito sinérgico dos polímeros. Os resultados obtidos demonstraram a factibilidade da produção dos AH por processos fermentativos, cuja descrição na literatura ainda é muito limitada, o que confere a este trabalho um caráter inovador.

Palavras chave: ácidos húmicos; empty-fruit-bunch (EFB); *Trichoderma*; fermentação submersa; fermentação em estado sólido.

ABSTRACT

Humic acids (HA) are organic macromolecules of high structural complexity. In agriculture, their direct effects on yield and quality of many crops have been exploited since 1940 and the last decade there was a large increase in their use in medicine and biology. Although they have attracted the attention of many researchers due to their numerous applications, commercials AH are still extracted from peat and coal, non-renewable carbon sources, indicating the necessity to develop a biotechnological process for their production. Empty-fruit-bunches (EFB) are waste of oil palm processing, which are underutilized or discarded, and their accumulation brings environmental problems due to the attraction and proliferation of pests. In addition, EFB fibers are composed of cellulose, hemicellulose and lignin, whose degradation can be achieved by the action of microorganisms with high capacity of lignocellulosic material degradation, such as *Trichoderma*. In this context, this work aimed to produce HA from the fermentation of EFB with *Trichoderma* species. The results showed that oats is a suitable substrate for the production of *Trichoderma* spores to be used as inoculum for application in submerged fermentations (SF) of EFB for the HA production. The investigation of the HA precursor among the constituents polymers from the EFB indicate a synergistic effect of these polymers. Regarding the replacement of yeast extract by vegetable peptones in the culture medium, for both nitrogen sources the results in the production of biomass and HA were very similar. Through the optimization of the HA production by SF using experimental design, were obtained 428.4 ± 17.5 mg/L of these acids, allowing an increase of 7-fold compared to initial conditions used in this work.. A comparison of the performance of *Trichoderma* species in the HA production by FS indicated the superiority of the *T. reesei* in the production of these acids. Finally, characterization of HA production process by solid state fermentation (SSF) using Raimbault columns, where were obtained 88.1 ±2.9 mg/L of these acids, confirmed the relationship between the fungal sporulation and the HA production as well as the hypothesis of synergistic effect of the polymers. The results demonstrated the feasibility of the HA production by fermentation process, whose description in the literature is still very limited, which confers an innovative character to this work.

Key words: humic acids; empty-fruit-bunch (EFB); *Trichoderma*; submerged fermentation; solid state fermentation.

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APRESENTAÇÃO

O presente trabalho foi redigido na forma de capítulos para uma melhor organização e discussão dos resultados obtidos, sendo este exemplar apresentado no seguinte formato:

- Introdução, apresentando a importância do trabalho;
- Objetivos e metas;
- Revisão bibliográfica, abordando os principais fundamentos considerados nos desenvolvimento deste trabalho;
- Resultados e Discussão, apresentados na forma de artigos científicos publicados ou submetidos a periódicos aos quais os assuntos estudados melhor se enquadravam;
- Conclusões;
- Sugestões para trabalhos futuros;
- Referências Bibliográficas.

1. INTRODUÇÃO

Substâncias húmicas (SH) são produtos da degradação oxidativa e subsequente polimerização da matéria orgânica animal e vegetal. Devido às inúmeras aplicações das substâncias húmicas na agricultura, na indústria, no meio-ambiente e na medicina (Peña-Mendez *et al.*, 2005), seu impacto na qualidade de vida humana tem sido cada vez mais reconhecido como um importante assunto para pesquisas (Klocking e Helbig, 2005). Elas são constituídas por uma mistura de compostos de elevada massa molar com uma grande variedade de grupos funcionais (Rauen *et al.*, 2002) e operacionalmente classificadas com base em sua solubilidade (Kipton, 1992). Huminas são a fração insolúvel em meio aquoso (Rauen *et al.*, 2002), ácidos fúlvicos (AF) são completamente solúveis em soluções aquosas e os ácidos húmicos (AH) são solúveis apenas em soluções básicas e que se precipitam em soluções ácidas. No entanto, esta divisão operacional entre AF e AH não é precisa, havendo um espectro de solubilidade resultante das diferenças de tamanho e peso molecular, além das distribuições de grupos funcionais destes ácidos (Kipton, 1992).

Os AH, um dos mais importantes componentes das SH, possuem propriedades notáveis que justificam suas aplicações em várias áreas. Na agricultura, onde suas aplicações são as mais estudadas, os AH ajudam a descompactar solos, auxiliam na transferência de micronutrientes do solo para as plantas, aumentam a retenção da água e as taxas de germinação das sementes, além de estimular o desenvolvimento da população de microflora nos solos e desacelerar evaporação de água destes (Peña-Mendez *et al.*, 2005). Nos últimos dez anos, os efeitos terapêuticos e profiláticos dos AH e AF foram identificados e o seu potencial uso em medicina vem sendo explorado com relação ao aumento da imunidade humana, atividades antiviral, antiinflamatória e estrogênica, como anticoagulante, na neutralização e eliminação de toxinas, na prevenção de problemas estomacais e intestinais, entre outros (Klocking e Helbig, 2005).

A potencialidade da produção de AH por fermentação em estado sólido (FES) foi estudada no trabalho de Umi Kalsom *et al.* (2006), utilizando EFB (empty-fruit-bunches), um resíduo da produção de óleo de palma, e fungos da espécie *Trichoderma viride*, isolados do próprio EFB. No entanto, o objetivo principal do trabalho de Umi Kalsom *et al.* (2006) foi somente caracterizar os AH produzidos. Nossa proposta de trabalho visa o desenvolvimento do processo biotecnológico a partir de resíduo agroindustrial para produção de AH, englobando o delineamento e estudo das variáveis importantes ao processo e sua otimização.

De acordo com Umi Kalsom *et al.* (2006), os EFB correspondem a mais de 20% do peso da fruta fresca e a abundância deste resíduo tem criado uma importante questão ambiental, devido à atração e proliferação de pragas (Law, 2007). Por isso, há uma necessidade crescente de pesquisas voltadas para aplicação dos EFB, sobretudo diante do incentivo ao aumento da produção do óleo de palma que vem ocorrendo recentemente no Brasil (AmbienteBrasil, 2010).

Com relação a processos fermentativos em geral utilizando *Trichoderma*, observa-se que há um grande número de trabalhos na literatura em que se emprega este fungo em fermentações submersas e em estado sólido, o que demonstra a sua atividade em ambos os processos. A utilização do gênero *Trichoderma* como agente degradante de lignoceluloses tem sido reconhecida desde o início de 1960 (Selby e Maitland, 1967). Dentre os trabalhos encontrados com a espécie *Trichoderma viride*, Ui-Haq *et al.* (2009) estudaram a biossíntese de endoglucanases e Zhou *et al.* (2008) produziram celulases, ambos utilizando FS. A otimização do cultivo de *Trichoderma viride* foi objeto de estudo do trabalho Al-Taweil *et al.* (2009) também por meio da FS. Estudos com FES foram realizados por Tan *et al.* (2008) utilizando resíduos de ervas com *Trichoderma koningii* para produção de proteínas e por Singhania *et al.* (2006) com resíduos lignocelulósicos e *Trichoderma reesei* para produção de celulases.

2. OBJETIVOS E METAS

O objetivo deste trabalho foi a produção de AH por fermentação, utilizando como substrato resíduos do processamento de óleo de palma (Empty Fruit Bunch – EFB) e espécies de *Trichoderma*.

O trabalho foi composto pelas seguintes metas:

- ✓ Desenvolvimento de um processo simples e de baixo custo, utilizando aveia como substrato, para produção de esporos em profundidade, visando sua aplicação como inóculo para as fermentações submersas (FS) de EFB para produção de AH;
- ✓ Investigação do(s) precursor(es) dos AH dentre os polímeros celulose, hemicelulose e lignina, através de FS independentes utilizando cepa de *Trichoderma viride*. Comparação dos resultados com a FS utilizando EFB como controle;
- ✓ Estudo do efeito da substituição do extrato de leveduras por peptonas vegetais, preservando a concentração total de nitrogênio no meio de cultura;
- ✓ Maximização da produção de AH através da otimização da composição inicial do meio de cultura e das condições de processo para FS, utilizando ferramentas de planejamento experimental;
- ✓ Comparação da cepa de *Trichoderma reesei* e da cepa de *Trichoderma viride* visando à seleção daquela com maior capacidade de produção de AH;
- ✓ Realização de FES com a cepa selecionada visando a caracterização do processo.

3. REVISÃO DE LITERATURA

3.1. Humificação

Humificação é o processo de deterioração envolvendo a transformação de biomoléculas, oriundas de organismos em decomposição, e atividade microbiológica onde substâncias húmicas são formadas e substâncias não húmicas são decompostas (Bernal *et al.*, 1998; Schnitzer, 1978). Em relação à contribuição para o ciclo global do carbono, a humificação é o segundo mais importante processo depois da fotossíntese (Hedges e Oades, 1997).

Embora atualmente o processo de formação de substâncias húmicas esteja sendo intensamente estudado, sua origem ainda não foi completamente elucidada. Algumas teorias permaneceram durante anos e, atualmente, supõe-se que substâncias húmicas sejam originadas a partir de lignina (Oglesby *et al.*, 1967). É evidente que o mecanismo de formação de substâncias húmicas pode ser diferente, dependendo das circunstâncias geográficas, climáticas, físicas e biológicas (Burdon, 2001; Davies *et al.*, 2001).

3.2. Substâncias húmicas

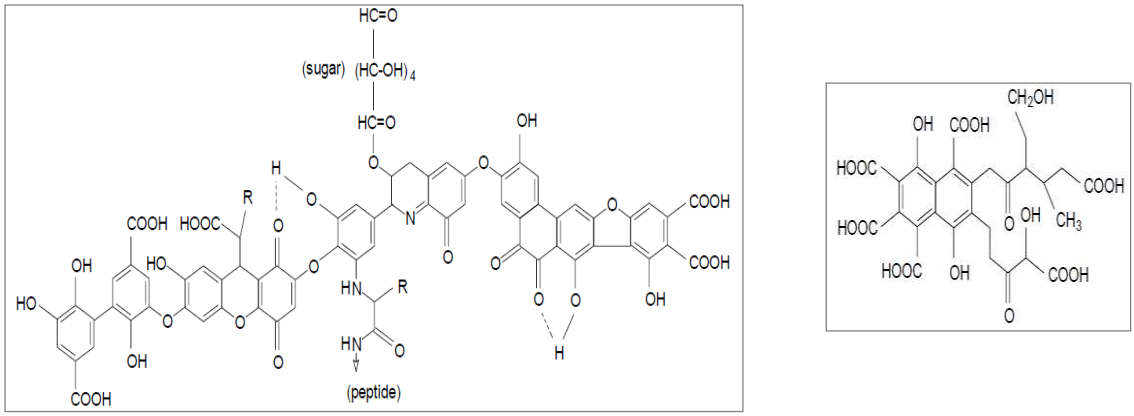
Maccarthy (2001) definiu amplamente substâncias húmicas (SH) como uma categoria de materiais encontrados naturalmente ou extraído de solos, sedimentos e águas naturais, resultante da decomposição de resíduos vegetais e animais.

Com o auxílio da técnica de espectroscopia de ressonância magnética nuclear, as SH foram definidas como compostos bi ou tridimensionais, formados por estruturas aromáticas, com porções de cadeias alifáticas estáveis, unidas por pontes de hidrogênio, contendo grupos carboxílicos, carbonilas, fenílicos, alcoólicos, hidroquinonas, entre outras (Schulten e Schnitzer, 1997). Apesar do importante papel das SH na sustentabilidade da vida, da sua natureza química e da sua reatividade, ainda há controvérsias no que diz respeito às suas estruturas moleculares (Hardie, 2008). Baseado na solubilidade, as SH podem ser divididas em três principais frações: AH, AF e huminas (Senesi e Loffredo, 2001). As huminas são insolúveis em água, os AF solúveis em qualquer faixa de pH e os AH solúveis em pH maior que 2 (Lain e Chen, 2001; Hur e Schlautman, 2004; Jones e Bryan, 1998).

A composição média de uma unidade básica para os AH e AF, em termos de fórmula química média, é C_{187} H_{186} O_{89} N_9 S_2 e C_{135} H_{182} O_{95} N_5 S_2, respectivamente. A razão C/N dos AH e AF é superior em 50% à média observada na matéria orgânica do solo, indicando seu menor grau

de degradação e conferindo-lhe maior estabilidade no ambiente (quanto menor a razão C/N maior é a degradabilidade no solo) (Santos e Camargo, 1999 *apud* Schnitzer, 1978).

A caracterização de misturas complexas de AF e AH constitui um dos mais importantes itens de pesquisas recentes relativas às substâncias húmicas. Graças ao desenvolvimento de técnicas analíticas e tecnologias computacionais, grandes esforços têm sido feitos para elucidar a estrutura molecular desses dois ácidos (Peña-Mendez *et al*., 2005). A Figura 1 (a,b) apresenta um modelo de estrutura molecular para os AH (Stevenson, 1982) e AF (Buffle *et al.*, 1977).



(a) (b)

Figura 1. Modelo de estrutura dos AH de acordo com Stevenson (1982); R pode ser alquil, aril ou aralquil (a). Modelo de estrutura dos AF de acordo com Buffle *et al*. (1977) (b).

3.2.1. Aplicações das substâncias húmicas

Além de seu tradicional uso como combustíveis e fertilizantes orgânicos, as SH são base para preparações farmacêuticas, nutracêuticas e também são materiais de partida para a síntese de vários produtos (Klocking e Helbig, 2005). Recentemente, as aplicações dessas substâncias têm sido divididas em quatro categorias principais: agricultura, indústria, meio-ambiente e biomedicina (Peña-Mendez *et al.*, 2005).

- **Agricultura**

A extensão da agricultura orgânica e da agricultura sustentável tem levado a um aumento das aplicações dos fertilizantes orgânicos. Estes fertilizantes orgânicos contêm uma quantidade significativa de matéria orgânica, com grande parte composta por SH (Deiana *et al.*, 1990). As SH participam de importantes reações que ocorrem nos solos, influenciando a fertilidade pela liberação de nutrientes, pela detoxificação de elementos químicos, pela melhoria das condições físicas e

biológicas (Santos e Camargo, 1999) e pela produção de substâncias fisiologicamente ativas (Guminski, 1968).

- **Indústria**

Húmus e materiais que os contêm, têm sido usados na construção civil como aditivos para controlar a taxa de fixação de concreto. As SH também são usadas em indústrias de couro, madeira e cerâmica.

- **Meio-ambiente**

A principal função das SH no meio-ambiente químico é a remoção de metais tóxicos, produtos químicos orgânicos e outros poluentes da água. (Shin *et al.*, 1999; Lofredo *et al.*, 2000).

- **Biomedicina**

Na última década houve um crescente interesse no emprego de SH na medicina (veterinária e humana) e na biologia (Mund-Hoym, 1981; Brzozowski *et al*., 1994). A principal razão deste interesse está na sua atividade antiviral, pró-fibrinolítica, antiinflamatória e estrogênica (Yamada *et al.*, 1998).

Os AH administrados em ratos diminuíram a extensão de danos gástricos induzidos pelo etanol (Brzozowski *et al*., 1994). Pflug e Ziechman (1982) relataram que AH são capazes de interagir com a bactéria *Micrococcus luteus*, protegendo-a contra a ruptura da parede celular pela enzima lisozima. Thiel *et al.* (1981) observaram que a pré-incubação de culturas de células com humato de amônio evitou a infecção pelo vírus da herpes. O potencial das SH para formar complexos de quelato com metais pesados (como cádmio) possibilita o seu uso na eliminação de metais pesados em organismos vivos (Klocking, 1992). Demonstrou-se que em sistemas aquáticos e seus sedimentos, as SH estão estreitamente relacionados com a eficácia da hidroterapia e balneoterapia (Gadzhieva *et al.*, 1991).

As propriedades antibacterianas das SH representam novas possibilidades para sua aplicação médica (Peña-Mendez *et al.*, 2005). Estudos realizados em hospitais mostraram que doenças respiratórias virais graves comuns em crianças são facilmente combatidas com AF na suplementação alimentar. As SH, principalmente os AF, atuam também na prevenção e tratamento de alguns tipos de câncer (Schneider *et al.*, 1996; Van Rensburg *et al*., 2002).

Na Tabela 1 estão resumidas as indicações mais frequentes da turfa (material de origem vegetal parcialmente decomposto e que contém SH), atualmente oferecidas por clínicas de saúde na Alemanha.

Tabela 1. Seleção de indicações de turfa em que substâncias húmicas estão provavelmente envolvidas (Klocking e Helbig, 2005).

Doenças	Indicações	Maiores efeitos terapêuticos
Doenças musculares e esqueléticas	Artroses degenerativas, Gota, Espondilopatias e osteoporoses, Reumatismo muscular, Poliartrites, Reabilitação após operações e acidentes	Hipertermia intensa melhora a circulação sanguínea e processo de regeneração
Doenças ginecológicas	Doenças de inflamação crônica, Desequilíbrio hormonal, Lombalgia, Adesões, Esterilidade, Queixas da menopausa	Hipertermia intensa Efeito estrogênico e apoio à produção de estrógeno endógeno Profilaxia da trombose pela liberação do ativador tecidual do plasminogênio
Doenças de pele	Eczema crônico, Neurodermatites, Psoríase	Ativação do metabolismo da pele e processo de regeneração, aumento da circulação sanguínea

3.3. Ácidos Húmicos

Dentre as três substâncias húmicas, os AH estão presentes em maior quantidade no meio ambiente. Estas macromoléculas orgânicas apresentam estrutura complexa constituída por carbono, hidrogênio, oxigênio, pequenas quantidades de nitrogênio e ocasionalmente fósforo e enxofre. Os conteúdos individuais dos diferentes elementos e as relações entre eles dependem da fonte de matéria orgânica, do grau de humidificação, da cadeia carbônica, do tipo de tratamento recebido e das condições ambientais (Terashima *et al*., 2004; Ramos-Tejada *et al*., 2003; Rauen *et al*., 2002).

Apesar dos AH possuírem estrutura complexa com elevado teor de aromáticos e grupamentos carboxílicos, não apresentam toxicidade, sendo muito importante para a fertilidade dos solos, a sua principal fonte. Os AH comerciais são extraídos da turfa e do carvão mineral, duas fontes não renováveis de carbono (Trompowsky, 2006). A extração mais eficiente dos AH é feita com álcalis, porém há riscos de alterações estruturais. Agentes de extração mais brandos como pirofosfato de sódio, agentes complexantes, ácido fórmico, misturas ácidas e solventes orgânicos também têm sido utilizados (Rosa *et al*., 2000).

3.4. Empty-fruit-bunch (EFB) da palma

O óleo de palma está em situação de destaque no mercado mundial em termos de consumo, ocupando atualmente o segundo lugar e com perspectivas de vir a ocupar o primeiro lugar já no início da próxima década. Assim como Malásia e Indonésia, que detêm 90% da produção mundial de palma, o Brasil tem as condições de clima e solo necessárias para o plantio desta oleaginosa, que se desenvolve bem em países de clima tropical (Informativo Rural, 2010). Por esta razão, aliada às pesquisas e inovações tecnológicas envolvendo este assunto, foi lançando no Pará em maio do corrente ano o Programa Nacional de Óleo de Palma que visa tornar o Brasil o maior produtor mundial desse vegetal nos próximos anos, garantindo o suprimento de combustível renovável (AmbienteBrasil, 2010).

Dos cachos de frutos frescos da palma podem ser extraídos o óleo de palma bruto (20%) e o óleo de palmiste (1,5%), resultando em: torta de palmiste (3,5%), cachos vazios (22%), fibras (12%), cascas (5%) e efluentes líquidos (50%) (BiodieselBR, 2010). A Figura 2 ilustra o fruto da palma.

Os EFB são compostos de 45-50% de celulose e quantidades iguais de hemicelulose e lignina, cerca de 25-35% (Deraman, 2010). Os cachos vazios (EFB), que restam após a produção do óleo são em geral descartados, havendo um crescente interesse na sua compostagem, a fim de agregar valor, e também de facilitar a redução do seu volume no meio ambiente (Yusri *et al.*, 1995; Umi Kalsom *et al*., 2006).

(a) (b)

Figura 2. Cacho do fruto da palma (a) e fruto aberto da palma (b).

3.5. *Trichoderma*

Trichoderma é um gênero complexo de fungos com importantes aplicações na agricultura e na medicina. Esta espécie é caracterizada pelo rápido crescimento de colônias, chegando ao diâmetro de 2 a 9 cm depois de 4 dias de crescimento à 20°C. *Trichoderma* ssp. são colonizadores onipresentes de material celulósico (Shuster e Schmoll, 2010). Este gênero é amplamente distribuído por todo o mundo e ocorre em quase todos os solos e outros habitats naturais, especialmente nos ambientes contendo material orgânico. É também encontrado na superfície de raízes de várias plantas e em cascas em decomposição, sobretudo quando estas se encontram danificadas ou contaminadas por outros fungos.

Como *Trichoderma* é um fungo cosmopolita, é capaz de crescer numa faixa ampla de diferentes condições ambientais e substratos. Esta característica confere ao gênero a possibilidade de ser utilizado em muitas situações de interesse biotecnológico (Eposito *et al*., 1998). Muitas espécies de *Trichoderma* têm sido utilizadas para a produção industrial de enzimas e degradação de material lignocelulósico (Nevalainen *et al.*, 1994). Com relação à demanda de oxigênio, processos fermentativos que envolvem *Trichoderma* são aeróbios (Verma *et al.*, 2006).

3.6. Otimização de processos biotecnológicos

Atualmente, técnicas estatísticas de planejamento experimental e análise de superfície de resposta representam uma estratégia muito útil para determinar as condições ótimas de processos biotecnológicos, por envolver um número reduzido de experimentos, permitir a quantificação da interação entre variáveis e a obtenção de um modelo matemático que explique estatisticamente os resultados experimentais. O planejamento de experimentos e otimização das variáveis é um processo sequencial. No cultivo de microrganismos, onde se busca nutrientes e condições físicas de maior influência, um grande número de fatores é envolvido, e neste caso, inicialmente um planejamento Plackett & Burman é o mais adequado para 5 a 12 fatores. Em uma segunda etapa, as variáveis significativas identificadas são otimizadas através de um planejamento fatorial completo. Finalmente, após a obtenção de um modelo matemático são determinados os valores ótimos preditos para as variáveis estudadas (Myers e Montgomery, 1995).

3.7. Peptonas vegetais

Peptonas são polipeptídeos que são produtos intermediários na hidrólise das proteínas. Dependendo do grau de hidrólise, peptonas podem fornecer nutrientes, componentes de adesão, ou

fatores de crescimento análogos. A substituição de todas as substâncias de origem animal por componentes de origem vegetal reduz o risco de reações imunogênicas (Sakai *et al.*, 2002).

De acordo com Weete (1980), fontes orgânicas de nitrogênio são as melhores para o crescimento de fungos. No entanto, fontes de nitrogênio de origem animal podem conter agentes que causam encefalopatias espongiformes transmissíveis (EET) (Vegetable Peptone, 2012), uma família de doenças em humanos e animais caracterizadas por degeneração esponjosa do cérebro com graves e fatais sinais e sintomas neurológicos (Zoonoses, 2012). Regulamentos destinados a prevenir o risco de contaminação com EET são de grande importância no indústria farmacêutica (Subramanian, 2012). Neste contexto, extrato de leveduras e peptonas vegetais são livres de componentes animais e são amplamente utilizados para formulações livres destes componentes. No entanto, extrato de leveduras possui endotoxinas, que causam doença em seres humanos e que são considerados contaminantes, devendo ser evitados ou minimizados na preparação de produtos farmacêuticos (Advanced Bioprocessing, 2012). Portanto, a utilização de peptonas vegetais na formulação de meios de cultura em linhas de produção industrial poderia reduzir o potencial de contaminação por agentes EET e endotoxinas.

