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ANDRÉIA ANSCHAU

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Produção de lipídeos por Lipomyces starkeyi: estratégia para obtenção de alta densidade celular a partir de xilose e glicose

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Supervisor/*Orientadora*: Prof. Dr. Telma Teixeira Franco

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RESUMO

Neste trabalho foram desenvolvidos estudos visando o estabelecimento de um processo de produção de lipídeos microbianos a partir de fontes renováveis, particularmente xilose, carboidrato derivado do processo de hidrólise de bagaço de cana-de-açúcar. Foi utilizada a levedura oleaginosa *Lipomyces starkeyi* DSM 70296, previamente selecionada no Laboratório de Engenharia Bioquímica, Biorefino e Produtos de Origem Renovável (LEBBPOR). A partir dos resultados preliminares em frascos agitados, partiu-se para estudos de batelada alimentada em biorreator (1,3 a 3L). Foram estudadas diferentes estratégias de alimentação, sendo que em batelada alimentada repetida, foram encontradas as maiores concentrações de células (85,4 g/L) e de lipídeos (41,8 g/L). Posteriormente foram estudados modos de operação em processos contínuos em meio sintético e meio contento o hidrolisado hemicelulósico (H-H). As maiores produtividades de células (0,443 g/g) e de lipídeos (0,236 g/g) foram encontradas em cultivo contínuo a $0,03h^{-1}$. Na vazão específica de alimentação de 0,06 h^{-1} foram obtidas as maiores produtividades de células (0,600 g/L.h) e de lipídeos (0.288 g/L.h). Análises de cromatografia em fase gasosa dos diferentes cultivos feitos revelaram que os principais constituintes deste complexo são os ácidos graxos de cadeia longa, como o ácido palmítico (C16:0), ácido esteárico (C18:0), ácido oleico (C18:1) e ácido linoleico. Foi estimado o número de cetano em torno de 61, muito próximo do biodiesel de palma. Também foram feitos estudos de balanço de massa e de energia em cultivo batelada alimentada utilizando somente xilose como fonte de carbono. O valor de calor de combustão (Q_c) de 25,7 kJ/g obtido após 142 h de cultivo representa aproximadamente 56% do conteúdo energético do óleo diesel (45,4 kJ/g), indicando o potencial da *L. starkeyi* para biodiesel. Cultivos contínuos subsequentes foram feitos para a compreensão do processo de acúmulo de lipídeos, utilizando a ferramenta estatística de reconciliação de dados para melhorar os dados experimentais obtidos em quimiostato, reduzindo os erros experimentais para posterior cálculo de análise de fluxos metabólicos (MFA). Nesse sentido, os lipídeos produzidos por *L. starkeyi* apresentam relevante importância do ponto de vista acadêmico e industrial, podendo ser utilizados como matéria-prima para biodiesel e indústria oleoquímica.

Palavras-chave: Lipídeos microbianos, biocombustível, hidrolisado hemicelulósico, estratégias de alimentação, balanço de massa e energia, reconciliação de dados de células.

ABSTRACT

Studies attempting the establishment of a microbial lipid production process from renewable resources, mainly xylose, were developed. This pentose, obtained from sugar cane bagasse hydrolysis. The oleaginous yeast *Lipomyces starkeyi* DSM 70296, previously selected at the Laboratory of Biochemical Engineering, Biorefining and Products from Renewable Sources (LEBBPOR), was used throughout this thesis. After preliminary studies in shake flasks, we started fed-batch studies in fermentor (1.3 to 3L). Among the strategies studied, the highest cell mass and lipid concentrations reached up to 85.4 and 41.8 g/L, respectively, when repeated fed−batch strategy was applied. Subsequently, continuous processes were studied in synthetic medium and media containing hemicellulosic hydrolysate (H-H). The highest overall cell mass (0.443 g/g) and lipid yields (0.236 g/g) were achieved at dilution rate of 0.03 h^{-1}. At dilution rate of 0.06 h^{-1}, were obtained the highest productivities of cell mass (0.600 g/L.h) and lipids (0.288 g/L.h). Gas chromatography of esterified lipids revealed that the major constituents of this complex are long-chain fatty acids, such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) with an estimated cetane (around 61) very close to the palm biodiesel. Also have been studies of mass and energy balances from fed-batch cultivation using xylose as sole carbon source. The combustion heat (Q_c) value 25.7 (kJ/g) obtained after 142 h of fed-batch cultivation, represents approximately 56% of the energy content of diesel oil (45.4 kJ/g), indicating the potential of *L. starkeyi* for biodiesel. Continuous cultures were made subsequently to understanding the process of lipid accumulation using a statistical tool for data reconciliation was used to improve the experimental data obtained in chemostat culture reducing the experimental errors for subsequent calculation of metabolic flux analysis (MFA). In this sense, lipids produced by *L. starkeyi* have relevant importance of academic and industrial point of view, as feedstock for biodiesel and oleochemical industry applications.

Keywords: Microbial lipids, biofuel, hemicellulose hydrolysate, feeding strategies, mass and energy balances, cell mass data reconciliation.

SUMMARY

Aos meus pais

Obrigada pela torcida e pelo apoio incondicional.

Ao Felipe

Sem você tudo seria mais difícil. Você é muito importante para mim!

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NOMENCLATURA

Abreviatura

AG	Ácido graxo
ACC	ATP citrato carboxilase (ATP citrate carboxilase)
ACL	ATP citrato liase (ATP citrate lyase)
AMP	Adenosina monofosfato (adenosine monophosphate)
ATP	Adenosina trifosfato (adenosine triphosphate)
CDW	Células em peso seco (cell dry weigth)
C/N	Razão carbono/nitrogênio
CO_2	Dióxido de carbono
D	Vazão específica de alimentação
DAG	Diacilglicerol (diacylglycerol)
DGA	Acil-CoA:diacilglicerol (Acyl-CoA:diacylglycerol
DGAT	DGA aciltransferase (DGA acyltransferase)
DHA	Ácido docosahexanóico
DHAP	Dihidroxiacetona fosfato (dihydroxyacetone phosphate)
DNA	Ácido desoxirribonucleico (deoxyribonucleic acid)
DO	Oxigênio dissolvido (Dissolved oxigen)
FAME	Methil éstere de ácido graxo (fatty acid methyl ester)
FAS	Ácido graxo sintetase (fatty acid synthase)
FTIR	Espectroscopia Infravermelho por Transformada de Fourier
G3P	Glicerol-3-fosfato (glycerol-3-phosphate)
GPAT	G3P aciltrasnferase (G3P acyltransferase)
GPD	G3P desidrogenase (G3P dehydrogenase)
H-H	Hidrolisado hemicelulósico (hemicellulosic hydrolizate)
HMF	Hidroximetilfurfural (hydroxymethylfurfural)
IMP	Inosina monofosfato (inosine monophosphate)
LPA	Ácido lisofosfatídico (lysophosphatidic acid)
MAG	Monoacilglycerol
MFA	Análise de fluxos metabólicos (metabolic flux analysis)
ME	Enzima málica (malic enzyme)

