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Maria Paula Cardeal Volpi

Produção de ácidos húmicos por *Trichoderma reesei* a partir de diferentes resíduos de óleo de palma

Production of humic acids by *Trichoderma reesei* from different palm oil residues

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RESUMO

Ácidos húmicos (AH) são compostos derivados da degradação da matéria orgânica, e encontrados apenas em fontes naturais não renováveis. Possuem grande aplicabilidade na agricultura, e atualmente vem sido explorado seu uso em aplicações farmacêuticas e biomédicas devido ás propriedades anti-inflamatórias, antibacterianas e estrogênicas dos seus grupos químicos. EFBs (*Empty Fruit Bunchs*) são os cachos vazios de onde foram retirados os frutos da palma, para a extração do óleo. São resíduos agroindustriais ricos em lignina, celulose e hemicelulose, além de lipídios remanescentes dos frutos retirados. Nos últimos cinco anos, processos de fermentação usando cepas de *Trichoderma,* têm sido estudados como uma fonte renovável de produção dos AH. O presente trabalho teve por objetivo caracterizar dois diferentes EFBs e estudar a influência da sua composição lignocelulósica e lipídica no crescimento de cepa de *Trichoderma reesei* e produção dos AH em fermentações submersa e em estado sólido. Os EFBs foram provenientes de diferentes solos (regiões nordeste e sudeste do Brasil) e de diferentes produtores de óleo de palma. A caracterização dos EFBs permitiu classificá-los em: tipo 1 (maior conteúdo lignocelulósico e lipídios residuais semelhantes aos do óleo de palma e de palmiste) e tipo 2 (menor conteúdo lignocelulósico e lipídios residuais semelhantes ao óleo de palma). As fermentações foram conduzidas com as fibras brutas e desengorduradas dos EFBs. Resultados das fermentações mostraram que houve crescimento fúngico e produção de AH em ambos os EFBs. As maiores produções de AH foram obtidas nas fermentações em estado sólido com fibras brutas de EFB do tipo 1 (350 mg .100 g^{-1} de EFB) e com as fibras desengorduradas do EFB tipo 2 (110 mg .100 g^{-1} de EFB). Para as fermentações submersas as maiores produções de AH foram 90 mg .L^{-1} para o EFB do tipo 1 e 70 mg .L^{-1} para o EFB do tipo 2 , ambas conduzidas com as fibras desengorduradas. Esses resultados são importantes por mostrarem a influência da procedência dos EFBs e do tipo de fermentação na produção de AH, assim como a possibilidade de agregar valor ao alto volume de EFB rejeitado da produção do óleo de palma.

Palavras-chave: Ácidos húmicos, *Empty Fruit Bunch*, *Trichoderma reesei,* resíduo agroindustrial, fermentação submersa e em estado sólido.

ABSTRACT

Humic acids (HA) are compounds derived from the degradation of organic matter, and found only in non-renewable natural sources. They have great applicability in agriculture, and its use has been explored in pharmaceutical and biomedical applications because of its anti-inflammatory, antibacterial and estrogenic properties of its chemical groups. EFBs (Empty Fruit Bunchs) are the empty bunches from fruits of the palm were extracted, for the extraction of the oil. They are agroindustrial residues rich in lignin, cellulose and hemicellulose, as well as remaining lipids from the removed fruits In the last five years, fermentation processes using *Trichoderma* strains have been studied, as a renewable source of AH production. The objective of the present work was to characterize two different EFBs and to study the influence of their lignocellulosic and lipidic compositions on growth of *Trichoderma reesei* strain and production of HA in submerged and solid state fermentations. The EFBs were from different soils (northeastern and southeastern regions of Brazil) and from different palm oil producers. Characterizations of the EFBs allowed classify them in: type 1 (higher lignocellulosic content and residual lipids similar to palm oil and palm kernel oil) and type 2 (lower lignocellulosic content and residual lipids similar to palm oil). The fermentations were carried out with raw and degreased EFB fibers. Results from fermentations showed fungal growth and HA in both EFBs. The highest production of HA were obtained in solid state fermentations using the raw fibers of type 1 EFB (350 mg .100 g^{-1} of EFB) and the degreased fibers of type 2 EFB (110 mg .100 g^{-1} of EFB). For submerged fermentations the highest productions of HA were 90 mg .L^{-1} with type 1 EFB and 70 mg .L^{-1} with type 2 EFB, both with the degreased fibers. These results are important because they show the influence of EFB origin and the type of fermentation in the HA production, as well as the possibility of adding value to the high volume of rejected EFB from the palm oil production.

Keywords: Humic acids, Empty fruit bunch, *Trichoderma reesei*, Agroindustrial residue, submerged and solid state fermentation.

ÍNDICE DE FIGURAS

ÍNDICE DE TABELAS

Sumário

1 INTRODUÇÃO

Substâncias húmicas (SH) são complexantes de ocorrência natural, que compõem a maior parte da matéria orgânica do solo, turfa e esgoto.

As SH são obtidas a partir da decomposição gradual dos compostos orgânicos realizada por fungos e actinomicetos, em que ocorre a oxidação e posteriormente polimerização da matéria orgânica (STEVENSON, 1982). Por este motivo, são retiradas de carvão mineral e turfas, as quais são fontes não renováveis (TROMPOWSKY, 2006).

Tais substâncias podem trazer vários benefícios para os sistemas onde são empregadas. Na produção agrícola, é citada a sua capacidade de quebrar argila, estruturar melhor o solo, transferir parte de micronutrientes do solo para plantas, aumentar retenção de água e aumentar taxas de germinação de sementes. Estas atividades estão associadas aos grupos funcionais que compõem os ácidos húmicos, ou seja, estruturas aromáticas, com porções de cadeias alifáticas, estáveis, unidas por ponte de hidrogênio, possuindo vários grupos orgânicos como carbonilas, fenóis, alcoóis, quinonas, grupos carboxílicos e outros (SCHULTEN; SCHNIZER, 1997).

As SH são divididas em três componentes principais: ácidos fúlvicos, ácidos húmicos (AH) e huminas. Os AH são formados durante a decomposição de matéria orgânica e, e por consequência, podem ser encontrados em praticamente todos os ambientes naturais em que os materiais orgânicos ou micro-organismos estiverem presentes (BALDOTTO; BALDOTTO, 2014).

A literatura tem apresentado aplicações dos AH nas mais diversas áreas. Busato et al. (2010), por exemplo, demonstraram que os AH provenientes de resíduos da cana-de-açúcar são estimuladores do crescimento de plantas, por promoverem o crescimento radicular. Van Rensburg et al. (2010), utilizaram AH em um estudo piloto para o tratamento de inflamação e osteoartrite do joelho. Devido às suas propriedades anti-inflamatórias, pacientes tratados com AH mostraram benefícios clínicos significativos em comparação com aqueles que receberam placebo. Mais recentemente, os AH vêm sendo também aplicados em nanomateriais para remoção de metais pesados de soluções aquosas, devido à capacidade de seus grupos funcionais para complexação com esses íons (TANG et al., 2014).

Resíduos sólidos frequentemente gerados de atividades agropecuárias possuem nutrientes que podem ser disponibilizados e convertidos em produtos comerciais por bioprocessos. Destaca-se a obtenção de enzimas por fungos filamentosos em processos conhecidos como "cultivo ou fermentação em estado sólido", principalmente devido à baixa disponibilidade de água destes materiais (COUTO; SANROMÁN, 2006).

A extração do óleo de palma leva à geração de um resíduo chamado de *empty fruit bunch* (EFB), o qual poderia representar uma fonte renovável para a produção microbiana de AH. Em média, os EFBs são compostos de 44,4% de celulose, 30,9% de hemicelulose e 14,2% de lignina. (FERRER et al., 2012; NAZIR et al., 2013). *Trichoderma* é um gênero de fungo de ocorrência ampla, sendo isolada do solo, madeira em decomposição e de matéria orgânica vegetal, e apresenta potencial de degradação de compostos lignocelulósicos (SELBY; MAITLAND, 1967).

Motta e Santana (2013) evidenciaram o potencial das espécies de *Trichoderma* para a produção de AH utilizando fibras desengorduradas de EFB como substrato. *T. reesei* apresentou uma produção três vezes maior de AH do que *T. viride*. Essa diferença pode ser explicada pela capacidade da espécie *T. reesei* de utilizar melhor a fonte de carbono e energia quando submetida a diferentes fontes de carbono (MOTTA; SANTANA, 2014a; SEIBOTH; IVANOVA; SEIDL-SEIBOTH, 2011). Também otimizaram condições operacionais do processo de fermentação submersa, tais como temperatura (MOTTA; SANTANA, 2014b) e produziram AH por fermentação em estado sólido (MOTTA; SANTANA, 2014c). A fermentação submersa foi inoculada com esporos de *Trichoderma* produzidos em meio não convencional de aveia (MOTTA; SANTANA, 2012).

Neste cenário, a produção de AH utilizando EFB e *T. reesei* apresenta-se promissora, uma vez que grandes quantidades deste resíduo são geradas pela indústria de óleo de palma. Na Malásia, cinco bilhões de toneladas de troncos de palmeira foram derrubados no ano de 2000. Aproximadamente 5,2 milhões de toneladas de EFB foram gerados em 2002, após a extração do óleo (TERASHIMA; FUKUSHIMA; TANAKA, 2004). Esse grande volume de resíduo gerado tem criado uma importante questão ambiental relacionada ao surgimento de pragas indesejáveis, e problemas relacionados à decomposição desses cachos (LAW; DAUD; GHAZALI, 2007). A Malásia e a Indonésia são responsáveis por 85% da produção mundial de óleo de palma. Nigéria, Tailândia, Colômbia, Equador e Papua Nova

Guiné representam juntas 6,6% da produção. O saldo de 8,4% divide-se entre outros 36 países, incluindo o Brasil. Em 2014, o Brasil estava na nona posição mundial de produção de óleo de palma (0,56%), com trezentos mil toneladas/ano (ABRAPALMA, 2016). Neste contexto, destaca-se a empresa Agropalma® como maior produtora de óleo de palma no Brasil, com sede no Pará e uma unidade de refinaria do óleo em Limeira-SP.

Como um resíduo agroindustrial, a qualidade do EFB depende do solo e condições climáticas da produção do fruto da palma, assim como do seu processamento para a remoção dos frutos frescos dos cachos do fruto da palma. Portanto, EFBs de origens distintas podem apresentar diferentes composições lignocelulósicas e de lipídios residuais associados às suas fibras. A influência desses fatores na fermentação com cepa de *T. reesei* foi estudada no presente trabalho em fermentações submersa e em estado sólido. Os EFBs foram provenientes de solos das regiões norte e nordeste do Brasil, e de diferentes indústrias produtoras de óleo de palma. As fibras dos EFBs usadas nas fermentações foram previamente caracterizadas quanto ao teor de lignocelulósicos e de lipídios residuais. Tanto quanto é do nosso conhecimento, os aspectos abordados no presente trabalho não foram anteriormente reportados na literatura. Portanto, os resultados obtidos deste trabalho contribuirão para o desenvolvimento de processos de produção do AH por fermentação, o que representa uma possibilidade de agregar valor a um resíduo agroindustrial gerado em grandes volumes pelas indústrias produtoras do óleo de palma.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Substâncias Húmicas (SH)

A matéria orgânica presente nos solos, sedimentos e turfas é formada por um sistema complexo de várias substâncias de diversas origens e passa por transformações contínuas com ação de fatores físicos, químicos e biológicos. Organismos da mesofauna, como cupins e formigas, fazem o primeiro ataque a estas substâncias, quando ocorre a degradação dos compostos orgânicos menos recalcitrantes, como celulose, açúcares, proteínas e amidos. Na fase seguinte, os produtos intermediários são biodegradados por uma nova variedade de microrganismos, que produzem nova biomassa e liberam gás carbônico. E no final, ocorre decomposição de compostos mais resistentes, pela atividade de actinomicetos e fungos (KONONOVA, 1994).

Esta matéria orgânica é dividida em dois grupos distintos: o das substâncias não húmicas e as substâncias húmicas. O primeiro grupo é formado por compostos bem definidos quimicamente, geralmente incolores, que são exclusivos do solo. Geralmente, são compostos de baixa massa molar e mais prontamente disponíveis aos microrganismos. Entre esses compostos, estão carboidratos simples e complexos, resinas, proteínas e aminoácidos auxinas, aldeídos e ácidos aromáticos e alifáticos. Eles representam de 10 a 15% da reserva total de carbono orgânico nos solos minerais (SENESI; LOFFREDO, 2001).

O segundo grupo, que são as substancias húmicas (SH), compõem de 85 a 90% da reserva total do carbono orgânico. Os produtos formados associam-se em estruturas complexas mais estáveis, de elevado peso molecular, coloração escura e separadas com base na solubilidade. Com base nesse último critério, as SH podem ser classificada em 3 frações: os ácidos fúlvicos, AH e humina (SENESI; LOFFREDO, 2001).

• Ácidos fúlvicos (AF): os AF representam a fração solúvel em meios alcalino e ácido, são constituídos por polissacarídeos, aminoácidos e compostos fenólicos. Apresentam alta quantidade de grupos carboxílicos, peso molecular baixo, e podem estar combinados com óxidos de Fe, Al, argilas e outros compostos. Além disso, podem formar complexos estáveis com Fe, Cu, Ca, M, e apresentar propriedades redutoras.

• Ácidos húmicos (AH): os AH compreendem a fração solúvel em meio alcalino e insolúvel em meio ácido (pH < 2). Em meio ácido ocorre precipitação e formação de produto escuro e amorfo. Estes são complexos quimicamente, formados por polímeros aromáticos e alifáticos com massa molar elevada e capacidade de troca iônica.

• Humina: é a fração insolúvel, em qualquer condição de pH e possui reduzida capacidade de reação.

Segundo Schnitzer (1978, apud SILVA FILHO; SILVA, 1999) a composição média de uma unidade para os AH e AF em sua fórmula química é C_{187} H_{186} O_{89} N_9 S_2 e C_{135} H_{182} O_{95} N_5 S_2. Os AH e AF possuem maior estabilidade no ambiente e menor degradação devido a sua razão C/N que é 50% superior à média observada na matéria orgânica do solo (BALDOTTO; BALDOTTO, 2014).

2.2 Importância e aplicações de substâncias húmicas

As SH têm um importante papel no meio ambiente, como em solos e sedimentos, ajudando na retenção de calor e induzindo a germinação de sementes o desenvolvimento de raízes. Além disso, possuem diversificados papéis em ambientes aquáticos, reações fotoquímicas, atuação de complexantes de íons metálicos e absorção de poluentes orgânicos (MESSIAS, 2004)

Dentre as áreas de maior aplicação das SH, a agricultura é a que mais se destaca pela utilização dos AH, principalmente porque aumentam a absorção de nutrientes, melhoram a estrutura do solo e geram benefícios na produção, como o incremento da produtividade. Por exemplo, muitas vezes o fósforo encontra-se no solo complexado com outros íons, como na forma de fosfato cálcico. Os AH complexam-se com o cálcio e liberam o fósforo para o uso vegetal, resultando em maior disponibilidade de fósforo para adsorção por plantas (BORSARI, 2013). Além disso, os AH possuem aplicabilidade no estímulo do desenvolvimento radicular de plantas. Segundo o estudo de Busato et al. (2010), os AH extraídos da torta de filtro, um resíduo da produção do açúcar, podem ser utilizados como estimuladores do crescimento de plantas.

Na construção civil, as SH e materiais contendo húmus são utilizados como controle da taxa de fixação. Na indústria de cerâmica, são usadas como aditivos para melhorar

a resistência do material. Segundo Majakova (1972) conforme citado por Peoa-méndez, Havel e Patočka (2005) na produção de plásticos, são aplicadas, principalmente como corantes de nylon 6 ou plástico PVC.

De estudos feitos usando AH em ratazanas, verificou-se que diminuiu significativamente a extensão de danos gástricos causados por etanol. A principal razão pela atenção dada aos AH é por eles possuírem atividade antiviral, profibrinolítica, anti-inflamatória e atividades estrogênicas (YAMADA; OZAKI; KIMURA, 1998).

As propriedades das SH, como citada acima por Yamada, Ozaki e Kimura (1998), também possuem aplicações potencialmente importantes na área médica. Estudos hospitalares realizados nas décadas de 1980 e 1990 já mostraram que doenças virais de crianças foram tratadas com sucesso com a utilização de ácido fúlvico (THIEL et al.,1981). Foi também observado que a pré-incubação de culturas de células *in vitro* com humato de amônio evitou infecção pelo vírus do herpes. Marcadores clínicos e laboratoriais de humato de potássio foram usados num estudo piloto em pacientes com osteoartrite de joelho, verificando-se a potencialidade dos AH na redução da inflamação causada por essa doença (VAN RENSBURG et al., 2010).

Estudos na área ambiental mostraram que os AH tem a capacidade de se associarem a íons metálicos e ácidos orgânicos sólidos, promovendo a remoção de metais pesados e xenobióticos orgânicos de soluções aquosas (VON WANDRUSZKA, 2000).

Mais recentemente, o AH também foi explorado como agente fototeranóstico para imagens fotoacústicas induzidas pela luz e por terapia fototérmica (MIAO et al., 2018).

Na área de nanotecnologia, estudos feitos com nanopartículas demonstraram a capacidade de AH se estruturar incorporando agentes antioxidantes. Além disso, os grupos fenólicos e carboxílicos da superfície das nanopartículas apresentaram atividade fungicida e antioxidante, demonstrando assim o vasto potencial de aplicação dos AH em produtos farmacêuticos e cosméticos (MELO; MOTTA; SANTANA, 2015).

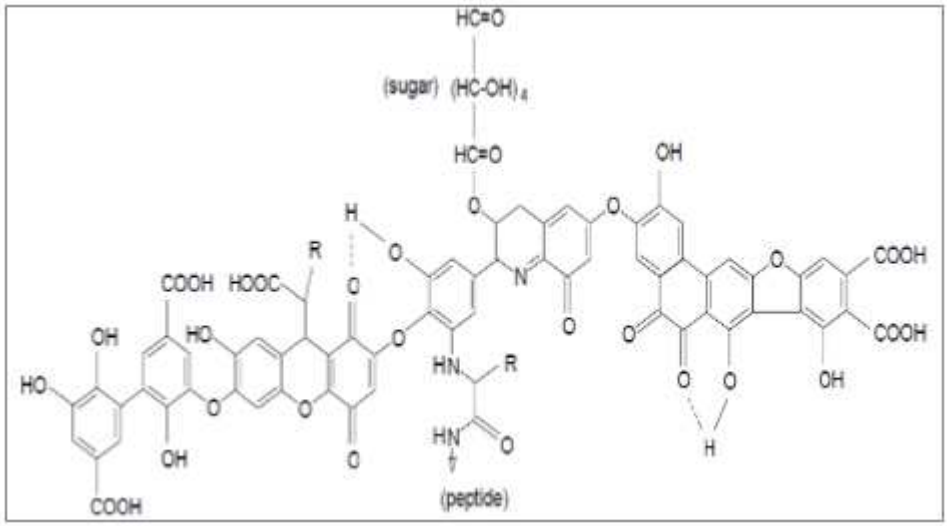
2.3 Ácidos Húmicos

Os AH são macromoléculas complexas, que constituem diferentes estruturas aromáticas e alifáticas, com funções fenólicas e carboxílicas. Tais estruturas se ligam a peptídeos e outros componentes que contém nitrogênio e carboidrato em pequenas quantidades (HAYES; CLAPP, 2001).