4. RESULTADOS E DISCUSSÃO

Esta seção será apresentada na forma de capítulos, contendo os artigos submetidos a periódicos científicos selecionados de acordo com a afinidade do periódico com o aspecto abordado.

O primeiro artigo apresentado (*Biomass Production from Trichoderma viride in Nonconventional Oat Medium*) trata-se da produção de esporos de *T. viride* por FS utilizando aveia como substrato. Este trabalho consistiu de um estudo para a produção de esporos a partir de substrato de baixo custo, para que os esporos produzidos fossem utilizados como inóculo nas fermentações posteriores empregando EFB, que constitui o objetivo principal deste trabalho.

O segundo artigo (*Production of humic acids from oil palm empty fruit bunch by submerged fermentation with Trichoderma viride: cellulosic substrates and nitrogen sources*) aborda o desenvolvimento do processo de FS para produção dos AH a partir do EFB, assim como a investigação do substrato celulósico para produção destes ácidos. Sendo o EFB composto por celulose, hemicelulose e lignina, foram testados meios de cultura contendo estes três polímeros isolados na busca pela identificação do precursor dos AH na fermentação de EFB.

O terceiro artigo (*Statistical optimization of the production of humic acids by Trichoderma viride under submerged fermentation, using the oil palm empty fruit bunch as a substrate*) apresenta a otimizaçao da produção de AH, partindo da composição do meio de cultura e das condições de fermentação propostas no segundo artigo, onde foi alcançado um aumento de cerca de 7 vezes na produção destes ácidos pela aplicação de ferramentas estatísticas.

O quarto artigo (*Comparison of humic acids produced by Trichoderma viride and Trichoderma reesei using the submerged fermentation of oil palm empty fruit bunch*) engloba a comparação das espécies de *Trichoderma*, tanto em relação à produção de esporos em meio de aveia quanto em relação à produção de AH por FS de EFB. Além disso, através do PB design, este estudo indicou as variáveis estatisticamente significativas na produção destes ácidos, assim como o efeito causado por elas.

O quinto artigo (*Solid state fermentation for humic acids production by a Trichoderma reesei strain using an oil palm empty fruit bunch as the substrate*) apresenta os resultados obtidos pela FES em colunas de Raimbault para produção de AH. Neste trabalho, foi feita a caracterização do substrato e da cinética da SSF em termos de umidade, pH, taxa global de transferência de oxigênio, proteínas celulares e constituintes do EFB (celulose, hemicelulose e lignina).

4.1. *Biomass Production from Trichoderma viride in Nonconventional Oat Medium*

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Biomass production from *Trichoderma viride* in non-conventional oat medium

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Abstract

Oatmeal, an alternative, renewable and low-cost substrate, was used for the production of *Trichoderma viride* spores by submerged fermentation. The non-conventional oat medium was only supplemented with potato peptone, which is a green source of nitrogen for the microorganism. Because particles are suspended in the non-conventional oat medium, the characterization was based on viscosity, average particle diameter, size distribution, and porosity of the particles. Due to the complexity of the fungal biomass extraction, the dry weight and protein content were used as methods for quantifying the growth of *Trichoderma viride*. The inversion between the proportion of mycelia and spores were captured in the microscopic image analysis during the fermentation process. After 60 hours, spores began to appear, accounting for most of the form present at 120 hours of fermentation. The decrease in pH and the increase in glucose concentration during fermentation indicate that glucan hydrolysis occurs and that glucose is released into the medium. The potential for industrial applications of submerged fermentation with oats for biomass production of *Trichoderma viride* are noted in the results. This simple and easily controllable process has several advantages, including the use of low cost substrates for the propagation of a microorganism that is widely used in scientific and commercial settings.

Keywords: *Trichoderma viride*, oat, submerged fermentation, biomass, sporulation.

1. Introduction

Trichoderma is a genus of asexually reproducing fungi with a high level of genetic diversity[1]. They are frequently found growing in soil as well as on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions[2]. This characteristic confers the possibility of the genus being used in many biotechnological applications[3]. Filamentous fungi have also received great attention due to their ability to produce thermostable enzymes that are of high scientific and commercial value, such as amylases, pectinases, xylanases, cellulases, chitinases, proteases, lipases, and β-galactosidases[4,5]. Moreover, in recent years, *Trichoderma* species have been used in commercial preparations for the biological control of fungal plant diseases, reducing the use of polluting chemicals[3]. *Trichoderma*-based biocontrol agents (BCAs) are better able to promote plant growth and soil remediation processes than their counterparts (viruses, bacteria, nematodes, and protozoa)[6]. Like the conidial mass of other fungal BCAs, the conidial mass of *Trichoderma* is the most proficient propagule, and it tolerates downstream processing (e.g., air drying)[7].

Trichoderma fermentation is a highly aerobic process[8], and the oxygen transfer in submerged fermentation is largely affected by the viscosity and morphology of the mycelia and the pellet formation[9,10]. These parameters create heterogeneity in the medium, resulting in the compartmentalization of the fermentation broth in terms of dissolved oxygen concentration, pH, and substrate availability[11,12].

Many authors have quantified the estimated biomass using indirect methods based on specific component measurements, such as protein content, glucosamine, ergosterol, and nucleic acids. Protein content is the most readily measured biomass component[13].

Although many studies have reported the use of conventional synthetic media for production of *Trichoderma* sp.[14,15], the high cost of the substrates normally limits their commercial use. This can be achieved by using low cost agro-industrial residues, such as wheat bran, oat bran, and rice straw[16].

Oatmeal (*Avena sativa*) is produced from oats and only the shells are removed. Therefore, oatmeal has all of the nutrients that are found in grains[17], such as a high concentration of soluble fiber, the β-glucanas[18]. These fibers are soluble in water with a tendency to form viscous aqueous solutions and gels. They also have a high viscosity even at low concentrations and are extremely pseudoplastic at concentrations of 0.5% or higher. The increase in the temperature causes the

decrease of viscosity of these solutions, causing these solutions to return to a thickened state upon cooling[19]. Then, the utilization of oatmeal as a substrate leads to a non-conventional submerged fermentation.

The aim of this study was to develop a practical and low cost submerged fermentation process for the production of *Trichoderma viride* spores. Whereas most of industrial costs are due to the media, oats are low cost and renewable substrate that can be supplemented with minimal nutrients. Because cereals usually have low concentrations of nutrients, such as nitrogen[20], vegetable peptone was used as a supplement to the culture medium.

2. Methods

2.1. Microorganisms

For storage, the *T. viride* strain culture was grown on potato dextrose agar plates at 24 °C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 ml cryotubes at −70 °C.

The average diameter of the spores was measured using a Zetasizer® Nano (Malvern Instruments, United Kingdom) at 25 °C.

2.2. Preparation and characterization of the fermentation media

The media was prepared by adding 30 g of oatmeal (Quaker Oats Company®, thin flakes; see Table 4.1.1 for detailed composition) into 1 L of distilled water and boiling the suspension for 90 min at 90 °C with constant stirring. Immediately after heating, the suspension was filtered through a sieve (0.150 mm diameter holes). Distilled water was added to the filtrate to achieve the final volume of 1 L. Then, 5 g of potato peptone purchased from Sigma-Aldrich was added, and the media was adjusted to pH 6.0[21] prior to autoclaving at 121 °C for 15 min.

Table 4.1.1. Composition of oatmeal thin flakes (data collected form manufacturer's product specification sheet, Quaker Oats Company®).

Constituent	Content per 100 g
Carbohydrate	56.7
Protein	14.3
Total fat	7.3
Fibers	9.7
Beta-glucan	4.0

2.2.1. Water activity

The water activities of the oatmeal used as the substrate and of the system during fermentation were measured using an Aqualab Series 3TE system (Decagon, USA), which uses the chilled-mirror dewpoint technique to measure the water activity of a sample. The experiments were performed in triplicate at 25 °C, and the standard deviations were calculated.

2.2.2. Media viscosity and solid content

Media viscosity assays were performed before inoculation and after 120 hours of fermentation using a SV-10 Vibro Viscometer (AND, Japan) at 25 °C. This instrument uses a constant frequency of 30 Hz at a constant amplitude. There is a damping effect related to the viscosity of the fluid, which reduces the amplitude, and the power required to keep the sensors vibrating at the original amplitude is measured and converted into the viscosity.

The total concentration of solids in the medium was measured before inoculation and after 120 hours of fermentation. Samples were dried at 105 °C to achieve constant weight and placed in a desiccator. Then, the concentration of solids was determined.

All experiments were performed in triplicate, and the standard deviations were calculated.

2.2.3. Particle size

The average mean diameter and particle size distribution of the medium before inoculation were determined by laser diffraction using a Mastersizer 2000 (Malvern Instruments, USA).

To verify the adhesion of the fungus to the oat particles, the particle size distribution was determined after 120 hours of fermentation. Because the spores are nanometric, the distribution

was determined by laser light scattering using a Zetasizer® Nano instrument (Malvern Instruments, United Kingdom).

The experiments were performed in triplicate at 25 °C, and the standard deviations were calculated.

2.2.4. Scanning electron microscopy (SEM)

The particle shape and the surface morphology were examined by scanning electron microscopy (Leo 440i, LEO Electron Microscopy, England) in high vacuum mode. The acceleration voltage was 10 kV. The powder was sprinkled onto an SEM-stub covered by adhesive carbon tape and sputter coated (SC7620, VG Microtech, England) with 92 A° thickness of Au. Because oatmeal particles are electrically nonconductive, the sample was subjected to low vacuum gold sputter coating in the presence of argon gas.

2.3. Submerged fermentation

The culture were performed in 500 mL Erlenmeyer flasks containing 300 mL of culture media inoculated with 1 mL of a spore suspension inoculum that was prepared as described in item 2.1. The culture flasks were incubated at 24°C at 150 rpm. During 120 hours of cultivation, samples were withdrawn every 12 hours for the analyses.

2.4. Biomass quantifying

2.4.1. Dry weight

According to the methodology adapted of Szijártó *et al.*[22], where optical density was used to evaluate *T. reesei* biomass behavior in delignified pine pulp, the dry biomass concentration of the *T. viride* was evaluated by reading the optical density at 600 nm in a spectrophotometer. The absorbance at 600 nm was correlated to the cell dry weight per culture volume. The dry weight was determined as follows: fungal biomass was extracted from the culture medium by heating an aliquot of known volume after the dilution in distilled water at a ratio of 1:10 at 85 °C under constant agitation for 10 min. After heating, the aliquot was filtered through coffee filter paper, and the filtrate was collected. The pie was retained, heated and filtered as described above until the green color (typical of the spores of *Trichoderma*) disappeared. The total volume of the filtrate was centrifuged at 10,000 g at 5 °C, and the supernatant was discarded. The precipitate was dried at

105 °C to achieve constant weight and placed in a desiccator, and then, the mass of fungus was determined.

2.4.2. Protein quantifying

Protein quantification was performed according to the adapted methodology of Callow and Ju[23] in order to quantify only the cellular proteins. 3.0 mL sample was collected and centrifuged at 10,000 g for 10 min to obtain a pellet. The supernatant was collected for further processing. The pellet was re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged and the water discarded. To release the intracellular proteins, the pellet was suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100 °C for 10 min for cell breakage. Following cooling, the digested sample was centrifuged at 10,000 g for 10 min to remove cell debris and other solids; the supernatant was then collected and protein concentration was determined using a commercial kit (Modified Lowry Protein Assay, Thermo Scientific, USA) based on the Lowry assay[24] and using a standard curve constructed with bovine serum albumin.

2.5. Glucose consumption

Glucose concentration was determined using an enzymatic glucose oxidase kit. The fermentation medium was centrifuged at 10,000 g for 10 min at 5 °C. A portion of the supernatant (25 µL) was mixed with the reagent (2.0 mL) and incubated at 37 °C for 30 min. The absorbance was measured at 505 nm using a spectrophotometer, and the glucose content was computed by least squares linear regression using a standard curve.

2.6. Image analysis

Approximately 10–50 µL samples were taken for smear preparation. Observations of mycelia and conidia were carried out using a microscope Reichert-Jung Series 150 (Reichert, USA) equipped with a digital camera.

2.7. Spore counts

The spores were enumerated by directly counting them using a Neubauer chamber to compare the initial concentration of spores inoculated to the spore concentration at the end of fermentation.

3. Results and Discussion

3.1. Media characterization

Particle size and SEM – The particle size distribution of the raw material is one of the key parameters affecting the growth of microorganisms during fermentation; particle size distribution influences the allocation of nutrients and metabolites as well as the provision of the surface areas for the biological activities[25]. Another important parameter is the porosity, which influences the water holding capacity of the substrate and varies significantly with particle size, as well as affects the diffusion of nutrients[26].

Figure 4.1.1 shows oatmeal particles used in the culture medium for *T. viride* strain growth. The average diameter of particles in the culture medium before inocultation was 16.8±0.1 µm, and particles size distribution (diameter versus volume percentage of the particles) is represented in Figure 4.1.2 (a). The average diameter of *Trichoderma viride* spores was 0.3±0.0 µm, which is approximately 60 times lower than that found for the particles of the medium. Moreover, if the average diameter of spores is compared to the diameter of the largest particles present in the culture medium (630.6 µm), these particles are approximately 2,200 times greater than these spores. The analysis of the particle size distribution of the growth medium after 120 hours of fermentation (Figure 4.1.2 (b)) revealed that there is a peak comprising particles with diameters between 0.2 and 0.6 µm. This peak most likely represents the spores present in the medium because the average diameter found for the spores was 0.3±0.0 µm. Although this histogram does not include the biggest oatmeal particles, because the maximum size range of the equipment used was 0.3 nm to 10 µm, the presence of two distinct peaks comprising particles with diameters typical of spores and diameters typical of oatmeal particles indicates that *T. viride* spores are present in a free state in the culture medium after 120 hours of fermentation.

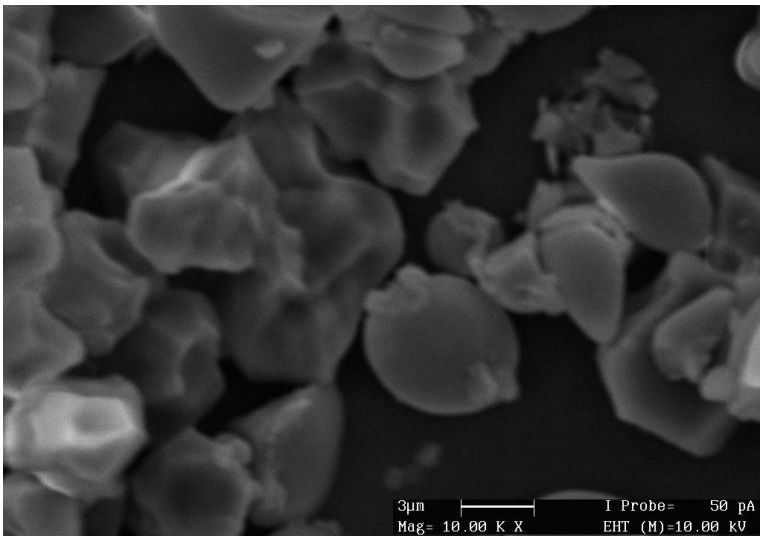

Figure 4.1.1. SEM micrographs of oatmeal particles before medium preparation.

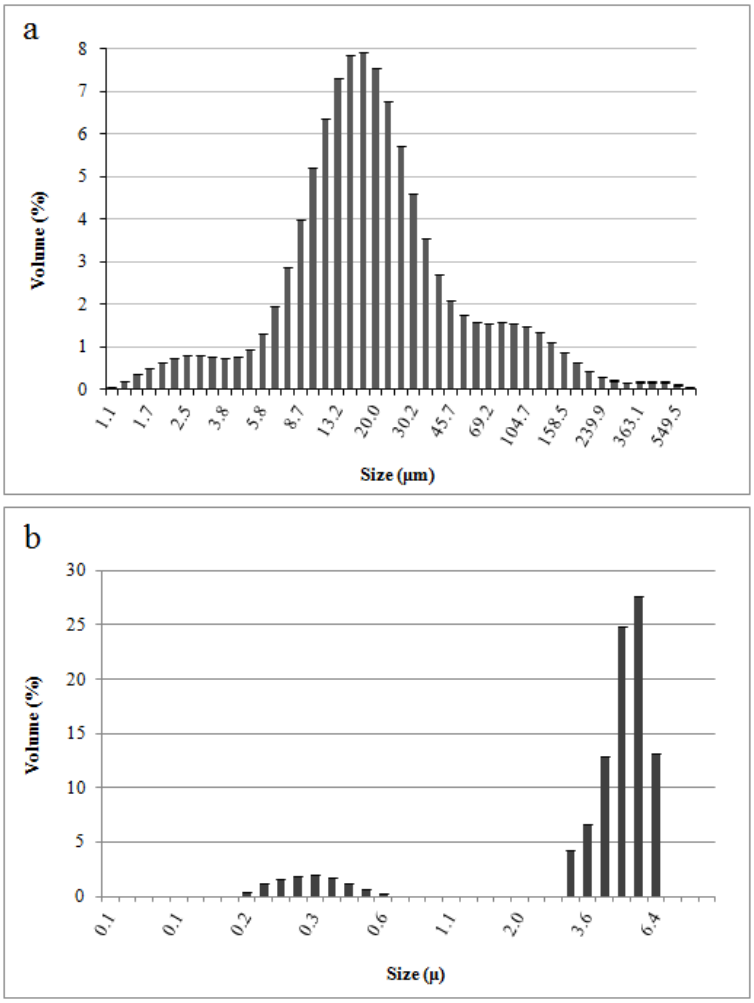

Figure 4.1.2. Particle size distribution (with standard deviation) of growth media before inoculation determined by laser diffraction using a Mastersizer 2000 (a) and of growth media at 120 hours determined by laser light scattering using a Zetasizer® Nano instrument (b) of submerged fermentation with *T. viride* strain.

Water activity - The water activity has a significant effect on the growth rates and the types of microorganisms that can grow in a given environment[27]. According to Hung *et al.*[28], the minimum water activity required for fungal growth is 0.61. The addition of solutes can reduce the water activity of a system[27]. In this study, although the water activity value obtained for the oatmeal used in the culture media was 0.49±0.01, the value for the medium throughout the fermentation process was 1.00±0.00, indicating that there was no decrease in the water activity during fermentation and that the water activity remained greater than the limit (0.61).

Viscosity and concentration of solids - In general, the high viscosity of fungal broths causes heterogeneity and poor mass transfer during the production phase, resulting in limited oxygenation inside the fermenter[29]. Because β-glucan is a hemicellulose that substantially increases the viscosity of aqueous solutions even at low concentrations[30], it was necessary to measure the viscosity of the media and the concentration of solids. According to the table of nutritional information supplied by the manufacturer (Table 4.1.1), the oatmeal used in this study is composed of 4.0% β-glucan. The media viscosity and the concentration of solids before inoculation were 4.14±0.02 mPa•s and 34.5±0.9 g/L, respectively. After 120 hours of fermentation, the values found were 0.91±0.01 mPa•s and 10.5±0.4 g/L for the media viscosity and the concentration of solids, respectively. The problems with submerged fermentation typically caused by high-viscosity media were not encountered with the process studied because the initial viscosity was only four times greater than the viscosity of water at 25 °C (0.90 mPa•s)[31] and the final viscosity was almost equal to that of water. Therefore, the concentration of solids decreased 3-fold during the fermentation, indicating that *Trichoderma viride* readily hydrolyzed the available substrates, for example, the β-glucans.

3.2. Dry biomass and protein content

Biomass is a fundamental parameter in the characterization of microbial growth, and its measurement is essential for kinetic studies on fermentation. Complete recovery of fungal biomass from the substrate is very difficult when the fungal hyphae penetrates into and binds tightly to the solid substrate particles[13]. The present study adopted two methods, one direct and one indirect, for biomass quantification.

Figure 4.1.3 shows that the biomass and protein concentration curves have the same behavior along the 120 hours of cultivation. Moreover, the results conclude that the concentration

of both protein and biomass remained almost constant from approximately 60 hours of fermentation. These results are in agreement with the analysis in Figure 4.1.6 and are discussed in item 3.4, which shows that from 60 hours of cultivation *Trichoderma viride* begins to sporulate.

Christias *et al*.[32] determined in their work, using the method of Lowry *et al*.[24], the amount of protein present in the biomass of five different genera of fungi, and they obtained a range of 30% to 40%. By applying these results in the present study, it is possible to observe that the ratio between protein and biomass concentration is within the range obtained by Christias *et al*.[32].

Figure 4.1.4 shows the relationship between *T. viride* biomass concentration and cellular protein concentration, where the evidence of a straight light line relationship and strong correlation value (0.980) were observed.

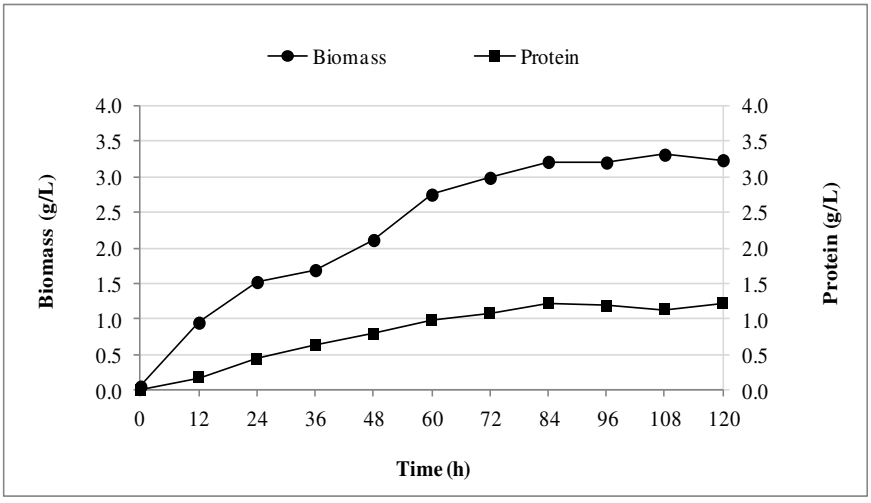

Figure 4.1.3. Biomass and cellular protein *versus* time during a submerged fermentation of oatmeal with *T. viride* strain.

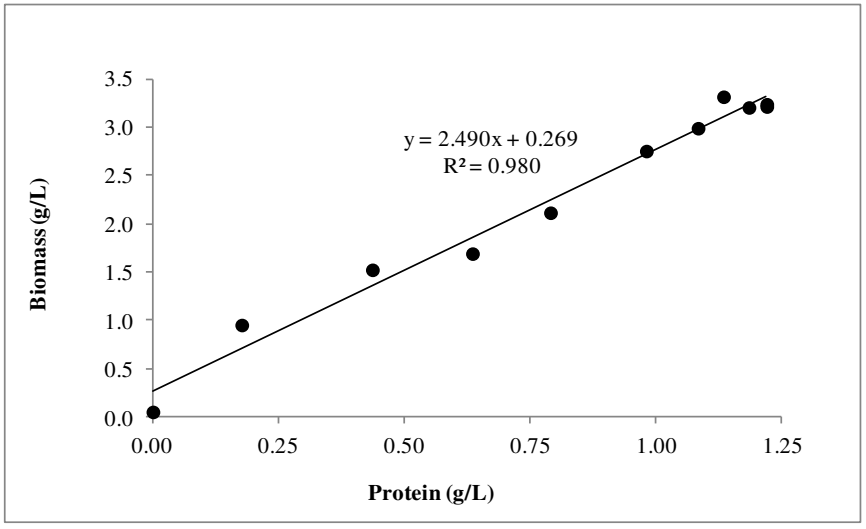

Figure 4.1.4. Relationship between cell biomass and cellular protein concentration in submerged fermentation of oatmeal with *T.viride* strain.