MPL	Matérias-primas lignocelulósicas
$NADP^+$	Nicotinamida adenina dinucleotídeo fosfato (Nicotinamide adenine dinucleotide phosphate)
NO_X	Óxido de nitrogênio
PA	Ácido fosfatídico (phosphatidic acid)
PAP	Fosfatidato fosfatase (phosphatidic acid phosphatase)
PDAT	Fosfolipídeo:diacilglicerol aciltransferase (phospholipid:diacylglycerol acyltransferase)
PUFA's	Ácidos graxos poli-insaturados (Polyunsaturated fatty acids)
RNA	Ácido ribonucleico (ribonucleic acid)
SCO	Single cell oil
TAG	Triacilglicerol (triacylglycerol)
TAC	Ciclo do Ácido Tricarboxílico (Tricarboxilic Acid cycle)
VVM	Vazão por volume por minuto (volume per volume per minute)
YE	Extrato de levedura (yeast extract)

Capítulo 1. Introdução

"Cada pessoa deve trabalhar para o seu aperfeiçoamento e, ao mesmo tempo, participar da responsabilidade coletiva por toda a humanidade".

Marie Curie

1.1 Escopo

A capacidade de alguns microrganismos em produzir quantidades significativas de lipídeos, chamados de *Single Cell Oil* (SCO), já é conhecida e vem recebendo grande atenção da comunidade científica. Lipídeos produzidos por fermentação possuem características de alto valor como ácidos graxos poli-insaturados e fosfolipídeos. A produção de óleo microbiano torna-se atraente para diferentes aplicações, desde a alimentação (propriedades nutracêuticas de ácidos graxos insaturados), até a produção de biodiesel e outros componentes da indústria oleoquímica.

A presente Tese faz parte de um projeto em parceria entre UNICAMP e Shell Brasil Petróleo Ltda. Os primeiros estudos estão relatados na Dissertação de Mestrado de Camilo Sixto López Garzón (2009) onde inicialmente foram testadas 25 leveduras oleaginosas quanto à capacidade de assimilação de xilose e produção de lipídeos, sendo selecionada a *Lipomyces starkeyi* DSM 70296. Posteriormente foram definidas as relações C/N 50 e utilização de sulfato de amônio e extrato de levedura como fontes de nitrogênio. O modelo de Mulchandani representou adequadamente a cinética de inibição. Um modelo não estruturado do cultivo em batelada simples foi desenvolvido baseado nos dados cinéticos obtidos, o qual foi validado para cultivo com limitação de nitrogênio em biorreator de dois litros.

Na Dissertação de Mestrado de Eulália Vargas Tapia (2012) foram desenvolvidos estudos visando o melhoramento genético da levedura *L. starkeyi* DSM 70296, por meio de mutagênese aleatória de DNA por irradiação ultravioleta. Os mutantes foram selecionados com o uso da cerulenina agente interferente ao metabolismo de interesse, de forma que fossem identificados os mutantes cujas alterações genéticas pudessem estar promovendo efeitos sobre este metabolismo. Após a seleção do mutante, foram feitos cultivos em batelada alimentada com xilose e glicose, nas condições otimizadas na presente Tese de Doutorado. Foram obtidas maiores concentrações celulares e de lipídeos na cepa mutante quando comparada à cepa padrão.

Na Dissertação de Mestrado de Ruth Verónica Sierra Aristizábal (2013) foi estudada a obtenção do hidrolisado hemicelulósico (H-H) após sete etapas sequenciais de extração de bagaço previamente explodido a vapor. Após, foi feita a adaptação da *Lipomyces starkeyi* em meio contendo H-H por engenharia evolutiva. Outra Tese de Doutorado em andamento, da aluna Michelle da Cunha Abreu Xavier, está sendo desenvolvida com o intuito de otimizar a extração de H-H para obter maiores concentrações de xilose e glicose com baixas concentrações de

inibidores. Cultivos em biorreator serão feitos nas melhores condições obtidas em meio sintético da presente Tese de Doutorado.

Na Dissertação de Mestrado em andamento de Alessandro Luis Venega Coradini foi feito estudo transcriptômico de genes relevantes ao acúmulo de lipídeos pela *L. starkeyi*, identificando os que possam servir como alvos de futuras modificações genéticas e metabólicas. Para isso foram utilizadas técnicas de transformação para deleção do gene URA3, presente no genoma da levedura (sequenciado e analisado com auxílio da Dr[a] Ana Carolina Deckmann e do Dr. Marcelo Carazzolle, LGE-IB/UNICAMP), de forma a obter um mutante auxotrófico para a uracila. Nenhuma das técnicas de transformação produziu transformantes URA3. Verificou-se, ainda, que a levedura parece não possui mecanismos responsáveis para replicação e expressão do DNA plasmidial, não sendo possível a construção do plasmídeo modificado YEp352. Por fim, foi feita análise da expressão de genes de interesse, por RT-PCR, visando complementar as informações obtidas pela análise do genoma e selecionar possíveis alvos de transformação genética.

Já a Dissertação de Mestrado de Érika Marques Reis (2013) relata a transesterificação convencional e *in situ* da massa celular de *L. starkeyi*, obtida dos cultivos na presente Tese de Doutorado, por rota etílica na presença de catalisadores (NaOH e H_2SO_4) para obtenção de biodiesel. Foram estudados os efeitos de solventes (hexano, clorofórmio, metanol e etanol) na extração de lipídeos por Soxlet e Butt. Verificou-se que a eficiência da extração está relacionada diretamente com a polaridade do solvente. Também foi feita a caracterização do lipídeo por métodos teóricos e experimentais.

Nesse contexto, a presente Tese de Doutorado possui sete capítulos além do Anexo que apresenta os estudos preliminares feitos em frascos agitados. Sendo o Capítulo 1 a presente introdução, os demais capítulos estão estruturados da seguinte forma:

Capítulo 2 fornece uma ampla revisão da literatura sobre o uso de materiais lignocelulósicos como energias alternativas para a produção de lipídeos microbianos e aspectos que influenciam o mecanismo de acúmulo de lipídeos, desenvolvendo assim a lógica da tese e destacando sua inovação e importância.

Capítulo 3 relata cultivos em batelada alimentada em biorreator que serviram de base para o capítulo seguinte.

Capítulo 4 sumariza os principais resultados obtidos em batelada, batelada alimentada, batelada alimentada repetida e cultivo contínuo em biorreator. Este estudo foi publicado no periódico *Bioresource Technology* (2014).

Capítulo 5 apresenta os estudos de balanço de massa e de energia feitos a partir de resultados em batelada alimentada utilizando xilose como única fonte de carbono, estimando-se assim, o rendimento energético e calor de combustão das células. Este estudo está sob revisão para porterior submissão a periódico.