A composição dos AHs existentes na natureza depende da matéria orgânica, do grau de humificação, do tratamento recebido, e das condições ambientais da cadeia carbônica (RAMOS-TEJADA et al., 2003; TERASHIMA; FUKUSHIMA; TANAKA 2004).

Atualmente, os AH comerciais são extraídos principalmente da turfa e do carvão mineral, que não são fontes não renováveis. Não existe ainda uma metodologia oficial usada para a extração destas substâncias do solo, mas a literatura reporta a utilização de agentes extratores como pirofosfato de sódio, agentes complexantes e ácido fórmico. Mesmo existindo riscos de alteração da estrutura, a extração mais completa dos AH utilizando álcalis é comumente utilizada (ROSA; ROCHA; FURLAN, 2000). Os AH possuem diversos grupos funcionais como quinonas, fenóis, ácidos carboxílicos, o que lhes conferem as propriedades antioxidante, anti-inflamatória, anti-bacteriana entre outras (SENESI; LOFFREDO, 2001; MELO; MOTTA; SANTANA, 2016). A Figura 1 representa um modelo de estrutura molecular para os AH ressaltando os seus grupos funcionais.

Figura 1. Modelo de estrutura dos AH



Fonte: Stevenson, 1982.

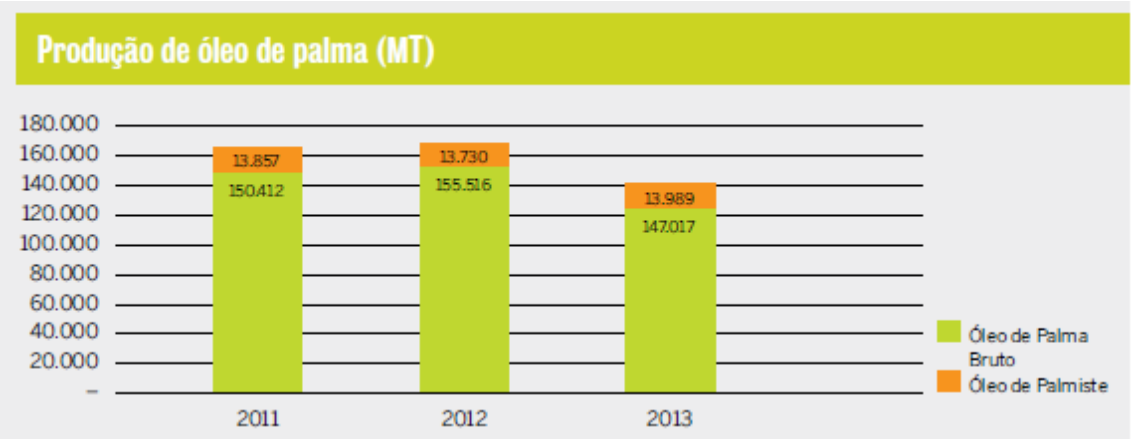
2.4 Produção de AH

Atualmente a procura por fontes alternativas e renováveis para produção de diversos produtos industriais vem aumentando. Alguns estudos dos últimos cinco anos (MOTTA; SANTANA, 2013; MOTTA; SANTANA, 2014a; MOTTA; SANTANA, 2014b) têm mostrado a factibilidade de produção de AH por via biotecnológica, a partir do resíduo agroindustrial do fruto da palma conhecido como cachos vazios ou EFBs (*empty fruit bunch*es) e cepas de fungos do gênero *Trichoderma.*

2.4.1 EFB (empty fruit bunch)

EFB é oriundo do processo de remoção dos frutos frescos usados para extração do óleo de palma. É considerado um resíduo lignocelulósico por não possuir um destino definido (BOCCHI, 2008). Constitui um problema econômico para a empresa geradora, visto que cada vez mais a demanda de produção de palma aumenta. As Figuras 2 e 3 apresentam dados da empresa Agropalma® sobre a produção de óleo de palma e produção do cacho de frutos frescos respectivamente, no Brasil.

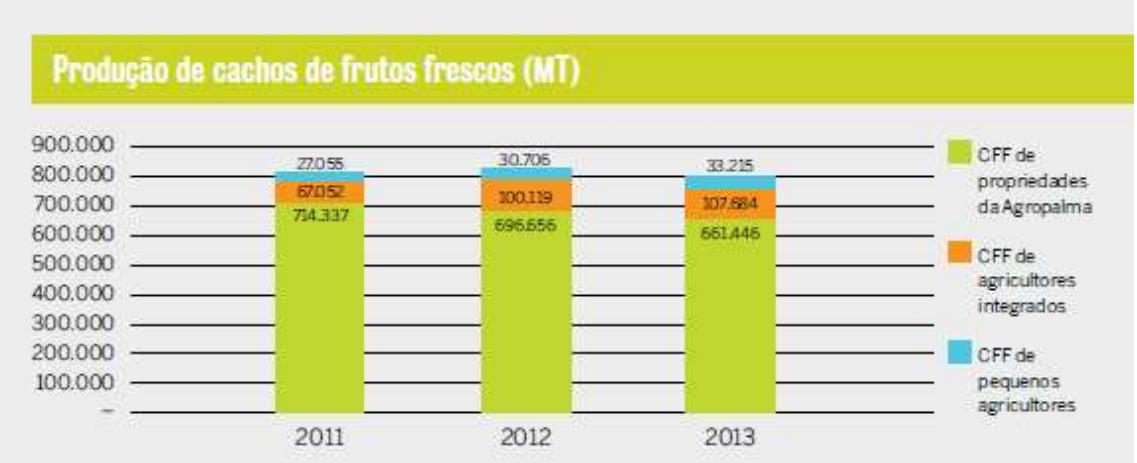
Figura 2. Dados de produção do óleo de palma bruto e do óleo de palmiste



MT: mil toneladas

Fonte: Relatório Sustentabilidade Agropalma (2013).

Figura 3. Produção de Cachos de Frutos Frescos



CFF: Cachos de frutos frescos; MT: mil toneladas

Fonte: Relatório Sustentabilidade Agropalma (2013).

A Figura 2 indica a crescente produção do óleo de palma nos anos de 2011, 2012 e 2013. Na Figura 3 é mostrada a produção dos cachos de frutos frescos, ou seja, aqueles cachos em que serão retiradas as frutas e darão origem ao EFB, o que representa uma proporção expressiva em comparação com a produção do óleo de palma.

A palma oleaginosa (*Elaeis guineensis* Jacq.), que pertence á família *Arecaceae*, originária da África Ocidental como na Indonésia Malásia e Tailândia, é conhecida popularmente no Brasil como dendê, uma espécie que cresce em clima quente e úmido com abundância de chuvas. O norte brasileiro apresenta condições ideais para o crescimento desta planta, e o estado do Pará é um dos maiores produtores (ALVES et al., 2011).

Pelas estimativas reportadas na literatura, o Brasil possui setenta milhões de hectares disponíveis para o plantio (LIMA et al., 2002). Em países tropicais o principal óleo utilizado pelas indústrias para o desenvolvimento de produtos, destacando o setor alimentício, é o óleo de palma, devido ao clima para o cultivo da palmeira.

A Malásia possui um total de 4,7 milhões de hectares de plantações de palmeiras, que produzem anualmente cerca de dezenove milhões de toneladas de óleo de palma e dois milhões de toneladas de óleo de palmiste por ano, gerando uma grande quantidade de resíduos, principalmente o EFB (HUNTER; BYRD, 2017).

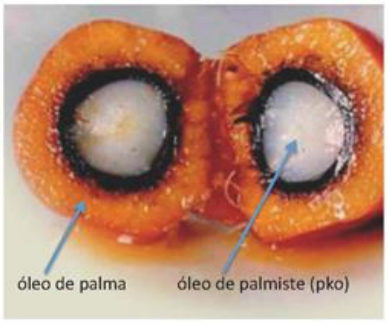
Mundialmente a cultura da palmeira ocupa 8% das terras alocadas para o cultivo das oleaginosas e fornece quase um terço da produção global de óleos vegetais (ABRAPALMA, 2016). Na Figura 4 pode-se observar o aspecto físico do óleo e do fruto da palmeira ou fruto da palma.

Figura 4. Óleo do fruto da palma

Fonte: Agropalma (2016)

Do fruto da palma, é possível obter dois tipos de óleos: o da polpa (mesocarpo) de onde é extraído o óleo de palma propriamente dito, e o da semente de onde é extraído o óleo de palmiste, conforme ilustrado na Figura 5.

Figura 5. Imagem do fruto de palmeira ilustrando de onde são extraídos os óleos de palma e de palmiste.



Fonte: Akira (2012).

Segundo Hoffman (1989), citado por Gioielli (1996), o processo de extração do óleo de palma ocorre por solvente, se o teor de óleo for menor que 25%, ou por prensagem, para maiores teores.

Para iniciar o processo de extração do óleo de palma, os cachos contendo os frutos frescos FFBs (f*resh fruit bunches*) são colhidos nas plantações e transportados até a unidade de extração (BRITO, 2006). Nessas unidades os FFBs são aquecidos com vapor durante uma hora, passando em seguida por debulhador para a separação dos frutos frescos dos cachos. Nesta etapa são gerados os cachos vazios ou EFBs, que são os primeiros resíduos da extração do óleo de palma.

Nas etapas posteriores do processo, os frutos são prensados para a retirada do óleo cru, o qual é transferido para desaerador para retirar as partículas pesadas em processos de clarificação e filtração, gerando assim o óleo bruto da palma. Nessa etapa surgem novos resíduos, como as fibras, nozes e outras impurezas. As nozes são transferidas para um moinho, que separa as amêndoas das casas, e das amêndoas é extraído o óleo de palmiste. Portanto existem dois tipos de fibras provenientes do processamento do fruto da palma: o primeiro tipo às fibras dos cachos vazios ou EFBs, e o segundo tipo às fibras da casca do mesocarpo da fruta (HASSAN et al., 2010). A figura 6 ilustra o EFB.

Figura 6. Empty Fruit Bunch (EFB)

Fonte: Alibaba

A composição em ácidos graxos nos óleos de palma e palmiste são diferentes. No óleo de palma, predominam os ácidos palmítico e oleico enquanto no óleo de palmiste, os principais são os ácidos graxos láurico e mirístico, sendo semelhante aos óleos de coco e coco de babaçu (BLOCK; BARRERA-ARELLANO, 2013). A principal diferença entre os dois óleos está no teor de ácido láurico, que é predominante no óleo de palmiste e praticamente ausente no óleo de palma.

O óleo de palma apresenta estado semissólido em temperatura ambiente, devido a sua composição balanceada entre ácidos graxos saturados e insaturados, como 50% de ácidos graxos saturados, 40% de monoinsaturados e 10% de polinsaturados. Além disso, o óleo de palma é estável à oxidação devido à presença de antioxidantes naturais (BLOCK; BARRERA-ARELLANO, 2013).

O óleo de palmiste possui ácidos graxos de cadeia curta, como 48% de ácido láurico (C14:0), e 16% de ácido mirístico. Devido a essa composição, esse óleo apresenta-se sólido à temperatura ambiente (PANTZARIS; BASIRON, 2011).

A extração dos óleos de palma e palmiste gera um grande volume de resíduos, principalmente os EFBs, que apresentam baixo valor comercial e tornam-se um problema ambiental para as indústrias de processamento da palma.

Os EFBs representam de 22 a 23% dos cachos usados nas indústrias de óleo de palma. Atualmente, os EFBs são queimados, e depois descartados em aterros sanitários, ou então, são usados em compostagem para adubo orgânico. Como o processo de queima gera problemas ambientais, são recomendadas novos processos para um uso sustentável desta biomassa de alto valor lignocelulósico, como a bioconversão em um produto que tenha alto valor agregado (ISROI et al., 2012).

O EFB é classificado como resíduo lignocelulósico, composto em média de 44,4% de celulose, 30,9% de hemicelulose e 14,2% de lignina. A alta concentração de celulose e baixa de lignina agrega valor ao produto (FERRER et al., 2012; NAZIR et al., 2013). Além disso, a literatura reporta que fibras do mesocarpo do fruto da palma contêm compostos bioativos, como caroteinóis, tocoferóis e fitoesteróis (CARDENAS-TORO et al., 2015; LAU et al., 2008). O EFB ainda pode possuir lipídios residuais do processo de remoção dos frutos usados na extração de óleo.

Estudos recentes mostram que o EFB pode ser utilizado como matéria prima para a fabricação de papel (HUNTER; BYRD, 2017). Rosazley et al. (2016) caracterizaram a celulose nanofibrilada (NFC) de EFB, e mostraram potencial para uma vasta gama de aplicações na fabricação de materiais reforçados. Rayung et al. (2014) realizaram o tratamento para branqueamento de fibras de EFB e as associaram ao polímero PLA. O

material compósito PLA / OPEFB (EFB do óleo de palma) apresentou maior resistência mecânica e uma superfície com sítios de maior adesão.

2.4.1.1 Óleo de Palma

O óleo de palma é utilizado nas indústrias alimentícias, químicas e de bicombustíveis, principalmente para a produção do biodiesel obtido pela transesterificação do óleo de dendê com etanol. A produtividade obtida a partir da palma é maior quando comparada com as outras oleaginosas, sendo cerca de cinco toneladas de óleo por hectare por ano, enquanto as outras produzem menos que um. Os pequenos produtores produzem a palma em busca de uma agricultura social e sustentável e, por este motivo, a produção deste fruto vem aumentando (BRASIL, 2010). Assim, a geração de resíduos, tanto sólidos como líquidos, tende a aumentar, assim como os estudos sobre a possibilidade do seu uso em biorrefinarias.

O óleo de palma apresenta uma composição de 90 a 92% de triacilgliceróis, aproximadamente 6% de diacilgiceróis, 0.1% de monoacilgliceróis, 2 a 3% de ácidos graxos livres, fosfolipídeos, água e outros componentes minoritários (BLOCK; BARRERA-ARELLANO, 2013).

Há um equilíbrio em sua composição de ácidos graxos saturados e insaturados, contendo aproximadamente 50% de ácidos graxos saturados, 40% de ácidos graxos monoinsaturados e 10% de ácidos graxos poli-insaturados (MANORAMA; SARITA; RUKUMINI, 1997). Dentre os ácidos graxos saturados, estão presentes são 42 a 40% do ácido palmítico, 4 a 5% do ácido esteárico, 39 a 40% do ácido oléico e 10 a 12 % do ácido linoléico (BLOCK; BARRERA-ARELLANO, 2013). Essa composição característica do óleo de palma, balanceada entre ácidos graxos saturados e insaturados, confere uma característica semi-sólida a temperatura ambiente, quando é possível observar duas frações com diferentes comportamentos térmicos, que são classificadas em estearinas (sólidas) e oleínas (líquida) (GEE, 2007). A estearina de palma é rica em triacilgliceróis (TAGs) de alto ponto de fusão e a oleína de palma é rica em TAGs de baixo ponto de fusão (BLOCK; BARRERA-ARELLANO, 2013).

A tabela 1 mostra a composição em ácidos graxos do óleo de palma e das frações saturadas e insaturadas, e na tabela 2 consta a composição em TAGs das mesmas porções.

Tabela 1. Composição em ácidos graxos do óleo de Palma e das frações saturada (estearina) e insaturada (oleína)

Composição de Ácidos Graxos (%)			
Ácidos Graxos	Óleo de Palma	Oleína de Palma	Estearina de Palma
C12:0 Láurico	0,10-0,40	0,20-0,40	0,10-0,30
C14:0 Mirístico	1,00-1,40	0,90-1,20	1,10-170
C16:0 Palmítico	40,90-47,50	36,80-43,20	49,80-68,10
C18:0 Esteárico	3,80-4,80	3,70-4,80	3,90-5,60
C18:1 Oleico	36,40-41,20	39,80-44,60	20,40-34,40
C18:2 Linoleico	9,20-11,60	10,40-12,90	5,00-8,90
C18:3 Linolênico	0,05-0,60	0,10-0,60	0,00-0,50
C20:0 Araquídico	0,20-0,70	0,30-0,50	0,00-0,50

Fonte: Codex Alimentarius, 2001

Tabela 2. Composição em triacilgliceróis (TAG) do óleo de Palma e das frações saturada (estearina) e insaturada (oleína)

Composição em Triacilgliceróis (%)			
	Óleo de Palma	Oléina de Palma	Estearina de Palma
Triinsaturados			
OOO	4,4	4,61	2,14
OOL	0,58	0,66	1,81
Monosaturados			
PLO	9,68	10,63	4,53
POO	23,26	25,6	9,4
OOS	2,24	2,58	2,47
Disaturados			
MPL	2,2	2,52	2,22
PPL	9,23	9,61	7,18
POP	29,62	29,64	23,36
POS	4,9	5,11	3,85
SOS	--	0,98	--
Trissaturados			
MMM	0,42	0,46	0,93
MMP	1,7	1,85	2,05
PPP	5,51	0,5	27,16

O= Oleico, L= linoleico, P=palmítico, S=esteárico, M=mirístico.

Fonte: Codex Alimentarius, 2001

2.4.1.2 Óleo de palmiste

A produção mundial de óleo de palmiste foi de 2,9 milhões de toneladas em 2001. Os maiores produtores são a Malásia e a Indonésia, que juntos somam 78% da produção e 90% das exportações (PANTZARIS; BASIRON, 2011).

Como exposto na Figura 5, o fruto da palma tem forma oval de aproximadamente 3 cm de eixo maior. A parte exterior do fruto, que é o mesocarpo fibroso, origina o óleo de

palma, enquanto que o núcleo, que está dentro de uma casca dura, origina o óleo de palmiste (PANTZARIS; BASIRON, 2011).