3.3. pH and glucose

Figure 4.1.5 represents the pH behavior and concentration of glucose in the medium during the submerged fermentation of oats with *Trichoderma viride*, where there is a clear decrease in pH and an increase in glucose concentration between 36 and 72 hours. Because the β-glucan polysaccharides are D-glucose monomers linked by β-glycosidic linkages[33], such behavior can be explained by the occurrence of a hydrolysis of polysaccharides, causing a decrease in the pH, which results in a large release of glucose into the culture medium.

These results demonstrate the applicability of this fungus in the fermentation of oats for the production of enzymes, such as β-1,3-glucanases. According to Giese *et al.*[34], the regulation of enzyme expression in *Trichoderma* can be influenced by the amount of β-glucan that is present in the culture medium, because β-glucan acts as a promoter.

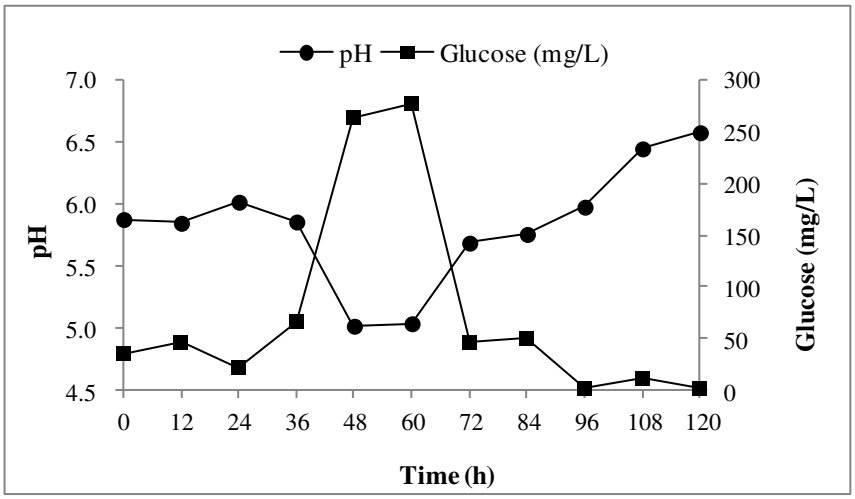

Figure 4.1.5. Glucose concentration and pH *versus* time during a submerged fermentation of oatmeal with the *T. viride* strain.

3.4. Sporulation

The images shown in Figure 4.1.6 refer only to the range 36 to 120 hours of fermentation because only mycelia were present at lower concentrations by 36 hours of fungal culture.

Observing these images, it is possible to observe the fungus sporulation and the reversal of the mycelia to spore ratio during the fermentation process. Sporulation began at approximately 60 hours of fermentation (Figure 4.1.6 (c)), and after 120 hours (Figure 4.1.6 (h)), the fungus was present in the form of spores.

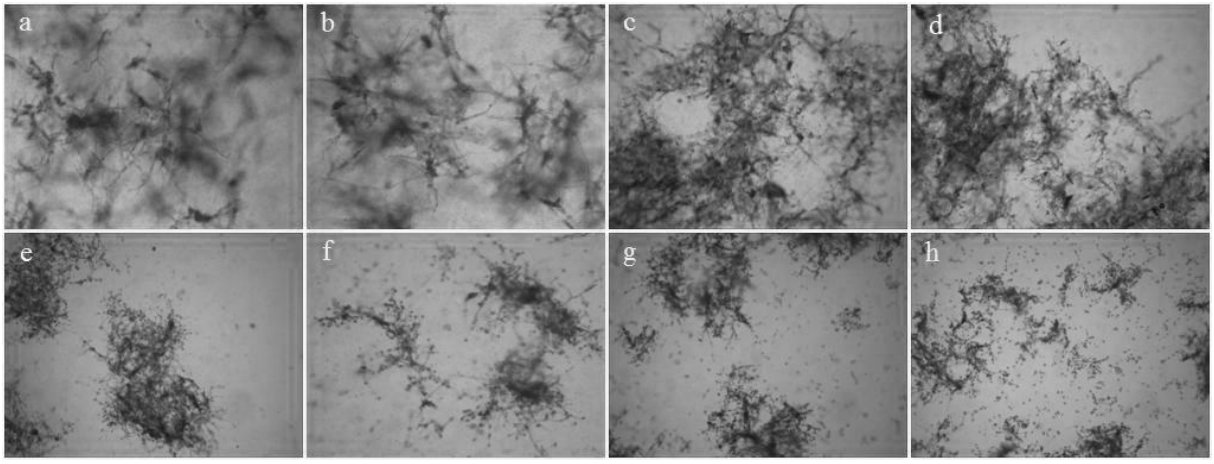

Figure 4.1.6. *Trichoderma viride* sporulation during submerged fermentation of oats at 36 hours (a); 48 hours (b); 60 hours (c); 72 hours (d); 84 hours (e); 96 hours (f); 108 hours (g); 120 hours (h).

3.5. Spore counts

The initial concentration of spores in the culture media was 4.20 x 10^4 spores/mL. After 120 hours of fermentation, the spore concentration found in the fermentation medium was 3.52 x 10^6 spores/mL, indicating that there were two logs of growth during the 5 days of oatmeal-based fermentation.

Watanabe *et al.*[35] used a culture medium containing soluble starch and soybean meal supplemented with KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ to produce *Trichoderma asperellum* spores by submerged fermentation. They obtained 7.8 x 10^8 spores/mL after 7 days of fermentation starting from an inoculum of 1.0 x 10^6 spores/mL. Jakubíková *et al.*[36] optimized *Trichoderma atroviride* sporulation in submerged fermentation on cellobiose supplemented with $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, and $FeSO_4$. The culture medium was inoculated with 1 x 10^6 spores/mL, and the spore concentration reached a maximum level of 2.68 x 10^8 spores/mL after 4 days of fermentation.

The comparison of the results obtained in the present work to past results indicates that the submerged fermentation of oatmeal is a good alternative for the production of *Trichoderma viride* spores, especially considering that the culture medium used in this study was supplemented only with peptone.

4. Conclusions

The oats were valuable substrates that can be inoculated for the biomass production from *Trichoderma viride* using a non-conventional heterogeneous submerged fermentation process, which is easily controllable. In addition, it showed ability to conidiate under submerged conditions, which makes it suitable for the fermentative production of spores and application in biocontrol. The protein concentration and dry biomass correlated with cell concentrations in oat medium, which was an adequately way to quantify the fungal biomass. Research on the enzymes produced by *Trichoderma viride* during the fermentation using oats as a substrate as well as the optimization of the medium composition and process conditions, according to the target product, are important topics for future works.

Acknowledgements

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4.2. *Production of humic acids from oil palm empty fruit bunch by submerged fermentation with Trichoderma viride: cellulosic substrates and nitrogen sources*

Artigo publicado no Periódico Científico *Biotechnology Progress*

Production of humic acids from oil palm empty fruit bunch by submerged fermentation with *Trichoderma viride*: cellulosic substrates and nitrogen sources

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Abstract

The novelty of this study was to produce humic acids by submerged fermentation of empty fruit bunch (EFB) with *Trichoderma viride* and to investigate the effects of the cellulosic substrates and the organic sources of nitrogen on the biotechnological production of these acids. The results obtained indicate the potential application of EFB, a waste of oil palm processing, for humic acids production. Because EFB contains cellulose, hemicellulose and lignin, fermentations were also performed using these polymers as carbon sources, separately or in combination. After 120 hours of fermentation, significant production of humic acids was observed only in cultures containing either EFB or a mixture of the three polymers. Use of either potato peptone or yeast extract as a nitrogen source yielded nearly identical patterns of fungal growth and production of humic acids. The data obtained from microscopic imaging of *Trichoderma viride* growth and sporulation in EFB, coupled with the determined rates of production of humic acids indicated that the production of these acids is related to *Trichoderma viride* sporulation.

Keywords: Humic acids, *Trichoderma viride*, submerged fermentation, cellulosic substrate, nitrogen source.

1. Introduction

Humification is the process of the decay of dead organisms through microbial activity, resulting in the production of humic substances (HS) and the decomposition of nonhumic substances.[1,2] The mechanisms of formation of HS differ depending on the geographical, climatic, physical and biological circumstances. These compounds can be formed in several ways, although lignin is important in the majority of the humification processes.[3,4] HS are divided into three main fractions based on their solubility in acids and alkalis: humic acid (HA), fulvic acid and humin.[5]

HA, one of the most important components of HS, consists of a heterogeneous association of molecules or small humic sub-units of different chemical natures and origins[2] containing a variety of functional groups, including carboxylic acid (COOH), phenolic hydroxy (OH), enolic (OH), alcoholic (OH), quinone, hydroxylquinone, lactone, and ether.[6] The remarkable properties of these acids and their applications in agriculture, industry, the environment, and biomedicine[7] have attracted the attention of many investigators.[8] They help break up clay in compacted soils, assist in transferring micronutrients from soil to plants, enhance water retention, increase seed germination rates, and stimulate the development of microflora populations in soils.[8] However, the main targets for the increased interest in HA are their pharmacological effects with potential use in medicine such as their antiviral, estrogenic and desmutagenic activities, the antiinflammatory effects and pro-inflammatory properties, and the influence on blood coagulation and fibrinolysis.[8,9] HA are extracted from peat and coal, two sources of non-renewable carbon, in industrial processes.[10] Liu and Huang[11] investigated the catalysis of hydroxy-aluminosilicate ions in the oxidative polymerization of catechol and the resultant formation of HS. Litvin *et al.*[12] developed a two-step chemical procedure for preparing synthetic HA that is similar to natural HA. However, very little research has been performed on the production of these acids by biotechnological processes. This is a major gap in the production processes of HA for pharmaceutical applications, which require sustainable production and use of controlled and reproducible processes. Therefore, the aim of this study was to develop a low-cost fermentation process for producing HA for pharmaceutical use. As far as we know, this is the first report of using EFB for HA production by *Trichoderma viride* in submerged fermentation.

For economic reasons, industrial fermentations involve complex, largely undefined substrates, which are often by-products of other industrial processes. In this context, empty fruit bunch (EFB), a left-over from palm oil mill processing, is a cellulosic material source consisting

of 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin[13], and is a strong candidate for use as a fermentation substrate. EFB, which comprises 23% of the fresh fruit bunch[14], is an abundant and capacious waste product of oil palm processing and has not been utilized effectively. Considering that the global production rate for oil palm has doubled over the last decade, and worldwide demand for it is expected to double again by 2020[15], there is a growing interest in adding value to EFB[16] and also in reducing the amount of this waste to diminish its environmental impact.[17]

The potential of *Trichoderma* species as lignocellulose-degrading agents was recognized in the early 1960s.[18] *Trichoderma* species are frequently found growing in soil as well as on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic capacities and their adaptability to various ecological conditions.[19] These characteristics make the genus useful for many biotechnological applications.[20] The conidial mass of *Trichoderma* is the most proficient propagule that tolerates downstream processing.[21] Because *Trichoderma* fermentation is a highly aerobic process[22], submerged fermentation, whereby the organism is grown in a liquid media that is vigorously aerated and agitated[23], is widely used for this genus. Furthermore, because liquid cultures are homogenous and easy to standardize, maintain, and monitor[24]; this fermentative process is usually preferred for large-scale fermentations.[25]

Because HA precursor(s) has(have) not yet been elucidated, this work also investigated the influence of different cellulosic substrate(s) for HA production by fermentation using *Trichoderma viride*. A study comparing the effect of different organic sources of nitrogen on both HA production and fungal growth was also performed. Yeast extract was replaced with vegetable peptone for the nitrogen source study because the latter is a green product and may be a safer supplement for culture media.[26]

2. Methods

2.1. Scanning electron microscopy (SEM) of EFB particles

The shapes and surface morphologies of particles were examined by scanning electron microscopy (Leo 440i, LEO Electron Microscopy, England) in the high-vacuum mode with an acceleration voltage of 10 kV. Powders were sprinkled onto SEM stubs topped with adhesive carbon tape, and sputter coated (SC7620, VG Microtech, England) with Au to a thickness of 92 A°. Because EFB particles are electrically nonconductive, the samples were subjected to low-vacuum gold sputter-coating in the presence of argon gas.

2.2. Size of EFB particles

The average mean diameter of EFB particles used in the culture media was determined by laser diffraction using a Mastersizer 2000 (Malvern Instruments, USA).

2.3. Microorganism cultures

For storage, the *T. viride* strain culture was grown on potato dextrose agar plates at 24 °C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 ml cryotubes at -70 °C.

The inoculum was prepared in 500 mL Erlenmeyer flasks containing 300 mL of culture media comprising 30 g L^{-1} oatmeal and 5 g L^{-1} potato peptone, as described in our previous paper.[27] The culture media was inoculated with 1 mL of the spore suspension and incubated for 120 hours at 24 °C with agitation of 150 rpm, to allow the formation of large quantities of spores.

2.4. Fermentation media

EFB provided by Oil Palm S/A Agro-industrial OPALMA (Bahia, Brazil), were milled to produce standardized particles with diameters between 125 and 500 μm (115 and 32 mesh of Tyler series, respectively). The composition of the EFB particles was determined with an elemental analyzer CNH (Perkin Elmer Series II 2400, USA) indicating that mass percentage of each of these elements is 48.0±0.7% of carbon; 2.6±0.1% of nitrogen and 6.1±0.2% of hydrogen. These results are expressed as the mean of triplicates and their average deviations.

Cellulosic substrates study: To investigate the influence of various cellulosic substrates for the production of HA by *T. viride,* five different culture media were prepared (see Table 4.2.1). Three separate culture media were prepared with only one of the three cellulosic components of EFB: commercial cellulose, hemicellulose or lignin. The fourth culture medium was prepared with a mixture of these three components in equal proportions, and the fifth, containing EFB, was the control medium. The concentrations of $(NH_4)_2SO_4$, K_2HPO_4 and yeast extract were based on the work of Kalsom *et al*.[28] The initial pH was adjusted to 6.0[21] prior to autoclaving at 121 °C for 15 min.

Table 4.2.1. Composition of the culture media used in the cellulosic substrates study.

Media	Carbon source (g L^{-1})		Components present in all fermentation media (g L^{-1})	
1	Cellulose	20.0		
2	Xylan*	20.0		
3	Lignin	20.0	$(NH_4)_2SO_4$	0.77
	Cellulose	6.67	K_2HPO_4	1.54
4	Xylan*	6.67	Yeast extract	3.85
	Lignin	6.67		
5	EFB	20.0		

*Xylan represents the hemicellulose carbon source.

Nitrogen sources study Two fermentations were carried out to investigate the effect on fungal growth and HA production of substituting yeast extract with vegetable peptone. Both culture media contained 0.77 g $L^{-1}(NH_4)_2SO_4$, 1.54 g L^{-1} K_2HPO_4 and 20 g L^{-1} EFB, with either yeast extract or potato peptone at 3.85 g L^{-1}. The pH was adjusted to 6.0[21] prior to autoclaving at 121 °C for 15 min.

2.5. Submerged fermentation

The cultures were grown in 500 mL Erlenmeyer flasks containing 270 mL of culture media and 30 mL of inoculum prepared as described above (see 2.3). The culture flasks were incubated at 30 °C with agitation at 150 rpm. Samples withdrawn every 12 hours throughout the 120 hours of cultivation were processed for determination of the protein and HA concentrations and for microscopic imaging. All fermentations were carried out in triplicate and the results represent means of three independent experiments ± standard deviation.

2.5.1. Protein quantification

Indirect estimation of the fungal biomass was carried out by determining the protein concentration. Protein quantification was performed according to the method adapted by Callow and Ju[23] to quantify only the cellular proteins. Culture samples (3.0 mL) were collected and centrifuged at 10,000 g for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged and the water discarded. To release the intracellular

proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100 °C for 10 min. After cooling, the digested samples were centrifuged at 10,000 g for 10 min to remove the cell debris and other solids; the supernatants were then collected and their protein concentrations were determined with the bicinchoninic acid assay[24], using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were constructed with bovine serum albumin.

2.5.2. HA quantification

Following methods adapted by Badis *et al.*[32], samples were centrifuged at 10,000 g for 15 min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany) and the supernatant fractions were filtered using the Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted five-fold with 0.5 M NaOH solution and the absorbances at 350 nm at pH 4.5±0.01 were measured. Standard curves were obtained from the absorbances at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in 0.5 M NaOH solution pH 4.5±0.01.

2.5.3. Image analysis

Samples of approximately 10–50 µL were taken for smear preparations. Images of the mycelia and conidia were obtained using a microscope (Reichert-Jung Series 150, Reichert, USA) equipped with a digital camera.

3. Results and Discussion

Figure 4.2.1 shows examples of the EFB particles that were utilized for HA production in the culture media (Medium 5, Table 4.2.1). These particles had an average diameter of 248.5±0.8 µm, and became surrounded by both fungal mycelia and conidia during fermentation (Figure 4.2.2).

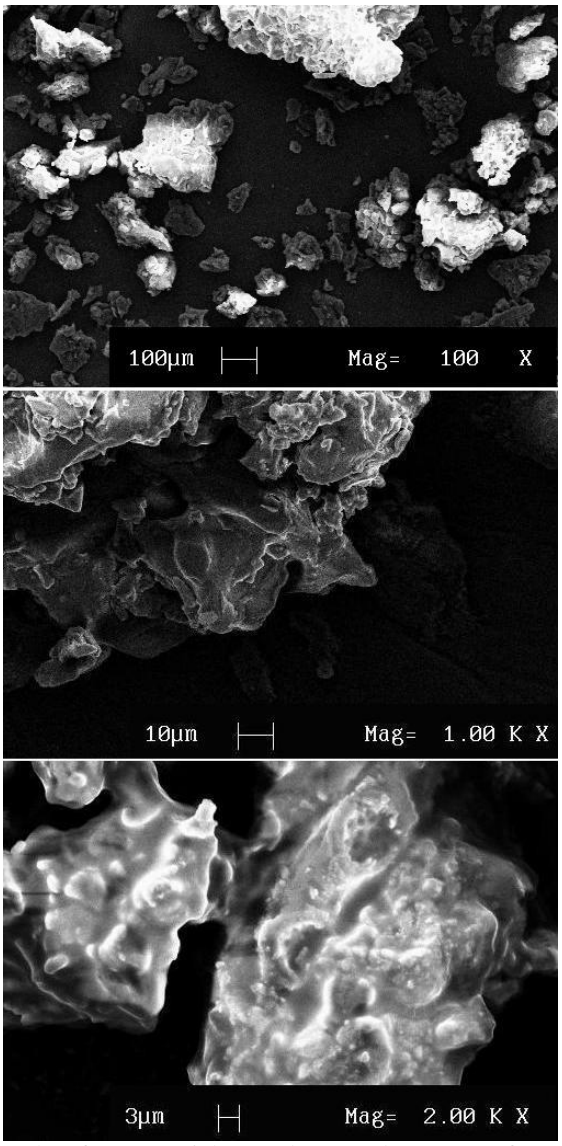

Figure 4.2.1. SEM micrographs of EFB particles used as a carbon source for HA production by *T. viride*.

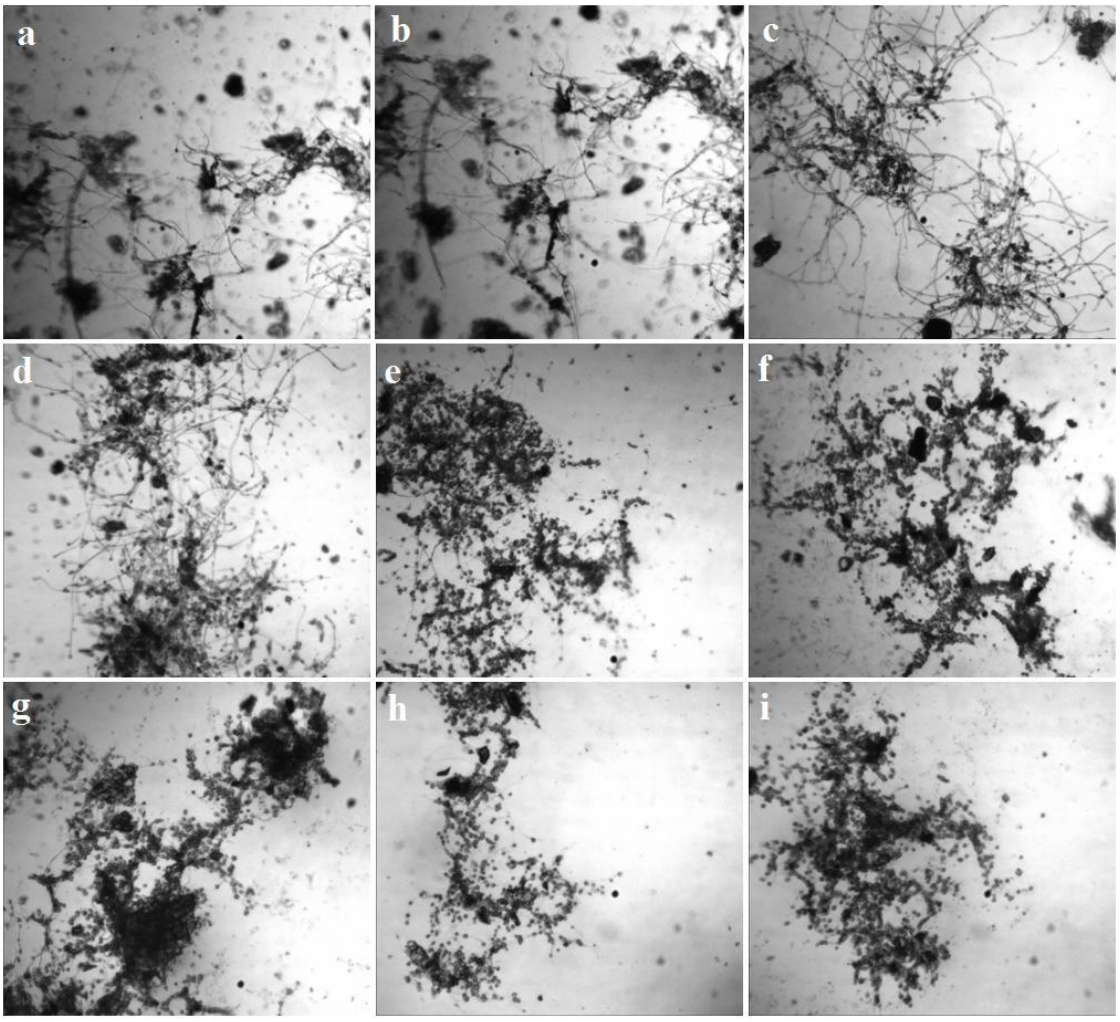

Figure 4.2.2. *T. viride* sporulation biomasses present after submerged fermentation of EFB and potato peptone for 24 hours (a); 36 hours (b); 48 hours (c); 60 hours (d); 72 hours (e); 84 hours (f); 96 hours (g); 108 hours (h); and 120 hours (i). Magnification 45x.