Capítulo 6 estuda a compreensão do processo de acúmulo de lipídeos através de cultivos em quimiostato, utilizando a ferramenta estatística de reconciliação de dados para melhorar os dados experimentais obtidos e melhor adaptá-los aos balanços de massa, reduzindo os erros experimentais para posterior cálculo de análise de fluxos metabólicos (MFA). Este estudo foi feito na Universidade Autónoma de Barcelona sob orientação do Prof. Dr. Pau Ferrer durante período sanduíche e estão sob revisão para porterior submissão a periódico.

Capítulo 7 resume as conclusões dos capítulos 3 a 6 e dá uma visão para futuras aplicações dos resultados obtidos. Além disso, os desafios são destacados e possíveis soluções discutidas antes de tirar uma conclusão geral do trabalho realizado no âmbito desta Tese.

Anexo aborda estudos preliminares feitos em frascos agitados, utilizando planejamentos experimentais para definição de alguns parâmetros. Os resultados desses estudos serviram de base para os estudos posteriores em biorreator.

1.2. Objetivo geral

Avaliar o potencial de obtenção de lipídeos por *Lipomyces starkeyi* DSM 70296 a partir de xilose e glicose, que estarão disponíveis através da hidrólise de materiais lignocelulósicos, como bagaço de cana-de-açúcar.

1.2.1. Objetivos específicos

Nas linhas seguintes estão discriminados os objetivos específicos da Tese que serão explicados de forma mais detalhada nos capítulos seguintes:

- Avaliar a produção de lipídeos e o crescimento celular por batelada alimentada utilizando diferentes estratégias de alimentação;

- Avaliar a produção de lipídeos e o crescimento celular por cultivos contínuos a diferentes vazões específicas de alimentação;
- Identificar a composição de ácidos graxos ao longo dos diferentes cultivos estudados;
- Analisar balanços de massa e de energia para o cultivo de *L. starkeyi*;
- Avaliar a composição macromolecular e o balanço de metabólicos extracelulares de *L. starkeyi* em cultivos contínuos para posterior análise de fluxos metabólicos (MFA).

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Capítulo 2. Revisão da Literatura

"A criação de algo novo é consumado pelo intelecto, despertado pelo instinto de uma necessidade pessoal. A mente criativa age sobre algo que ela ama."

Carl Jung

2.1. Perspectivas energéticas e necessidade de energias alternativas

O petróleo é a maior fonte de energia consumida mundialmente, superando o carvão, gás natural, nuclear, hidrelétrica e energias renováveis. O intenso consumo de energia está rapidamente esgotando o petróleo bruto global, aumentando a concentração de gases de efeito estufa em nosso meio e dificultando a reciclagem de resíduos. No entanto, havendo forte correlação entre o consumo de energia, o nível de vida e o crescimento da população mundial, espera-se que aumente ainda mais a demanda por petróleo bruto e de produtos à base de petróleo (HIRSCH, 2005).

A necessidade de fornecer bens, utilizando recursos renováveis, combinados com os avanços da ciência e da tecnologia, tem proporcionado um ambiente receptivo para o desenvolvimento de sistemas de biorrefinarias sustentáveis. Biorrefinarias oferecem a promessa de usar menos recursos naturais não renováveis, reduzindo as emissões de CO_2, criando novos empregos e estimulando a inovação, utilizando tecnologias limpas e eficientes (WELLISCH *et al.*, 2010). As preocupações com a segurança energética, alterações climáticas e aumento dos preços do petróleo estão impulsionando políticos e cientistas para as alternativas energéticas que permitam romper dependência do petróleo fóssil. Combustíveis derivados de recursos renováveis são uma das melhores opções para liderar a transição dos combustíveis de petróleo à curto prazo.

Biodiesel é o combustível derivado de óleos vegetais ou de gordura animal que pode substituir total ou parcialmente o óleo diesel derivado de petróleo (SANTACESARIA *et al.*, 2012). Pode ser obtido por diferentes processos, como craqueamento, transesterificação ou esterificação, tendo glicerina como subproduto. Além de ser obtido de fontes renováveis, tem como vantagens a menor emissão de gases poluentes e a menor persistência no solo. Entretanto, o custo de produção atualmente é maior e o balanço energético, apesar de variar com o sistema de produção utilizado, é menos favorável em relação ao óleo diesel (DA SILVA e DE FREITAS, 2008).

2.2. Matérias-primas para o Biodiesel

No Brasil, o biodiesel é produzido principalmente a partir de óleo de soja (BUNYAKIAT *et al.*, 2006), óleo de palma (AL-WIDYAN e AL-SHYOUKH, 2002), óleo de girassol (ANTOLIN *et al.*, 2002), óleo de canola (PETERSON *et al.*, 1996), óleo de pinhão manso (HAWASH *et al.*, 2009) e óleo residual de restaurantes (DEMIRBAS, 2009).

Óleos e gorduras são compostos principalmente de triacilgliceróis (TAG), três moléculas de ácidos graxos (AG) ligadas a uma molécula de glicerol por ligações éster. Estes podem conter menor quantidade de diacilgliceróis (DAG) e também monoacilglicerol (MAG). Os AG do biodiesel podem apresentar diferentes comprimentos de cadeia. AG de cadeia longa resultam em biodiesel com alto número de cetano e reduzida emissão de óxidos de nitrogênio (NOx) no escapamento de motores (LANG *et al.*, 2001; ANTOLIN *et al.*, 2002; VICENTE, MARTINEZ e ARACIL, 2004). Resíduos de óleos e gorduras alimentares também podem ser usados como matérias-primas, mas estes podem conter grandes quantidades de AG livres e requerem processamento adicional (DEMIRBAS, 2003).

A rápida expansão da produção de biodiesel estendeu o potencial de recursos potenciais que podem ser utilizados como matérias-primas. É crescente a preocupação sobre o impacto do aumento dos preços das *commodities* no sistema alimentar global (COYLE, 2007). O governo chinês identificou várias matérias-primas potenciais (não grãos) como mandioca e batata doce para a produção de biocombustíveis a fim de evitar a alta nos preços de alimentos. O governo da Indonésia aumentou o imposto de exportação de óleo de palma bruto, também usado na produção de biodiesel, em meados de 2007, para desacelerar o aumento do custo de óleo comestível (WISE, 2007). Medidas como estas têm diminuído os preços, mas aumentaram os debates de alimentos *versus* biodiesel.

Biodiesel derivado de oleaginosas ou gordura animal pode suprir apenas uma pequena fração da demanda existente para combustíveis de transporte, sem comprometer excessivamente grandes áreas cultivadas de terras agrícolas de qualidade para o cultivo de oleaginosas, alvo da produção de alimentos (CHISTI, 2007). Portanto, é necessário explorar matérias-primas que: (1) proporcionem benefícios ambientais superiores sobre os materiais fósseis que irão substituir, (2) sejam economicamente competitivos, (3) possam ser produzidos em quantidades suficientes para satisfazer as exigências energéticas, (4) proporcionem ganho líquido de energia sobre as fontes de energia utilizadas para produzi-lo, e (5) também não concorram com a produção de alimentos (MENG *et al.*, 2009).