Na produção do óleo de palma, o fruto fresco é prensado para obter o óleo de palma do mesocarpo. As nozes são duras e resistem à pressão, não sendo quebradas no processamento. Por esse motivo, elas são separadas das fibras e rachadas para remover a casca. Logo após são secas para impedir o crescimento de micro-organismos e posteriormente são submetidas à extração do óleo de palmiste. Esse óleo, mesmo sendo da mesma fruta, possui composição de ácidos graxos diferente do óleo de palma (PANTZARIS; BASIRON, 2011).

Com relação à composição de ácidos graxos, o óleo de palmiste possui 48% de ácido láurico, 16% de ácido mirístico e 15% de ácido oleico. Esses são os principais ácidos graxos encontrados, enquanto os outros não passam de 10% da composição. Eles possuem um alto ponto de fusão devido à preponderância de ácidos graxos saturados combinados com baixos níveis de instauração (PANTZARIS; BASIRON, 2011). A tabela 3 apresenta a composição em ácidos graxos do óleo de palmiste encontrada na literatura. Os TAGs encontrados no óleo de palmiste compreendem TAG com cadeias de carbonos variando de C_{28} a $C_{52,}$ sendo o do C_{32} a C_{42} os TAGs predominantes (PANTZARIS; BASIRON, 2011). A tabela 4 mostra a principal composição em triacilglicerol presente no óleo de palmiste.

Tabela 3. Composição ácido graxo (%) do óleo de palmiste

Ácido Graxo	Faixa
C6:0 Capróico	ND-0,8
C8:0 Caprílico	2,4-6,2
C10:0 Cáprico	2,6-5,0
C12:0 Laúrico	45,0-55,0
C14:0 Mirístico	14,0-18,0
C16:0 Palmítico	6,5-1,0
C16:1 Palmitoleico	ND-0,2
C18:0 Esteárico	1,0-3,0
C18:1 Oleico	12,0-19,0
C18:2 Linoleico	1,0-3,5
Índice de iodo	14,1-21,0

ND: Não detectável.

Fonte: Códex Alimentarius, 2001

Tabela 4. Composição de triacilgliceróis do óleo de palmiste

Número de Carbonos	Valor em % de massa
28	0,6
30	1,4
32	6,5
34	8,5
36	21,6
38	16,4
40	9,8
42	9,1
44	6,6
46	5,4
48	6,1
50	2,6
52	2,7
54	2,7

Fonte: Códex Alimentarius, 2011.

2.4.2 Fermentação de EFB - Produção biotecnológica de AH

Por tratar-se de uma fonte rica em lignocelulósicos, o EFB vem sendo estudado como substrato para produção biotecnológica de AH. Motta e Santana (2013) realizaram cultivos submersos de *Trichoderma viride* com fibras de EFB suspensas no meio líquido. Como este resíduo é fonte de celulose, hemicelulose e lignina, esses três polímeros também foram usados como fonte de carbono, separados e em combinação, para investigar os efeitos dos substratos celulósicos e das fontes orgânicas de nitrogênio na produção biotecnológica de AH. A maior produção foi obtida em culturas que continham o EFB e a mistura dos três polímeros, inferindo que os diferentes substratos celulósicos apresentam um efeito sinérgico na produção de AH.

Para a otimização da produção de AH em fermentação submersa com *Trichoderma viride*, Motta e Santana (2013) estudaram nutrientes adicionais e variáveis operacionais da fermentação, através de programas estatísticos. O estudo apontou que o uso de peptona de batata, $(NH_4)_2SO_4$, 50 $g.L^{-1}$ de EFB e 30°C foram as condições que maximizaram a produção de AH. Essas informações representam ferramentas valiosas para o desenvolvimento de meios de fermentação industrial econômicos, particularmente porque a fonte de carbono é um resíduo subutilizado.

Em estudos do mesmo grupo de pesquisa pôde-se verificar que a espécie *T. reesei* apresentou uma produção de três vezes mais de ácido húmico quando comparado ao *T. viride*, em fermentação submersa, utilizando como fonte de carbono o EFB. Para a maximização da produção por *T reesei*, as condições de cultivo foram: 20 $g.L^{-1}$ de EFB, temperatura de 30°C, 0,77 $g.L^{-1}$ de $(NH_4)_2SO_4$, 1,54 $g.L^{-1}$ de K_2HPO_4 e pH de 6 (MOTTA; SANTANA, 2014a).

Na fermentação em estado sólido de EFB com *T. reesei* para produção do AH, ocorre uma esporulação rápida, havendo consumo significativo de oxigênio. A produção de AH foi efetiva indicando uma utilização eficiente do EFB. Isso torna o cultivo em estado sólido um processo atraente para a produção de ácido húmico, já que mimetiza o habitat natural dos fungos (MOTTA; SANTANA, 2014c).

2.5 *Trichoderma*

O gênero *Trichoderma* engloba espécies de fungos filamentosos que habitam no solo e está distribuído pelo mundo em quase todos os ambientes naturais, principalmente naqueles que contêm matéria orgânica e na rizosfera de muitas plantas. Este gênero é um dos mais estudados entre os fungos filamentosos, assim como *Neurospora*, *Penicillium* e *Aspergillus*, por suas aplicações industriais e biotecnológicas (SAMUELS, 1996).

A capacidade de proliferação em um amplo espectro de substratos e condições ambientais é o que torna esse gênero de fungo de grande interesse biotecnológico (ESPOSITO; SILVA, 1998).

Espécies desse gênero produzem enzimas hidrolíticas, como por exemplo, celulases (HAAB et al., 1990), e o *T. reesei* se destaca especificamente por produzir enzimas celulolíticas e hemicelulolíticas, e as secreta constituindo um sistema multienzimático capaz de hidrolisar polissacarídeos.

As espécies de fungos filamentosos mais estudados na literatura científica, com a capacidade de produção dessas enzimas são: *T. reesei, Penicillium pinophilum, Humicola insolens, T. koningii, P. funiculosum, Fusarium solani, Myrothecium verrucaria, Sporotrichum pulverulentum* e *Aspergillus niger* (RABELO, 2007).

Devido à capacidade desses fungos colonizarem-se em substrato sólido pelo crescimento apical e conseguirem penetrá-lo, eles possuem maior vantagem para uso em fermentação em estado sólido (RAIMBAULT, 1998).

No período de crescimento, as colônias se espalham radialmente sobre a superfície do substrato, e formam o micélio. Existem ainda, as hifas aerais, que podem se prolongar na fase gasosa, com ramificações, e também as hifas penetrativas, que conseguem entrar na matriz sólida, até o ponto em que oxigênio e nutriente são limitados (HÖLKER; LENZ, 2005).

Para o desenvolvimento das espécies de *T. reesei* e *T. viride*, foi estudado um meio de cultura de baixo custo, que foi aveia suplementada com peptona de batata. Esse meio de aveia não convencional mostrou que, em 60 horas de crescimento, de um total de 120

horas, aparecem esporos, que são essenciais para o uso em fermentações com EFB e produção de AH (MOTTA; SANTANA, 2012).

3 OBJETIVO E METAS

3.1 Objetivo

Estudar a influência da composição lignocelulósica e dos lipídios residuais na produção de AH por *T. reesei* utilizando diferentes fontes do resíduo agroindustrial do fruto da palma, EFB (*Empty Fruit Bunch*).

3.2 Metas

– Caracterizar os compostos ligninocelulósicos dos EFBs;

– Caracterizar os lipídios residuais presentes nos EFBs;

– Estudar a influência dos compostos ligninocelulósicos e dos lipídios residuais das fibras dos EFBs, no crescimento de cepa de *T. reesei* e a produção de AH em fermentação submersa;

– Estudar a influência dos compostos ligninocelulósicos e dos lipídios das fibras dos EFBs, no crescimento de cepa de *T. reesei* e produção de AH na fermentação em meio sólido;

– Determinar a influência dos lipídios residuais na recuperação dos AH produzidos por fermentação submersa e em estado sólido utilizando as fibras de EFBs.

4 RESULTADOS E DISCUSSÃO

Esta sessão será apresentada na forma de artigos científicos que foram submetidos a periódicos especializados.

O primeiro artigo apresentado, “Characterization of lignocellulosic composition and residual lipids in empty fruit bunches from palm oil processing”**,** trata da caracterização lignocelulósica de dois EFBs que foram provenientes de duas diferentes fontes, e também da caracterização dos lipídios que ficam associados as fibras desses EFBs.

O segundo artigo, “The role of lignocellulosic composition and residual lipids in empty fruit bunches on the production of humic acids in submerged fermentations*”* aborda o estudo do cultivo submerso de *T. reesei* no EFB, para a obtenção de AHs. Foram utilizados EFBs de duas diferentes fontes, que foram cultivados na presença e na ausência dos lipídios residuais. E foi analisada a influência do material lignocelulósico e dos lipídios dos EFBs para o crescimento fungico e produção de AHs.

O terceiro artigo, “Production of humic acids in solid-state cultivation by *Trichoderma reesei* from different palm oil residues” aborda também o cultivo do *T. reesei* em dois EFBs provenientes de processamentos diferentes, para a obtenção de AH, porém em cultivo em estado sólido em colunas de Raimbault.

Adicionalmente, são apresentados dois apêndices com os seguintes conteúdos:

No apêndice A, encontram-se os primeiros resultados obtidos do cultivo submerso, a partir dos quais decidiu-se modificar a metodologia de análise do AH no caldo fermentado, em que se passou a centrifugar o extrato fermentado antes de ser submetido às analises.

No apêndice B, encontra-se o trabalho que foi apresentado no SINAFERM 2017, com os resultados preliminares de cultivo submerso com *T. reesei* em diferentes EFBs na presença e na ausência de lipídios, visando caracterizar o potencial desse fungo para a produção de AH.

4.1 Characterization of lignocellulosic composition and residual lipids in empty fruit bunches from palm oil processing

Sumbetido ao periódico Applied Biochemistry and Biotechnology

Characterization of lignocellulosic composition and residual lipids in empty fruit bunches from palm oil processing

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Abstract

Empty fruit bunches (EFB) are an agroindustrial residue discarded in the environment when the palm fresh fruits are removed for extraction of the oil. EFB are abundant in countries producing palm oil, generating environmental problems. Therefore, the developments of new technologies for its use have been recommended. Besides its high content of lignocellulosics, EFB also contain a significant amount of residual lipids from separation process of the fresh fruits. Because the palm fruit has two main types of oil, from the pulp (palm oil) or the seeds (kern oil), the residual EFB lipids may have different compositions. Thus, this work aimed to characterize the lignocellulosic content and the residual lipids in two EFBs from different palm oil producers. The EFBs were classified as type 1 and type 2 according to their source. The results shown type 1 EFB had higher lignocellulosic and fatty acids composition similar to palm and kernel oils, while the type 2 EFB had lower lignocellulosic content and fatty acids composition similar to the palm oil. The relevance of these results is both for secondary recovery or for the use of EFBs in new technologies such as in fermentation processes.

Keywords: Empty Fruit Bunch, Agroindustrial residue, Palm oil, Lipids, Lignocellulosic material.

1 Introduction

The palm oil industry is one of the largest oil producers in the world. The oleaginous palm (Elaeis guineensis Jacq.), belongs to the *Arecaceae* family, originated in West Africa as in Indonesia, Malaysia and Thailand, and also in Brazil [1]. In 2015, the Directorate of Processing and Marketing of Agricultural Products of the Indonesian Ministry of Agriculture, reported the total palm plantation reached 11.4 million of hectare with produced 20–24 tons of oil palm/hectare approximately [2].

The main residues of palm oil processing are the empty fruit bunches (EFB), which are discarded in the environment after removal of the fresh fruits used for extraction of the palm oil. The amount of EFB is much greater than the other residues such as the kernel shells and mesocarp fibers [3,4].

EFBs are classified as a lignocellulosic residue, composed on average of 44.4% of cellulose, 30.9% of hemicellulose and 14.2% of lignin. The high concentration of cellulose and low lignin add value to the product [5,6].

The oil extraction process initially involves the collection of fresh bunches in plantations and transportation to the extraction unit where they are treated by steam, followed by passing through a threshing machine to separate the fresh fruits and the empty bunches [7]. In the separation process a residual oil may remain in EFBs. According to Hoffman, as cited by Gioielli [8], the extraction process of palm oil fruit may occur by solvent, when the oil content in the fruits is less than 25%, or by pressing for the highest concentrations. Two types of oil are extracted from the fruits: the palm oil from the pulp or mesocarp and the palm kernel oil from the seeds. The fatty acid composition of these oils is completely different. In palm oil, the predominant fatty acids are palmitic and oleic acids, while the palm kernel oil, similar to coconut and babassu coconut, primarily contains lauric, myristic and oleic fatty acids [9].

In terms of stability, palm oil is one of the most resistant vegetable oil to oxidation due to the presence of natural antioxidants. It is in a semi-solid state at room temperature because of its composition of fatty acids (50% saturated, 40% monounsaturated and 10% polyunsaturated) [10].

The average composition of the fatty acids in palm kernel oil is 48% lauric acid, 16% myristic acid and 15% oleic acid. They have a high fusion point because of the preponderance of saturated fatty acids combined with low levels of unsaturation and are solid at room temperature [10].

In addition, the literature reports the fibers of the palm fruit mesocarp contain bioactive compounds such as caroteinols, tocopherols and phytosterols [11, 12].

Studies have shown the potential of EFB for use in some areas such as papermaking and fabrication of reinforced materials [13,14]. Motta and Santana [15] evidenced the capacity of the EFB to produce humic acids through submerged fermentation and solid-state fermentation, using fungus of *Trichoderma* genus.

Thus, the aim of this research was to characterize the the lignocellulosic content and the residual lipids present in EFBs from different palm oil producers. The results are useful for secondary recovery of the oil, as well as for development of novel technologies.

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2 Materials and Methods

2.1 Materials

Type 1 EFBs were provided by the Agropalma® industry, Limeira, São Paulo, Brazil, and type 2 EFBs were from the cooperative of small palm oil producing industries (Muniz Ferreira, Bahia, Brazil).

2.2 Methods

2.2.1 Moisture and Lignocellulose Characterization of EFBs

EFB samples were stored at 5°C and characterized by moisture content according to Approved Methods of the American Association of Cereal Chemists International (AACCI) 44-15.02 [16]. The quantification of cellulose, hemicellulose and lignin was performed using neutral detergent fiber (NDF) and acid detergent fiber (ADF) from the Association of Official Analytical Chemists (AOAC) [17].

2.2.2 Extraction of Lipids from EFBs

The residual lipids were extracted from EFB fibers for characterization and quantification. Extraction was carried out in a Soxhlet Extractor using petroleum ether according to the American Oil Chemists' Society (AOCS) method AM 2-93 [18].

2.2.3 Characterization of residual lipids of EFBs

2.2.3.1 Free fatty acids compostion

Free fatty acids were determined by the Ca 5a-40 AOCS method [18]. The analyses were performed after esterification on a capillary gas chromatograph (CGC Agilent 6850 Series GC System) according to the method of Hartman and Lago [19]. The methyl esters of the fatty acids were separated according to the procedure of AOCS Ce 1f-96 [18] on an Agilent DB-23 capillary column (50% Cyanopropyl-methylpolysiloxane) with the following dimensions: 6.0 m x 0.25 mm x 0.25 m. The operating conditions of the chromatograph were as follows: column flow rate = 1.0 mL min^{-1}; linear velocity = 24 cm $.s^{-1}$; detector temperature = 280°C; injector temperature = 250°C; oven temperature = 110-215°C (5°C $.min^{-1}$), 215°C for 24 min; drag gas - helium; and volume of injection = 1.0 μL.

2.2.3.2 Iodine and Peroxide indices

The iodine indices were determined from the composition of fatty acids by the methodology of AOCS Cd 1c-85 [18]. The peroxide index was obtained by the method Cd

8b-90 [18] based on titration with 0.1 M sodium thiosulphate and the use of a starch solution as an indicator.

2.2.3.3 Classes of lipidis

The classes of lipids was determined by size exclusion chromatography (HPSEC) on a Perkin Elmer liquid chromatograph 250; Sicon Analytic refractive index detector; Column 1: Jordi Gel dvb 300 x 7.8 mm, 500 Å; Column 2: Jordi Gel dvb 300 x 7.8 mm, 100 Å. Conditions: mobile phase: THF; flow rate: 1 mL min^{-1}; and volume injected: 20.0 µL of 20 mg / mL THF diluted sample.

2.2.3.4 Triacylglycerols composition

Triacylglycerol (TAG) composition was analysed after the TAG fraction was isolated from diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids (FFA). Thus, the samples were purified by passing the oil through silica SPE column, according to Moreda et al [20]. Briefly, the column was placed in a vacuum elution apparatus and washed with 6.0 mL of hexane, under atmospheric pressure. The lipid matrix (0.12 g) was diluted in 0.5 mL of hexane and loaded into the column. The solution was pulled through and then eluted with 10.0 ml of hexane-diethylether (87:13 v:v) under atmospheric pressure. The eluted solution was then evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. The residue was split into two parts for TAG and fatty acid composition determinations. TAG compositions were analyzed according to the method AOCS Ce 5-86 [18] in a capillary gas chromatograph (CGC Agilent 6850 Series Gc System). The capillary column used was the DB-17 HT Agilent Catalog: 122-1811 (50% phenyl-methylpolysiloxane) with dimensions of 15.0 m, Ø int: 0.25 mm and 0.15-µm film. The operating conditions of the chromatograph were as follows: flow column = 1.0 mL / min; linear speed = 40 cm / sec; detector temperature: 375°C; injector temperature: 360ºC; oven temperature: 280 - 340°C - (2°C / min), 340°C - 40 minutes; drag gas: helium; injected volume: 1.0 µL, split 1: 100; and sample concentration: 10.0 mg mL^{-1} tetrahydrofuran. The fatty acid composition from TAG fraction was determined according to IUPAC Standard Methods [21], as the composition of fatty acid methyl esters (FAME) by GC. Trans-

esterification of the oils was carried out with KOH in methanol at a concentration of 2.0 mol L^{-1}. For FAME separation, the chromatographic conditions above described were used.

2.2.3.5 Phytosterols composition

To determine the phytosterols composition, the unsaponifiable matter was initially extracted by the AOCS Ca 6a-40 method [18]. The sterol profile in the samples was obtained by the Ch 6-91 method, and the total sterols content was quantified by use of an internal standard a-cholestanol (Sigma-Aldrich).

2.2.3.6 Carotenoids content

The total carotenoid content was obtained using the method described by Porim [22]. Briefly, the samples (0.8 g) were diluted in hexane (25.0 mL), and the absorbance was read in a spectrophotometer at 466 nm (UV / VIS Lambda20 - Perkin Elmer).