Figure 4.2.3 shows the changes in the concentrations of cellular proteins, which represent the *T. viride* biomass, during fermentation in the five different culture media used for the cellulosic substrates experiment. Fungal growth was observed in all media employed, as expected because strains of *Trichoderma* can degrade complex substrates such as cellulose, chitin, xylan, and lignin.[33] The data reveal that the highest rate and amount of cell growth was achieved in the culture media containing EFB, whereas the lignin media and the media containing an equal mixture of the three polymers supported increasingly slower growth rates. The higher fungal growth found in the EFB medium can be related, among other factors, to the residual oil content in EFB, which represents at least 0.5% m/m of theses fibers and can be easily recovered[34]. The lowest cellular protein concentrations were reached at similarly slow rates in media containing either cellulose or

xylan. *Trichoderma* produces large quantities of hydrolytic enzymes[35], including chitinases, β-1,3-glucanases[36], cellulases, amylases and proteases.[37] Moreover, this filamentous fungus is an exceptionally efficient producer of cellulases and hemicellulases, which act in synergy to degrade lignocellulosic materials.[38] Therefore, the high growth rates observed in EFB media and in the media containing an equal mixture of the three polymers compared to that in media with a single cellulosic substrate source can be explained by the greater diversity of cellulosic material allowing the fungus to synergistically use a wider variety of enzymes for its growth.

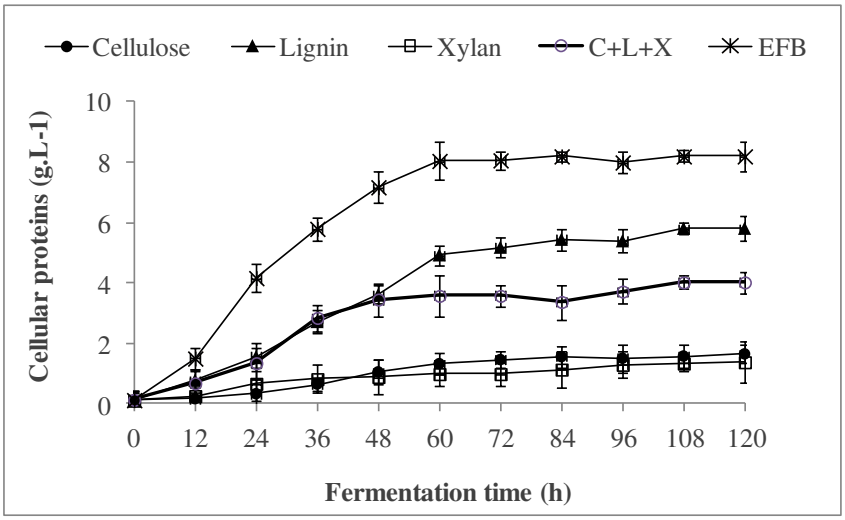

Figure 4.2.3. Cellular protein concentration *versus* time during submerged fermentations of different cellulosic substrates by the *T. viride* strain. C+L+X: mixture of cellulose, lignin and xylan.

Figure 4.2.4 demonstrates that fungal synthesis of HA occurred only in media containing either the polymer mixture or EFB. The HA concentrations in the media containing cellulose, lignin or xylan, separately, was close to zero throughout 120 hours of fermentation, negligible compared to that achieved in the other two culture media, where the three polymers were present in the culture medium composition. These results indicate the synergistic effect of the three polymeric components of EFB on the production of HA during the fermentation processes evaluated in this study. Furthermore, the HA production in the media containing the polymer mixture was lower than that in the media containing EFB, 42.3 mg L^{-1} and 52.2 mg L^{-1}, respectively. These results indicate that the ratio of cellulose, hemicellulose and lignin in the media influences the rate of synthesis of HA by *T. viride*.

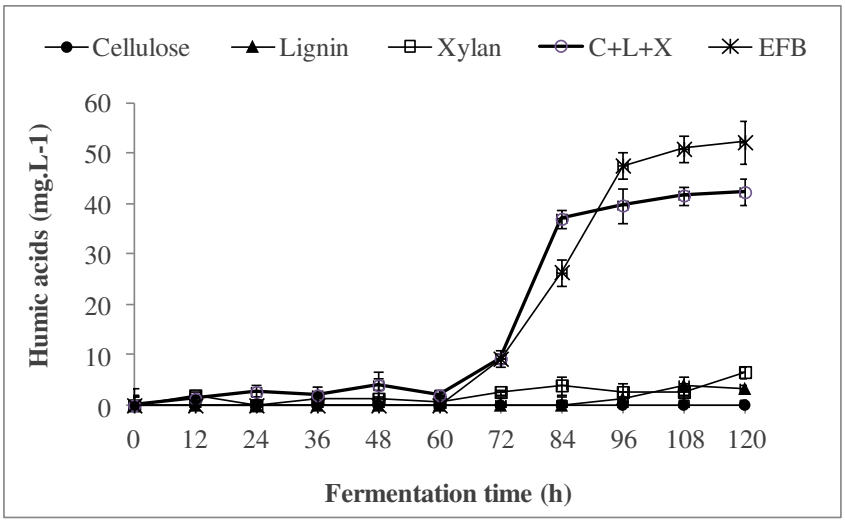

Figure 4.2.4. HA concentration *versus* time during submerged fermentations of different cellulosic substrates by *T. viride*. C+L+X: mixture of cellulose, lignin and xylan.

Kalsom *et al.*[28] produced HA by solid state fermentation using yeast extract as the nitrogen source. According to Weete[39], organic nitrogen sources are best for the growth of many fungi. However, nitrogen sources of animal origin may contain agents that cause transmissible spongiform encephalopathies (TSE)[40], a family of diseases of humans and animals characterized by spongy degeneration of the brain with severe and fatal neurological signs and symptoms.[41] Regulations aimed at preventing the risk of TSE contamination are highly important in the pharmaceutical industry.[42] In this context, yeast extract and vegetable peptone are animal-free components and are used extensively for many animal-free formulations. However, yeast extract have endotoxins, which cause illness in humans and are considered contaminants that must be avoided or minimized in the preparation of pharmaceutical products.[43] Therefore, using vegetable peptone in the culture media on industrial production lines would reduce the potential for contamination by TSE agents. Therefore, in the present study, yeast extract was replaced by potato peptone, an organic source of nitrogen from vegetables, because the ultimate aim of this work is establishing methods to produce HA for pharmaceutical use.

According to the suppliers' data, the yeast extract (Oxoid, England) used in this study contains 10-12.5% w/w of total nitrogen and 5.1% w/w of amino nitrogen, whereas the potato peptone used (Fluka, France) contains 9.5% w/w of total nitrogen and 4.5% w/w of amino nitrogen. These values indicate that both nitrogen sources would provide similar amounts of nitrogen to *T. viride*, and are consistent with the similar rates of growth (Figure 4.2.5) and HA production (Figure 4.2.6) in media containing either yeast extract or potato peptone. The cellular protein and HA

concentrations reached by 120 hours of fermentation were, respectively 8.7 g L^{-1} and 64.7 mg L^{-1} in the media containing yeast extract, and 9.3 g L^{-1} and 62.1 mg L^{-1}, in the media containing potato peptone. These results demonstrate that replacing yeast extract peptone with vegetable peptone, in addition to achieving the main goal of reducing the risks of contamination, does not compromise HA production. In the culture media containing peptone, HA production relative to the mass of EFB used was 3.1 mg of HA / g of EFB.

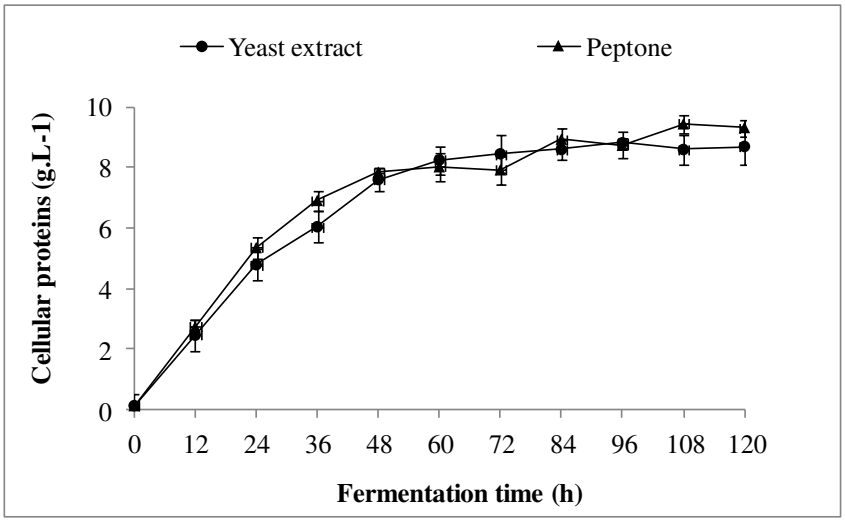

Figure 4.2.5. Cellular protein concentrations *versus* time during submerged fermentations of EFB by *T. viride* using different nitrogen sources.

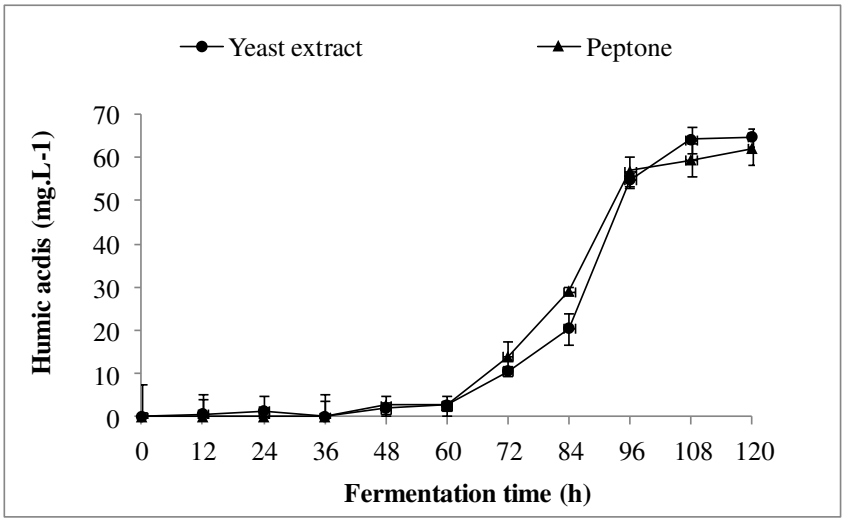

Figure 4.2.6. HA concentrations *versus* time during submerged fermentations of EFB by *T. viride* using different nitrogen sources.

Figure 4.2.4 and Figure 4.2.6 show that production of HA by *T. viride* began at approximately 60 hours of fermentation. To investigate the basis for this pattern, samples of culture media containing peptone and EFB were examined by microscopy. The microscopic images shown in Figure 4.2.2 demonstrated that sporulation was occurring by 60 hours of fermentation and was completed by approximately 96 hours. After 96 hours, only spores were observed; mycelia were not observed. Comparing the microscopic images with the graphs of HA concentration *versus* time during fermentation with potato peptone for which the images were captured (Figure 4.2.2 and Figure 4.2.6) reveals that HA production and *T. viride* sporulation surge at a very similar time point during fermentation, indicating a relationship exists between these processes. As reported by Brodhagen and Keller[44], who studied the relationship between *Aspergillus* and *Fusarium* sporulation and mycotoxin production, both regulated by G protein signalling pathways, some secondary metabolites are associated temporally and functionally with sporulation. Slepecky and Law[45] concluded in their work that the synthesis of poly-3-hydroxybutyric acid is connected with *Bacillus megaterium* strain sporulation. Scribner *et al.*[46] investigated the production of pigment by *Streptomyces venezuelae* where found that the medium pigmentation appeared to be closely associated with sporulation. Moreover, Siddiqui *et al.* confirmed through their experiments the fungicidal activity of HA isolated from empty fruit bunch of oil palm compost[47], which indicates that the similarity between the time duration of sporulation and HA production might be a niche preservation strategy of the *T. viride*.

According to the International Humic Substances Society (IHSS)[48], the HA purification is not labor intensive and involves inexpensive reagents. The methodology proposed by IHSS comprises the acidification of the alkaline extract causing the precipitation of these acids, which are removed from the acidified extract by centrifugation and purified (i.e. de ashed) using an HCl/HF ash.

Propagules, like submerged spores, must retain a high level of viability under adverse and variable conditions[49], where the conidial mass of *Trichoderma* is the most proficient propagule, tolerating downstream processing.[50] Moreover, reduction of the mycelia in the liquid culture, increasing the spore population, would also be desirable, since mycelia create separation and disposal problems.[51]

Apart from the extremely low cost of EFB and the advantages of the purification process of HA, only spores are present at the end of the studied submerged fermentation, allowing a simple

one-step centrifugation promotes the separation of the fungus and the target product, factors that increase the economic benefits of the proposed process.

4. Conclusions

This study provides crucial information on the production of HA by *T. viride*. This is the first report of using EFB for HA production by *T. viride* in submerged fermentation and the results obtained indicate its potential for industrial applications. Such use offers a valuable alternative to simply recycling EFB, not only adding value to this abundant, underutilized waste material but also reducing its environmental impact. The studies employing different cellulosic substrates suggest that the presence of cellulose, hemicellulose and lignin has synergistic effects because none of these polymers used singly supported the production of HA. In addition, the results showed that peptone potato can be substituted for yeast extract without compromising HA production; this would reduce the risks of contamination caused by the use of animal sources of nitrogen. Further improvements in the proposed process are necessary before testing large-scale applications, including optimization of the media components and the fermentation conditions, as well as more detailed investigations into the inoculum size and the duration of its culture, because HA production seems to be related to *T. viride* sporulation.

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4.3. *Influence of medium components and fermentation conditions on the production of humic acids by Trichoderma viride*

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Influence of medium components and fermentation conditions on the production of humic acids by *Trichoderma viride*

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Abstract

Statistical analysis were used to optimize the production of humic acids (HA) by a *Trichoderma viride* strain under submerged fermentation, in a medium based on the oil palm empty fruit bunch (EFB). The media nutrients and the fermentation conditions were optimized using a sequential statistical optimization process. First, the factors that affected the HA production were screened out by a Plackett & Burman design (PB). The most significant factors (the temperature and the EFB, potato peptone and $(NH_4)_2SO_4$ concentrations) were further studied by a central composite rotatable design (CCRD), which allowed the construction of a prediction model and a response surface. A temperature of 40 °C and concentrations of 50 g L^{-1} EFB, 5.7 g L^{-1} potato peptone and 0.11 g L^{-1} $(NH_4)_2SO_4$ were indicated by the contour curves as the levels that would maximize HA production. The designed model was then validated using those levels and comparing the results with predicted values. The HA concentration obtained was 428.4±17.5 mg L^{-1}, compared to a prediction of 412.0 mg L^{-1}. The results indicate the success of the statistical optimization—HA production can be enhanced by optimizing the media and the fermentation conditions.

Keywords: Statistical analysis; humic acids; *Trichoderma viride;* submerged fermentation; empty fruit bunch.

1. Introduction

Humic acids (HA), part of the organic matter in soil, are the soil fraction that is most resistant to microbial degradation. They are complex polymeric organic acids with a wide range of molecular weights, and they can be heterogeneous mixtures of a variety of organic compounds, including aromatic, aliphatic, phenolic, and quinolic functional groups [1]. In addition, HA's size, chemical composition, structure, and functional groups may vary greatly, depending on its origin and the age of the material [2]. They are one of most active fractions of organic matter and affect a variety of chemical, physical, and biological reactions. From an agricultural point of view, they improve the absorption of nutrients by both plants and soil microorganisms, positively affect the dynamic of nitrogen and phosphorus in soil, stimulate plant respiration and photosynthesis, and favor the formation of soil aggregates, among other benefits [3]. Moreover, they have deservedly received growing attention from the field of biomedicine, primarily due to their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities [9], which are of great importance for pharmaceutical and biomedical applications [7]. An important source of HA is leonardite, which contains 40% of these acids, as well as black peat and brown coal, both of which contain 10% HA [6]. However, harvesting humic acids from such non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peatlands, which are important for biodiversity, carbon storage and flood risk management [7]. It is thus desirable to use more ecologically sustainable precursors/feedstocks for HA and develop cheaper and cleaner methods for the extraction of this valuable product.

For economic reasons, industrial fermentations involve complex, almost undefinable substrates that are often the by-products of other industries. Empty fruit bunch (EFB), a cellulosic material source containing 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin [13], is a strong candidate for use as a fermentation substrate. EFB is the product of oil palm processing, waste that has not been completely utilized, and it is produced in large quantities: 23% of the fresh fruit bunch [14] used in oil palm production remains as EFB. The global production of oil palm has doubled over the last decade, and demand is expected to double again by 2020 [15]. Thus, there is a growing interest in making EFB [16] useful, reducing the large volume of waste and its ensuing environmental problems [17].

The potential of the *Trichoderma* species as lignocellulose degrading agents has been recognized since early 1960 [18]. They are frequently found in soil as well as on substrates like wood,

bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions [19]. This characteristic allows the genus to be used in many and varied biotechnological applications [20]. Moreover, the conidial mass of *Trichoderma* is a proficient propagule and tolerates downstream processing [21]. Because *Trichoderma* fermentation is a highly aerobic process [22], submerged fermentation, in which the organism is grown in a vigorously aerated and agitated liquid medium [23], is widely used. Furthermore, liquid cultures are homogenous, making them easier to control, maintain, and monitor [24], so this fermentative process is often preferred for large-scale fermentations [25].

The classical method of medium optimization, changing one variable while fixing the others at a certain level, is laborious and time-consuming, particularly when the number of variables is large. An alternative and more efficient approach for microbial systems is the use of statistical methods [21]. The most popular statistical tools are the Plackett & Burman (PB) [22] design and the central composite rotatable design (CCRD) [23]. The PB design is a powerful tool that rapidly determines the effects of different variables on the yield of the required product. It is used to minimize the number of screening variables needed for optimization studies. Response surface methodology is an optimization method primarily based on statistical techniques, and it is a powerful and efficient mathematical approach applied to fermentation processes, where CCRD is the most popular choice for fitting a second order model [23].

In our previous article [24], we proposed a submerged fermentation process with *T. viride* as a biotechnological route for HA production from EFB. In the present study, an investigation was carried out to understand the optimum media composition and fermentation conditions for HA production, based on the results obtained in our previous work. For this purpose, PB was used to screen for the significant parameters, followed by the application of CCRD to determine the response surface. Once the culture medium and fermentation conditions were optimized, assays were performed in triplicate to validate the results. From these assays, the HA production as well as the profiles of the cellular proteins representing the fungal biomass were determined.

2. Material and Methods

2.1. Microorganisms and inoculum

For storage, the *T. viride* strain culture, purchased from Fundação Tropical (São Paulo, Brazil), was grown on potato dextrose agar plates at 24 °C for 10 days. After sporulation, the spores

were resuspended in a sterile 20% glycerol solution, which was stored in 1.2-mL cryotubes at -70 °C.

The inoculum was prepared in 500 mL Erlenmeyer flasks, each of which contained 300 mL of culture media with 30 g L^{-1} oats and 5 g L^{-1} peptone, prepared as described in our previous article [27]. The culture flask was inoculated with 1 mL of the spore suspension (1.26 x 10^7 spores) and incubated at 24 °C and 150 rpm for 120 hours to produce a large number of spores.

2.2. Submerged fermentation

EFB (provided by Oil Palm S/A - Agro-industrial OPALMA, Bahia, Brazil) was milled to achieve a standardized particle size of between 125 and 500 μm (115 and 32 Tyler series mesh, respectively). The composition of the EFB particles was determined by a CNH elemental analyzer (Perkin Elmer Series II 2400, Maryland, USA). The mass percentages were 48.0±0.7% carbon, 2.6±0.1% nitrogen and 6.1±0.2% hydrogen. These results are the means of triplicates with their average deviations. Potato peptone was purchased from Fluka Analytical (France); $(NH_4)_2SO_4$ and K_2HPO_4 were purchased from Ecibra (Brazil).

The fermentation cultures were housed in 500 mL Erlenmeyer flasks that contained 270 mL of culture media inoculated with 30 mL of inoculum (3.52 x 10^6 spores mL^{-1}), prepared as described in section 2.3. The culture flasks were incubated at 150 rpm. Over the course of 120 hours of cultivation, samples were withdrawn every 24 hours for analysis.

2.3. Statistical analysis

Plackett & Burman design: This design was used to screen for the selected variables that significantly affected the HA production. Six variables were screened: pH, temperature (°C), EFB (g L^{-1}) as a carbon source, potato peptone (g L^{-1}) as an organic nitrogen source, and K_2HPO_4 (g L^{-1}) and $(NH_4)_2SO_4$ (g L^{-1}) as inorganic nitrogen sources (Table 4.3.1). Each factor of this statistical analysis was examined at three levels—low (−), high (+) and central (0)—to evaluate both the linear and curvature effects of the variables. The samples that were withdrawn every 24 hours throughout the cultivation were processed to determine their HA concentrations, and the values obtained after 120 hours of fermentation were used as the response variables. Table 4.3.2 shows the PB experiment design, with 16 trials that varied each of the six factors, and the resulting HA amounts produced. This statistical design does not consider the interactions between the selected variables and follows

a linear approach to screen the factors [22]. The effects of the variables are summarized in Table 4.3.3, as obtained by Statistica software, version 8.0 (Statsoft, Oklohoma, USA). The variables that were considered to have a significant effect on the response (p-value<0.1) were then optimized by CCRD, a response surface methodology.

Table 4.3.1. The lower (-1), higher (+1) and central (0) levels of the six variables screened by the PB design.

Variables	Units	Experimental values		
		-1	0	+1
EFB	g L^{-1}	10.0	20.0	30.0
Peptone	g L^{-1}	1.00	3.85	6.70
pH	-	4.0	6.0	8.0
Temperature	°C	25.0	30.0	35.0
K_2HPO_4	g L^{-1}	0.28	1.54	2.80
$(NH_4)_2SO_4$	g L^{-1}	0.24	0.77	1.30

Table 4.3.2. The experimental PB design used to screen six variables, with their real and code values (in parentheses) and the observed values of the HA production response.

Trial	Experimental values						HA ($mg\ L^{-1}$)[a]
	EFB ($g\ L^{-1}$)	Peptone ($g\ L^{-1}$)	pH	Temperature (°C)	K_2HPO_4 ($g\ L^{-1}$)	$(NH_4)_2SO_4$ ($g\ L^{-1}$)	Observed
1	30.0 (+1)	1.00 (-1)	8.0 (+1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	12.6
2	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	0.28 (-1)	0.24 (-1)	85.2
3	10.0 (-1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	0.24 (-1)	48.9
4	30.0 (+1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	38.3
5	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	107.0
6	30.0 (+1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	44.3
7	10.0 (-1)	6.70 (+1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	46.9
8	10.0 (-1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	46.9
9	10.0 (-1)	1.00 (-1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	1.30 (+1)	10.3
10	30.0 (+1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	3.3
11	10.0 (-1)	6.70 (+1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	1.30 (+1)	47.6
12	10.0 (-1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	20.2
13	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	58.8
14	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	58.1
15	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	57.5
16	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	60.8

[a] HA concentrations were obtained after 120 hours of fermentation.

Table 4.3.3. A summary of the estimated effects of the PB-tested variables.

Factor	Estimated effect	Standard error	t(8)	*p*-value
Mean/Interc.	42.6	3.1	13.6	<0.0001
Curvature	32.3	12.5	2.6	0.0323
EFB	11.6	6.3	1.9	0.0998
Peptone	41.4	6.3	6.6	0.0002
pH	-6.0	6.3	-1.0	0.3671
Temperature	26.3	6.3	4.2	0.0030
K_2HPO_4	1.7	6.3	0.3	0.7984
$(NH_4)_2SO_4$	-21.7	6.3	-3.5	0.0085

Central composite rotatable design: After screening the significant variables, a CCRD was used to optimize four variables: the temperature (°C) and the levels of EFB (g L^{-1}), potato peptone (g L^{-1}), and $(NH_4)_2SO_4$ (g L^{-1}). Each variable was studied at five coded levels (−2, −1, 0, +1, +2), and the model included 28 runs with four replicates at the center value, as shown in Table 4.3.4. A multiple regression analysis of the model and the construction of response surface graphs were performed by Statistica, version 8.0 (Statsoft, Oklohoma, USA). The quality of the regression equation was determined by the coefficient of determination (R^2), and its significance was judged by an F test. The fitted second order polynomial equation was explained in the form of three-dimensional graphs to show the relationship between the response and the experimental variables. The point optimization method was used to optimize the maximum response of each variable. The samples withdrawn every 24 hours throughout the cultivation were processed to determine their HA concentrations, and the values obtained after 120 hours of fermentation were used as the response variables.