2.3. Produção microbiana de lipídeos

Lipídeos microbianos se referem a óleos e/ou gorduras produzidos por fermentação a partir de algas, fungos filamentosos e leveduras. Os TAG são os principais componentes lipídicos

encontrados nestes microrganismos (RATLEDGE, 1991), compostos majoritariamente por AG de cadeias de 14 a 20 carbonos (ZHAO *et al.*, 2008) de grande interesse industrial.

O termo *Single-cell oil* (SCO) foi adotado pela comunidade científica para descrever lipídeos, particularmente TAG, produzidos microbiologicamente. Nesta definição estão excluídas outras classes de lipídeos mais complexos como fosfolipídeos, glicolipídeos e carotenóides. Esse termo é utilizado em analogia ao termo *Single-cell protein* proposto para nomear as proteínas extraídas de microrganismos (bactérias, leveduras, fungos e algas) de resíduos agroindustriais. Assim, os SCO também são gerados a partir desses materiais (MORETON, 1987). SCO possuem estrutura similar à óleos vegetais e distribuição semelhante de AG nas três posições centrais do glicerol (RATLEDGE, 1993). Devido ao conteúdo de ácidos graxos poli-insaturados (PUFA's) produzidos, SCO também podem ser usados como nutracêuticos, farmacêuticos, suplementos alimentares e alimentação em aquicultura (RATLEDGE, 1993; LEWIS, NICHOLS and MCMEEKIN, 2000; SPOLAORE *et al.*, 2006).

A similaridade química de SCO produzidos por leveduras, em relação a óleos vegetais com potencial produção industrial de biodiesel, aumentou a exploração desses lipídeos nos últimos anos (XUE *et al.*, 2006; DAI *et al.*, 2007; LIU e ZHAO, 2007; ANGERBAUER *et al.*, 2008; PENG e CHEN, 2008; XIONG *et al.*, 2008; ZHAO *et al.*, 2008).

O alto custo de produção de SCO é a maior barreira para a comercialização desse produto, entretanto, para fins nutracêuticos (PUFA), a produção de SCO é economicamente viável. Assim como na maioria dos processos de produção de produtos químicos por fermentação, o maior impacto no custo de produção está relacionado com o custo da matéria-prima. Somente com a utilização de matérias-primas de baixo custo disponíveis em grandes volumes, a produção de SCO para fins industriais pode ser transformada em realidade (RATLEDGE, 2004).

Alguns microrganismos pertencentes às famílias de microalgas e fungos possuem a capacidade de produzir e acumular uma grande fracção de lipídeos em sua massa seca, sendo chamados de "oleaginosos" quando o teor de lipídeos ultrapassa os 20% (RATLEDGE and WYNN, 2002). O acúmulo de lipídeos é uma característica de desequilíbrio do metabolismo: quando todos os nutrientes estão presentes no meio de crescimento, ocorre a síntese de novas células, com níveis mínimos de lipídeos. Somente quando a célula esgotar, ou for deliberadamente privada de um nutriente essencial, começará a acumular lipídeos (RATLEDGE

e COHEN, 2008). Vários microrganismos com potencial para a produção microbiana de lipídeos estão listados na Tabela 2.1.

Tabela 2. 1 Conteúdo de lipídeos de alguns microrganismos oleaginosos (MENG *et al.*, 2009).

Microrganismo	Conteúdo lipídico (% peso seco)
Microalga	
Botryococcus braunii	25–75
Chlorella sp.	28–32
Nannochloropsis sp.	31–68
Schizochytrium sp.	55-70
Bactéria	
Arthrobacter sp.	>40
Rhodococcus opacus	24–25
Acinetobacter calcoaceticus	27–38
Bacillus alcalophilus	18–24
Levedura	
Candida curvata	58
Cryptococcus albidus	65
Lipomyces starkeyi	64
Rhodotorula glutinis	72
Fungo filamentoso	
Aspergillus oryzae	57
Mortierella isabellina	86
Humicola lanuginose	75
Mortierella vinacea	66

Para os microrganismos com maior capacidade de acúmulo de lipídeos, as condições ótimas são aquelas em que existe excesso de carbono e limitação de nitrogênio. Esta limitação garante que o crescimento celular cesse em algum ponto na fase exponencial de crescimento, devido à falta de nitrogênio. No entanto, as espécies oleaginosas têm propriedades metabólicas que permitem o consumo do substrato após o esgotamento da fonte de nitrogênio, o qual é

convertido em lipídeos e estes são armazenados como reservas de energia dentro da célula (RATLEDGE, 1991; RATLEDGE e COHEN, 2008). Outros nutrientes, como ferro, fósforo e oxigênio podem afetar a produção de óleos e gorduras, sendo este efeito dependente da espécie de levedura (GRANGER *et al.*, 1993; HASSAN *et al.*, 1996; ZHAO *et al.*, 2008).

A representação ideal do processo de acúmulo de lipídeos em microrganismos oleaginosos está apresentado na Figura 2.1. O meio deve ser formulado com elevada proporção C/N (cerca de 50:1) para assegurar que o nitrogênio se esgote e o carbono permaneça em excesso. A composição do meio de cultura é formulada de modo que o fornecimento de nitrogênio limite o crescimento. Após seu esgotamento, as células não se multiplicam mais, mas continuam a assimilar a glicose (matéria-prima usual de carbono). Este é então canalizado para a síntese de lipídeos de armazenamento dentro das células (RATLEDGE, 2005).

Na transição entre a fase de crescimento (crescimento com produção de massa celular) e a fase de acúmulo de lipídeos (diminuição na velocidade específica de crescimento, limitação de nutrientes e o desvio de carbono em excesso para o acúmulo de lipídeos), algumas vias são reprimidas (ácido nucléico e síntese de proteínas), ao passo que outras vias são induzidas (AG e síntese de TAG). Esta transição é induzida pela limitação de nitrogênio (Figura 2.1). Além disso, durante a fase de acúmulo de lipídeos, os precursores (acetil-CoA, a malonil-CoA e glicerol) e energia (ATP, NADPH) são necessários para a síntese de lipídeos (BEOPOULOS *et al.*, 2009).

Devido à imposição de limitação de nitrogênio, a velocidade específica de crescimento não é alta. Espera-se o consumo total de nitrogênio e o incremento na massa celular total após esse momento devido ao acúmulo de lipídeos (MORETON, 1987). Assim, é evidente que as células aumentam em tamanho e massa similarmente ao conceito de obesidade observada em animais. Para produzir maior número de células, a concentração de nitrogênio e de carbono pode ser aumentada, mantendo-os na mesma proporção, permitindo que a fase de crescimento equilibrado possa continuar até que a concentração máxima de células possa ser atingida em fermentador, antes do início da fase de acúmulo de lipídeos (RATLEDGE, 2005).