2.2.3.7 Tocopherols composition

Tocopherols were determined according to the IUPAC Standard Method 2432 [21]. Samples of oil were diluted in hexane (10.0 mg mL^{-1}) and followed by injection directly into the liquid phase chromatography system equipped with an Si column (250 mm × 4.0 mm x 4 μm) and a fluorescence detector (Shimadzu RF-10AXL fluorescence detector) adjusted to excitation and emission at wavelengths 290 and 330 nm, respectively. Hexane: 2-propanol mixture (99: 1, v:v) at a flow rate of 1.0 mL min^{-1} was used as a diluent. Tocopherols concentrations (mg kg^{-1}) were determined from a calibration curve using tocopherol standards in hexane in the range of 4 to 6 μg mL^{-1}.

3 Results and discussion

3.1 Lignocellulosic characterization of EFBs

Table 4.1.1 shows the lignocellulosic characterization of the two EFBs.

Table 4.1.1. Lignocellulosic composition of the EFBs

EFB*	Cellulose (C)	Hemicellulose (H) (%)	Liginin (L)	Proportion C:H:L
type 1	31.9	19.8	24.1	1.3:0.8:1
type 2	17.9	15.2	13.7	1.3:1.1:1

*type 1 (higher lignocellulosic content and fatty acids composition of this oil was similar to a mixture of palm oil and palm kernel) and type 2 (lower lignocellulosic content and fatty acids composition of this oil was similar to palm oil).

Although similar proportions exist among the components, the lignocellulosic content was higher in type 1 EFB. Figure 4.1.1 shows the visualization of the EFBs after extraction of the residual lipids. The darker color in type 1 EFB corresponds to the higher lignocellulosic concentration. The moisture content in the EFBs was 11.45% (type 1) and 18.94% (type 2), and the residual lipids content was 6.87% in type 1 EFB and 38.4% in type 2 EFB.

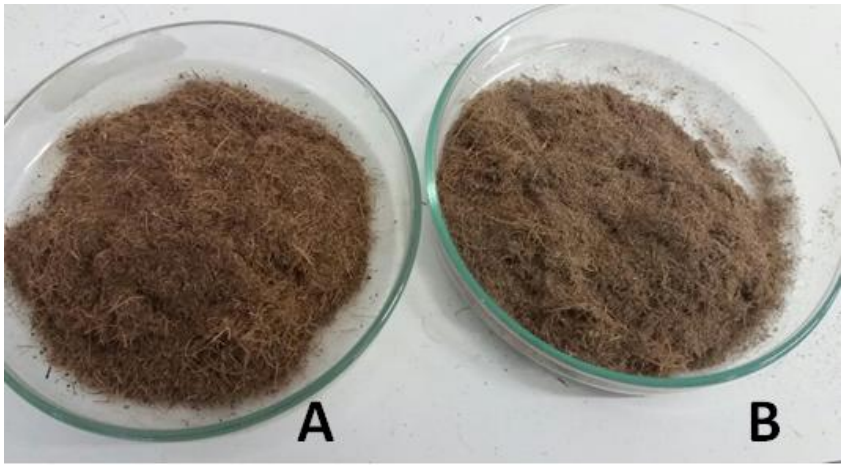

Figure 4.1.1. Visualization of type 1 (A) and type 2 (B) EFBs after lipid extraction

3.2 Characterization of residual lipids in EFBs

The composition of the residual fatty acids in the EFBs is presented in Table 4.1.2.

Table 4.1.2. Fatty acid composition in EFBs

Fatty Acids	Type 1 EFB [a] (%)	Type 2 EFB[a] (%)
C8:0 – Caprylic Acid	0.73 ± 0.06	--
C10:0 - Capric Acid	0.71 ± 0.05	--
C12:0 - Lauric Acid	9.57 ± 0.55	--
C14:0 - Myristic Acid	3.93 ± 0.14	1.44 ± 0.15
C16:0 - Palmitc Acid	32.58 ± 1.62	65.90 ± 4.01
C18:0 - Stearic Acid	4.44 ± 0.24	8.49 ± 0.19
C18:1 - Oleic Acid	37.60 ± 2.39	20.91 ± 2.69
C18:2 - Linoleic Acid	8.77 ± 0.54	0.85 ± 0.83
C18:3 - Linolenic Acid	--	--
C20:0 - Arachidonic Acid	--	0.63 ± 0.05
∑ Saturated	51.96	76.46
∑ Unsaturated	46.37	21.76
I V	110.2	82.1
FFA (%)	9.31 ± 0.16	51.63 ± 1.12
PV	12.41 ± 1.27	67.49 ± 2.9

[a]Mean of three replicates ± Standard Deviation; Values below 0.5 % were omitted from the table. --: not detected; IV: iodine value, PV: peroxid value, FFA: free fatty acids.

The data show that type 2 EFBs present a higher concentration of saturated fatty acids (76.46%) compared with type 1 EFBs (51.96%). The percentage of saturated lipids in type 2 EFBs is predominantly composed of palmitic (66%) and stearic fatty acids (8%),

followed by smaller amounts of myristic and arachidonic acids. In type 1 EFBs, the predominant saturated fatty acids were palmitic acid (32%), lauric acid (9%), stearic acid (4%) and myristic acid (4%) as well as 1% short-chain fatty acids such as caprylic acid and capric acid. The unsaturated fractions correspond to the oleic and linoleic fatty acids in the residues, adding a total of 21.76% in the lipid content of type 2 EFBs and 46.37% in type 1 EFBs.

The iodine value refers to the active sites of unsaturations in the fatty acids that compose the oils [23]. According to Santos [24], the calculation of the iodine content, obtained from the composition in fatty acids, provided a value for palm oil of 52.7, indicating high levels of unsaturation. In Table 4.1.2, the iodine value in type 1 EFBs was higher than in type 2, confirming that the first has more instaurations, is more susceptible to oxidation.

The differentiated chemical composition between saturated and unsaturated fatty acids gives the lipids differentiated physical properties. It was observed that type 2 EFBs were solid at room temperature and therefore can be considered a fat, while type 1 EFBs remained liquid under the same conditions and can be considered oil according to the basic classification of oils and fats [9].

Palm oil has equilibrium in saturated and unsaturated fatty acids, presenting semisolid at room temperature, since it consists of approximately 50% saturated fatty acids and 50% unsaturated fatty acids [25]. According to Block and Barrera-arellano [9], the saturated fatty acids present in palm oil are between 42-40% palmitic acid, 4-5% stearic acid, 39-40% oleic acid and 10-12% linoleic acid. Correlating the values obtained in this study with those found in the literature, it is possible to affirm that the verified characteristics are directly related to the fractions obtained from the palm, such as, stearin and olein. The concentration of oleic acid in palm oil is approximately 36%, with palm olein being 39%, which is a liquid fraction at room temperature [26]. According to Table 4.1.2, oleic acid is present in 37% of the oil extracted from type 1 EFB, that is liquid at room temperature. In addition, type 1 EFB also presented approximately 9 % of lauric acid, which is the main component (48%) of the palm kernel oil [10]. Therefore, type 1 EFB residual oil contains fractions of the palm and kernel oils. The residual oil in type 2 EFB was solid at room temperature due to the high concentration of saturated fatty acids (76.46 %), according to the stearin fraction of palm oil

[10]. As palmitic was the predominant fatty acid (65%) and the lauric acid wasn't found, it could be inferred that the residual fatty acids of type 2 EFB are similar to palm oil only.

The table 4.1.3 presents results about TAGs composition of the oils extracted from the both EFBs.

Table 4.1.3. Tryacilglycerols composition of the oils extracted from the EFBs

Carbon Number	Triaciglycerols	Type 1 EFB [a] (%)	Type 2 EFB [a] (%)
C40	LaMM-LaPLa	3.45 ± 0.06	--
C48	SLaS-PPP-SMP	6.09 ± 0.12	--
C48	MPS-PPP	--	16.31 ± 0.14
C50	SOM-POP	28.12 ± 0.22	36.7 ± 0.28
C50	SLM-MOO-PLP	6.28 ± 0.16	6.17 ± 0.1
C52	POO-SLP	26.03 ± 0.55	17.57 ± 0.15
C52	PLO	6.76 ± 0.08	4.59 ± 0.07
C52	SOP	--	6.55 ± 0.03
C54	SLO-OOO	4.68 ± 0.05	3.45 ± 0.18
Others > 3 %		18.6 ± 0.6	8.68 ± 0.6

[a]Mean of three replicates ± Standard Deviation; Values below 0.5 % were omitted from the table. --: not detected; La: Lauric Acid, M: Miristic Acid, P: Palmitic Acid, S: Stearic Acid, O: Oleic Acid

TAG analysis showed that the predominant TAGs in EFBs were SOM-POP and POO-SLP. Analyzing the type 2 EFB, the quantity of myristic acid was very low (1%), while the amount of oleic acid (20%) and palmitic acid (65 %) are quite high, so it is assumed that the most probable TAG present is POP. In the case of POO-SLP, in both samples the probability of having POO is higher than SLP, since the levels of linoleic acid (0.85 and 3%) and stearic acid (8 and 4%) are also very low. According to Chee Man et al. [27], palm oil has high amounts of POO (23%) and POP (29%) TAGs, which agrees with the data obtained in

this work, showing that both EFBs has characteristics of palm oil. However, it can also be seen that in type 1 EFB there is lauric acid (LaPLa, 3%), that is characteristic of kernel oil, where lauric acid compouses around of 50% of fatty acid composition. Thus, it was considered the residual oil in type 2 EFB machs to palm oil, while type 1 EFB contains both palm and kernel oils.

In relation the qualitative characterization of the oils and fats, the determinations of FFA and peroxides were done. The value of the FFA obtained for the lipid content extracted from the type 2 EFB oil was much higher than that from type 1 EFB oil, being, on average, 51.63 and 9.31, respectively (Table 4.1.2). These values were confirmed by lipid class analysis, where similar values of MAG and FFA were obtained (Table 4.1.4).

Table 4.1.4. Glyceridic classes of oils extracted from type 1 and 2 EFBs

Glyceridic classes	Type 1 EFB[a] (%)	Type 2 EFB[a] (%)
Triacylglycerols	74.91 ± 1.46	45.53 ± 1.16
Diacilglicerois	18.23 ± 0.69	7.17 ± 0.31
Monoacylglycerols+FFA	6.84 ± 1.14	47.28 ± 1.47

[a]Mean of three repetitions ± standard deviation; FFA = free fatty acids.

The presence of high amounts of DAG, MAG and FFA in the samples indicated a high hydrolysis degree, the action of lipolytic enzymes on the TAG molecules releases DAG, MAG and fatty acids. The high lipolytic activity of palm lipases is already known, therefore, in the case of an industrial residue this was clearly expected. Due to the high amount of MAG+FFA into the type 2 EFB, is possible to affirm that it presented a higher degradation level than type 1 EFB. This kind of situation can be related to the conditions of palm fruit management as well as the time and conditions of the residue storage that is not commonly controlled [28]. On the other hand, the fact that it is a material with high content of FFA, may be interesting to facilitate microbial conversion of this type of substrate into biomass and metabolites.

In addition, it can be inferred, through the visual observation of the residues (Figure 4.1.2), that the processes of extraction of the palm oils probably followed different procedures and/or storage times and conditions considering their particularities in terms of composition and appearance. The type 1 EFB residual oil shows a red coloration, which corresponds to the presence of high amounts of carotenoids, whereas the type 2 EFB was obtained only a white fraction, indicating that this residue has a low concentrations of carotenoids. This visual difference was confirmed by the analysis of the carotenoids concentrations. The amount of carotenoids found in the sample extracted from the type 1 EFB was 890.57 mg kg^{-1}, being tenfold higher than content presents on the type 2 EFB, which was 81.57 mg kg^{-1} (Table 4.1.5). These compounds are considered bioactive, because of the connections that are easily oxidized, and for this reason, they presented antioxidant activity [29]. The low amounts of carotenoids in type 2 EFB indicates a high level of oxidation of this sample, since the antioxidant molecules (carotenoids) were degraded. A similar behavior was noticed for the tocopherols content. The tocopherol known as Vitamin E, is a lipid-soluble nutrient with the main function of protecting long-chain polyunsaturated fatty acids from cell membranes and lipoproteins against oxidation [30]. According to Wong et al. [31] the total tocopherol found in crude palm oil was 794 mg kg^{-1} and in refined palm oil is 563 mg kg^{-1}. The lipid fraction extracted from type 1 EFB presented 1188.70 mg kg^{-1} of tocopherol, while in the lipid extracted from the type 2 EFB the presence of tocopherol was not detected (Table 4.1.5). The total tocotrienols of lipid fraction extracted from type 1 EFB was 563.80 mg kg^{-1} (Table 4.1.5), papers from the literature show that palm oil is composed for 188 mg kg^{-1} of tocotrienols [9]. The Table 4.1.6 represents tocopherols and tocotrienols profile from EFB type 1. Both the tocopherol and tocotrienol dominant present in the sample were from the alpha form.

Table 4.1.5. Carotenoids, phytosterols, tocopherols and tocotrienols from residual lipids of type 1 and 2 EFBs

	Type 1 EFB[a]	Type 2 EFB[a]
Carotenoids (mg kg^{-1})	890.24 ± 0.00	81.53 ± 5.43
Phytosterols (mg kg^{-1})	5270.65 ± 150.67	1102.35 ± 13.03
Tocopherols (mg kg^{-1})	1188.70 ± 5.46	--
Tocotrienols (mg kg^{-1})	563.90 ± 1.51	--

[a]Mean of three repetitions ± standard deviation; --: not detected

Table 4.1.6. Tocopherols and Tocotrienols profile from residual lipids of Type 1

	Tocoferol [a] (mg kg^{-1})	Tocotrienol [a] (mg kg^{-1})
Alpha	92.40 ± 0.43	52.27 ±0.78
Beta	4.94 ± 0.29	--
Gamma	2.66 ± 0.20	38.15 ± 0.65
Delta	--	9.58 ± 0.47

[a]Mean of three repetitions ± standard deviation; --: not detected

Finally these entire oxidation hypotheses were confirmed by the peroxide value. The values obtained for the peroxide value (Table 4.1.2) are higher for the type 2 EFB than the type 1 EFB (67.49% and 12.41%, respectively). These values confirm the primary oxidation state of oils or fats, which can be influenced by factors such as time and storage conditions after processing [9]. It is worth mentioning that the substrate is a residue of the palm oil processing, in other words, with raw material that has already undergone several treatments, with later uncontrolled storage of the residue.

Figure 4.1.2. Visualization of the residual lipid fractions of type 1 (A) and type 2 (B) EFBs.

The phytosterols content on lipid from type 1 EFB is also higher than the lipid from type 2 EFB (Table 4.1.5). According to Choo et al. [32] palm skin has a significant amount of carotenoids ranging from 4000 to 6000 mg $.kg^{-1}$, vitamin E about 2400-3500 mg

$kg^{-1}$and sterols 4500-8500 mg $.kg^{-1}$. The results of the literature are closer to type 1 EFB lipid data, indicating a low degradation level of this residue, unlike the type 2 EFB.

The Table 4.1.7 represents phytosterols profile of lipids from EFB type 1 and type 2. Is possible to observe that the phytosterols found in major quantity in lipids from both EFB were β-sitosterol+sitostanol (61.48 and 63.86%), flowed by campesterol (14.26 and 17.52%) and stigmasterol (13.37 and 10.26%), respectively. These phytosterols are generally found in palm oil in greater amounts, being in agreement with the literature [33].

Table 4.1.7. Phytosterols profile from residual lipids of type 1 and 2 EFBs

Phytosterols	Type 1 EFB [a] (%)	Type 2 EFB [a] (%)
Cholesterol	2.78 ± 0.04	3.15 ± 0.02
Brassicasterol	0.20 ± 0.01	--
Ergosterol	4.31 ± 0.08	--
Campesterol	14.26 ± 0.02	17.52 ± 0.06
Stigmasterol	13.37 ± 0.01	10.26 ± 0.03
Clerosterol	1.00 ± 0.00	2.00 ±0.09
β+sitosterol+sitostanol	61.48 ± 0.15	63.86 ± 0.15
Δ5-avenasterol	2.19 ± 0.01	2.28 ± 0.01
Δ5,24-stigmastadienol	0.23 ± 0.01	0.44 ± 0.20
Δ7-stigmatenol	0.08 ± 0.02	0.5 ± 0.19
Δ7- Avenasterol	0.08 ± 0.05	--

[a]Mean of three replicates ± standard deviation;. --: not detected

4 Conclusions

The lipids extracted from the two EFB types had different composition and characteristics. The content of cellulose (31%), hemicellulose (19%) and lignin (24%) of type 1 EFB was higher than type 2 EFB (17% cellulose, 15% hemicellulose and 13% lignin). The lipid content from type 1 EFB presented fatty acids composition similar to the palm oil and palm kernel oil, higher amount of carotenoids than lipid from type 2 EFB. The lipid extracted from type 2 EFB presented similar characteristics only to palm oil, and a higher degree of free fatty acids and more oxidized. Therefore, the soil type and the palm fresh-fruit separation process provide different lignocellulosic compositions and residual lipids in EFBs.

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4.2 The role of lignocellulosic composition and residual lipids in empty fruit bunches on the production of humic acids in submerged fermentations

Sumbetido ao periódico Applied Biochemistry and Biotechnology

The role of lignocellulosic composition and residual lipids in empty fruit bunches on the production of humic acids in submerged fermentations

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Abstract

The aim of this research was to study the production of humic acids (HA) by the fermentation of *Trichoderma reesei* in empty fruit bunches (EFBs) from palm oil processing with a focus on the effects of lignocellulosic content and residual lipids. Fermentation represents a renewable and controlled process for HA production. EFBs from two different soils and palm oil producers were previously characterized regarding their lignocellulosic composition and sized by milling in the range of 710 to 355 μm. Submerged fermentations were inoculated with *T. reesei* spores from pre-cultivation and carried out in Erlenmeyer flasks in the presence or absence of residual lipids. The results showed that the soil and the processing for removal of the palm fresh fruits were crucial to EFB quality. Thus, EFBs were classified as type 1 (higher lignocellulosic and fatty acids composition similar to the palm oil and palm kernel oil) and type 2 (lower lignocellulosic content and fatty acids composition similar to palm oil). The fungal growth at 120 h was similar for both EFB types but with different profiles. HA production was associated with fungal growth, and it was higher without lipids for both EFBs. The highest HA productivity (approximately 90 mg L^{-1} 48 h) was obtained from type 1 EFB.

Therefore, the lignocellulosic composition and the nature of the residual lipids in EFBs play an important role in HA production by submerged fermentation.