Table 4.3.4. The experimental CCRD design of the media nutrients and physical parameters, with both the real and coded values (in parentheses) and the observed values of the HA production response.

Trial	Experimental values				HA (mg L^{-1})[a]	HA (mg L^{-1})	Relative deviation (%)
	EFB (g L^{-1})	Peptone (g L^{-1})	Temperature (°C)	$(NH_4)_2SO_4$ (g L^{-1})	Observed	Predicted	
1	20.0 (-1)	2.00 (-1)	25.0 (-1)	0.44 (-1)	45.4	89.1	49.0
2	20.0 (-1)	2.00 (-1)	25.0 (-1)	1.10 (+1)	51.1	67.2	24.0
3	40.0 (+1)	2.00 (-1)	25.0 (-1)	0.44 (-1)	89.8	49.8	-80.3
4	40.0 (+1)	2.00 (-1)	25.0 (-1)	1.10 (+1)	16.9	27.9	39.3
5	20.0 (-1)	5.70 (+1)	25.0 (-1)	0.44 (-1)	70.3	69.8	-0.7
6	20.0 (-1)	5.70 (+1)	25.0 (-1)	1.10 (+1)	79.0	47.9	-65.1
7	40.0 (+1)	5.70 (+1)	25.0 (-1)	0.44 (-1)	44.9	83.6	46.3
8	40.0 (+1)	5.70 (+1)	25.0 (-1)	1.10 (+1)	27.4	61.7	55.6
9	20.0 (-1)	2.00 (-1)	35.0 (+1)	0.44 (-1)	112.3	79.4	-41.5
10	20.0 (-1)	2.00 (-1)	35.0 (+1)	1.10 (+1)	75.4	57.5	-31.1
11	40.0 (+1)	2.00 (-1)	35.0 (+1)	0.44 (-1)	87.5	127.5	31.4
12	40.0 (+1)	2.00 (-1)	35.0 (+1)	1.10 (+1)	91.7	105.6	13.1
13	20.0 (-1)	5.70 (+1)	35.0 (+1)	0.44 (-1)	157.2	145.6	-7.9
14	20.0 (-1)	5.70 (+1)	35.0 (+1)	1.10 (+1)	61.0	123.7	50.7
15	40.0 (+1)	5.70 (+1)	35.0 (+1)	0.44 (-1)	239.9	246.9	2.8
16	40.0 (+1)	5.70 (+1)	35.0 (+1)	1.10 (+1)	269.6	225.0	-19.8
17	30.0 (0)	3.85 (0)	20.0 (-2)	0.77 (0)	46.3	40.2	-15.1
18	30.0 (0)	3.85 (0)	40.0 (+2)	0.77 (0)	172.2	193.8	11.2
19	30.0 (0)	0.15 (-2)	30.0 (0)	0.77 (0)	6.8	1.0	-600.7
20	30.0 (0)	7.55 (+2)	30.0 (0)	0.77 (0)	117.4	101.0	-16.2
21	10.0 (-2)	3.85 (0)	30.0 (0)	0.77 (0)	74.8	86.0	13.1
22	50.0 (+2)	3.85 (0)	30.0 (0)	0.77 (0)	152.7	148.0	-3.2
23	30.0 (0)	3.85 (0)	30.0 (0)	0.11 (-2)	142.3	138.9	-2.4
24	30.0 (0)	3.85 (0)	30.0 (0)	1.43 (+2)	98.5	95.1	-3.6
25	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	137.5	117.0	-17.5
26	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	136.3	117.0	-16.5
27	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	139.4	117.0	-19.2
28	30.0 (0)	3.85 (0)	30.0 (0)	0.77 (0)	136.8	117.0	-16.9

[a] HA concentrations were obtained after 120 hours of fermentation.

Statistical model validation: To validate the optimization of the media composition and fermentation conditions that were determined by the statistical analysis, an experiment using the optimized variables was conducted in triplicate for 120 hours. The mean values were used to confirm the results of the CCRD analysis. Moreover, the samples withdrawn every 24 hours throughout the cultivation were processed to determine their HA and cellular protein concentrations. Graphs were constructed to represent the behavior of the parameters during the optimized fermentation process.

2.4. HA quantification

The following methods were adapted from Badis et al. [26]. The samples were centrifuged at 10,000 g for 15 min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant fractions were filtered using the Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted 5-fold with a 0.5 M NaOH solution at pH 4.5±0.01, and the absorbances at 350 nm were measured. Standard curves were obtained from the absorbances at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in a 0.5 M NaOH solution at pH 4.5±0.01.

2.5. Protein quantification

An indirect estimation of the fungal biomass was made by determining the protein concentration. Protein quantification was performed according to the method adapted by Callow and Ju [23] to quantify only the cellular proteins. Culture samples (3.0 mL) were collected and centrifuged at 10,000 g for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged and the water discarded. To release the intracellular proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100 °C for 10 min. After cooling, the digested samples were centrifuged at 10,000 g for 10 min to remove the cell debris and other solids; the supernatants were then collected and their protein concentrations were determined via the bicinchoninic acid assay [28], using a commercial kit

(BCA Protein Assay, Thermo Scientific, USA). Standard curves were constructed using bovine serum albumin.

3. Results and Discussion

Selection of critical media components and fermentation conditions: Six variables were screened by PB, as shown in Table 4.3.1. The temperature and the concentrations of EFB, peptone and $(NH_4)_2SO_4$ were the four of those screened variables that were considered to have a statistically significant effect on the HA production by presenting a *p*-value<0.1 (Table 4.3.3). Among these four variables, only the $(NH_4)_2SO_4$ concentration had a negative effect on HA production. The peptone concentration had the greatest positive effect on HA production, followed by temperature and EFB concentration. These four variables were selected for further optimization by CCRD to maximize the HA production. K_2HPO_4 concentration and pH had no significant effect on HA production at the confidence level studied (90%). According to Bhattiprolu [29], *T. viride* is able to grow under wide range of pH (4.0 to 9.0), which may explain the fact that this factor have not been statistically significant. Regarding to the K_2HPO_4 concentration, the average chemical formula of HA elucidated by Schnitzer and Khan [30] is $C_{187}H_{186}O_{89}N_9S_2$, which do not contain phosphorus and potassium, explaining the absence of significant effect of this factor.

Media components and fermentation conditions optimization: Following the variable screening, a CCRD optimization including 28 experiments was performed to determine the optimal levels of the four significant factors (the temperature and the EFB, peptone and $(NH_4)_2SO_4$ concentrations) that affected HA production. The real and coded values of each variable used in the trials, as well as the resulting HA concentration obtained after 120 hours of fermentation, are shown in Table 4.3.4. The values of the parameters considered not statistically significant by the PB (the pH and K_2HPO_4 concentration) were fixed at the central level (code 0) shown in Table 4.3.1.

According to the regression analysis of the CCRD (Table 4.3.5), the model terms temperature (Q), EFB (Q), $(NH_4)_2SO_4$ (L), $(NH_4)_2SO_4$ (Q), temperature (L) by $(NH_4)_2SO_4$ (L), peptone (L) by EFB (L), peptone (L) by $(NH_4)_2SO_4$ (L) and EFB (L) by $(NH_4)_2SO_4$ (L) were not

significant (p>0.10). However, the terms $(NH_4)_2SO_4$ (L) and peptone (L) by EFB (L) were ultimately included in the model because their *p*-values were very close to 0.10.

Table 4.3.5. A summary of the regression coefficients for the CCRD.

Factor	Regression coefficients	Standard error	t(13)	*p*-value
Mean/Interc.	137.5	18.0	7.6	<0.0001
Temperature (L)	38.4	7.3	5.2	0.0002
Temperature (Q)	-8.1	7.3	-1.1	0.2879
Peptone (L)	25.0	7.3	3.4	0.0047
Peptone (Q)	-19.9	7.3	-2.7	0.0178
EFB (L)	15.5	7.3	2.1	0.0549
EFB (Q)	-7.0	7.3	-1.0	0.3573
$(NH_4)_2SO_4$ (L)	-10.9	7.3	-1.5	0.1598
$(NH_4)_2SO_4$ (Q)	-5.3	7.3	-0.7	0.4793
Temperature (L) by Peptone (L)	21.4	9.0	2.4	0.0333
Temperature (L) by EFB (L)	21.9	9.0	2.4	0.0303
Temperature (L) by $(NH_4)_2SO_4$ (L)	-1.5	9.0	-0.2	0.8732
Peptone (L) by EFB (L)	13.3	9.0	1.5	0.1635
Peptone (L) by $(NH_4)_2SO_4$ (L)	1.5	9.0	0.2	0.8671
EFB (L) by $(NH_4)_2SO_4$ (L)	3.9	9.0	0.4	0.6717

Explained variance (R^2) = 83.8%

The linear effect of the temperature variable was the most significant, with a *p*-value < 0.0002, which can be explained by the fact that enzyme activities as well as regulation and transport systems are in generally affected enormously by the temperature in microbial systems [31]. The linear effect of EFB concentration, as well as of temperature, was positive, once this component was the main carbon source for HA production by *T. viride* in the studied media. Regarding the nitrogen sources, both peptone (L) and $(NH_4)_2SO_4$ (L) concentration had significant effect on HA production although the first had a positive effect and the second a negative effect. Juwon and Emmanuel [32] tested in their work the influence of nitrogen sources in growth and enzymes production by *T. viride* strain, where they observed that organic nitrogen substrates, like peptone, supported better biomass yield and enzyme activity of the fungus as compared to the inorganic nitrogen substrates tested.

The interactions temperature (L) by peptone (L), temperature (L) by EFB (L) and peptone (L) by EFB (L) had positive coefficients, increasing HA production, whereas the $(NH_4)_2SO_4$ (L) and Peptone (Q) terms had negative coefficients, decreasing HA production.

A nine-variable quadratic polynomial regression model (Eq. 4.3.1) was used to predict the HA production as a function of the four process parameters, including the temperature (T, ◦C), peptone concentration (P, g L^{-1}), EFB concentration (EFB, g L^{-1}) and $(NH_4)_2SO_4$ concentration ($(NH_4)_2SO_4$, g L^{-1}). The equation was constructed from the statistically significant terms and includes $(NH_4)_2SO_4$ (L) and peptone (L) by EFB (L). The values for the independent variables in Eq. 4.3.1 are the coded values.

$$HA\ (g\ L^{-1}) = 117.02 + 38.40*T + 25.01*P - 16.50*P^2 + 15.49*EFB - 10.95*(NH_4)_2SO_4 + 21.40*T*P + 21.86*T*EFB + 13.28*P*EFB \quad \textbf{Eq. 4.3.1}$$

The statistical significance of the model was checked by an F test (ANOVA), and the results are shown in Table 4.3.6. The *F* test value (10.27) for the regression was highly significant (5.1 times higher than the critical value of 2.02), and the percent of variation in the model was suitable (R^2=81.2%). Taking into account the inherent variability of bioprocesses, the model could be considered predictive and was therefore used to generate a contour plot and a response surface (Figure 4.3.1) for HA production.

Table 4.3.6. ANOVA results for the proposed model.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	*F* test[a]
Regression	84,304.38	8	10,538.05	10.27
Residual	19,501.66	19	1,026.40	
Total	103,806.04	27		

Explained variance (R^2) = 81.2%; [a]$F_{0.10;\ 8;\ 19}$ ($F_{tabulated}$) = 2.02

Eq. 4.3.1 was used to plot 3-D response surfaces and their corresponding 2-D contours in Statistica 8.0 to show how the HA production would be affected by different levels of the four process variables. The surface and contour plots are shown in Figure 4.3.1. The response surfaces can be used to explain how two process parameters interact with each other when the other two

parameters are fixed at their central levels. Each 3-D response surface curve has a corresponding 2-D contour curve, representing an infinite number of points for two independent process parameters. In the contour curves, the color level represents the different responses; the darker area demonstrates the conditions that lead to a higher HA production. Figure 4.3.1 (f) and (l) show that there are no significant interactions between the $(NH_4)_2SO_4$ concentration and either the temperature or the EFB concentration, when the other two process parameters are fixed at their central levels. Figure 4.3.1 (b), (d) and (f) show that the highest temperature level (+2) is required to achieve a high HA production. The same analysis can be performed for the EFB concentration, and according to Figure 4.3.1 (d), (h) and (l), a high HA production is achieved when 50 g/L EFB is used. On the other hand, the best $(NH_4)_2SO_4$ concentration level is -2, both economically and for HA production. No increase is seen when the peptone concentration is raised from 5.70 g L^{-1} (+1) to 7.55 g L^{-1} (+2), justifying its use at the +1 level to reduce the fermentation cost.

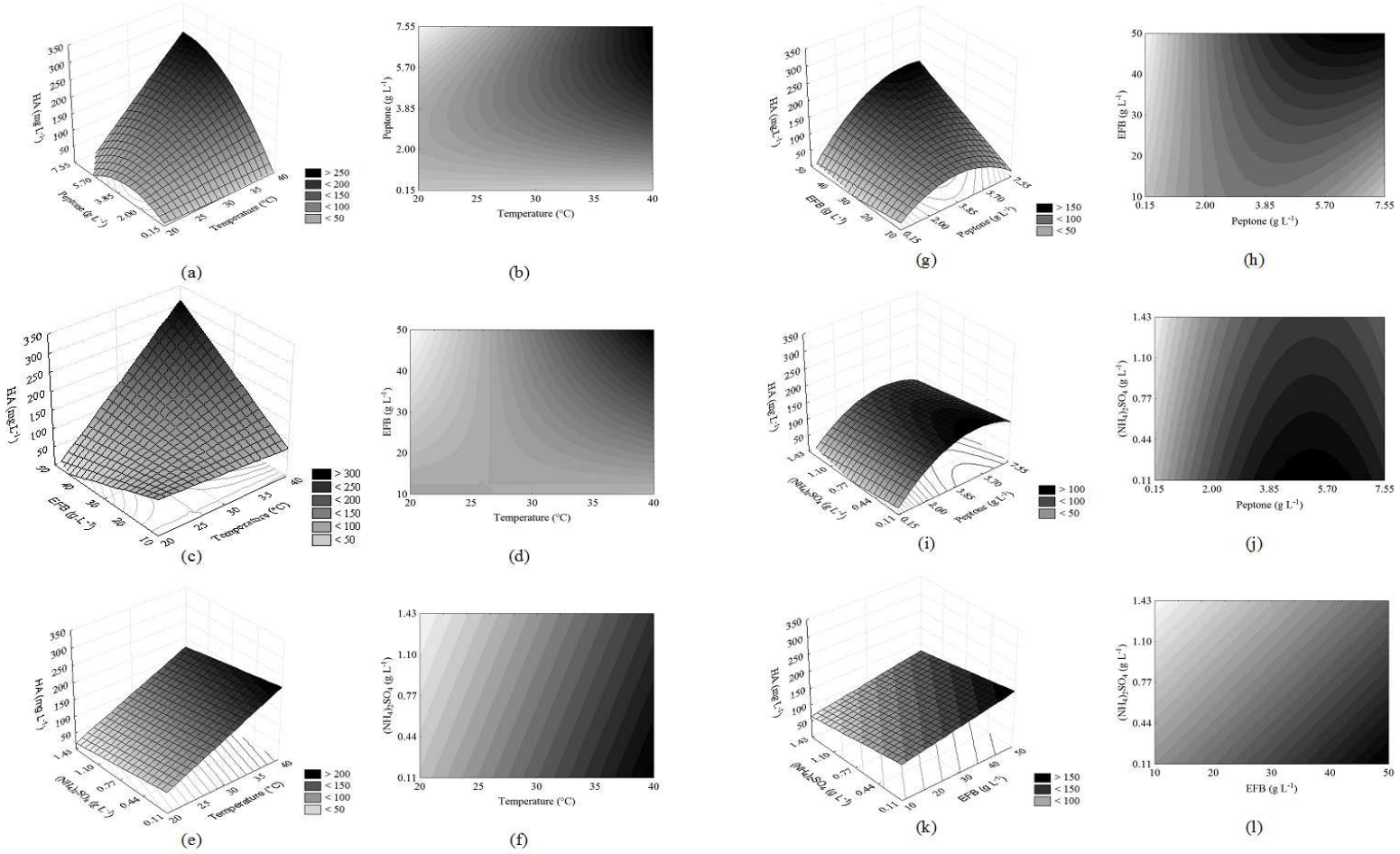

Figure 4.3.1. Response surface plots for HA production showing the interactive effects of:
(a) the temperature and the peptone concentration, keeping the EFB and $(NH_4)_2SO_4$ concentrations at central values (3D); (b) the temperature and the peptone concentration, keeping the EFB and $(NH_4)_2SO_4$ concentrations at central values (2D); (c) the temperature and the EFB concentration, keeping the peptone and $(NH_4)_2SO_4$ concentrations at central values (3D); (d) the temperature and the EFB concentration, keeping the peptone and $(NH_4)_2SO_4$ concentrations at central values (2D); (e) the temperature and the $(NH_4)_2SO_4$ concentration, keeping the peptone and EFB concentrations at central values (3D); (f) the temperature and the $(NH_4)_2SO_4$ concentration, keeping the peptone and EFB concentrations at central values (2D); (g) the peptone and EFB concentrations, keeping the temperature and the $(NH_4)_2SO_4$ concentration at central values (3D); (h) the peptone and EFB concentrations, keeping the temperature and the $(NH_4)_2SO_4$ concentration at central values (2D); (i) the peptone and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the EFB concentration at central values (3D); (j) the peptone and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the EFB concentration at central values (2D); (k) the EFB and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the peptone concentration at central values (3D); (l) the EFB and $(NH_4)_2SO_4$ concentrations, keeping the temperature and the peptone concentration at central values (2D).

Statistical model validation: With the help of special CCRD features, the point prediction and optimum values of the four fermentation variables were determined to maximize the production of HA. According to the 2D contour curves shown in Figure 4.3.1, the highest levels (+2) of temperature and EFB and the lowest level (-2) of $(NH_4)_2SO_4$ lead to the highest HA production. Both the +1 and +2 levels of peptone lead to a higher HA production. Therefore, the model validation was carried out using the +2 levels for the EFB concentration and temperature, -2 for the $(NH_4)_2SO_4$ concentration and +1 for the peptone concentration, which is the best option economically. The real values used were 40 °C , 50 g L^{-1} EFB, 5.7 g L^{-1} peptone and 0.11 g L^{-1} $(NH_4)_2SO_4$. Experiments were carried out in triplicate with the predicted conditions and concentrations to validate the model. The similarity between the predicted response of HA production (412.0 mg L^{-1}) and the experimental value (428.4±17.5 mg L^{-1}) proves the validity of the model. Moreover, the relative deviations in the Table 4.3.4 trials that had similar levels to those used in the model validation (trials 18, 20, 22 and 23) were low, with a high degree of similarity between the predicted and experimental values, further validating the model.

Comparing the amount of HA produced after optimization with that produced in our previous work [24], which is, as far we know, the first submerged fermentation process proposed for HA production, a 7.3-fold increase was achieved. This is a high value when compared to the other bioprocess optimization methods using *Trichoderma* seen in literature. Singhania et al. [33] used process optimization to improve the cellulase production by *T. reesei* RUT C30 under solid-state fermentation, resulting in a 6.2-fold increase in production. El-Sayed [34] increased by 2.2-fold the production of L-glutaminase by *T. koningii*, using wheat bran as a substrate. Alan et al. [35] reached an approximately 1.5-fold increase in cellulase activity by statistically optimizing the process conditions in a liquid state bioconversion using *T. harzianum*.

The time profiles of HA production are represented in Figure 4.3.2 which shows that the production of HA by *T. viride* in the optimized culture media increases substantially after 96 hours of fermentation. Moreover, it is possible to observe that the concentration of these acids increases throughout the fermentation time due to its accumulation in the medium. According to Aiken et al. [1], HA are the fraction of the organic matter that is most resistant to microbial degradation, which may explain the observed accumulation. This confirms that the HA concentration after 120 hours of fermentation was the appropriate variable to choose as the response in the statistical analysis (both PB and CCRD).

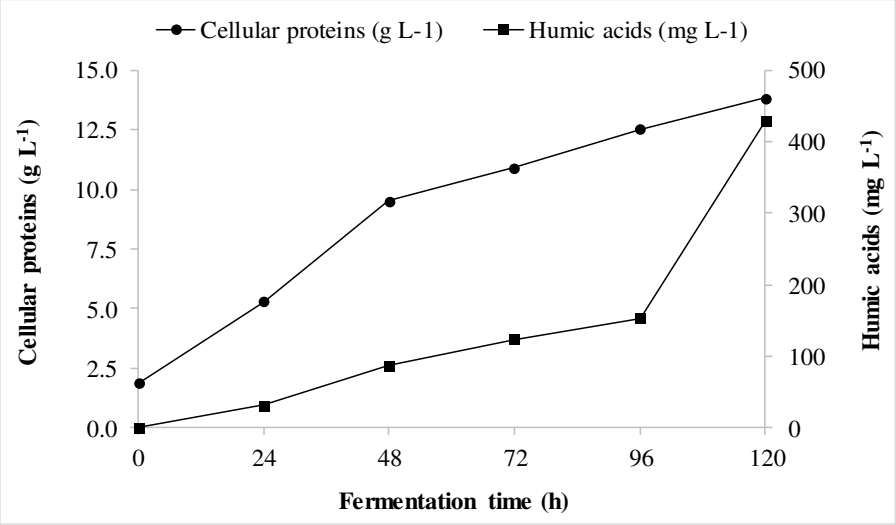

Figure 4.3.2. The cellular proteins and HA concentration over time during submerged fermentations using the *T. viride* strain and the parameters optimized by CCRD.

The concentration of cellular proteins, an indirect method of biomass estimation, indicates fast growth of the fungus between 0 and 48 hours of fermentation and mild growth between 48 and 120 hours (Figure 4.3.2). Christias et al. [32] determined in their work the amount of protein present in the biomasses of five different genera of fungi, obtaining a range of 30% to 40%. By applying these results to the present study, it is estimated that the *T. viride* biomass began at approximately 4.7-6.2 g L^{-1}, relative to the biomass from the inoculum, and reaches 34.6-46.1 g L^{-1}, a population increase of nearly 7.4-fold.

According to the Figure 4.3.2, the HA formation seems to be not coupled with energy metabolism, since the cellular proteins and HA concentration curves have different behavior along the 120 hours of cultivation.

4. Conclusions

The present study demonstrated that *T. viride* successfully produced HA via the submerged fermentation of EFB. HA production levels were influenced by the temperature as well as the EFB, peptone and $(NH_4)_2SO_4$ concentrations, and these parameters were successfully optimized using a statistics-based response surface model. The screening and optimization methodologies described here represent valuable tools for the development of cost-effective industrial fermentation media, particularly because the carbon source is an underutilized residue, adding value to the procedure. The profile of HA production obtained by the validation assays throughout the fermentation

duration indicates that cultivation studies beyond 120 hours can yield even more of these acids. Furthermore, the production of HA in a bioreactor as well as its purification would aid the characterization of this process.