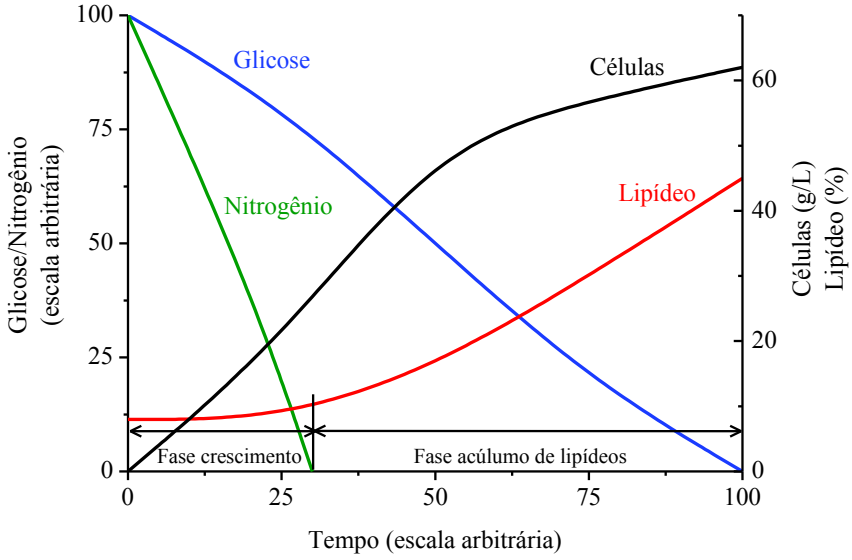

Figura 2. 1 Representação ideal para processo de acúmulo de lipídeos em microrganismos oleaginoso (RATLEDGE, 2005).

2.3.1. Leveduras oleaginosas

Leveduras oleaginosas são fungos unicelulares que possuem ao menos 20% do seu peso seco constituído de lipídeos (RATLEDGE e WYNN, 2002). Estes microrganismos podem crescer em em diversas de fontes de carbono (glicose, xilose, arabinose, manose, glicerol e outros resíduos agrícolas e industriais). Muitas leveduras podem acumular lipídeos em níveis superiores a 40% do seu peso seco e, chegando a 70% em condições limitantes de nutrientes (BEOPOULOS *et al.*, 2009). Porém, o conteúdo e o perfil de AG diferem entre as espécies (LI *et al.*, 2008; BEOPOULOS *et al.*, 2009; MENG *et al.*, 2009).

2.3.2. Biorecursos disponíveis para a produção microbiana de lipídeos

A produção comercial de lipídeos é prejudicada pelo alto custo dos substratos utilizados. Em vista disso, matérias-primas alternativas, eficazes e de baixo custo têm sido exploradas, como: resíduo de sorgo sacarino, resíduo de batata doce, resíduo de tomate, águas residuais municipais e de fazendas de gado bovino, glicerol derivado de biodiesel, efluente de produção de fertilizante, lodo de esgoto, uréia, materiais lignocelulósicos, melaço de beterraba, amido solúvel, melaço de cana-de-açúcar, extratos de casca de laranja, glicerol industrial, bioprodutos

agroindustriais, água de maceração de milho, amido de mandioca, palha e farelo de trigo, palha de arroz e amido de águas residuais (SUBRAMANIAM *et al.*, 2010). A maioria desses substratos está disponível localmente, necessitando somente pequenas instalações de produção.

2.3.2.1. Matérias-primas lignocelulósicas (MPL) para produção microbiana de lipídeos

MPL consistem em três frações químicas básicas (Figura 2.2): i) hemicelulose, contendo principalmente polímeros de açúcar de cinco carbonos, ii) celulose, contendo polímeros de açúcar de seis carbonos, e iii) lignina, polímeros de fenol (TYSON *et al.*, 2005).

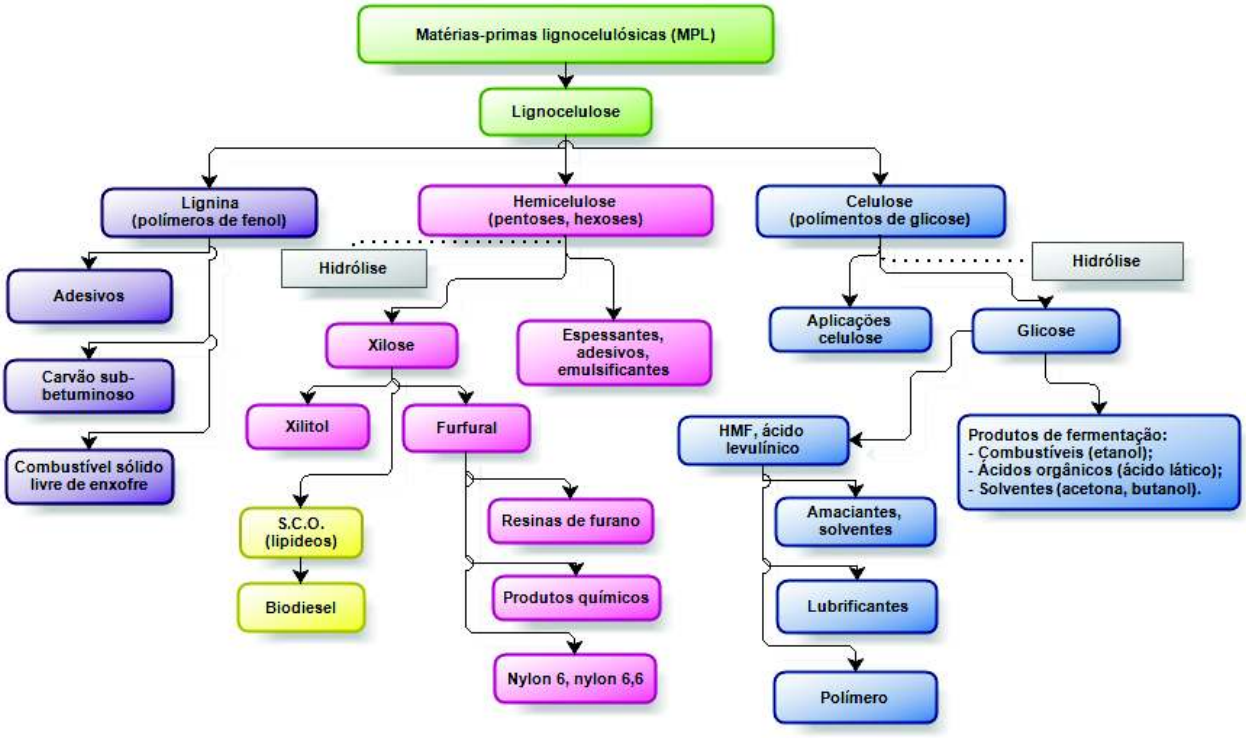

Figura 2. 2 Representação de processo de biorrefinaria utilizando MPL e respectivos produtos. Adaptado de Fernando *et al.* (2006).