Keywords: Humic acids, Empty Fruit Bunch, Agroindustrial residue, *Trichoderma reesei*, Submerged fermentation

1 Introduction

Humic substances (SH) are natural complexes that represent approximately 85% of the earth's total carbon resources [1]. SH are formed by the microbial degradation of animal and plant residues and exist in organic soil materials, seawater, peat and sewage [2]. They are composed of fulvic acids, humic acids (HA) and humins that differ primarily in their water solubility [3].

HA are soluble in alkaline media but partially soluble in water and insoluble in acidic media [4,5]. Although their chemical structure still is not well defined, HA contain various functional groups such as phenol, carboxyl, and quinone, which give them antioxidant, anti-inflammatory, metal-chelant, and regenerable electron acceptor properties [4,5,6].

HA from natural sources have been widely used in agriculture, the environment, biomedicine and energy production [7-10]. Since ancient times, peat has been known for its healing properties. Medical applications also include the prevention and treatment of musculoskeletal, gynecological and skin diseases, and HA-based formulations have been developed [11]. More recently, HA also have been explored as phototheranostic agents for light-induced photoacoustic imaging and photothermal therapy [12].

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Although an abundance of HA is from non-renewable sources, its controlled production is sought to meet the reproducibility requirements of products, especially for uses in medical applications.

The microbial production of HA was initially demonstrated with *Trichoderma* and empty fruit bunches (EFBs) from palm oil processing as a substrate. EFBs are an agroindustrial residue of the extraction of palm oil from the fresh fruit bunches, which are rich in lignin (14.2%), cellulose (44.4%) and hemicellulose (30.9%). They represent 22-23% of the total bunches used by the palm oil industries. Currently, EFBs are an economic problem for companies producing palm oil as its demand is continuously increasing. In general, EFBs are burned and then deposited in landfills, or they are used in composting as an organic fertilizer. Because the burning process generates environmental problems, novel technologies have been recommended as sustainable uses of this high-value lignocellulosic biomass such as bioconversion in an added value product [13,14].

Depending on the processing for separation of fresh fruits, different compositions of residual lipids are associated with EFBs [15]. Two types of oil are extracted from the palm fruit: the palm oil from the pulp or mesocarp and the palm kernel oil from the seeds. They present different characteristics and fatty acid composition. The predominant lipids in palm oil are palmitic and oleic acids and it is semi-solid at room temperature The palm kernel oil is solid at room temperature and rich in the lauric and myristic acids [16].

Trichoderma is a species of fungus with the potential to degrade lignocellulosic compounds [17]. They occur worldwide in soils, decomposing wood and places with organic plant matter.

Motta and Santana effectively studied the production of HA by fermentation, focusing on *Trichoderma* strains and operational variables [18, 19]. The authors found that *T. reesei* produced three times more HA than *T. viride*, probably because *T. reesei* are able to assimilate the carbon source and energy better due to the expression of the genes involved in carbon degradation [17, 19]. In this scenario, HA production using EFB and *T. reesei* is promising, since large quantities of EFB residue are generated by the palm oil industry.

The objective of this work was to extend previous findings by studying the production of HA by *T. reesei* in a submerged fermentation using EFBs from different palm oil producers and to determine the effects of the lignocellulosic content and the residual lipids of EFBs in the production of HA.

2 Materials and Methods

2.1 Materials

Type 1 EFBs were provided by the Agropalma® industry, Limeira, São Paulo, Brazil, and type 2 EFBs were from the cooperative of small palm oil producing industries (Muniz Ferreira, Bahia, Brazil). A characterized strain of *T. reesei* (CCT 2768) was purchased from the Collection of Tropical Cultures (CCT), Campinas, São Paulo, Brazil.

2.2 Methods

2.2.1 Inocolum

A strain of *Trichoderma reesei* was used for HA production in EFBs. The cultivations were carried out at the best operational conditions reported by Motta et al. [20]. Initially, the cultures were sporulated in test tubes containing lean potato dextrose agar for two weeks at 24°C until a large number of spores developed. A solution of glycerol in water (5 mL of 20% m/v) was added to each tube and then gently scraped off the surface of the medium to release the spores. The glycerol solution containing the spores was stored in 1.2 mL cryotubes in an ultrafreezer at -70°C.

T. reesei was pre-cultivated in an oat medium according to Motta et al. [20]. Briefly, the medium was prepared by adding 30.0 g of oats to 1.0 L of water and then boiled at 90°C for 90 minutes. This suspension was filtered, and distilled water was added to the filtrate to achieve a final volume of 1 liter. Potato peptone (5.0 g), as a nitrogen source, was added to the medium, and the pH was adjusted to 6.0.

The microorganism stored in the cryotubes was then propagated along 120 h in 500 mL Erlenmeyer flasks containing 300 mL of the oat culture medium and 0.4 mL of the suspension spore solution.

2.2.2 Culture medium

The culture medium for submerged fermentation was composed of 0.77 $g.L^{-1}$ of $(NH_4)_2SO_4$; 1.54 $g.L^{-1}$ K_2HPO_4; 20.0 $g.L^{-1}$ EFB; and 3.85 $g.L^{-1}$ potato peptone. The pH was then adjusted to 6.0 and autoclaved at 121°C for 15 minutes. EFBs were milled to produce standardized particles with sizes in the range of 355 and 710µm [21].

The residual lipids were extracted from EFB fibers for characterization and use in the control fermentations. Extraction was carried out in a Soxhlet Extractor using petroleum ether according to the method AM 2-93 [22]. Table 4.2.1 presents the composition of the EFBs in terms of lignocellulosic content, residual lipids and other components

Table 4.2.1. Composition of lignocellulosics, oil residual lipids and other components in the EFBs

Component	Type 1 EFB [a] (%)	Type 2 EFB [a] (%)
	LIGNOCELLULOSIC (%)	
Cellulose	31.9	17.9
Hemicellulose	19.8	15.2
Lignin	24.1	13.7
	LIPIDS* (%)	
Fatty Acids	Oleic Acid - 37.60 ± 2.39	Palmitic Acid- 65.90 ± 4.01
Triacylglycerols	POP - 28.12 ± 0.22	POP - 36.7 ± 0.28
Diacilglicerois	18.23 ± 0.69	7.17 ± 0.31
Monoacylglycerols+ Free Fatty Acids	6.84 ± 1.14	47.28 ± 1.47
Free Fatty Acids	9.31 ± 0.16	51.63 ± 1.12
Peroxid Value	12.41 ± 1.27	67.49 ± 2.9
Iodine Value	110.2	82.1
	OTHERS* (mg kg^{-1})	
Carotenoids	890.24 ± 0	81.53 ± 5.43
Phytosterols	5270.65 ± 150.67	1102.35 ± 13.03
Tocopherols	1188.70 ± 5.46	--

*Mean of three repetitions ± standard deviation; P: Palmitic Acid, O: Oleic Acid; The lignocelullosic was determined by AOAC [23] and lipids analyses were determined by AOCS method [22].

2.2.3 Submerged Fermentation

Submerged fermentations were carried out in 125 mL Erlenmeyer flasks containing 25.0 mL of the culture medium and 2.77 mL of the inoculum (10^4 spores) in an orbital shaker at 120 rpm at 30˚C. Samples were withdrawn at 24 hour intervals along the fermentation time. The samples were analyzed for protein and HA concentrations.

2.2.4 Quantitative analysis

HA concentration was determined by optical density, as described by Badis et al. [24]. Samples in triplicate were centrifuged (5000 rpm for 15 min), and the supernatant was filtered using a Microfilter syringe filter (Thomapor®-Membranfilter, 5FP 025/1). The supernatant fraction (0.2 mL) was diluted five times with a 0.5 M NaOH solution, and the absorbance was measured at 350 nm (A350) and pH 4.5 ± 0.01. HA concentrations were determined using a previously constructed standard curve.

The indirect estimation of the fungal biomass was performed by protein concentration according to the method adapted by Callow and Ju [25] to quantify cellular proteins. Samples of the fermented liquid (3 mL) were collected and centrifuged at 10,000 rpm for 10 minutes to obtain pellets. The supernatant was collected, and the pellets were re-suspended and washed twice with distilled water. For the release of the intercellular proteins, the pellets were suspended in 3 mL of 1 N NaOH and heated at 100°C for 10 minutes. After cooling, the protein concentration in the supernatant was determined after centrifugation of the samples at 10.000 rpm at 5°C and recovery of the supernatant. The protein concentration was determined with the bicinchoninic acid assay developed by Smith et al. [26], using a commercial Pierce BCA kit (Thermo Scientific, USA).

2.2.5 Recovery of HA

Recovery of AH followed the protocol of International Humic Substances Society (IHSS), which consists in some steps: alkaline extraction, separation of HA precipitate, removal of HA silicates and salts and ovened for remotion of inorganic compounds. The

oven step was not performed due to the absence of inorganic compounds from the fermented extract. Thus, HA was calculed by Equation 4.2.1:

$$HA = \frac{HA\ mass(g)}{total\ \ sample\ (g)} x\ 100 \qquad (4.2.1)$$

3 Results and discussion

3.1 Submerged fermentations

Figure 4.2.1 presents the protein and HA profiles from submerged fermentation showing EFBs containing residual lipids and in degreased EFB fibers used as a control. Protein indicates cell growth during EFB cultivation from *T. reesei*. *Trichoderma* growth at 120 h was similar for both EFB types, but with different profiles. For type 1 EFB, the maximum growth occurred at 72 h in with lipid condition (Figure 4.2.1A). For type 2 EFB, both growth profiles, with and without lipids (control) were similar up to 72 h. Thereafter, the protein concentration continued to grow in the condition with lipids, while it declined in control (without lipids) (Figure 4.2.1B). These results suggest that metabolic activity is also sustained by the lipids as well as by the greater presence of structural carbohydrates, especially cellulose. Species of *Trichoderma*, such as *T. harzianum,* are prominent producers of hydrolytic enzymes, including lipase that hydrolyse oils and fats in glycerol as well as free fatty acids at the oil-water interface [27, 28]. It can also be seen that the fraction of cellulose and hemicellulose (more available) of type 1 EFB is much higher (51.7% vs. 33.1%) than that from type 2 EFB (Table 4.2.1). In addition, the fact that the lignin content is nearly twice as great in type 1 EFB as in type 2 EFB may suggest a more stable physical structure for type 1 EFB, which can sustain growth from the structural polysaccharides.

In type 2 EFB, fungal growth seems to occur by another route due to greater availability of FFA (Table 1) that is ready to be used for growth and HA production. Musa et al. [29] found that EFB can be used as an inexpensive substrate to improve lipase secretion by *Trichoderma*.

As known, diacylglycerol and monoacylglycerol form micelles that can incorporate oxygen and make it more available for fungal growth in submerged fermentation.

Figure 4.2.1 (C and D) show the kinetic profiles for HA production. The highest HA productivity (approximately 90 mg L^{-1}) was obtained for type 1 EFB in the control (EFB without lipid) at 48 hours. HA production was associated with *Trichoderma* growth and was higher without lipids for both EFBs. Comparing the protein (Figures 4.2.1A and 4.2.1B) and HA production profiles (Figures 4.2.1C and 4.2.1D), it appears that *T. reesei* uses the available carbon content in the lipid to metabolic cell growth route while it beneficially uses carbon in fibers for the production of HA. The difference in HA production in both EFB can be explained by the variation of lignocellulosic content in the fibers (Table 4.2.1) since type 1 EFB contain higher amounts of cellulose, hemicellulose and lignin, providing more carbon for growth and HA production.

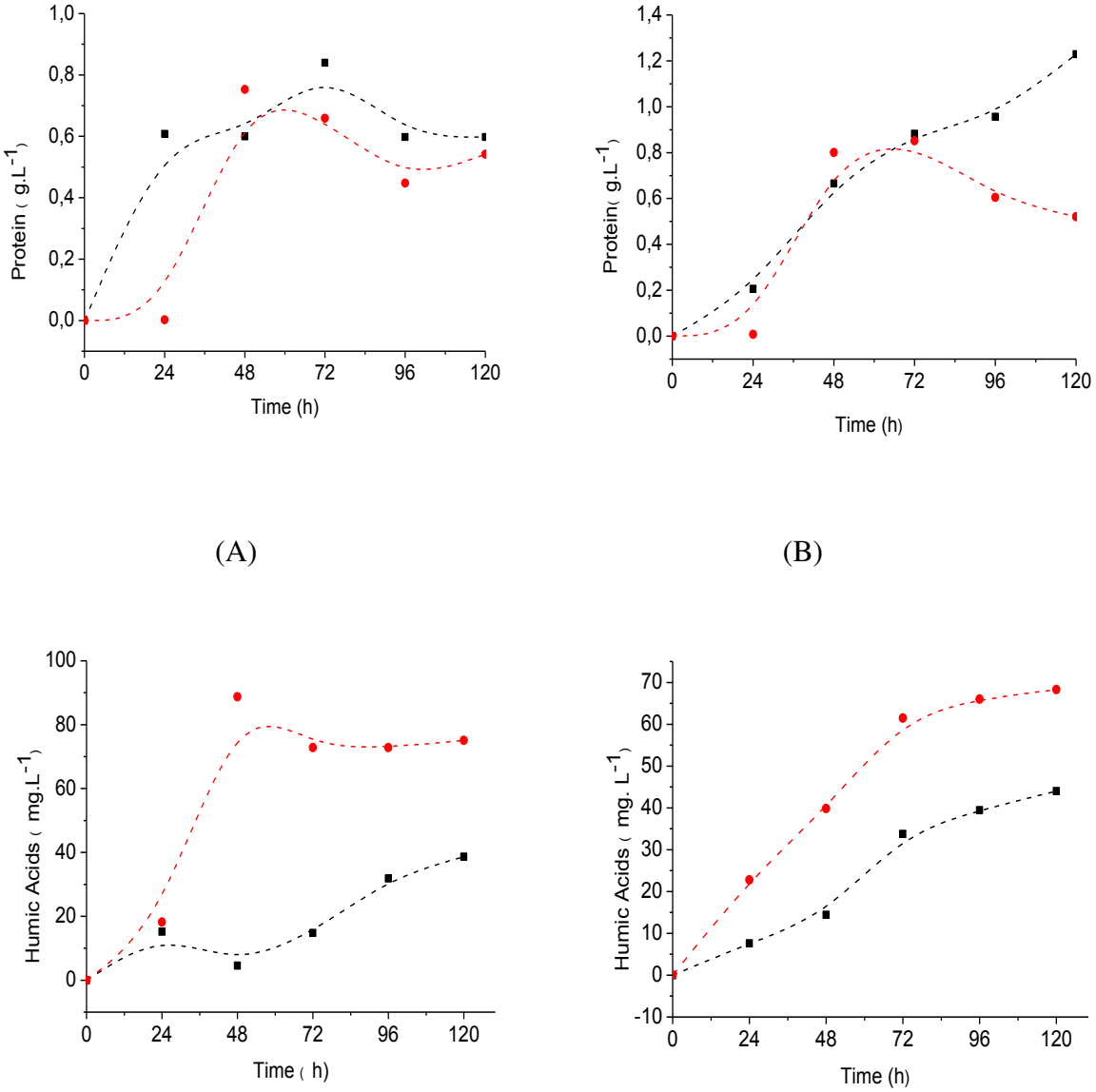

Figure 4.2.1. Protein and HA production profiles of submerged fermentations for *Trichoderma reesei* in from type 1 (A, C) and type 2 (B, D) EFBs. (●) control (without lipids), (■) with lipids

Table 4.2.2 shows the specific growth rates for the evaluated conditions. The highest rates were obtained without lipids at 0.0072 and 0.0079 h^{-1} for type 2 and 1 EFB, respectively. Additionally, the differences in log-phase intervals should be considered, as the events were slower and longer with lipids. In addition, the slightly higher growth rate for type

2 EFB with lipids (0.0064 h^{-1}) may be due to the higher amount of FFA, which is more easily assimilated in these fibers, compared with type 1 EFB (Table 2).

Table 4.2.2. Specific growth rates and log-phase period for T. reesei from types 1 and 2 EFBs with and without lipids, based on protein profiles

EFB	With lipids	Without lipids
type 2	0.0064 h^{-1}(0 - 120 h)	0.0072 h^{-1}(0 – 72 h)
type 1	0.0059 h^{-1} (0 – 72 h)	0.0079 h^{-1}(0 – 48 h)

Although a different source of EFBs was used, the results are consistent with those reported by Motta et al. [21] regarding lignocellulosic concentration in EFBs.

3.2 HA Recovery

Table 4.2.3 shows that the best HA recovery was from the fermentations in type 1 EFBs. The nature of the lipids influenced HA recovery, and it was higher in the condition without lipids (control) for both EFBs.

Table 4.2.3. HA recovery from the best fermentation conditions for EFBs type 1 and 2.

EFB	Recovery HA (g/g)
Type 1 (with lipid, 72 h fermentation)	12.15
Type 1(without lipid, 48 h fermentation)	15.49
Type 2 (with lipid, 120 h fermentation)	2.16
Type 2 (without lipid, 96 h fermentation)	5.44

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5 Conclusions

EFBs from palm oil extraction are a valuable substrate for HA production by *Trichoderma reesei*. The fermentation process assures the controlled and sustained production of HA. It is possible to notice that there is a consumption of lipid from EFB by *Trichoderma*, mainly used in its growth. Further research should include the characterization of the chemical groups present in the HA molecule, with the goal of defining its functionality and best applications.

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4.3 Production of humic acids by solid-state fermentation of *Trichoderma reesei* in raw oil palm empty fruit bunch fibers

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Production of humic acids by solid-state fermentation of *Trichoderma reesei* in raw oil palm empty fruit bunch fibers.

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Abstract

Humic acids (HA) are organic macromolecules of high structural complexity and are primarily obtained from non-renewable carbon sources such as peat and coal. HA is widely used in agriculture but is known to have therapeutic properties, which are still poorly explored. In both cases, HA production from renewable sources is desirable for ensuring sustainability and reproducibility. Previous studies have shown the potential of biotechnological processes in the production of HA in submerged (SF) and solid-state (SSF) fermentations, using pre-treated fibers of oil palm of empty fruit bunch (EFB) for the cultivation of *Trichoderma* strains. EFB is an agro-industrial residue that is readily available at a low cost. The present study aimed to study the production of HA by *Trichoderma reesei* in the SSF of raw fibers of EFBs from two different palm oil producers. The previous characterization allowed the classification of fibers by their lignocellulosic content and composition of residual oil. The fermentation processes were characterized along time, by the profiles of HA production, cellular protein, pH, glucose, moisture, and oxygen transfer. SEM images were obtained before and after SSF of the raw EFBs, and degreased EFB fibers were used as a control. The results showed efficient HA production in the raw fibers of the EFBs. HA production followed the cellular protein evolution of the fermentations in the absence of lipids, while the composition of lipids greatly affected its production. The best HA production

(350 mg HA .100 g^{-1} fibers) was obtained from EFB that was richer in lignocellulosics and the residual lipids were similar to the fractions of palm and kernel oils.