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4.4. *Comparison of humic acids produced by Trichoderma viride and Trichoderma reesei using the submerged fermentation of oil palm empty fruit bunch*

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Comparison of humic acids produced by *Trichoderma viride* and *Trichoderma reesei* using the submerged fermentation of oil palm empty fruit bunch

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Abstract

The remarkable properties of humic acids (HA) have generated a broad spectrum of applications in pharmaceutical, cosmetic and agricultural fields, and encouraged fermentation studies focusing on HA production. This work compares the HA production of *Trichoderma* (*viride* and *reesei*) species using empty fruit bunch (EFB) as the substrate during submerged fermentation. The performance of each species was compared by examining spore production in oat medium, and the significant medium components and fermentation conditions were identified using Plackett and Burman statistical design. For both *Trichoderma* species, the results indicated that HA production can be enhanced by increasing the temperature, EFB and peptone concentrations and by decreasing the $(NH_4)_2SO_4$ concentration. *T. reesei* performed better than T. *viride*, generating 3-fold more HA.

Keywords: humic acids; *Trichoderma reesei*; *Trichoderma viride*; submerged fermentation; empty fruit bunch; Plackett & Burman

1. Introduction

Trichoderma is a genus of asexually reproducing fungi with a high level of genetic diversity (Harman et al. 2004). They are frequently found growing in soil as well as on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic potential and their adaptability to various ecological conditions (Druzhinina 2011). These characteristics indicate that this genus could be used in many biotechnological applications (Esposito and Silva 1998). The genus *Trichoderma* is widely used in industrial applications, because they produce extracellular lignocellulose-degrading hydrolases in large amounts (Paulo and Gubitz 2003), which can be useful for recycling cellulosic waste materials as well as producing useful by-products (Samuels 1996).

Humic acids (HA), a component of the organic matter in soil, are the soil fraction that is most resistant to microbial degradation. They are complex polymeric organic acids with a wide range of molecular weights and exist as heterogeneous mixtures of a variety of organic compounds, including aromatic, aliphatic, phenolic, and quinolic functional groups (Aiken 1985). They are one of the most active fractions of organic matter and affect a variety of chemical, physical, and biological reactions. Previously recognized for agricultural applications, they have received growing attention from the biomedical field due primarily to their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities (Yamada et al. 1998), which are of great importance for pharmaceutical and biomedical applications (von Borstel et al. 1994). Important sources of HA are leonardite, which contains 40% HA, and black peat and brown coal, both of which contain 10% HA (Lester 2009). However, harvesting HA from non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peatlands, which are important for biodiversity, carbon storage and flood risk management (Alexander 2010). Thus, it is desirable to use more ecologically sustainable precursors/feedstocks for HA and to develop cheaper and cleaner methods for the extraction of this valuable product.

For economic reasons, industrial fermentation involves complex, almost indefinable substrates that are often the by-products of other industries. Empty fruit bunch (EFB), a cellulosic material source containing 43.8% cellulose, 35.0% hemicelluloses, and 16.4% lignin (Hamzah et al. 2011), is a strong candidate for use as a fermentation substrate. EFB is the product of oil palm processing and is produced in large quantities, but it is a waste product that has not been completely

utilized: 23% of the fresh fruit bunch (Law et al. 2011) used in oil palm production remains unused as EFB. Thus, there is a growing interest in identifying uses for EFB (Thambirajah et al. 1995) and reducing the large volume of waste and ensuing environmental problems (Sudiyani 2009).

Although no quantitative studies have been performed previously, *Trichoderma* spp. is a suitable genus for the production of HA from EFB. These fungi have been recognized for their extreme facility in producing a large variety of extracellular enzymes and the degradation of lignocellulose (Kirk and Farrell 1987). Furthermore, *T. viride* and *T. reesei* are the most extensively studied fungi in the field of cellulosic material degradation (Cullen and Kersten 1992).

In our previous work, we demonstrated the production of HA from EFB using a *T. viride* strain (Motta and Santana 2013). This work extends our previous findings by comparing the performance of *T. reesei* and *T. viride* in HA production from EFB, as well as evaluating medium components and fermentation conditions. Spores from both species were produced by submerged fermentation in oat medium as previously described for *T. viride* (Motta and Santana 2012). The effects of medium components and fermentation conditions were compared using Plackett and Burman (PB) statistical design (Plackett & Burman 1946).

2. Materials and Methods

2.1. Microorganism maintenance

For storage, the *T. reesei* culture, purchased from Fundação Tropical (São Paulo, Brazil), was grown on potato dextrose agar plates at 24 °C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 ml cryotubes at −70 °C.

2.2. Production of T. reesei spores in oat medium

The spore production and fermentation analysis for *T. reesei* were performed as previously described by Motta and Santana (2012) for *T. viride*.

The medium was prepared by adding 30 g of oatmeal (Quaker Oats Company®, thin flakes; see Table 4.4.1 for detailed composition) into 1 L of distilled water and boiling the suspension for 90 min at 90 °C with constant stirring. Immediately after heating, the suspension was filtered through a sieve (0.150 mm diameter holes). Distilled water was added to the filtrate to achieve a final volume of 1 L. Then, 5 g of potato peptone purchased from Sigma-Aldrich was

added, and the media was adjusted to pH 6.0 (Al-Taweil et al. 2009) prior to autoclaving at 121 °C for 15 min.

Table 4.4.1. Composition of oatmeal thin flakes (data collected from the manufacturer's product specification sheet, Quaker Oats Company®).

Constituent	Content per 100 g
Carbohydrate	56.7
Protein	14.3
Total fat	7.3
Fibers	9.7
β-glucan	4.0

The cultures were grown in 500 mL Erlenmeyer flasks containing 300 mL of oatmeal culture media inoculated with the spore suspension prepared as described in the previous section (*2.1. Microorganism maintenance*). To obtain the same initial concentration of spores as in our previous work with *T. viride*, 0.4 ml of the spore suspension were used. The culture flasks were incubated at 24°C at 150 rpm. During 120 hours of cultivation, samples were collected every 24 hours for dry biomass estimation and cellular protein quantification, which are direct and indirect method for fungal biomass determination, respectively. Image analysis was performed every 24 hours, and spore counts were performed at 0 and 120 hours of fermentation.

2.2.1. Dry weight biomass

Based on the methodology adapted by Szijártó et al. (2004), in which the optical density was used to evaluate *T. reesei* biomass behavior in delignified pine pulp, the dry biomass concentration of *T. reesei* was evaluated by reading the optical density at 600 nm in a spectrophotometer. The absorbance at 600 nm was correlated to the cell dry weight per culture volume. To determine the dry weight, the fungal biomass was extracted from the culture medium by heating an aliquot of known volume at 85 °C under constant agitation for 10 min after dilution in distilled water at a ratio of 1:10. After heating, the aliquot was filtered through coffee filter paper, and the filtrate was collected. The solids were retained, heated and filtered as described above until the green color (typical of *Trichoderma* spores) disappeared. The total volume of the filtrate was centrifuged at 10,000 g at 5 °C, and the supernatant was discarded. The precipitate was dried at

105 °C to achieve a constant weight and placed in a desiccator; then, the fungal mass was determined.

2.2.2. Cellular protein quantification

Indirect estimation of the fungal biomass was performed by determining the protein concentration in each sample. Protein quantification was performed according to the method adapted by Callow and Ju (2012) to quantify only the cellular proteins. Culture samples (3.0 mL) were collected and centrifuged at 10,000 g for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were resuspended and washed twice with deionized water. After each wash step, the biomass was centrifuged and the water was discarded. To release intracellular proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100 °C for 10 min. After cooling, the digested samples were centrifuged at 10,000 g for 10 min to remove the cell debris and other solids; the supernatants were then collected, and the protein concentrations were determined by the bicinchoninic acid assay (Smith et al. 1985) using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were generated with bovine serum albumin.

2.2.3. Spore counts

The spores were directly counted using a Neubauer chamber. After mycelia and conidia were observed with a Reichert-Jung Series 150 microscope (Reichert, USA), indicating that fungal sporulation had been completed at 120 hours of fermentation, the spore count was performed at 0 and 120 hours to compare the initial concentration of spores to the spore concentration at the end of fermentation.

2.3. HA production

The HA production for both species was analyzed and compared according to the results obtained with the PB assay as well as the effect of medium components and fermentation conditions.

2.3.1. Submerged fermentation

EFB was provided by Oil Palm S/A - Agro-industrial OPALMA (Bahia, Brazil) and was milled to a standardized particle size between 125 and 500 μm (115 and 32 mesh in the Tyler series,

respectively). The composition of the EFB particles was determined with an elemental analyzer CNH (Perkin Elmer Series II 2400, USA) and indicated that the elemental mass percentage is 48.0±0.7% carbon, 2.6±0.1% nitrogen and 6.1±0.2% hydrogen. These results are expressed as the mean of triplicates and the average deviation. Potato peptone was purchased from Fluka Analytical (France); $(NH4)_2SO_4$ and K_2HPO_4 were purchased from Ecibra (Brazil).

T. reesei and *T. viride* were cultured in 500 mL Erlenmeyer flasks containing 270 mL of culture media inoculated with 30 mL of inoculum, which consisted of spores produced in oat medium for both species (item 2.3). The culture flasks were incubated at 150 rpm, and samples were withdrawn at 120 hours of fermentation for HA quantification.

2.3.2. HA quantification

According to the methods adapted by Badis (2010), the samples were centrifuged at 10,000 g for 15 min (Rotina 380 R Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany), and the supernatant fractions were filtered using the Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted five-fold with 0.5 M NaOH solution, and the absorbances at 350 nm at pH 4.5±0.01 were measured. Standard curves were obtained from the absorbance at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in 0.5 M NaOH solution, pH 4.5±0.01.

2.3.3. PB design

The PB design was used for screening the selected variables, which had significant effects on HA production. Six variables were screened: EFB (g L^{-1}) as a carbon source; potato peptone (g L^{-1}) as an organic nitrogen source; pH; temperature (°C); and K_2HPO_4 (g L^{-1}) and $(NH_4)_2SO_4$ (g L^{-1}) as inorganic nitrogen sources (Table 4.4.2). Each factor in this experimental design was examined at three levels: low (−), high (+) and central (0) to evaluate the linear and curvature effects of the variables (Table 4.4.2).

Table 4.4.2. Six variables screened using PB design at lower (-1), higher (+1) and central (0) levels.

Variables	Units	Experimental values		
		-1	0	+1
EFB	g L^{-1}	10.0	20.0	30.0
Peptone	g L^{-1}	1.00	3.85	6.70
pH	-	4.0	6.0	8.0
Temperature	°C	25.0	30.0	35.0
K_2HPO_4	g L^{-1}	0.28	1.54	2.80
$(NH_4)_2SO_4$	g L^{-1}	0.24	0.77	1.30

Table 4.4.3 shows the design with 16 PB trials along the levels. This statistical design does not involve the interactions between the selected variables and follows a linear approach for screening the factors (Plackett and Burman 1946).

Table 4.4.3. Experimental PB design used to screen six variables with real and code values (parentheses) for the response of HA production along with its observed values.

Trial	Experimental values						HA (mg L^{-1})*	
	EFB (g L^{-1})	Peptone (g L^{-1})	pH	Temperature (°C)	K_2HPO_4 (g L^{-1})	$(NH_4)_2SO_4$ (g L^{-1})	Observed for *T. reesei*	Observed for *T. viride*
1	30.0 (+1)	1.00 (-1)	8.0 (+1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	128.1	12.6
2	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	0.28 (-1)	0.24 (-1)	318.7	85.2
3	10.0 (-1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	0.24 (-1)	112.6	48.9
4	30.0 (+1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	189.1	38.3
5	30.0 (+1)	6.70 (+1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	182.9	107.0
6	30.0 (+1)	6.70 (+1)	8.0 (+1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	159.8	44.3
7	10.0 (-1)	6.70 (+1)	8.0 (+1)	35.0 (+1)	0.28 (-1)	1.30 (+1)	113.7	46.9
8	10.0 (-1)	1.00 (-1)	8.0 (+1)	35.0 (+1)	2.80 (+1)	0.24 (-1)	75.4	46.9
9	10.0 (-1)	1.00 (-1)	4.0 (-1)	35.0 (+1)	2.80 (+1)	1.30 (+1)	27.1	10.3
10	30.0 (+1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	2.80 (+1)	1.30 (+1)	1.1	3.3
11	10.0 (-1)	6.70 (+1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	1.30 (+1)	85.5	47.6
12	10.0 (-1)	1.00 (-1)	4.0 (-1)	25.0 (-1)	0.28 (-1)	0.24 (-1)	0.6	20.2
13	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	143.4	58.8
14	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	142.3	58.1
15	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	139.7	57.5
16	20.0 (0)	3.85 (0)	6.0 (0)	30.0 (0)	1.54 (0)	0.77 (0)	147.1	60.8

***** HA concentration obtained after 120 hours of fermentation.

3. Results

3.1. Spores production in oatmeal medium

As shown in Figure 4.4.1, the biomass and protein concentration curves have the same behavior over 120 hours of cultivation for both *T. viride* (Motta and Santana 2012) and *T. reesei*. Moreover, it is possible to observe by both biomass estimation methods that there is effectively no fungal growth after 96 hours of fermentation, with the largest growth rate observed between 24 and 72 hours of fermentation.

With regard to the relationship between *T. viride* (Motta and Santana 2012) and *T. reesei* biomass concentration and cellular protein concentration, as described by Eq. 4.4.1 and Eq. 4.4.2 respectively, there is a strong correlation value (0.98) for both species.

$$\text{Biomass (g L}^{-1}) = 3.78 * \text{Protein (g L}^{-1}) + 0.11 \qquad \textbf{Eq. 4.4.1}$$

$$\text{Biomass (g L}^{-1}) = 2.33 * \text{Protein (g L}^{-1}) + 0.23 \qquad \textbf{Eq. 4.4.2}$$

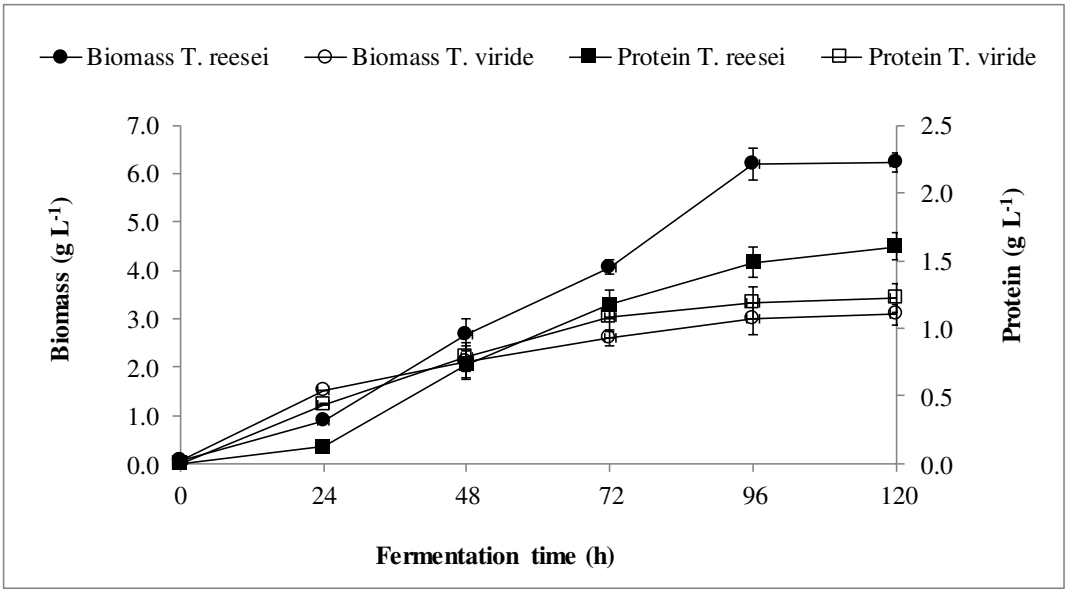

Figure 4.4.1 Biomass and cellular protein concentration over time during the submerged fermentation of oatmeal with *T. viride* (Motta and Santana 2012) and *T. reesei.*

Using image analysis with optical microscopy, it was observed that at 120 hours of fermentation the *T. reesei* strain completed its sporulation phase, and only spores are present in the culture medium at 120 hours, as was observed for the *T. viride* strain by Motta and Santana (2012). The initial concentration of spores in the culture medium was 4.54 x 10^4 spores mL^{-1}, and after 120 hours of fermentation, the spore concentration found in the fermentation medium was 6.36 x 10^6 spores mL^{-1}.

3.2. HA production

Six variables were screened using PB, as shown in Table 4.4.3, which reports the HA concentration for each trial and for both species. Trials 13 to 16 consisted of the center points, which were the same conditions used in our previous work in which we demonstrated the production of HA using EFB and the *T. viride* strain (Motta and Santana 2013). According to the values obtained for these center points, the average production of HA was 143.1±3.0 mg L^{-1} and 58.8±1.4 mg L^{-1} for *T. reesei* and *T. viride*, respectively. The largest HA production by *T. reesei* was observed in Trial 2 (318.7 mg L^{-1}), which used the highest level for EFB, peptone and temperature, and the lowest level (-1) for pH, K_2HPO_4 and $(NH_4)_2SO_4$. For *T. viride*, Trial 5 generated the highest HA production (107.0 mg L^{-1}), which used the highest level for EFB, peptone, temperature and K_2HPO_4, and the lowest level (-1) for pH and $(NH_4)_2SO_4$.

From the summary of effects shown in Table 4.4.4 and obtained using Statistica version 8.0 (Statsoft, Oklahoma, USA), the temperature and the concentration of EFB, peptone, $(NH_4)_2SO_4$ and K_2HPO_4 were the statistically significant variables with an effect on HA production in *T. reesei* (p-value<0.1). Among these five variables, $(NH_4)_2SO_4$ and K_2HPO_4 concentration had a negative effect on HA production, presenting similar estimated effect values (-40.3 and -46.1, respectively). The EFB concentration had the greatest positive effect on HA production, followed by peptone concentration and temperature. EFB, peptone and temperature have a highly significant effect on HA production, with a *p*-value much smaller than 0.1. For *T. viride*, four of the six variables screened were significant, because unlike *T. reesei*, K_2HPO_4 levels were not statistically significant. For *T. viride*, $(NH_4)_2SO_4$ concentration had a negative effect on HA production (-21.7), and the peptone concentration had the greatest positive effect on HA production, followed by temperature.

Table 4.4.4. Summary of estimated effects from the PB.

Factor	*T. reesei*				*T. viride*			
	Estimated effect	Standard error	t(8)	*p*-value	Estimated effect	Standard error	t(8)	*p*-value
Mean/Interc.	116.2	10.2	11.4	<0.0001	42.6	3.1	13.6	<0.0001
Curvature	53.8	40.7	1.3	0.2229	32.3	12.5	2.6	0.0323
EFB	94.1	20.3	4.6	0.0017	11.6	6.3	1.9	0.0998
Peptone	92.0	20.3	4.5	0.0019	41.4	6.3	6.6	0.0002
pH	27.1	20.3	1.3	0.2188	-6.0	6.3	-1.0	0.3671
Temperature	69.9	20.3	3.4	0.0089	26.3	6.3	4.2	0.0030
K_2HPO_4	-46.1	20.3	-2.3	0.0530	1.7	6.3	0.3	0.7984
$(NH_4)_2SO_4$	-40.3	20.3	-2.0	0.0828	-21.7	6.3	-3.5	0.0085

Using the Pareto Chart for both *Trichoderma* species (Figure 4.4.2), we can identify the variables that do or do not significantly affect HA production by comparing the value of the statistic (t_{cal}) for each variable, represented by the length of the bar, and the critical value (t_{crit} = 1.860), represented by the red line. Variables with $t_{cal} > t_{crit}$ have a statistically significant effect on HA production, and the variables with $t_{cal} < t_{crit}$ are not considered statistically significant. With respect to the curvature, it was not statistically significant for *T. reesei* (t_{calc} = 1.322 < t_{crit} = 1.860) but was statistically significant for *T. viride* (t_{calc} = 2.586 > t_{crit} = 1.860).

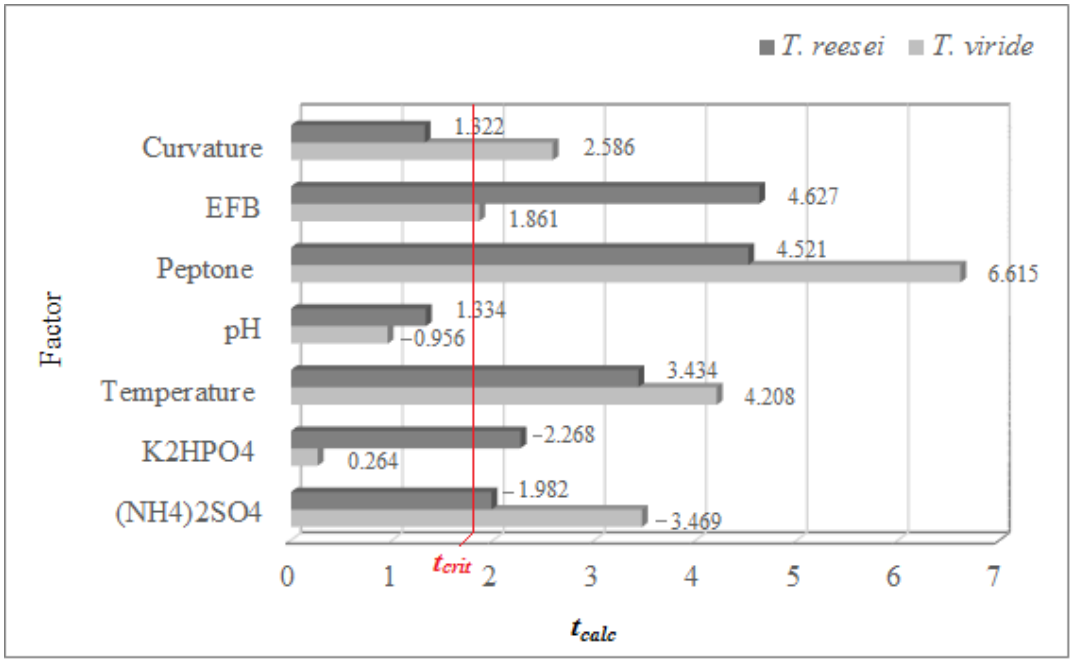

Figure 4.4.2 Pareto chart of the standardized effects of independent variables. Response HA mg L^{-1} and alpha = 0.10.

4. Discussion

Biomass is a fundamental parameter in the characterization of microbial growth, and its measurement is essential for kinetic studies on fermentation. Complete recovery of fungal biomass from the substrate is very difficult when the fungal hyphae penetrates into and binds tightly to the solid substrate particles (Abd-Aziz et al. 2008). Based on our previous work (Motta and Santana 2012), substrate particles are suspended in the oat medium used for *Trichoderma* spores production, creating a non-conventional heterogeneous medium based on the viscosity, average particle diameter, size distribution and porosity of the particles. The present study, as well as our previous work, adopted two methods for biomass quantification, one direct and one indirect, consisting of dry biomass and cellular proteins quantification, respectively.

Christias et al. (1974) determined in their work the amount of protein present in the biomass of five different genera of fungi, and they obtained a range of 30% to 40%. By applying these results to the present study, it is possible to observe that the ratio between protein and biomass concentration is within the range obtained by Christias et al. (1974).

According to the optical microscopy image analysis, after 120 hours of fermentation the sporulation phase is complete, and only spores are present in the culture medium. Therefore, the spore counts were performed at 0 and 120 hours of fermentation to determine the increase in the fungal population using oat medium for submerged fermentation. The comparison between the initial concentration of spores in the culture and after 120 hours of fermentation indicated that there were two phases of log growth during the 5 days of oatmeal-based fermentation.

Watanabe et al. (2006) used a culture medium containing soluble starch and soybean meal supplemented with KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ to produce *T. asperellum* spores by submerged fermentation. They obtained 7.8×10^8 spores/mL after 7 days of fermentation from a starting inoculum of 1.0×10^6 spores/mL. Jakubíková et al. (2006) optimized *T. atroviride* sporulation in submerged fermentation on cellobiose supplemented with $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, and $FeSO_4$. The culture medium was inoculated with 1×10^6 spores/mL, and the spore concentration reached a maximum level of 2.68×10^8 spores/mL after 4 days of fermentation.