A biorrefinaria de MPL ilustrada na Figura 2.2 utiliza materiais vegetais fibrosos onde, inicialmente, o material é limpo e separado nas três frações (hemicelulose, celulose e lignina) por digestão química ou hidrólise enzimática. Celulose e hemicelulose podem ser obtidos por via alcalina (soda cáustica) e sulfito (ácida, bissulfito, alcalina, etc.) A lignina é decomposta por enzimas como ligninases, lignina peroxidases, lacases e enzimas xilanolíticas (KAMM e KAMM, 2004).

A hemicelulose consiste em cadeias curtas altamente ramificadas de açúcares. Contrastando com a celulose, polímero composto somente por glicose, a hemicelulose é um polímero composto por açúcares de cinco carbonos (normalmente D-xilose e L-arabinose), açúcares de seis carbonos (D-galactose, D-glucose e D-manose) e de ácido urônico. As seguintes reações químicas (Figura 2.3) fornecem uma visão geral das conversões que ocorrem em uma biorrefinaria de MPL (FERNANDO *et al.*, 2006).

$$Lignocelulose + H_2O \rightarrow Lignina + Celulose + Hemicelulose$$

$$Hemicelulose + H_2O \rightarrow Xilose$$

$$Xilose\ (C_5H_{10}O_5) + ácido/catalisador \rightarrow Furfural\ (C_5H_4O_2) + 3H_2O$$

$$Celulose\ (C_6H_{10}O_5) + H_2O \rightarrow Glicose\ (C_6H_{12}O_6)$$

Figura 2. 3 Equação geral para conversão de precursores de biorrefinaria de MPL (KAMM e KAMM, 2004).

Algumas leveduras oleaginosas são conhecidas por usarem estes dois açúcares simultaneamente (RATLEDGE e COHEN, 2008). Xilose é o segundo mais abundante açúcar presente na biomassa lignocelulósica. (LEE, PRASHANT e TORGET, 1999). Bagaço de cana-de-açúcar é uma potencial fonte de xilose, cuja hidrólise para a obtenção de soluções de xilose tem dupla consequência: a eliminação de desperdício e a geração de um produto agregado, aumentando assim a economia do processo (RODRIGUEZ-CHONG *et al.*, 2004). Hidrolisados hemicelulósicos resultam em uma solução contendo principalmente açúcares como xilose, glucose e arabinose. Outros componentes tóxicos como hidroximetilfurfural, furfural e ácido acético também são liberados. Todos exibem forte inibição do crescimento de microrganismos e o efeito sinérgico desses componentes pode reduzir consideravelmente a concentração mínima crítica de inibição, afetando o crescimento celular e acúmulo de lipídeos (ZHAO *et al.*, 2012).

Devido à sua abundância, xilose bruta pode ser considerada como substrato modelo para a produção de SCO, uma vez que as vias envolvidas na assimilação são diferentes das vias de assimilação de glucose (Figura 2.4), o substrato mais estudado para a produção de SCO. A estequiometria do metabolismo de glucose é em torno de 1,1 moles de acetil-CoA por 100 g de glucose utilizada (EVANS e RATLEDGE, 1984). Xilose pode ser metabolizada tanto pela reação de fosfocetolase, que é a via metabólica mais eficiente com rendimento de 1,3 moles de acetil-

CoA por 100 g de xilose utilizada, ou pela via das pentoses fosfato, que resulta em 1 mol de acetil-CoA por 100 g de xilose utilizada (EVANS e RATLEDGE, 1984).

$$\text{Glicose} \xrightarrow{\text{citrato/ciclo malato}} \frac{1{,}1\ moles\ de\ acetil - CoA}{100\ \text{g glicose utilizada}}$$

$$\text{Xilose} \xrightarrow{\text{reação fosfocetolase}} \frac{1{,}3\ moles\ \ acetil - CoA}{100\ \text{g xilose utilizada}}$$

$$\text{Xilose} \xrightarrow{\text{via das pentoses fosfato}} \frac{1{,}0\ mol\ \ acetil - CoA}{100\ \text{g xilose utilizada}}$$

Figura 2. 4 Estequiometria do metabolismo de xilose e glicose. (EVANS e RATLEDGE, 1984).

2.3.3. Perfil de AG em lipídeos microbianos

Óleos e gorduras são compostos principalmente de TAGs possuindo função de armazenamento de carbono e de energia em microrganismos (HU *et al.*, 2008). A composição de AG reflete no número de saponificação, valor de iodo (KALAYASIRI, JEYASHOKE e KRISNANGKURA, 1996) e tem influência em parâmetros de qualidade do biodiesel, como o número de cetano, calor de combustão, estabilidade oxidativa, ponto de nuvem e lubricidade (KNOTHE, 2009).

Alguns microrganismos são capazes de produzir quantidades significativas de ácido γ-linoleico (C18:2), araquidônico (C20:4) e docosahexaenóico (C22:6). Ácido mirístico, palmítico, esteárico, oleico, linoleico e linolênico são os principais ácidos graxos encontrados em lipídeos produzidos por leveduras (LI, XU and WU, 2007; LI, ZHAO and BAI, 2007; LIU and ZHAO, 2007). O perfil de lipídeos de *L. starkeyi* (Tabela 2.2.) e outras leveduras foram descritos por diversos pesquisadores (YAMAUCHI *et al.*, 1983; ANGERBAUER *et al.*, 2008). Aproximadamente 25-45% dos AG são saturados e 50-55% são insaturados. Assim, a proporção de AG insaturados e saturados em lipídeos microbianos varia entre 1 e 2, sendo semelhante à de óleos vegetais.

Tabela 2. 2 Comparação de perfil de ácidos graxos produzidos por *Lipomyces starkeyi* e óleo de palma.