Keywords: Humic acids, Empty Fruit Bunch, agroindustrial residue, *Trichoderma reesei*, solid-state fermentation.

1 Introduction

Humic substances (HS) compose 85-90% of the total organic carbon reserve present in the soil and are characterized by stable complexes of dark staining, high molecular mass and different solubilities [1]. Humic acids (HA) are a fraction of HS that are soluble in alkaline medium and precipitate in an acid medium (pH < 2), forming dark crystalline and amorphous structures. HA presents a complex molecular structure with various chemical groups, which define their properties. Agriculture is the one that stands out most as using HA, mainly because they increase the absorption of nutrients, stimulate plant growth, improve soil structure and generate benefits for productivity [2,3].

Currently, commercial HA is extracted from non-renewable carbon sources such as peat and coal non-peat and coal. Solid residues generated from agricultural activities have available nutrients that can be converted into commercial products by fermentation. Mimicking their natural environment, filamentous fungi have the capacity to grow in low water levels, and thus, solid-state fermentation is a promising process for these residues [4].

The processing for the production of palm oil generates two types of fibers, including those from the empty fruit bunch (EFB) after separation of the fresh palm fruits, and the mesocarp fibers, which are waste materials left after the oil extraction. The fibers from EFB are commonly used for composting materials and various other applications because they are readily available at a low cost [5,6]. The raw EFB fibers contain residual oil whose composition depends on the processing for the removal of palm fresh fruits.

EFB is an agroindustrial residue composed of 45-50% cellulose, approximately 25-35% hemicellulose and 10-15% lignin in addition to minor components like xylose, mannose, galactose, silica, copper, calcium and manganese [5,7,8]. *Trichoderma* is a complex

genus of fungus characterized by the rapid growth of colonies, reaching a diameter of 2 to 9 cm after four days of growth at 20 °C. *Trichoderma* ssp. is ubiquitous colonizers of cellulosic material [9].

The oil palm EFB fibers are a promising renewable source to produce HA by *Trichoderma* strains. Motta and Santana [10] studied the production of HA in submerged fermentation (SF) by *Trichoderma reesei* and *Trichoderma viride* strains cultured in pre-treated oil palm EFB fibers. The HA production was higher than the microbial growth in both cases, and *Trichoderma reesei* produced three times more HA than *T. viride*. A relationship between *Trichoderma* sporulation and HA production was observed [11,12]. In subsequent studies, Motta and Santana [13] also used the same EFB fibers for HA production in solid-state fermentation (SSF). In this case, fast sporulation occurred, and no vegetative form was observed throughout the fermentation time.

The present study extended the previous findings of the potential HA production from oil palm EFB in SSF by evaluating the use of the raw EFB fibers for the production of HA by *T. reesei*. The fermentations were conducted with EFB fibers from two different palm oil producers. The palm trees were cultivated in different soils and weather conditions of north and northeast Brazil, and the EFBs resulted from different processing conditions. The raw EFB fibers were previously classified as type 1 (higher lignocellulosic content and residual oil similar to the palm oil and kernel oil fractions), and type 2 (lower lignocellulosic content and residual oil similar to palm oil).

2 Materials and Methods

2.1 Inoculum

The *Trichoderma reesei* strain CCT 2768 from the Tropical Culture Collection-CCT (Campinas, São Paulo, Brazil) was used in SSF. The cultures were initially sporulated in test tubes containing potato-dextrose agar tilted for two weeks at 24 °C. After that 5.0 mL of 20% glycerol solution in water was added to each tube, which was then gently scraped of the middle surface for the release of the spores. The solution of glycerol containing the spores was stored in cryotubes of 1.2 mL in an ultrafreezer at -70°C [10].

2.2 Solid support

The raw EFB fibers used in SSF were provided by Agropalma industry in the city of Limeira (São Paulo, Brazil) and by the cooperative of small palm oil producing industries (Muniz Ferreira, Bahia, Brazil). Table 4.3.1 shows the lignocellulose content and the initial moisture of the fibers, and the main residual lipids are presented in Table 4.3.2 and Table 4.3.3.

Table 4.3.1. Lignocellulosic and moisture compound contents of Type 1 and Type 2 EFBs

EFB	Cellulose (%)	Hemicellulose (%)	Liginin (%)	Moisture (%)
Type 1 EFB	31.9	19.8	24.1	11.45
Type 2 EFB	17.9	15.2	13.7	18.94

The lignocelullosic method was performed using the neutral detergent fiber (NDF) and acid detergent fiber (ADF) from the Association of Official Analytical Chemists AOAC [14], and the moisture content according to AACCI methods 44-15.02 [15].

Table 4.3.2. Composition of the oil residual lipids in the EFBs

Composition of Lipid	Type 1 EFB [a] (%)	Type 2 EFB [a] (%)
Fatty Acids	Oleic Acid - 37.60 ± 2.39	Palmitc Acid- 65.90 ± 4.01
Triacylglycerols	POP - 28.12 ± 0.22	POP - 36.7 ± 0.28
Diacilglicerois	18.23 ± 0.69	7.17 ± 0.31
Monoacylglycerols+ Free Fatty Acids	6.84 ± 1.14	47.28 ± 1.47
Free Fatty Acids	9.31 ± 0.16	51.63 ± 1.12
Peroxid Value	12.41 ± 1.27	67.49 ± 2.9
Iodine Value	110.2	82.1

[a]Mean of three repetitions ± standard deviation; P: Palmitic Acid, O: Oleic Acid; all analyses were determined by AOCS method [16].

Table 4.3.3. Content of Carotenoids, Phytosterols and Tocopherols in the residual lipids in the EFBs

	Type 1 EFB [a]	Type 2 EFB [a]
Carotenoids (mg kg^{-1})	890.24 ± 0	81.53 ± 5.43
Phytosterols (mg kg^{-1})	5270.65 ± 150.67	1102.35 ± 13.03
Tocopherols (mg kg^{-1})	1188.70 ± 5.46	--
Tocotrienols (mg kg^{-1})	563.90 ± 1.51	--

[a]Mean of three repetitions ± standard deviation; --: not detected; all analyses were determined by AOCS Methods [16].

The fibers from Agropalma were classified as type 1, and the fibers from Bahia were classified as type 2.

The range of sizes of the EFB fibers used in the fermentations (355 to 710 μm) was obtained for those fibers retained in the 24 and 42 Tyler sieves.

The residual lipids were extracted from EFB fibers for characterization and use in the control fermentations. Extraction was carried out in a Soxhlet Extractor using petroleum ether according to the method AM 2-93 [16].

2.3 Solid-State Fermentations

The SSFs were performed in packed-bed column bioreactors (Raimbault columns), as previously established in Motta and Santana's studies [13]. The system was composed of 5 cylindrical glass columns 15 cm high and 3 cm in diameter, coupled to an air humidifier at the entrance of the column. The air supply was through a compressor. The airflow at the column input was adjusted to 0.4 L min^{-1} and monitored by air flow meter. The measurement of the inlet and outlet oxygen was performed in a YSI® Model 5300 oximeter. The columns were arranged in a microbiological oven to keep at 30°C. Each packed-bed bioreactor, previously autoclaved, was filled with 30 g of culture medium, 100 g of EFB with 100% (m/m) distilled water, 2.5% peptone, 1.0% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, pH adjusted to 6.0, and then inoculated with 1.0 mL of 10^6-10^7 spores of glycerol suspension. The columns were withdrawn as samples at 24-hour intervals, and the soluble fermented materials were extracted with distilled/deionized water, where 2.0 grams of sample was weighed and 4.0 mL of water was added.

2.4 Fungal growth and HA production

2.4.1 Cell protein concentration

The indirect estimation of the fungal biomass was performed by determining the protein concentration. Protein was quantified according to the method adopted by Callow and Ju [17] to quantify only the cellular proteins. Samples of the fungal extract (3.0 mL) were collected and centrifuged for 10 minutes at 10,000 rpm to obtain the pellets. The supernatant was collected, and the pellets were resuspended and washed twice with distilled water. The pellets were suspended in 3.0 mL of 1.0 N NaOH and heated at 100 °C for 10 minutes for the release of intracellular proteins. After cooling, the solution was centrifuged at 10,000 rpm at 5°C, the supernatant was recovered, and the protein was determined with the bicinchoninic acid assay developed by Smith et al. [18] using a commercial Pierce BCA kit (Thermo

Scientific, USA). Two mL of standard reagent was added to 0.1 mL of sample, and the mixture was incubated at 37°C for 30 minutes. After cooling to room temperature, the absorbance was read at 562 nm. The protein concentration was determined from a calibration curve previously constructed with bovine serum albumin (BSA).

2.4.2 Quantification of humic acids

The quantification of humic acids was performed as described by Badis et al. [19], where the samples were centrifuged for 15 min at 5000 rpm and the supernatant was filtered using the Microfilter syringe filter (Thomapor®-Membranfilter, 5FP 025/1). The supernatant was diluted five times in 0.5 M NaOH solution and then diluted ten times in water (pH 4.5 ± 0.01), and the absorbance was measured at 350 nm. The concentration of HA was determined by a standard curve that was previously constructed.

2.4.3 Oxygen transfer

Oxygen transfer was evaluated by the gaseous balance at the inlet and outlet of the columns, according to Equation 4.3.1 [20]:

$$NO_2 = (F_{in} * C_{O2in} - F_{out} * C_{O2out})/V \quad \text{(Eq. 4.3.1)}$$

where F_{in} is the air flow at the inlet of the column, F_{out} is the air flow at the outlet, C_{O2in} is the oxygen concentration at the column inlet, C_{O2out} is the oxygen concentration at the outlet, and V is the volume of liquid in the fermenter, which was calculated considering the moisture content (found in item 2.1.1.6) present in the volume of medium.

2.4.4 pH

The pH of the fungal extract was measured after fermentation by a pH meter after vigorous shaking of 5.0 g of fermented medium and 5.0 mL of distilled water.

2.4.5 Glucose

The glucose concentration of the fungal extract was determined using the enzymatic glucose-oxidase kit, LABORLAB. Aliquots of 10 μL of the extract were added to 1 mL of the standard reagent and incubated at 37°C for 5 minutes. After cooling to ambient temperature, the absorbance was read at 505 nm. The assays were performed in triplicate.

HA productivity was calculed by difference of production in the time.

2.4.6 Moisture content

The moisture content on wet basis was performed by drying approximately 3 grams of the fermented sample in a 105 °C until constant weight.

2.4.7 Scanning electron microscopy (SEM)

The crude and fermented fibers after 72 hours were submitted to 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 Å.

2.5 Recovery of HA

Recovery of AH was performed according to the protocol of the International Humic Substances Society (IHSS), which consists of three steps: alkaline extraction, separation of HA precipitates and removal of HA silicates and salts and oven-drying for removal of inorganic compounds. The oven step was not performed due to the absence of inorganic compounds from the fermented extract. Thus, HA was calculated by Equation 4.3.2:

$$HA = \frac{HAmass}{\text{total sample mass}} x\ 100 \quad \text{(Eq. 4.3.2)}$$

3 Results and Discussion

3.1 Characterization of the microbial assay process

Figure 4.3.1 shows the kinetic profiles of SSF in terms of HA production, cellular protein, pH, glucose, moisture of the solid medium and oxygen transfer rate for the fermentations with type 1 EFB fibers.

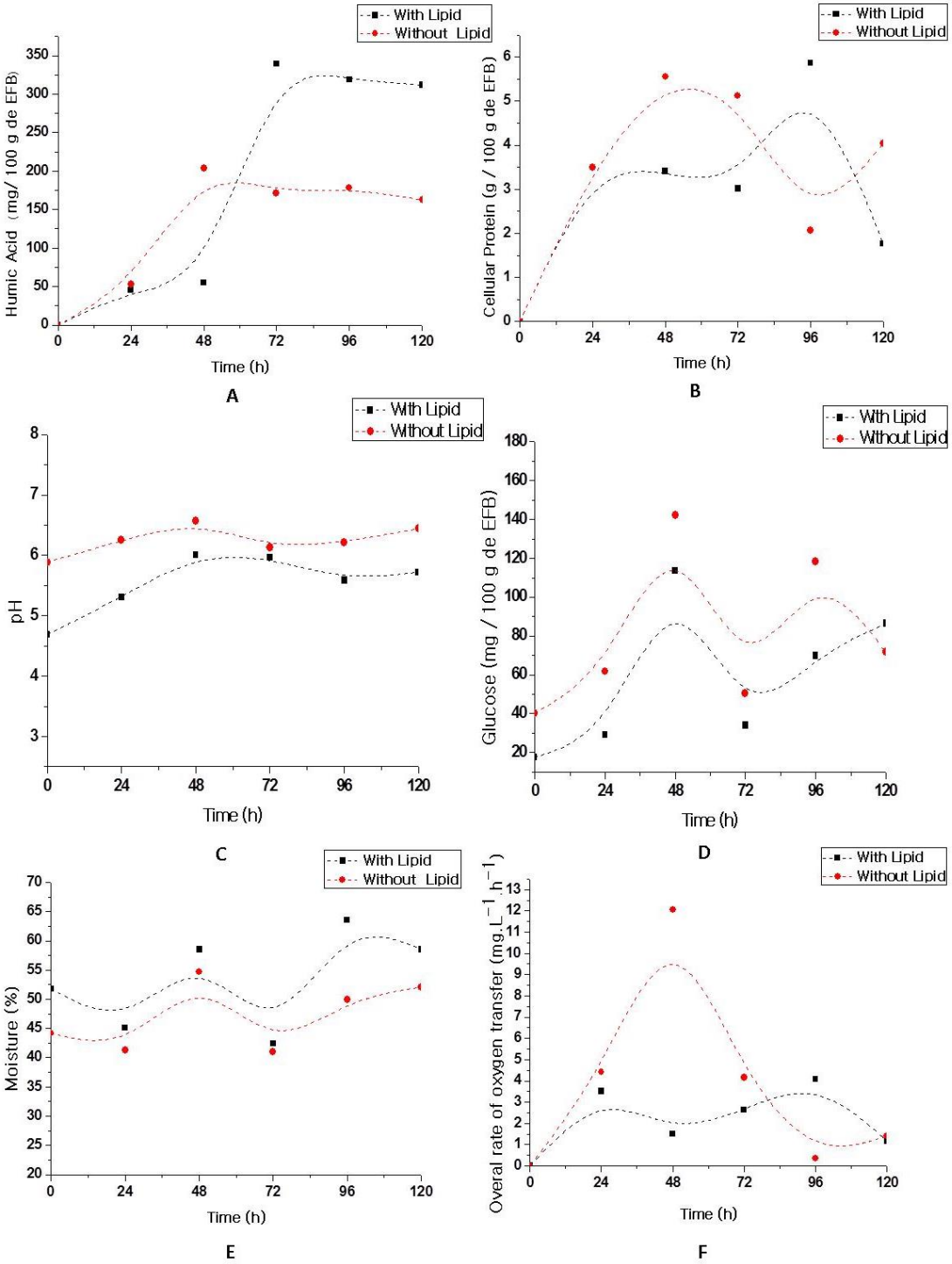

Figure 4.3.1. Profiles of the AH content, cell protein, pH, glucose concentration, solid moisture and oxygen transfer for SSF of Type 1 EFB with *T. reesei*

According to Figure 4.3.1a, HA was produced in the raw EFB fibers containing residual lipids and in degreased EFB fibers used as a control. In both cases, HA production was higher than cellular protein, evidencing the efficient use of the EFB fibers. The cellular protein evolution (Figure 4.3.1b) was similar for both fermentations, but the maximum concentration (6.0 g protein .100 g^{-1} of fibers) was delayed (96 h) in the presence of lipids compared with control (5.5 g protein .100 g^{-1} of fibers in 48h). HA production followed the cellular protein evolution, but the highest concentration (350 mg HA .100 g^{-1} of fibers) was obtained in the presence of lipids, at approximately 72 hours. Although there were mass transfer limitations in the presence of lipids, the utilization of these lipids as an additional carbon source may justify the higher HA production obtained.

Figure 4.3.1c shows that the pH of the microbial HA production ranged from 4.5 to 6.6, a range that is optimal for fungal growth (2-6 approximately) [21].

Figure 4.3.1d presents the glucose excess in the medium along the fermentation time. Due to imprecision in the measurements, similar behavior for both EFBs was considered. However, in both cases, the greatest drop in glucose occurred around 72 h, suggesting that the glucose from the breaking of the lignocellulosic compounds was preferentially consumed in the presence of lipids.

The moisture in the fibers remained around 65%, even for the highest HA production (Figure 4.3.1e). This is in agreement with fungal SSFs as reported by Bastos et al. [22]. A low moisture content may lead to poor nutrient accessibility and poor oxygen availability, resulting in poor microbial growth [23,24].

Figure 4.3.1f shows the highest oxygen consumption for the control condition (without lipid), at 48 hours, in agreement with fungal growth. In the presence of lipids, the overall rate of oxygen transfer remained around 3 mg L^{-1} h^{-1} along fermentation time, suggesting the storage of oxygen by the residual lipids in EFB.

Figure 4.3.2 shows the kinetic characterization of SSF in terms of HA production, cellular protein, pH, glucose, moisture and oxygen transfer for type 2 EFB.