Comparing the results obtained in the present work to past results indicates that the submerged fermentation of oatmeal is a good alternative for the production of *T. reesei* spores. Moreover, the culture medium used in this study for spore production was supplemented only with peptone, a safe supplement for culture media that is animal- and endotoxin-free. These compounds

can cause illness in humans and are considered contaminants that must be avoided or minimized in the preparation of pharmaceutical products.

Because both species were grown under identical conditions, *T. reesei* in this work and *T. viride* in a previous study (Motta and Santana 2013), and are completely transformed into spores after 120 hours of fermentation, it is possible to make a comparison between the two species regarding growth in oat medium. For *T. viride*, the initial concentration of spores (0 h) was 4.20 x 10^4 spores mL^{-1}, reaching 3.52 x 10^6 spores mL^{-1} at the end of the fermentation (120 hours) (Motta and Santana 2012). With respect to the *T. reesei* spore concentration, the initial and final values were 4.54 x 10^4 and 6.36 x 10^6, respectively. Therefore, we observed that there was an 84-fold increase in the population of *T. viride* and a 140-fold increase in the population of *T. reesei* after 120 hours of cultivation under the same conditions. The highest growth potential for *T. reesei* with respect to *T. viride* can also be observed by comparing the biomass concentration (g L^{-1}) throughout the fermentation for both species (Figure 4.4.1). *Trichoderma* species produces large quantities of hydrolytic enzymes (Papavizas 1985) including chitinases, β-1,3-glucanases (Howell et al. 2000), cellulases, amylases and proteases (Bastos 1996), which explains the high growth capability of each species. However, *T. reesei* is regarded in the literature as one of the main producers of endoglucanases, secreting at least five types of these enzymes and justifying the increased growth in culture compared to *T. viride*, because the oat media is comprised of β-glucans that can be hydrolyzed to glucose, providing the fungus with a readily usable carbon source for growth (Herpoël-Gimbert et al. 2008).

The *T. reesei* and *T. viride* spores were then used in the submerged fermentation of EFB for HA production. By performing the PB trials, the species were compared in terms of the HA produced as well as analyzed for of the effect of fermentation conditions on the production of HA.

By comparing the central points of the PB for both species, we observed that *T. reesei* HA production was 2.4-fold greater than *T. viride*. With respect to the highest HA production for each species (Trial 2 for *T. reesei* and Trial 5 for *T. viride*), this value increases to 3-fold. Although many species of *Trichoderma* have previously been used for industrial enzyme production and lignocellulosic degradation (Nevalainen et al., 1994), *T. reesei* is known to be the main producer of cellulases and hemicellulases acting in synergy to degrade lignocellulosic materials (Herpoël-Gimbert et al. 2008), which explains its increased production of HA.

To understand the effect of the variables involved in the proposed process, we used PB statistical analysis, in which the response variable (HA concentration) was examined at 120 hours, because HA accumulated in the culture medium, due to the resistance of HA to microbial degradation compared to other organic materials (Aiken 1985). This idea is confirmed by the work of Motta and Santana (2013), in which the production of HA by submerged EFB fermentation was studied. The profile obtained for HA concentration increased as fermentation time increased, demonstrating accumulation in the media.

The Pareto chart (Figure 4.4.2) distinguishes the statistically significant variables from the statistically insignificant among the factors studied, as previously discussed, by comparing the value of the statistic (t_{cal}) for each variable to the critical value (t_{crit} = 1.860).

Center points were added in the statistical analysis, allowing the performance of explicit statistical significance tests of curvature. These values were not statistically significant for *T. reesei*, indicating that there is a linear relationship between the factors and the dependent variable. For *T. viride*, the curvature was statistically significant, meaning that at least one variable is involved at an order higher than one.

Among the analyzed variables, only pH was considered to have no effect on HA production at the confidence level studied (90%) for both *Trichoderma* species. According to Bhattiprolu (2008), *T. viride* is able to grow in wide range of pH (4.0 to 9.0), which may explain the fact that this factor was not been statistically significant for either species in this work.

Regarding the nitrogen sources, peptone (L) and $(NH_4)_2SO_4$ (L) concentration had a significant effect on HA production by both species, although the first had a large positive effect and the second a negative effect. Juwon and Emmanuel (2012) tested the influence of nitrogen sources on growth and enzyme production in *T. viride* and observed that organic nitrogen substrates, like peptone, supported increased biomass yield and enzyme activity of the fungus compared to the inorganic nitrogen substrates tested.

The variable that affected HA production the least in *T. reesei* was K_2HPO_4 concentration, and its effect was not significant in *T. viride*. According to the average chemical formula elucidated by Schnitzer and Khan (1978) (C_{187} H_{186} O_{89} N_9 S_2) HA does not contain phosphorus and potassium, explaining the minimal influence of this compound on the production of HA.

Temperature has a large effect on both species, likely because enzyme activity as well as regulation and transport systems are generally largely affected by temperature in microbial systems

(Anastassiadis 2006). The linear effect of EFB concentration was larger in *T. reesei* than in *T. viride* and had the largest effect on this species. Although this component is the main carbon source for HA production in the studied media for both species, its effect was greater for *T. reesei*, possibly because this species is capable of utilizing the best carbon and energy source available when exposed to a mixture of carbon sources and downregulates the expression of genes involved in the degradation of less favorable and complex carbon sources (Seiboth et al. 2011).

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4.5. ***Solid state fermentation for humic acids production by a Trichoderma reesei strain using an oil palm empty fruit bunch as the substrate***

Artigo submetido ao Periódico Científico *Bioresource Technology*

Solid state fermentation for humic acids production by a *Trichoderma reesei* strain using an oil palm empty fruit bunch as the substrate

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Abstract

Empty fruit bunch (EFB), an underutilized waste product of oil palm processing, was studied as a substrate for the production of humic acids (HA) by a *Trichoderma reesei* strain by solid state fermentation (SSF) in Raimbault columns. HA are one of the most widely spread groups of natural organic substances that have attracted the attention of many investigators due to their applications in agriculture, industry, the environment, and biomedicine. Commercial HA are currently chemically extracted from peat and coal, which are non-renewable carbon sources. Biotechnological processes are important for its sustainable and controlled production, with SSF being especially promising for mimicking the natural habitat of fungi. *Trichoderma* sporulation and HA production are related, and the results of this study showed that SSF stimulated fast sporulation, and the mycelia were not detected since the beginning of fermentation. Significant oxygen consumption was also observed. The productivity related to HA was much higher than that of the biomass, indicating an efficient utilization of EFB. These findings, added to the low-cost of EFB, make SSF an attractive process for HA production.

Keywords: humic acids, *Trichoderma reesei*, solid state fermentation, empty fruit bunch.

1. Introduction

Humic acids (HA), one of the most important components of humic substances, consist of a heterogeneous association of molecules or small humic sub-units of different chemical natures and origins (Schnitzer, 1978) containing a variety of functional groups, including carboxylic acid (COOH), phenolic hydroxyl (OH), enolic (OH), alcoholic (OH), quinone, hydroxylquinone, lactone, and ether (Sposito, 1986). The remarkable properties of these acids and their applications in agriculture, industry, the environment, and biomedicine (von Borstel et al., 1994) have attracted the attention of many investigators (Peña-Mendez et al., 2005). These acids help break up clay in compacted soils, assist in transferring micronutrients from the soil to plants, enhance water retention, increase seed germination rates, and stimulate the development of microflora populations in the soil (Peña-Mendez et al., 2005). However, the main target for the increased interest in HA is their pharmacological effects with potential uses in medicine, such as their antiviral, estrogenic and desmutagenic activities; their anti-inflammatory effects; their pro-inflammatory properties; and their influence on blood coagulation and fibrinolysis (Peña-Mendez et al., 2005; Yamada, 1998). An important source of HA is leonardite, which contains 40% of these acids, and other sources are black peat and brown coal, both of which contain 10% HA (Lester, 2009). However, harvesting HA from such non-renewable carbon resources can be expensive and environmentally/ecologically unsustainable. Moreover, the extraction of peat leads to the destruction of peatlands, which are important for biodiversity, carbon storage and flood risk management (Alexander, 2010). Liu and Huang (2002) investigated the catalysis of hydroxy-aluminosilicate ions in the oxidative polymerization of catechol and the resultant formation of HS. Litvin et al. (2012) developed a two-step chemical procedure for preparing synthetic HA that are similar to natural HA. However, very little research has been performed on the production of these acids by biotechnological processes. This omission is a major gap in the production of HA for pharmaceutical applications, which require sustainable production and the use of controlled and reproducible processes.

Solid state fermentation (SSF) has emerged as a potential technology for the production of microbial products, such as feed, fuel, food, industrial chemicals and pharmaceutical products. Currently, with a better understanding of biochemical engineering aspects, particularly mathematical modeling and the design of bioreactors, SSF processes can be scaled up, and some designs have been developed for commercialization. The utilization of agro-industrial residues as

substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilized residues (Pandey, 2003).

In this context, empty fruit bunch (EFB), a by-product of palm oil mill processing, is a strong candidate for use as a fermentation substrate. EFB, which comprises 23% of the fresh fruit bunch (Law et al., 2011), is an abundant and capacious waste product of oil palm processing and has not been utilized effectively. Considering that the global production rate for oil palm has doubled over the last decade and that its worldwide demand is expected to double again by 2020 (Levin, 2012), there is a growing interest in adding value to EFB (Thambirajah et al., 1995) and in reducing the amount of this waste to diminish its environmental impact (Sudiyani, 2009).

Commonly, SSF involves the cultivation of filamentous fungi on natural solid substrates in which the carbon source constitutes their structure (Mitchell et al., 2004; Saucedo-Castañeda et al., 1994; Ooijkaas et al., 1998). The potential of *Trichoderma* species as lignocellulose-degrading agents was recognized in the early 1960s (Selby and Maitland, 1967). *Trichoderma* species are frequently found growing in soil and on other substrates, such as wood, bark, and other fungi, demonstrating their high opportunistic capacity and their adaptability to various ecological conditions (Druzhinina, 2011). These characteristics make the genus useful for many biotechnological applications (Esposito and Silva, 1998). The conidial mass of *Trichoderma* is the most proficient propagule that tolerates downstream processing (Al-Taweil, 2009). Because *Trichoderma* fermentation is a highly aerobic process (Verma, 2006), oxygen transfer is undoubtedly the most important phenomenon to sustain microbial activity. The rate of oxygen transfer to the cells is often the limiting factor that determines the rate of biological conversion. An insufficient oxygen transfer leads to a decrease in the microbial growth and product formation (Thibault et al., 2000).

Therefore, the aim of this study was to develop a low-cost fermentation process for producing HA using *Trichoderma reesei*, a microorganism with a high capacity for lignocellulosic material degradation. The promise of a higher yield by SSF involves the creation of an environment similar to the natural habitat of *Trichoderma*. In addition, the SSF system was incremented with forced aeration to favor both fungal growth and the production of the target compound. The performance of the production of HA from the EFB substrate was analyzed in terms of the main fermentation parameters.

2. Methods

2.1. Microorganism and maintenance

For storage, the *T. reesei* culture, purchased from Fundação Tropical (São Paulo, Brazil), was grown on potato dextrose agar plates at 24 °C for 10 days. After sporulation, the spores were resuspended in a sterile 20% glycerol solution. This mixture was stored in 1.2 mL cryotubes at -70 °C.

2.2. Solid state substrate

The EFB, provided by Oil Palm S/A Agro-industrial OPALMA (Bahia, Brazil), was characterized according to the criteria and methods outlined below.

2.2.1. EFB composition

The composition of the EFB particles was determined with a CNH elemental analyzer (Perkin Elmer Series II 2400, USA), indicating the mass percentages of carbon, nitrogen and hydrogen. The results are expressed as the mean of triplicates and their average deviations.

2.2.2. Particle size

The EFB average diameter and particle diameter distribution were determined by Tyler sieves with mesh sizes of 16, 24, 32 and 42, corresponding to opening sizes of 1000, 710, 500 and 355 µm, respectively.

2.2.3. Water activity (a_w)

The water activity of the EFB used as the substrate was measured using an Aqualab Series 3TE system (Decagon, USA), which uses the chilled-mirror dew point technique to measure the water activity of the sample. The experiments were performed in triplicate at 25 °C, and the standard deviations were calculated.

2.2.4. Moisture content

The EFB moisture, on a dry basis, was determined by drying a known amount of EFB to a constant weight in an oven at 105 °C.

2.2.5. Apparent density

The EFB was dried at 105 °C to achieve a constant weight and then placed in a desiccator. The dry weight was determined, and its density was calculated as the ratio of weight/volume.

2.2.6. Maximum capability for water absorption

The EFB was submerged in distilled water at 30 °C to saturation. The retained water was determined by subtracting the weight of the dry EFB from the weight of the saturated EFB. The maximum capability of water absorption was the ratio between the retained water and the total weight of the EFB before saturation, which was expressed as a percentage.

2.3. Solid state fermentation

The SSF was performed in the experimental setup outlined in Figure 4.5.1, composed of six fixed bed columns (modified Raimbault columns) (Raimbault and Alazard, 1980) with a diameter of 3 cm and height of 15 cm, and each one was coupled to a primary humidifier (Figure 4.5.2). The system was aerated with saturated air supplied by a dental compressor and humidified by bubbling into water before entering the column. The air flow was calibrated by a rotameter placed at the outlet of the system. To control the temperature, the columns were placed in a bacteriological incubator at 30 °C. An internal fan was used to better homogenize the temperature. The dissolved oxygen concentration was previously measured after passing through the primary humidifier to determine its concentration in the column inlet and during the fermentation at the outlet of the columns.

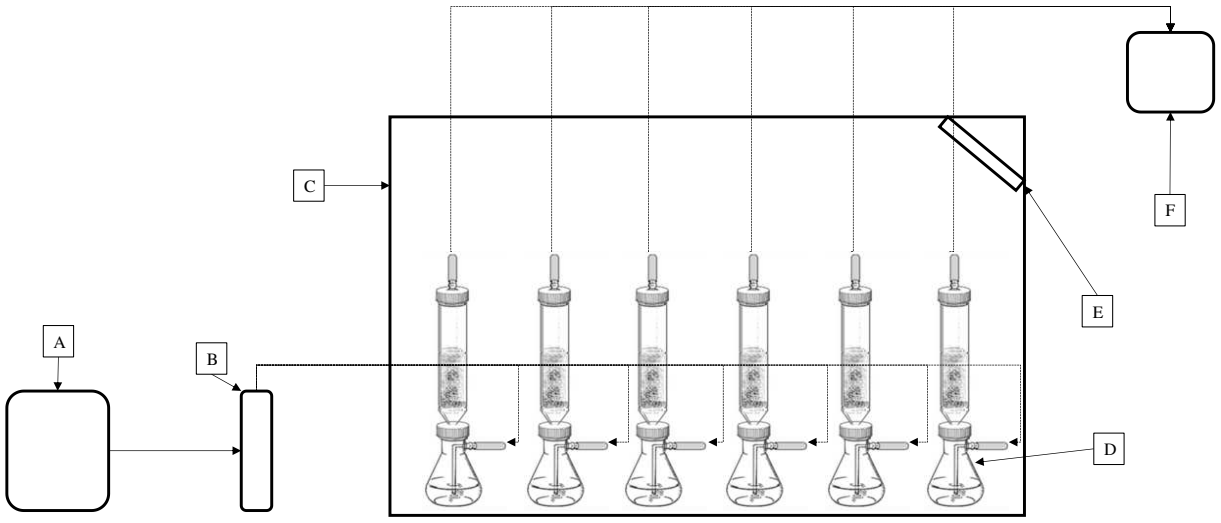

Figure 4.5.1. Schematic of the experimental setup. A: dental compressor; B: rotameter; C: bacteriological incubator; D: packed bed columns and air humidifier; E: fan; F: oximeter.

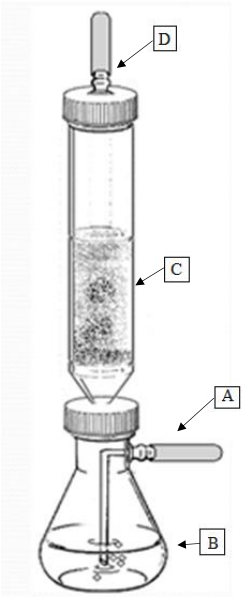

Figure 4.5.2. Packed bed with forced aeration system. A: inlet air in moisturizer; B: distilled water; C: EFB bed; D: outlet of air from the columns.

Each column received 30 g of culture medium prepared as follows: 100 g of the EFB was amended with 100% (v/w) distilled water, 2.5% (w/w) peptone, 1% (w/w) $(NH_4)_2SO_4$ and 0.2% (w/w) K_2HPO_4, and the pH was adjusted to 6.0. The columns were sterilized by autoclaving at 121 °C for 15 min, and after cooling at room temperature, the substrate mixture was inoculated with 1 mL of a spore suspension that was prepared as described in item 2.1. The fixed bed of each column

was measured, resulting in a value of 7.4 ± 0.6 cm. The cultivations were conducted for 120 hours, and the samples were withdrawn every 24 hours for the analysis, which were performed in triplicate.

2.3.1. Moisture content

The moisture content, on a wet basis, was determined by drying a known amount of fermented sample to a constant weight in an oven at 105 °C.

2.3.2. pH

The pH of the medium was measured by a pH meter after vigorously shaking 5.0 g of fermented medium and 5.0 mL of distilled water, pH 7.0, in vortex equipment.

2.3.3. Protein quantification

The indirect estimation of the fungal biomass was performed by determining the protein concentration. The protein quantification was performed according to the method adapted by Callow and Ju (2012) to quantify only the cellular proteins. The culture samples (5.0 g) were mixed with 5.0 mL of distilled water, pH 7.0, and agitated vigorously in vortex equipment. Then, they were centrifuged at 10,000 g for 10 min to obtain pellets. The supernatants were collected for further processing. The pellets were re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged, and the water was discarded. To release the intracellular proteins, the pellets were suspended in 3.0 mL of 1 N sodium hydroxide and heated at 100 °C for 10 min. After cooling, the digested samples were centrifuged at 10,000 g for 10 min to remove the cell debris and other solids; the supernatants were then collected, and their protein concentrations were determined with the bicinchoninic acid assay developed by Smith et al. (1985) using a commercial kit (BCA Protein Assay, Thermo Scientific, USA). Standard curves were constructed with bovine serum albumin.

2.3.4. HA quantification

Following the methods adapted by Badis *et al.* (2010), the supernatant fractions obtained after the first centrifugation described in the item 2.3.3 were filtered using the Microfilter syringe system (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fractions were diluted five-fold

with a 0.5 M NaOH solution, and the absorbances at 350 nm at pH 4.5±0.01 were measured. Standard curves were obtained from the absorbances at 350 nm of known concentrations of commercial HA (Sigma-Aldrich, United Kingdom) in a 0.5 M NaOH solution, pH 4.5±0.01.

2.3.5. Cellulose, Hemicellulose and lignin quantification

The determination of the cellulose, hemicellulose and lignin contents was made using the Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) methods of the AOAC (Association of Official Analytical Chemists).

2.3.6. Overall rate of oxygen transfer

The oxygen concentration at the inlet and outlet of the column was measured by a oximeter (YSI Model 5300, Yellow Springs, USA), calibrated for the temperature and humidity used. The rate of oxygen consumption was calculated from the oxygen balance in the column according to Eq. 4.5.1 adapted from Doran (1995).

$$N_{O2} = \frac{(F_{in} \times C_{02in} - F_{out} \times C_{02out})}{V_L} \qquad \textbf{Eq. 4.5.1}$$

where F_{in} and F_{out} are the air flow in the inlet and outlet, respectively, which have the same value in this process; C_{o2in} and C_{o2out} are the oxygen concentrations in the inlet and outlet column, respectively; and V_L is the volume of liquid in the fermenter, which was calculated considering the moisture content (found by item 2.3.1) present in the volume of medium.

2.3.7. Scanning electron microscopy (SEM)

The samples were examined by scanning electron microscopy (Leo 440i, LEO Electron Microscopy, England) in the high-vacuum mode with an acceleration voltage of 10 kV. Powders were sprinkled onto SEM stubs topped with adhesive carbon tape and were sputter coated (SC7620, VG Microtech, England) with Au to a thickness of 92 A°. Because EFB particles are electrically nonconductive, the samples were subjected to low-vacuum gold sputter-coating in the presence of argon gas.

2.3.8. Spore counts

The spores were enumerated by direct counting with a Neubauer chamber to compare the initial concentration of inoculated spores to the spore concentration at the end of fermentation.

2.3.9. Optical microscopy

Images of the EFB particles throughout the fermentation were obtained using a microscope (Reichert-Jung Series 150, Reichert, USA) equipped with a digital camera.

2.3.10. Fermentation productivity

The yield parameters were obtained by the slopes of the curves relating the product and substrate ($Y_{P/S}$), the biomass and substrate ($Y_{X/S}$), and the product and biomass ($Y_{P/X}$).

2.3.11. Fermentation productivity

The evaluation of the fermentation productivity was performed in terms of the biomass and product, according to Eq. 4.5.2 and Eq. 4.5.3, respectively.

$$P_x = \frac{X_{max} - X_0}{t_f} \qquad \textbf{Eq. 4.5.2}$$

$$P_p = \frac{P_{max} - P_0}{t_f} \qquad \textbf{Eq. 4.5.3}$$

where P_x is the fermentation productivity in terms of the fungal biomass; X_0 and X_{max} are the initial and maximum biomass concentrations achieved at time t_f, respectively; P_p is the fermentation productivity in terms of the product (HA); and P_0 and P_{max} are the initial and maximum product concentrations achieved at time t_f, respectively.

3. Results and Discussion

3.1. Solid substrate characterization

Generally, in SSF, the substrate is a non-soluble material that acts both as a physical support and nutrient source, and it may be the byproducts of agricultural activities or the products

obtained after the processing of agricultural materials (Manpreet et al., 2005). In this work, the cellulosic substrate utilized was a waste of oil palm processing that was utilized as substrate for *T. reesei* growth and HA production and as a solid support for fermentation.

Although some of the substrates used in SSF may require pretreatment to increase the accessibility of the carbon source to the microorganism, our previous work on HA production by submerged fermentation (SF) from EFB demonstrated that none of the three constituent polymers (cellulose, hemicellulose and lignin) of this fiber alone was able to promote the fungal growth and HA production (Motta and Santana, 2013a). In addition, the medium consisting of EFB promoted a greater production of HA and biomass than the medium composed of a blend of the three polymers. Therefore, EFB has been proven to be an adequate substrate for HA production by SSF because, in addition to having a very low cost, it promotes HA production and acts as a solid support.

Table 4.5.1 expresses the elemental composition of the EFB used as the substrate, demonstrating a high concentration of carbon, which is essential to HA production and fungal growth. In addition, the latter is favored by the presence of nitrogen in the EFB.

Table 4.5.1. Elemental composition of EFB used as substrate of the SSF.

Element	**(%m/m)**
Carbon	48.0 (±0.7)*
Nitrogen	2.6 (±0.1) *
Hydrogen	6.1 (±0.2) *

*Results expressed as the mean of triplicates and their average deviations.