Perfil de ácidos graxos de *L. starkeyi* produzidos em diferentes substratos (%, w/w)[a]							
Substrato	**Referência**	**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**
Fécula batata	(WILD *et al.*, 2010)	<1	39,0	3,0	3,0	55,0	ND
Lodo esgoto	(ANGERBAUER *et al.*, 2008)	0,9	55,9	13,8	1,8	25,8	0,1
Etanol	(YAMAUCHI *et al.*, 1983)	ND	31,8	6,7	2,3	53,2	5,0
Água residual	(LIU *et al.*, 2012)	16,0	19,9	NI	16,7	35,9	NI
Glicose	(LIU e ZHAO, 2007)	0,4	33,0	4,7	4,8	55,1	1,6
Glicose	(LIN *et al.*, 2011)	0,7	36,3	5,0	4,2	50,1	3,7
Xilose	(GARZÓN, 2009)	0,8	20,4	10,8	ND	56,1	11,9
Glicose:xilose	(GONG *et al.*, 2012)	NI	37,7	5,4	3,1	49,6	3,9
Glicose:xilose	(TAPIA *et al.*, 2012)	0,4	33,9	7,5	3,4	50,6	3,9
Perfil de ácidos graxos de óleos vegetais (%, w/w)[a]							
Vegetal	**Referência**	**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**
Cacau	(PAPANIKOLAOU *et al.*, 2001)	ND	13	78	ND	2	ND
Cacau	(LIPP *et al.*, 2001)	ND	26	36	ND	33	2,8
Palma	(LIU *et al.*, 2010)	1,3	50,4	4,7	0.1	33,5	8,2
Palma	(MALEKI, AROUA e SULAIMAN, 2013)	1,2	43,6	4,6	ND	40,5	10,1
Milho	(MA e HANNA, 1999)	ND	11,7	1,8	ND	25,2	60,6
Soja	(MA e HANNA, 1999)	ND	11,7	3,1	ND	23,3	55,5
Girassol	(MA e HANNA, 1999)	ND	6,1	3,3	ND	16,9	73,7

[a] Áreas de pico inferior a 0,1% foram considerados insignificantes e foram apresentados como não detectado (ND).; NI: valores não informados.

2.3.4. Influência das fontes de carbono

Easterling *et al.* (2009) exploraram a produção de lipídeos de *Rhodotorula glutinis* em diferentes fontes de carbono (glicose, xilose, glicerol, e misturas de glicose:xilose, xilose:glicerol e dextrose:glicerol). O acúmulo de lipídeos chegou a 34% quando utilizado dextrose:glicerol

como fontes de carbono. A fração de AG insaturados foi dependente da fonte de carbono, sendo a mais alta obtida com glicerol (53%) e o valor mais baixo obtido com xilose como única fonte de carbono (25%). A levedura *L. starkeyi* é a única conhecida por reutilizar minimamente os lipídeos produzidos por ela mesma devido à incapacidade de formar peroxissomas (HOLDSWORTH, VEENHUIS e RATLEDGE, 1988) e produzir carbohidrolases extracelulares (KANG *et al.*, 2004). Peroxissomas são associados com a via metabólica de β-oxidação para degradação de ácidos graxos em leveduras. Outro estudo explorou a produção de lipídeos por *L. starkeyi* utilizando lodo de esgoto (ANGERBAUER *et al.*, 2008). Somente houve crescimento celular e acúmulo de lipídeos em lodo tratado por diferentes métodos.

A produção de lipídeos microbianos a partir de glicose e fécula de batata doce também foi estudada (WILD *et al.*, 2010). Esses autores confirmaram os resultados de outro estudo (ANGERBAUER *et al.*, 2008) em relação à razão C/N na produção de lipídeos por *L. starkeyi*, onde condições que favorecem o acúmulo de lipídeos resultam na diminuição de crescimento celular. As células podem consumir fécula de batata doce não sacarificado produzindo células com 40% de lipídeos e rendimento de células a partir de fécula de 0,41 g/g (WILD *et al.*, 2010). Nesse estudo, o rendimento de células foi maior utilizando fécula como fonte de carbono em relação à glicose.

2.3.5. Razão C/N, pH, temperatura e outros parâmetros ambientais

A levedura *L. starkeyi* resulta em alto conteúdo lipídico quando utilizada razão C/N 150 (Tabela 2.3) comparada a razão C/N 60 em meio contendo lodo de esgoto (ANGERBAUER *et al.*, 2008). O acúmulo de lipídeos por *Cryptococcus curvatus* requer razão C/N 50 em cultivos em batelada e batelada alimentada (HASSAN *et al.*, 1996). *Cryptococcus curvatus* e *L. starkeyi* destacam-se como microrganismos capazes de consumir diferentes fontes de carbono, desde glucose até os mais complexos, como águas residuais e xilose (ANGERBAUER *et al.*, 2008; ZHAO *et al.*, 2008).

A definição das condições de cultivo é um fator muito importante para o crescimento celular, acúmulo de lipídeos e perfil de AG produzidos. Alguns estudos revelam que tanto o crescimento celular, quanto o acúmulo de lipídeos depende da fonte de nitrogênio (YOON e RHEE, 1983). Outros estudos mostraram que as enzimas desaturases são mais ativas em baixas temperaturas, aumentando o grau de insaturação dos AG. Em condições de oxigênio limitantes, a

porcentagem de AG insaturados diminuiu (MORETON, 1987; RATLEDGE, 1991). Também foi relatado que utilizando glicose, nitrogênio inorgânico no meio de cultivo deve ser suplementado com fontes de nitrogênio orgânico para aumentar o acúmulo de lipídeos (SATTUR e KARANTH, 1989).

Uma explicação bioquímica para a necessidade de baixos níveis de nitrogênio ou sua exaustão para o acúmulo de lipídeos foi dada por Evans e Ratledge (1985). Olhando para a sequência de eventos bioquímicos que ocorrem em um microrganismo oleaginoso, os autores relataram que AMP deaminase, a enzima que divide AMP em IMP e NH_4^+ é ativada pelo esgotamento do nitrogênio extracelular, desencadeando assim a sequência de eventos que levam ao acúmulo de lipídeos. Isto significa que em alta razão C/N, o esgotamento dos níveis de nitrogênio é rápido e o açúcar seria seletivamente conduzido para a síntese de lipídeos, resultando, assim, em altos coeficientes de lipídeos.

Tabela 2. 3 Produção de lipídeos por leveduras em diversos substratos e modos de cultivo.

Levedura	Substrato	Modo cultivo	Células (g/L)	Lipídeo (g/L)	Lipídeo (%)	Referência
Cryptococcus curvatus	Glicerol	Batelada alimentada	91	29,1	32	(MEESTERS *et al.*, 1996)
Cryptococcus curvatus	Glicerol	Batelada alimentada	118	29,5	25	(MEESTERS, HUIJBERTS e EGGINK, 1996)
Rhodosporidium toruloides	Glicose	Batelada alimentada	106,5	71,9	67,5	(LI, ZHAO e BAI, 2007)
Candida curvata	Xilose	Contínuo	15	5,6	37	(EVANS e RATLEDGE, 1983)
Apiotrichum curvatum	Soro leite	Batelada fermentador	19,7	11,4	58	(YKEMA *et al.*, 1988)
Apiotrichum curvatum	Glicose	Batelada fermentador	14,5	6,6	45,6	(HASSAN *et al.*, 1993)
Yarrowia lipolytica	Estearina	Batelada frasco	15,2	7,9	52	(PAPANIKOLAOU *et al.*, 2007)
Lipomyces starkeyi	Glicose + Xilose	Batelada frasco	20,5	12,6	61,5	(ZHAO *et al.*, 2008)
Lipomyces starkeyi	Xilose	Batelada frasco	46,7	21,3	45,7	(ZHAO *et al.*, 2008)
Lipomyces starkeyi	Lodo esgoto (C/N 150)	Batelada frasco	N.I.	6,4	68	(ANGERBAUER *et al.*, 2008)
Lipomyces starkeyi	Lodo esgoto (C/N 60)	Batelada frasco	N.I.	5,9	40	(ANGERBAUER *et al.*, 2008)
Rhodotorula glutinis	Glicose	Batelada fermentador	29,8	14,7	49,3	(DAI *et al.*, 2007)
Rhodotorula glutinis	Glicose	Contínuo	38,6	23,4	60,7	(DAI *et al.*, 2007)
Rhodotorula mucilaginosa	Glicose	Batelada alimentada	19,5	10,2	52,2	(ZHAO *et al.*, 2010)
Rhodosporidium toruloides	Glicose	Batelada alimentada	106,5	71,8	67,5	(LI,ZHAO e BAI, 2007)

N.I.: não informado.