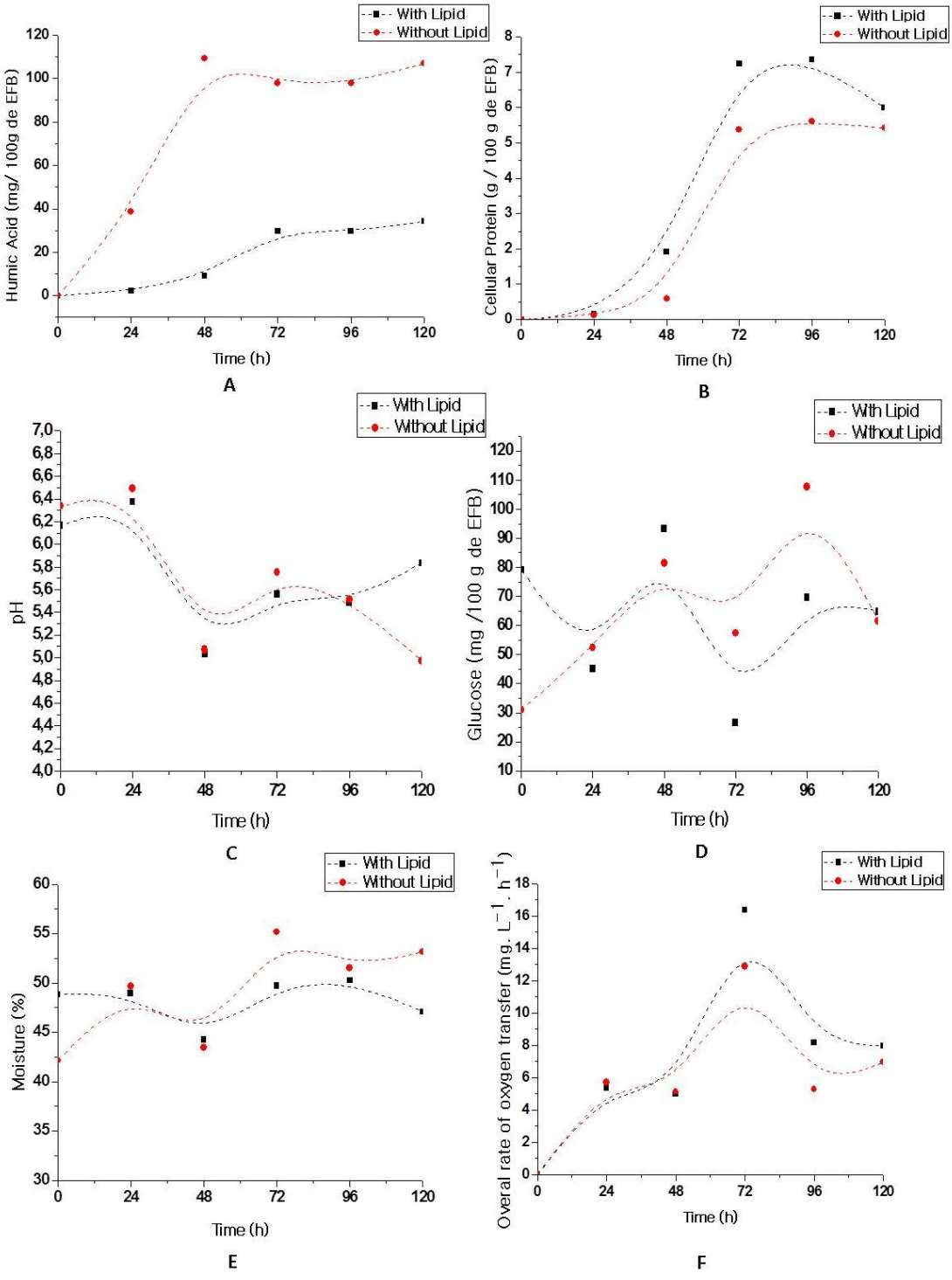

Figure 4.3.2. Profiles of the AH content, cell protein, pH, glucose concentration, solid moisture and oxygen transfer for SSF of Type 2 EFB with *T. reesei*

Figure 4.3.2a shows that the highest HA production was obtained for the control (EFB without lipid) at 48 hours, with approximately 110 mg .100 $g^{-1}$of fibers. Conversely, maximum fungal growth (7.5 g of protein .100 g^{-1} of fibers) was observed for EFB with lipid (Figure 4.3.2b). The pH curves were similar (Figure 4.3.2c), dropping in 48 h and remaining in the range of 5-6 up to 96 h. This concurred with the fungal growth (Figure 4.3.2b) and glucose consumption (Figure 4.3.2d). The moisture remained constant at around 50% during fermentation (Figure 4.3.2e). Moreover, higher oxygen consumption at approximately 72 h was in agreement with fungal growth (Figure 4.3.2f).

Table 4.3.4 shows specific patterns of growth for *T. reesei* from type 1 and type 2 EFB.

Table 4.3.4. Specific growth rates* and log-phase period for *T. reesei* from Type 1 and Type 2 EFBs, with and without lipids

EFB	With lipids	Without Lipids
Type 1 EFB	$0.0082h^{-1}$(0-96 h)	$0.019h^{-1}$ (0-48 h)
Type 2 EFB	$0.0080h^{-1}$(0-96 h)	$0.0057h^{-1}$(0-96 h)

For both, fibers with lipids showed approximately the same specific growth rates of 0.0080 h^{-1}.The highest specific growth rate was obtained for the type 1 EFB without lipid (0.019 h^{-1}) and as can be seen in Figure 4.3.1b, for the type 1 EFB an intense fungal growth in 48 h. In fiber with lipids, the behavior of the fungus is almost constant, showing peak growth at 96 h. For type 2 EFB, the highest specific growth rate was in the fiber with lipid, which had the same behavior as the cellular protein production (Figure 4.3.2b). Kupski [25] reported that *T. reesei* in SSF with bark and rice bran produced cellulase at a specific growth rate of 0.002 h^{-1}, that was lesser than that obtained in this study.

Comparing the behavior of type 1 and type 2 EFBs (Figures 4.3.1 and 4.3.2), the best HA production was with lipid for type 1 EFB and without lipids for type 2 EFB. Therefore, the composition of the residual lipids plays an important role in HA production. Apparently, *T. reesei* utilizes the lipids from type 2 EFB preferentially for growth instead of

HA production. Regarding Motta and Santana's work [13], *T. reesei* produced 75 mg AH .100 g^{-1} of fibers in 72 hours, while the highest HA production in type 1 EFB fibers was 350 mg AH .100 g^{-1} of fibers in 72 h in the presence of lipids. For type 2 EFB, the highest production (110 mg .100 $g^{-1}$of fibers in 48 h) was similar to that reported by Motta and Santana because of the absence of lipids. The cellular protein did not vary significantly for the best conditions, as it was 7.5 g .100 g^{-1} of fibers for type 2 EFB in the presence of lipids and 6 g .100 g^{-1} of fibers for type 1 EFB also with lipids. These values were higher than those obtained by Motta and Santana [13], which were 4.92 g .100 $g^{-1}$of fibers. Therefore, the lignocellulosic content and lipid composition contribute to both fungus growth and HA production.

The productivity of HA for type 1 EFB in the best growing condition with lipid was 2.6 mg .100 g^{-1} of fibers $.h^{-1}$, while for type 2 EFB in the best growing condition without lipid, it was 2.27 mg .100 g^{-1} of fibers $.h^{-1}$. Motta and Santana's [13] results (0.73 mg .100 g^{-1} of fibers $.h^{-1}$) were 3 times lower than these. In relation to the biomass productivity for type 1 EFB, it was 0.06 g .100 g^{-1} of fibers $.h^{-1}$ for fiber with lipids and 0.1 g .100 g^{-1} of fibers $.h^{-1}$ for the type 2 EFB with lipids, which were higher than those reported by Motta and Santana [13]. Thus, type 2 EFB functions better as a solid support for fungal growth, as reported in previous studies.

3.2 Images of EFB fibers performed in SEM

The fiber degradation process during the microbial growth could be observed by Scanning Electron Microscopy (SEM) through comparison of the images before and after the SSF for both EFBs (Figures 4.3.3 and 4.3.4).

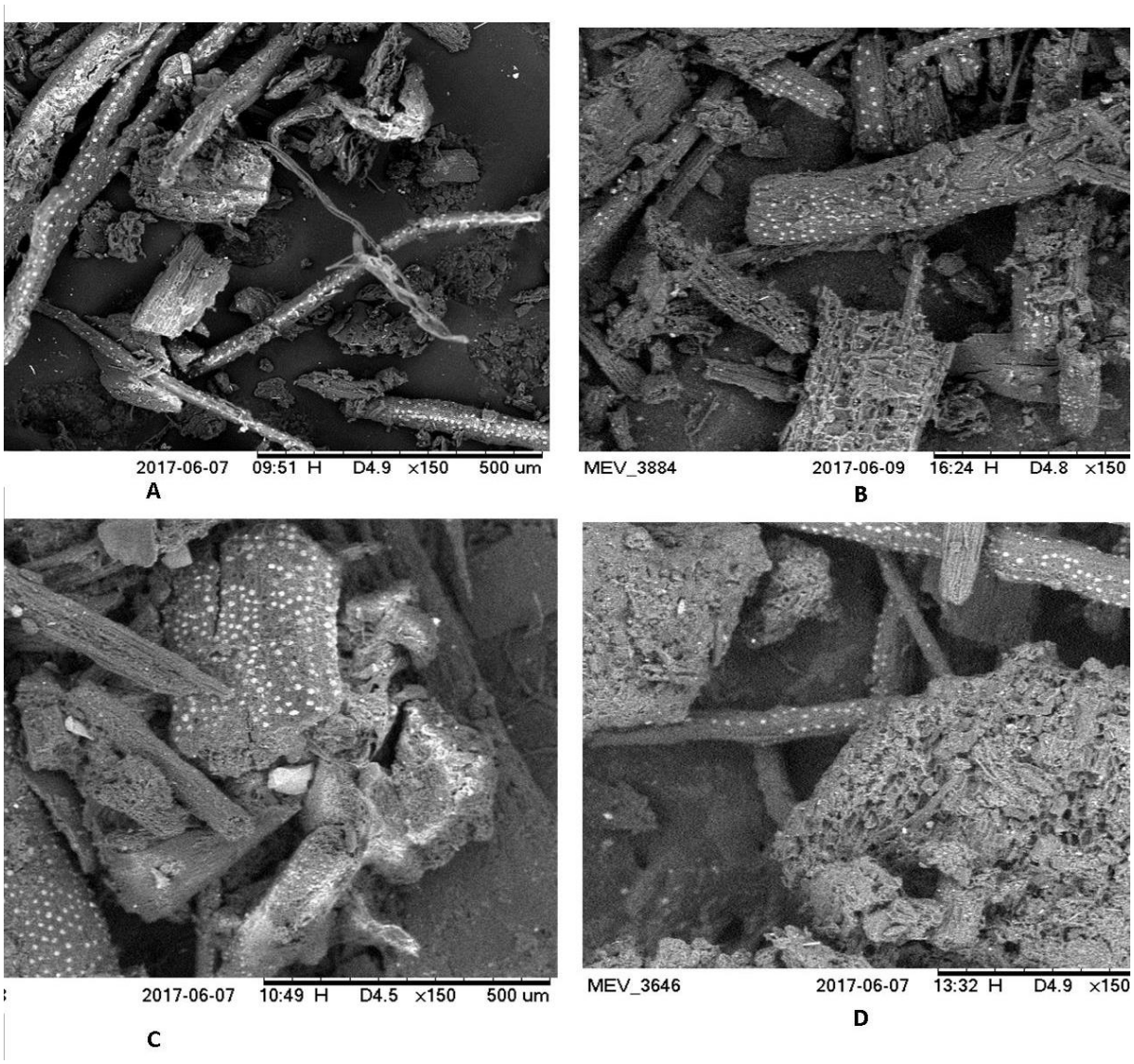

Figure 4.3.3. SEM images of type 1 EFB fibers before SSF: control (without lipids) (A) and with lipids (B). Fibers after 72 hours of SSF: control (without lipids) (C) and with lipids (D).

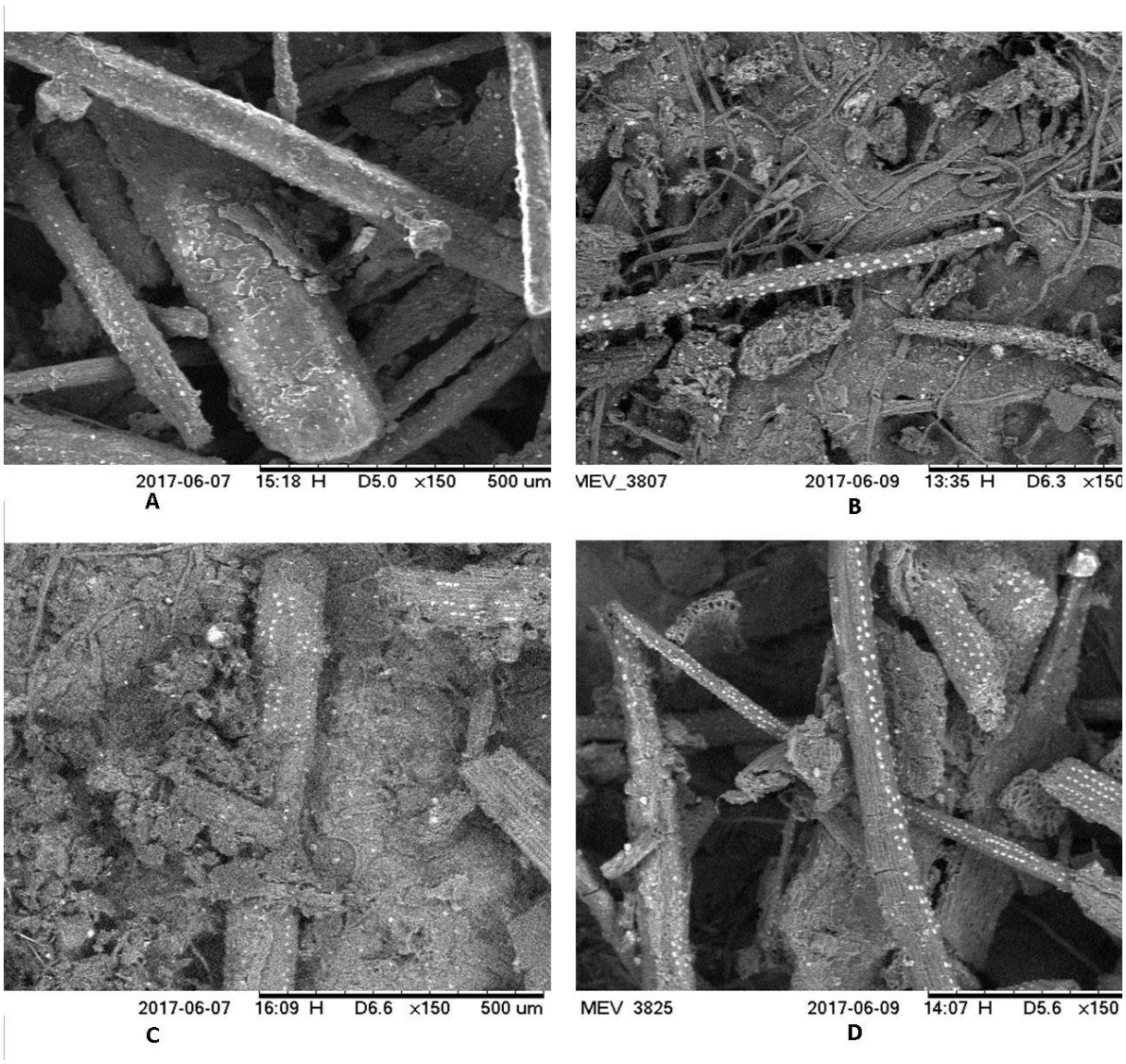

Figure 4.3.4. SEM images of type 2 EFB fibers before SSF: control (without lipids) (A) and with lipids (B). Fibers after 72 hours of SSF: control (without lipids) (C) and with lipids (D).

The images show no difference between the fibers with and without lipids before cultivation. The white dots present in the EFB fibers are silica bodies, which are generally found in EFBs. They are usually circular, uniform, and are present on the surface of the fibers [26]. After SSF, pores appeared on the fibers indicating consumption of the lignocellulosic compounds (Figures 4.3.3D and 4.3.4C). These images are similar to those obtained by Motta and Santana [13].

3.3 AH Recovery

The recovery of HA obtained from the SSF for the two EFBs varied around 50 to 60% as shown in the Table 4.3.5.

Table 4.3.5. AH recovery produced in SSF

EFB (better SSF condition)	Recovery HA (%)
Type 1 EFB with lipid 72 hours	63.11
Type 1 EFB without lipid 48 hours	60.82
Type 2 EFB with lipid 72 hours	49.35
Type 2 EFB without lipid 48 hours	58.75

For type 1 EFB, no great difference in HA recovery was obtained for the fibers with or without lipids. For type 2 EFB, the highest HA recovery was for the fibers without lipids. The difference in HA recovery was mainly due to the lower HA production in type 2 EFB fibers.

4 Conclusion

Raw fibers from palm oil EFBs are efficient substrates for the production of HA by *Trichoderma reesei* cultivation in SSF. The main importance of this finding is in making the production of HA even cheaper, by not requiring pre-treatment of the fibers for greater adhesion of microorganisms or absorption of water, as in other processes. Moreover, the use of fibers in SSF adds value to the high volume of EFB wasted from palm oil processing.

The removal process of palm oil fresh fruits from the bunches plays an important role in the composition of the residual lipids in the raw fibers. Thus, the presence of palmitic acid, carotenoids, lower free fatty acids, and lower oxidation in type 1 EFB benefited HA production compared with lipids in type 2 EFB.

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5 CONCLUSÕES

Assim, nas condições experimentais, conclui-se que:

- As fibras dos EFBs utilizadas neste estudo têm potencial de crescimento e produção de AH por *Trichoderma reseei*, tanto na presença como na ausência de lipídios.
- O conteúdo de celulose (31%), hemicelulose (19%) e lignina (24%) do EFB de tipo 1 foi maior do que o EFB tipo 2 (17% de celulose, 15% de hemicelulose e 13% de liginina).
- Os EFBs também apresentaram níveis diferentes de lipídios remanescentes, de 38,4 e 6,87% para a EFB tipo 2 e EFB tipo 1, respectivamente. Estes lipídios apresentaram características físicas diferentes, diretamente relacionadas às composições de ácidos graxos. Os lipídios extraídos do EFB tipo 2 foram sólidos à temperatura ambiente devido ao alto teor de ácidos graxos saturados e sua composição lipídica foi similar ao óleo de palma, enquanto os lipídios do EFB tipo 1 permaneceram líquidos devido ao equilíbrio entre os ácidos graxos saturados e insaturados, tendo sua composição lipídica de frações intermediárias, tanto do óleo de palma como do óleo de palmiste. Observou-se também que o conteúdo lipídico extraído do EFB tipo 1 apresentou coloração avermelhada, que está relacionada à presença de carotenóides no processo de obtenção de óleo de palma refinado, evidenciando que essa amostra se encontra com menor grau de oxidação.
- Foi observado que o cultivo em estado sólido foi mais adequado que o cultivo submerso em relação à obtenção de AH e crescimento fúngico, para ambos os EFBs. No cultivo submerso, a obtenção de AH foi melhor em EFBs controle (na ausência de lipídios), com uma produção de 70 mg $.L^{-1}$ no EFB tipo 2 e 90 mg $.L^{-1}$ no EFB tipo 1 devido ao alto teor de lingocelulose. Já para o cultivo em estado sólido, a obteve-se 110 mg .100 g^{-1} de EFB no controle (ausência de lipídio) para o EFB tipo 2 e 350 mg .100 g^{-1} de EFB na presença de lipídio para o EFB tipo 1.
- Fica evidente que para o EFB tipo 2, seria melhor um cultivo na ausência de lipídios levando em consideração a obtenção de AH, mesmo o lipídio tendo beneficiado o crescimento. Já para o EFB tipo 1, seria melhor o cultivo com lipídios, tomando por base as concentrações de AH obtido e também o crescimento celular, além do que há uma melhor relação custo/benefício do processo, evitando gastos com a extração dos lipídios.
- Além disso, as características dos lipídios presentes no EFB tipo 1, ajudam na

produção de AH, por serem líquidos à temperatura ambiente, possuírem maiores quantidades de compostos minoritários, e terem frações tanto do óleo de palma como do óleo de palmiste.