Physical factors affecting the utilization of a solid substrate include the accessibility of the substrate to the microrganisms (with porosity and particle size affecting the amount of surface area accessible by the organisms), film effect and mass effect. The size of the substrate particles affects the extent and rate of microbial colonization, aeration, CO_2 removal and downstream extraction. The optimum particle size often represents a compromise between the accessibility of the nutrients and the availability of oxygen (Manpreet et al., 2005). The particle size of EFB used as the substrate was measured by laser diffraction, revealing a mean value of 634.1 µm, with the largest population between 500 and 1,000 µm, especially between 710 and 1,000 µm (Figure 4.5.3). According to Pandey (1991), the enzyme productivities in SSF using a genus of fungi were higher with a

substrate that contains particles of mixed size varying from 180 µm to 1.4 mm, showing that the range and average diameter of the EFB particles herein possibly favored the production of HA.

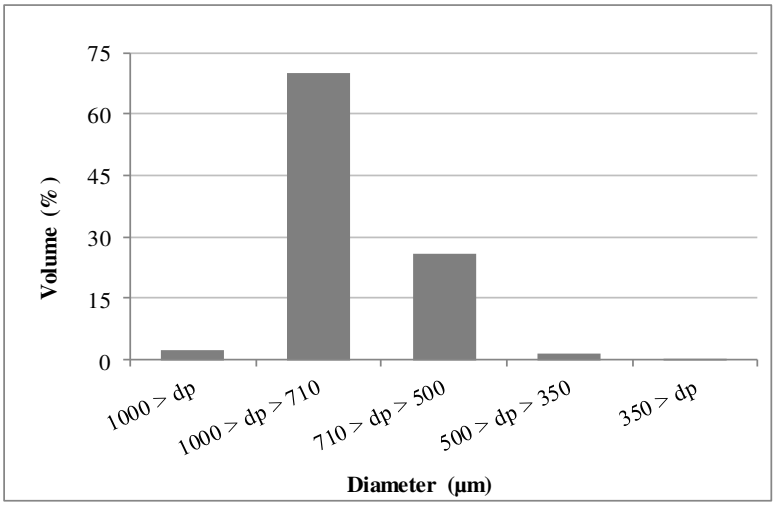

Figure 4.5.3. Particle size distribution of the EFB used as a substrate in SSF determined by laser diffraction using a Mastersizer 2000.

A low apparent density was determined for EFB (0.356 g cm^{-3}), which indicated the presence of irregularly sized particles that leads to the formation of a large number of empty spaces in the fermentation bed (Sousa and Correia, 2010). The irregular size of the EFB particles was confirmed by the SEM images (Figure 4.5.4). Low-density substrates tend to compact more difficultly, a feature that facilitates their water absorption capacity and possibly the transport of enzymes and metabolites between the medium and the microorganism (Viniegra-Gonzalez, 1997).

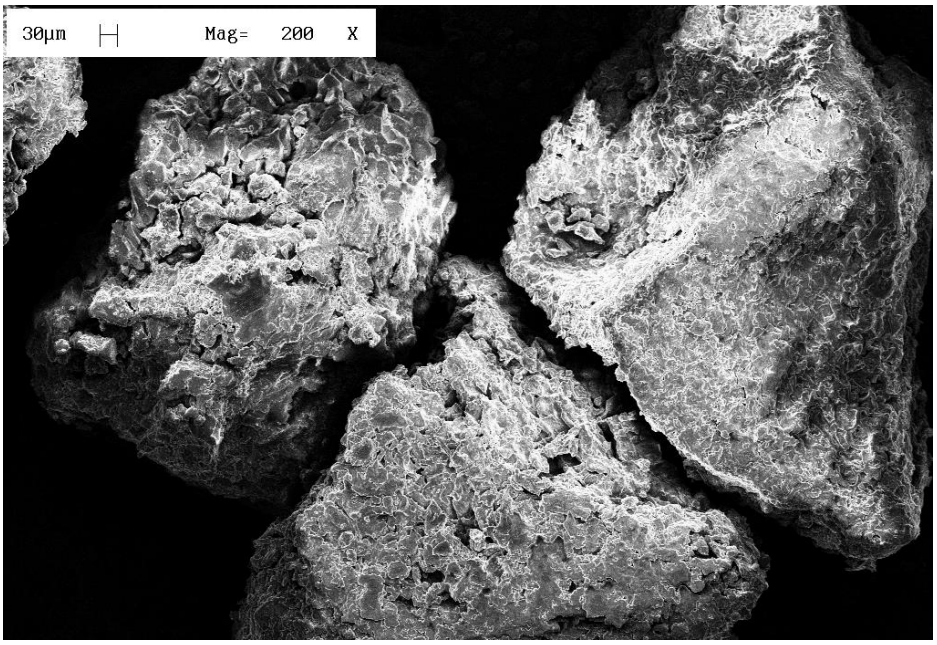

Figure 4.5.4. SEM micrographs of EFB particles before medium preparation.

The EFB used as the substrate had its moisture content measured, which was 8.93% with respect to its dry mass. Considering this value, the maximum capability for the water absorption of EFB was measured, resulting in a value of 258%, which means that each 100 g of EFB is capable of absorbing 258 mL of distilled water, considering a water density of 1 g/cm^3. Thus, the culture medium was prepared by adding 100 mL to each 100 g of EFB (100%) to respect the SSF principle of the absence of free water.

The water activity is also a determinant environmental variable for the growth of microorganisms in SSF. According to Hung et al. (2005), the minimum water activity required for fungal growth is 0.61. In this study, although the water activity value obtained for the EFB used in the culture media was 0.60±0.01, the capability of water absorption by the EFB ensured a large amount of water available for *T. reesei*, enhancing the conversion of the substrate to fungal biomass.

3.2. Characterization of the fermentation process

Figure 4.5.5 shows the kinetic characterization of the SSF in terms of the moisture content, pH, overall rate of oxygen transfer, cellular proteins, and EFB constituent polymers (cellulose, hemicellulose and lignin).

According to Figure 4.5.5 (a), the moisture content in the fermented medium remained between 64 and 72% during the cultivation, which agreed with the advised range, between 40 and 80% (w/w) (Doelle et al., 1992). Moreover, an increase in the moisture content was observed over the 120 hours of fermentation, which was due to the metabolism of the fungi as the forced humidification of the used system was constant.

According to Kredics et al. (2003), *Trichoderma* isolates are capable of growing at pH levels of 2.0 to 6.0. Figure 4.5.5 (b) shows that the pH ranged from 4.55 to 5.87 during the fermentation for HA production, which is within the range of the fungal growth capacity. Moreover, our previous work on the statistical optimization of HA production by *T. viride* using EFB (Motta and Santana, 2013b) demonstrated that there was no significant influence of pH on the production of these acids.

During microbial growth, the fungi penetrate into and bind tightly to the solid substrate particle, making it usually impossible to independently weigh the biomass of the residual substrate. Thus, indirect growth measurements have been described in the literature based on specific component measurements, such as protein, glucosamine, ergosterol, and nucleic acid contents (Abd-Aziz et al., 2008). In this study, the cellular protein content was measured by the BCA assay to estimate the fungal biomass. The cellular proteins increased between 0 and 72 hours of fermentation, with a sharp increment within 48 to 72 hours, which represents the region of greatest slope of the curve of the cellular protein concentration versus time (Figure 4.5.5 (c)). After 72 hours, the *T. reesei* cellular protein concentration became almost constant at 4.92 g / 100 g of EFB in 120 hours.

The overall rate of oxygen transfer is presented in Figure 4.5.5 (d). Initially (0 hours of fermentation), the high rate of transfer was due to the saturation of the liquid films around the particles in the bed. Between 24 to 72 hours, an increment of the rate of oxygen transfer was observed, due to the fungal growth in accordance with the kinetic behavior of the estimated biomass (Figure 4.5.5 (c)). After 72 hours, the fungal growth appeared to cease once the oxygen transfer starts to decrease, which may be an indication of cell death.

The HA production basically occurred between 0 and 72 hours, which appeared to be associated with the fungal growth. In our previous work (Motta and Santana, 2013a), the comparison of the microscopic images with the graphs of HA concentration *versus* time during SF revealed that the HA production and *T. viride* sporulation surged at a very similar time point,

indicating a clear relationship between growth and sporulation. However, although sporulation was observed only after 60 hours in SF, both sporulation and HA production were observed at the beginning of the fermentation in SSF (Figure 4.5.6). Other works have already described the relationship between fungal sporulation and metabolite production. Moreover, Siddiqui et al. (2009) confirmed through their experiments the fungicidal activity of HA isolated from the EFB of oil palm compost, which indicated that the similarity between the time duration of sporulation and HA production might be a fungal niche preservation strategy.

Figure 4.5.5 (f) represents the behavior of the polymer constituents of the EFB (cellulose, hemicellulose and lignin) *versus* time. The profiles showed there was a decrease in the cellulose and hemicellulose concentrations and an increase in the lignin concentration throughout fermentation. In reality, the lignin remained constant or decreased slightly because according to the methodology used, the amount of lignin was considered to be that that remained after digestion of the sample with sulfuric acid and relative to the total weight of the sample. Thus, if the amount of cellulose and hemicellulose decreased over time, the quantity of lignin would increase.

In a previous work (Motta and Santana, 2013a), we demonstrated the fungal synthesis of HA occurs only in media containing either the mixture of the three commercial polymers or EFB. The HA concentrations in the media containing cellulose, lignin or hemicellulose separately was close to zero throughout 120 hours of fermentation, which was negligible compared with the concentrations that were achieved in the other two culture media. In the other two culture media, the three polymers were present in the culture medium composition, indicating a synergistic effect of the three polymeric components of EFB on the production of HA by fermentation with *T. viride*. In the present work, similar conclusions can be drawn, given that cellulose and hemicellulose are consumed, although lignin requires further investigation for a more conclusive analysis.

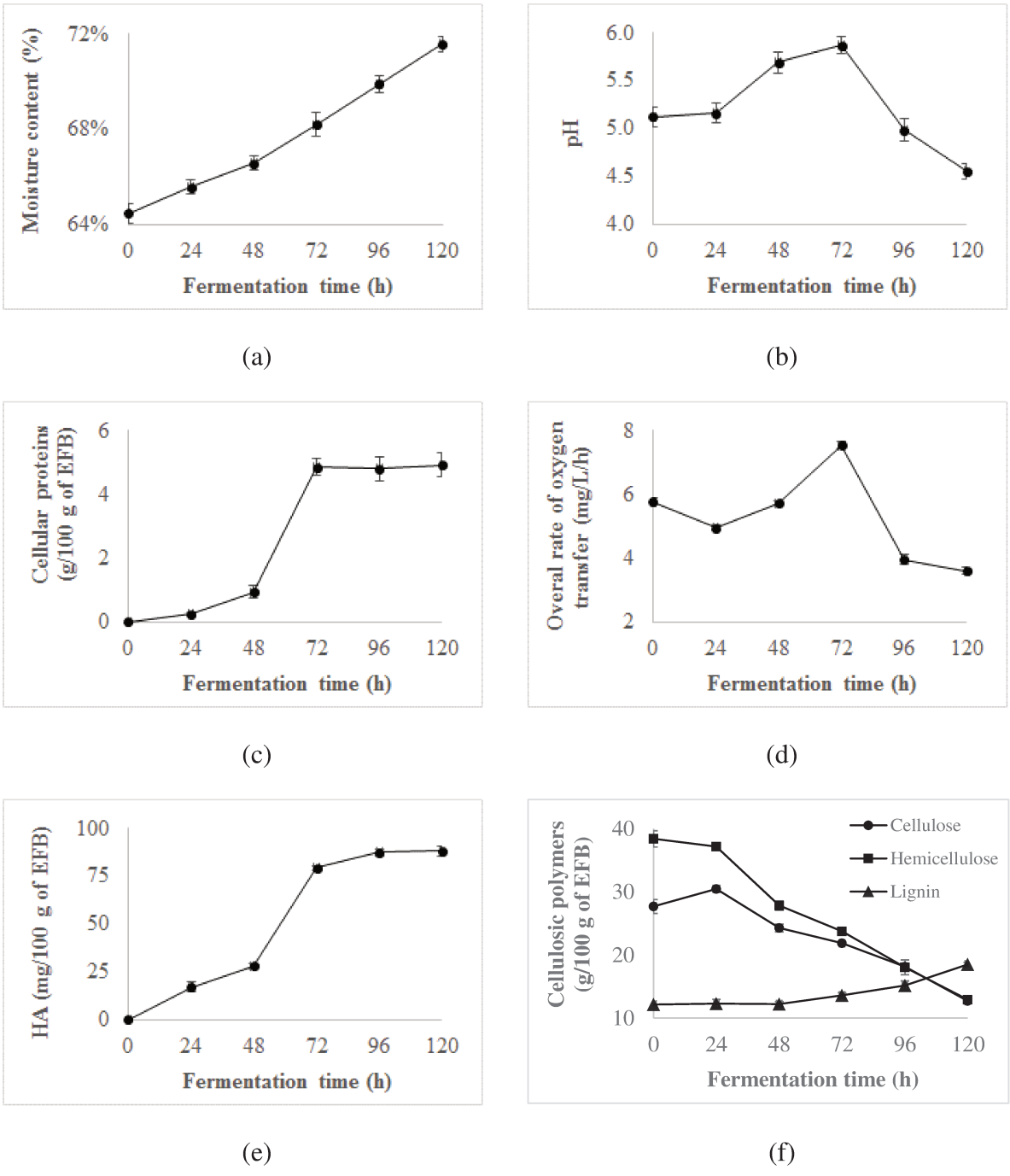

Figure 4.5.5. moisture content (a), pH (b), cellular proteins concentration (c), overall rate of oxygen transfer (d), HA concentration (e) and cellulosic polymers concentration (f) *versus* time during the SSF of EFB with the *T. reesei* strain.

By analyzing the images captured by SEM (Figure 4.5.6), only spores from the time of inoculation until the end of fermentation were visualized. The mycelial phase of *T. reesei* was likely short enough to not be visualized as the forced aeration may have created a condition that stimulates

fungal sporulation. Figure 4.5.6 also demonstrates that the increase in the spore's population was greater between 0 and 72 hours of fermentation, with little change in this population after 72 hours. Moreover, a spore counts was conducted immediately after inoculation and after 120 hours of fermentation, showing 2.16 x 10^8 spores/100 g of EFB and 4.41 x 10^{11} spores/100 g of EFB, respectively. Therefore, three logs of growth were achieved after five days of EFB-based fermentation, which represents good growth, especially compared with that observed in other works in the literature. Santos-Villalobos et al. (2012) produced *T. asperellum* spores in a mango industrial waste medium, obtaining two logs of growth after eight days of SSF. Sargin et al. (2013) utilized wheat bran to produce *T. harzianum* spores by SSF, and after four days of cultivation, they obtained two logs of growth.

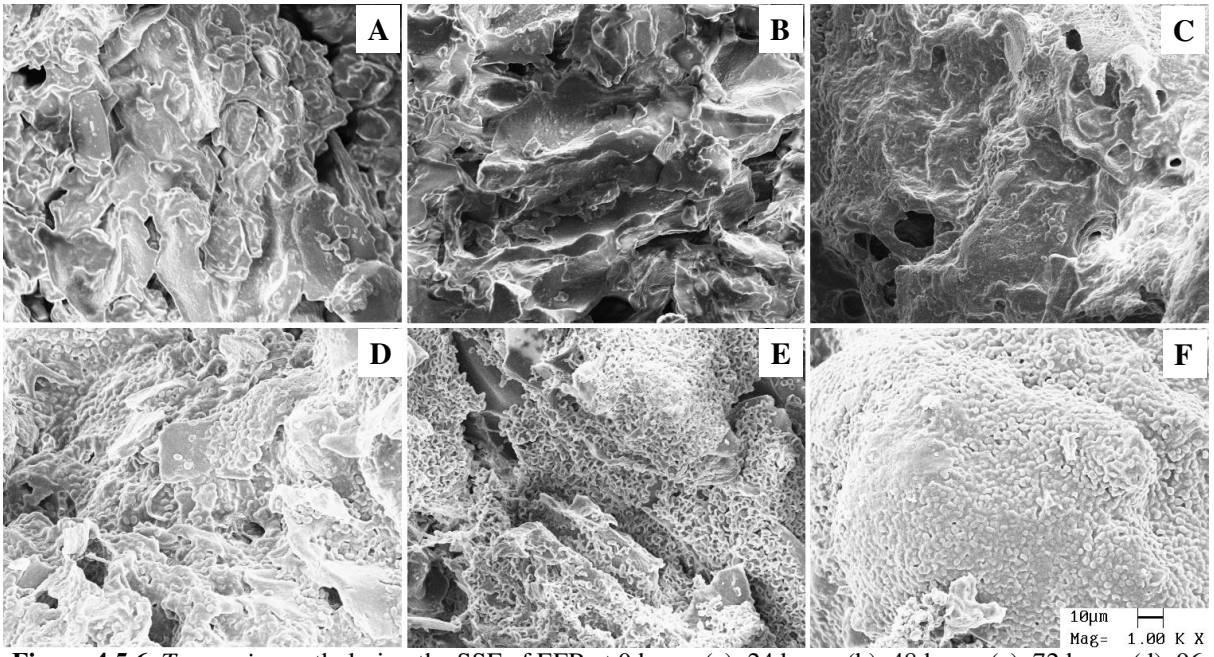

Figure 4.5.6. *T. reesei* growth during the SSF of EFB at 0 hours (a), 24 hours (b), 48 hours (c), 72 hours (d), 96 hours (e), and 120 hours (f).

Figure 4.5.7 shows darkening of the EFB particle throughout the SSF, which may be due to the HA production, as shown in Figure 4.5.5 (e), given that HA are dark brown to black in color.

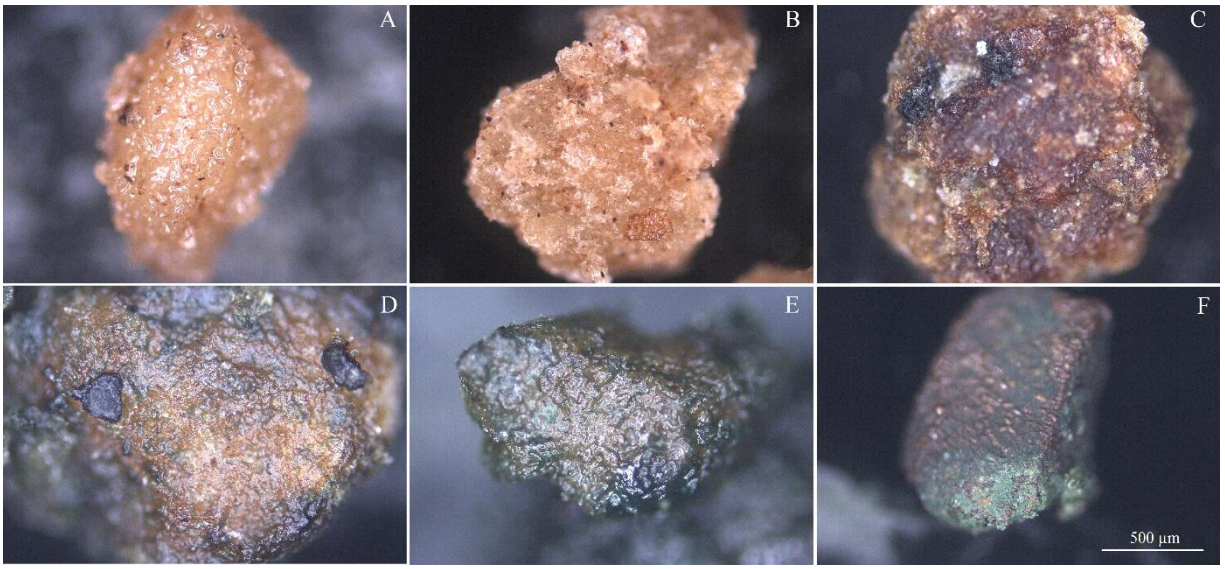

Figure 4.5.7. Optical microscopy of the EFB particles during SSF for HA production at 0 hours (a), 24 hours (b), 48 hours (c), 72 hours (d), 96 hours (e), and 120 hours (f). Mag 50 X.

The productivity was calculated at 72 hours of fermentation to compare the present results with those obtained by SF in our previous work. In terms of the product, the productivity was 0.73 mg of HA / 100 g of EFB / h, and in terms of the biomass, the estimation was 0.07 mg of cellular proteins / 100 g of EFB / h. Regarding the productivity of the product with respect to the productivity of the biomass, it was possible to observe that there was a good use of the EFB substrate by *T. reesei*, given that the product productivity was eleven times greater than the biomass productivity.

4. Conclusions

The characterization of the EFB in terms of its main physical-chemical parameters related to its use in SSF indicated that it is suitable for use as a substrate and solid support. Both the HA production and fungal growth occurred between 0 and 72 hours of fermentation. A decrease in the cellulose and hemicellulose was observed during the process, whereas lignin showed a slight decrease or constant behavior. After 120 hours, three log cycles of spore production were achieved, and a product productivity greater than the biomass productivity was achieved, indicating an effective use of the substrate by the fungus in the SSF.

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5. CONCLUSÕES

Os resultados obtidos para o crescimento do fungo em aveia demonstraram que este meio de cultura pode ser utilizado para produção em profundidade de esporos de *Trichoderma*. Além disso, de acordo com estes resultados, o tempo de cultivo deve ser de 120 horas para que apenas esporos estejam presentes no meio, servindo de inóculo para as fermentações seguintes com EFB.

Nos ensaios de produção dos AH, os resultados demonstraram-se promissores, uma vez que os valores encontrados indicaram que o EFB é o melhor substrato celulósico, dentre os estudados neste trabalho, para produção destes ácidos. Além disso, a substituição do extrato de leveduras por peptonas vegetais não teve influência sobre o crescimento do fungo e sobre a produção dos AH, demonstrando que o objetivo principal deste projeto, que é a produção de AH para aplicações farmacêuticas, pode ser atingido pela utilização de peptonas vegetais, as quais reduzem o risco da ocorrência de reações imunogênicas.

A otimização do meio de cultivo e das condições da FS para a cepa de *T. viride* permitiu que a produção fosse aumentada de aproximadamente 7 vezes, uma vez que nas condições centrais do PB a produção média foi de 58,8±1,4 mg/L e passou para 428,4±17,5 mg/L após a otimização pelo DCCR, indicando a eficiência do planejamento experimental empregado.

A comparação das espécies de *Trichoderma* em termos da produção de esporos em aveia por FS e da produção de AH por FS de EFB indicaram que a espécie *T. reesei* é a mais adequada nos dois aspectos estudados. Além disso, para ambas espécies os resultados obtidos pela análise do PB indicaram que a produção de AH pode ser favorecida pelo aumento da temperatura e da concentração de EFB e peptona e pela diminuição da concentração de $(NH_4)_2SO_4$.

Na FES, a caracterização do EFB em termos dos principais parâmetros físico-quimicos indicou que essas fibras são adequadas ao uso como suporte sólido. Além disso, foram observados durante o processo um significante consume de oxigênio e um decréscimo na concentração de celulose e hemicelulose, enquanto a lignina apresentou uma concentração constante ou um pequeno decréscimo. A produtividade em relação aos AH foi muito maior que a produtividade em relação à biomassa, indicando uma utilização eficiente do EFB pelo *T. reesei*.

Os resultados obtidos deste trabalho contribuirão para o aproveitamento de resíduos industriais na produção de compostos de expressivo valor agregado e aplicações nas áreas de cosméticos e farmacêutica, além de abrir oportunidades de estudos adicionais sobre AH produzidos por processos fermentativos, cuja descrição na literatura ainda é muito limitada.

6. SUGESTÕES PARA TRABALHOS FUTUROS

- Otimização da produção de AH por *T. reesei* por FS e FES e seleção do melhor processo;
- Estudo da influência da aeração forçada, da concentração de substrato (inibição) e do diâmetro das partículas de EFB utilizadas como substrato no processo selecionado;
- Estudo da influência do tamanho e do tempo de produção de inóculo;
- Estudos de modelagem do processo fermentativo selecionado.

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