6 SUGESTÕES PARA TRABALHOS FUTUROS

– Realizar um escalonamento e planejamento experimental do processo tanto para fermentação em estado sólido como para submersa, para tentar otimizar as produções de AH;

– Realizar uma análise de todos as substâncias húmicas produzidas e quantificar também os ácidos fúlvicos e huminas;

– Aumentar o tempo de fermentação para 120 horas.

– Caracterizar os grupos químicos presentes nos AH produzidos nas várias fermentações, após purificação com protocolo do International Humic Substance Society (IHSS) - Natural Organic Matter Research.

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8 APÊNDICE A

Resultados exploratórios dos cultivos submersos de *Trichoderma reesei* em EFB

Nos primeiros resultados de cultivo submerso, foram utilizados as metodologias 1 e 2 descritas abaixo para determinação de proteína e AH respectivamente.

1 Concentração celular (Metodologia 1)

Nas fermentações submersas, a concentração celular foi medida por método indireto através da análise do conteúdo de proteína. Amostras do líquido fermentado foram filtradas em papel filtro, e o filtrado foi submetido à análise de proteínas pelo kit comercial Pierce BCA (Thermo Scientific, USA), o qual emprega o método de LOWRY et al. (1951). Foram adicionados 2.0 mL do reagente padrão a 0,1 mL de amostra e a mistura incubada a 37 °C por 30 minutos. Após resfriamento até temperatura ambiente, foi feita leitura da absorbância a 562 nm.

9 Quantificação dos AH (Metodologia 1)

A quantificação de AH para fermentação submersa foi feita por densidade ótica, em que amostras em triplicata foram filtradas com filtro de café, a fração sobrenadante (0,2 ml) foi diluída cinco vezes com solução de NaOH 0.5 M e depois diluída dez vezes em água pH 4,5 e então a absorbância foi medida a 350 nm (A350). A concentração dos AH é determinada utilizando curva padrão previamente construída.

Porém, foram detectados problemas de transferência de massa do AH e da proteína para o sobrenadante, o que sugeriu algumas modificações nas análises pós fermentativas.

As Figuras 1.1 e 1.2 mostram os perfis de proteínas e AH durante o cultivo de *Trichomerma reesei* no EFB tipo 1.

Figura 1.1. Concentração de Proteína Celular da fermentação do EFB tipo 1, (○) sem e (□) com lipídios

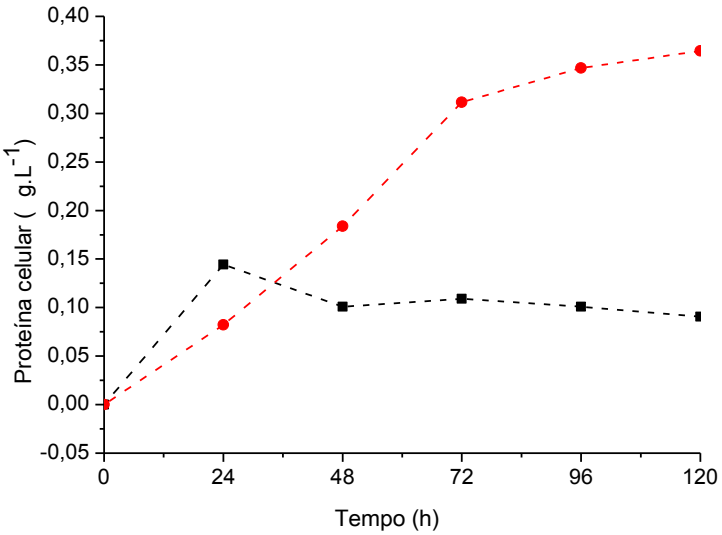
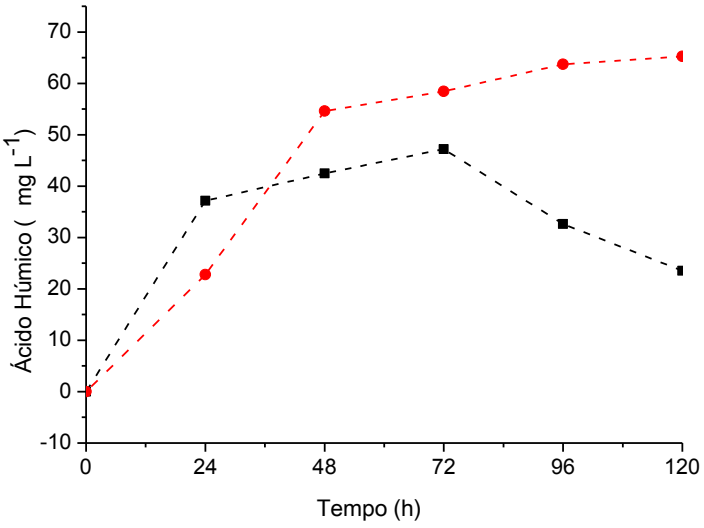


Figura 1.2. Concentração de AH da fermentação do EFB tipo 1 (○) sem e (□) com lipídios



Ao comparar a concentração de proteínas do EFB do tipo 1 (Figura 1.1), observa-se que o crescimento do fungo ocorreu até 120 horas na ausência de lipídios, ao passo que o crescimento na presença de lipídios não ocorre após 24 horas. Alguns fatores podem contribuir para estes resultados, tais como a dificuldade de aderência dos fungos nas fibras e limitação de transferência de massa de proteínas e AH para o sobrenadante durante as

análises. De acordo com a Figura 1.2 a concentração de AH após 72 horas diminuiu na presença de lipídios, o que não ocorreu para EFB sem lipídios.

As figuras 1.3 e 1.4 mostram os perfis de proteínas e AH obtidas da fermentação do EFB tipo 2 , na presença e ausência de lipídios.

Figura 1.3. Concentração de Proteína Celular da fermentação do EFB tipo 2 (○) sem e (□) com lipídios

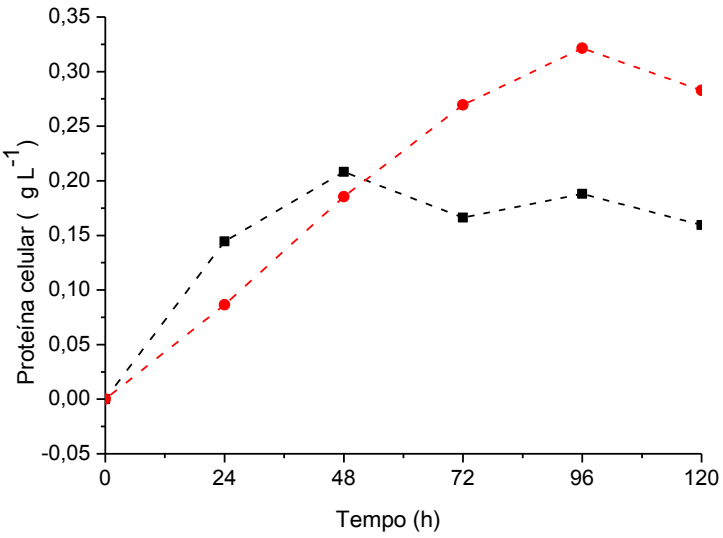
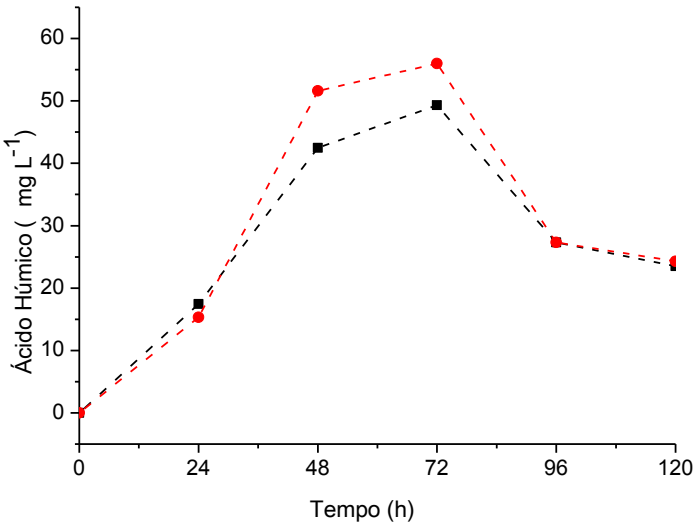


Figura 1.4. Concentração de AH da fermentação do EFB tipo 2 (○) sem e (□) com lipídios



Os perfis de crescimento mostraram a tendência para o EFB com lipídios atingir um patamar mais cedo, enquanto que para EFB sem lipídios continua a crescer ao longo do tempo (Figura 1.3). A tendência de concentração de AH foi semelhante para ambas as condições, porém havendo queda dos AHs no final da fermentação (Figura 1.4).

Nesses resultados foi detectado um problema de metodologia de análise das fermentações (metodologia 1), uma vez que o lipídio presente no EFB poderia estar impedindo que os AHs produzidos fossem para o sobrenadante, assim como a proteína, já que na maioria das condições com lipídio, há quedas do produto na fase final. Por este motivo, nos experimentos posteriores, que são os que estão nos artigos, as amostras foram centrifugadas antes das análises, para garantir que todos os metabólitos fossem para o sobrenadante e fossem quantificados. Dessa maneira o perfil de crescimento e produção de AH aumentou significativamente do que se tinha nos resultados preliminares, passando de concentrações de 0.4 g L^{-1} de proteína para 0.85 g L^{-1} e de 65 mg L^{-1} de AH para aproximadamente 90 mg L^{-1}, para o EFB tipo 1. Já para o EFB tipo 2 passou de 0.33 g L^{-1} de proteína para 1.3 g L^{-1} e de 55 mg L^{-1} de AH para 70 mg L^{-1}.

9 APÊNDICE B

EFFECTS OF THE LIPID CONTENT IN EMPTY FRUIT BUNCH ON THE HUMIC ACIDS PRODUCTION BY *Trichoderma reesei*

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ABSTRACT

Humic acids are substances derived from the organic matter degradation, and they are found only in natural and non-renewable sources. Many different fields utilize these acids, such as agriculture, medical areas, due to its anti-inflammatory, antibacterial, estrogenic and therapeutic properties. Empty fruit bunch (EFB) are residues from the palm oil processing, rich in lignin, cellulose, hemicellulose and reminiscing lipids from the oil extraction process. Considering previous studies about humic acids production from the EFB fermentation like a renewable source, this current work aims to study the effects of the lipids content on production of humic acids from the submerse EFB fermentation utilizing a Trichoderma strain. The fibers from EFB were obtained from two different industrial sources , and the fermentations were carried out in the presence and absence of lipids. Results show that the fiber of the both sources, with and without lipids, is potentially important to produce humic acids.

1. INTRODUCTION

Humic substances (HS) are natural complexes, components of a large portion of organic matter, since they are present in the soil, peat and sewage. These substances are divided into three main components: fulvic acids, humic acids (HA) and humins (Baldotto et al., 2014).Humic acids are formed during the decomposition of organic matter. Due to this fact, they can be found in all natural environments in which there is the presence of organic materials or microorganisms (BALDOTTO; ESTRELA; BALDOTTO, 2014).

Fermentation of agroindustrial residues has been studied as an alternative way to obtain these kinds of products, especially humic acids. The production of HA by fungi of the *Trichoderma* species from residue from palm oil production, called Empty Fruit Bunch (EFB), was studied by Motta & Santana (2014).In this study, researchers verified the potential of *Trichoderma reesei* in humic acids production.

The EFB byproduct derived from the palm oil extraction is a lignocellulosic residue because it does not have a defined destination (Bocchi, 2008). This generated residue presents great potential of use for the humic acids production. In addition, EFB often have large amounts of residual lipids that could be important for the future use of the industrial waste (MOTTA; SANTANA, 2013).

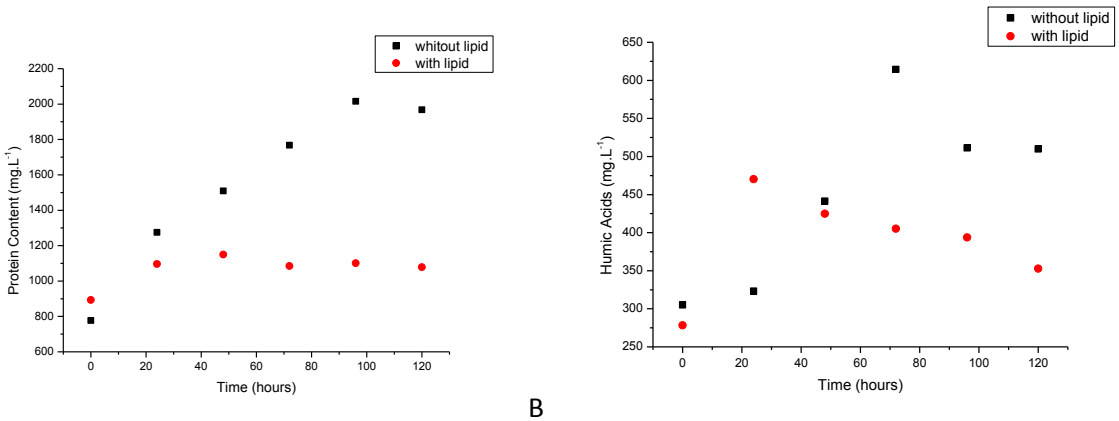
Since the studies in this field are scarce, this work demonstrates the influence of fiber industrial sources and the presence of lipids for the production of humic acids.

2. MATERIAL AND METHODS

The mesocarp fibers from EFB were kindly provided by the brazilian industries: Agropalma S. A. (Limeira city -São Paulo) and the small industries cooperative producing crude oil palm (Muniz Ferreira city- Sergipe).The microorganisms utilized in the fermentative process are *Trichoderma reesei* from CCT (*Coleção de Culturas Tropicais*), Campinas, São Paulo – Brazil. The EFB samples were stored at 5°C and, in triplicate, they were characterized by moisture contents according to AACCI 44-15.02 (AACCI, 2010). The particles were selected in the size range between 710 and 355 µm, characterized in terms of cellulose, hemicellulose and lignin. The degreased particles underwent solvent extraction in a Butt extractor using petroleum ether according to AM 2-93 (AOCS, 2009). Submerged fermentation assays were performed in 120 rpm orbital shaker in 125 mL Erlenmeyer flasks containing 25 mL culture medium, in which 2.77 mL of inoculum were added (MOTTA; SANTANA, 2013). During the assays, cell concentration was monitored every 24 hours via protein and humic acids analysis.

3.RESULTS AND DISCUSSION

The moisture content obtained from EFB samples from Sergipe industries was 18.94%, while the moisture content of Agropalma's sample was 11.45%. The amount of lipids remaining in Sergipe's fiber was 38.4% and, Agropalma's 6.87%. The Figure 1 shows profiles of protein and humic acids by *Trichomerma* from EFB Agropalma source.

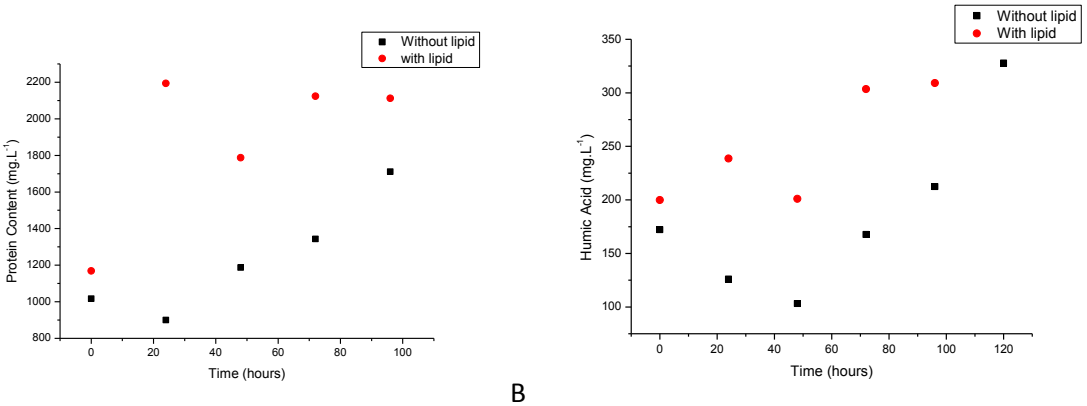


A B

Figure 1. Concentration of Algropalma's protein EFB (A) and Agropalma's humic Acids EFB (B) (○) with lipids and (□) without lipids

When comparing the protein concentration from Agropalma's EFB (Figure 1A), it is noted that the fungus growth increased up to 96 hours in the absence of lipids, while the growth in the presence of lipids is nearly constant after 24 hours. Some factors may contribute to these results, such as the difficulty of adherence of the fungus in the fiber, the problem of mass transfer of proteins and humic acids to the supernatant for analysis. According to Figure 1 (B) the concentration of humic acids after 48 hours decreased in the presence of lipids which did not occured for EFB without lipids. These results should be investigated in later studies.

Figure 2 shows the performance of EFB fermentation from Sergipe source. Growth profiles showed the tendency for EFB with lipids to reach a plateau earlier, while EFB without lipids continues to grow along time (Figure 2A). The trend of humic acids concentration was similar for both EFBs, reaching 200 mg L^{-1} in the presence of lipids and 225 mg L^{-1} without lipids (Figure 2B).



A B

Figure 2. Protein concentration (A) and Humic Acid concentration (B) of the EFB fermentation from Sergipe, (○) with lipids (□) and without

4.CONCLUSION

The results showed that both sources of EFB are promising for the production of humic acids both in the presence and absence of lipids, even with different fungus growth. These results are important for further metabolic studies as function of lignocellulosic composition in the fibers, as well the lipids composition from the sources.

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