2.3.6. Estequiometria do carbono para conversão em lipídeo

O processo de biossíntese de lipídeos é o mesmo quando se está lidando com leveduras oleaginosas, fungos filamentosos ou microalgas fotossintéticas. Para as algas, o substrato carbônico é o CO_2, mas este é, em essência, fixado em um hidrato de carbono de modo que, com

ambos os conjuntos de organismos é razoável considerar a eficiência de conversão de unidades de glicose em TAG (CHISTI, 2007).

Em cultivos contínuos foi desenvolvido um modelo baseado em balanço estequiométrico para carbono, nitrogênio, oxigênio e hidrogênio (YKEMA *et al.*, 1986). Supõe-se que o intermediário não contém nitrogênio. Todos os substratos e os produtos são convertidos em fórmulas que contêm um mol de carbono, levando à equação do balanço geral de massa celular, a produção de intermediários e de possíveis produtos (Eq. 2.1):

$$CH_kO_l + aNH_3 + bO_2 = Y_xCH_pO_nN_q + Y_iCH_tO_u + Y_lCH_rO_s + cH_2O + dCO_2 \qquad \text{Eq. (2.1)}$$

substrato *célula* *intermediário* *produto*

Nessa equação, CH_kO_l, $CH_pO_nN_q$, CH_tO_u e CH_rO_s representam a composição elementar do substrato orgânico, massa celular microbiana, intermediário e o produto, respectivamente. Os valores subscritos *k, l, r* e *s* podem ser calculados a partir da fórmula molecular do substrato orgânico e do produto. Os valores de *p, n* e *q*, e de *t* e *u*, podem ser determinados após análise elementar da célula microbiana e do respectivo intermediário (YKEMA *et al.*, 1986).

O máximo valor teórico da estequiometria de conversão de glicose em TAG é de 33% sem produção de nenhum outro material celular. A estequiometria global de é aproximadamente (Eq. 2.2):

$$15Glicose \rightarrow TAG + 36CO_2 \qquad \text{Eq. (2.2)}$$

Assim, se a glicose não fosse utilizada para a síntese de nenhum outro produto, o rendimento de glicose em lipídeos seria de aproximadamente 32 g por 100 g de glicose quando uma molécula geral de TAG ($C_{55}H_{98}O_6$) for considerada. Em termos aproximados, seriam necessários cinco toneladas de açúcar para produzir uma tonelada de lipídeo assumindo condições ótimas de crescimento (RATLEDGE, 2004; RATLEDGE e COHEN, 2008). A maior conversão de glucose em lipídeo relatada na prática foi de 22,4 g de lipídeo a partir de 100 g de glicose em cultivo contínuo e este deve ser o provável máximo rendimento (RATLEDGE e COHEN, 2008). Uma conversão ótima de 100 g de glicose deve resultar em 20 g de lipídeo somados a 30 g de massa celular livre de lipídeos, resultando num total de 50 g de células. Com isso sugere-se que em torno de 40% de lipídeos é provavelmente o valor ótimo obtido na maximização do rendimento de células. Para encontrar conteúdos de lipídeos de 60-70% (Tabela

1.2), não significa que se está produzindo mais lipídeos, e sim menos células livre de lipídeos. (RATLEDGE, 2004; RATLEDGE e COHEN, 2008).

2.3.7. Mecanismo de produção de TAG em leveduras oleaginosas

Em leveduras oleaginosas, os lipídeos podem ser acumulados por duas vias: (i) síntese *de novo*, onde a síntese ocorre a partir dos blocos construtores acetil-CoA e malonil-CoA e (ii) via *ex novo*, que envolve a captação de AG, óleos e TAG do meio de cultivo para o interior da célula para seu acúmulo. Essa última via requer hidrólise do substrato hidrofóbico e incorporação desses AG liberados. Na síntese *de novo*, a limitação de nitrogênio provoca desregulação do ciclo do ácido cítrico (TCA), resultando em superprodução de citrato, precursor imediato de acetil-CoA e malonil-CoA (BEOPOULOS e NICAUD, 2012), conforme Figura 2.5. Além dos precursores, também é requerida energia na forma de ATP e NADPH para a síntese de AG (BEOPOULOS *et al.*, 2009).

Leveduras não oleaginosas produzem acetil-CoA a partir da glicólise de açúcares fermentáveis através da quebra do piruvato na mitocôndria e pela via piruvato citossólico-aldeído-acetato. Em leveduras oleaginosas, há uma fonte adicional de acetil-CoA: o excesso de citrato produzido no ciclo do TCA é exportado para fora da mitocôndria pelo ciclo citrato-malato. O citrato é então clivado no citoplasma pela ATP citrato liase (ACL), uma enzima presente em microrganismos oleaginosos (RATLEDGE, 2004), conforme Eq. 2.3:

$$Citrato + CoA + ATP \rightarrow acetil - CoA + oxaloacetato + ADP + Pi \quad \text{Eq. (2.3)}$$

Em relação ao malonil-CoA, este é gerado pela enzima acetil-Coa carboxilase (ACC) pela condensação de uma unidade de acetil-CoA com um ânion bicarbonato (Eq. 2.4):

$$Acetil - CoA + HCO_3^- + ATP \rightarrow malonil - CoA + ADP + Pi \quad \text{Eq. (2.4)}$$

A síntese *de novo* de lipídeos em leveduras é conduzida pelo complexo enzimático AG-sintetase (FAS) no citoplasma e requer o fornecimento constante de acetil-CoA e malonil-CoA (BEOPOULOS e NICAUD, 2012). Acetil-CoA é a unidade biossintética inicial e malonil-CoA é a unidade de alongamento, promovendo dois carbonos em cada etapa do crescimento da cadeia de AG até um nível intermediário de (C14-C16), dependendo do arsenal enzimático de cada microrganismo. O principal AG sintetizado pela FAS é o palmitato, que é liberado pela enzima e pode ser alongado e/ou dessaturado para produzir outras moléculas de AG. Para cada etapa de alongamento da cadeia de carbono, o complexo FAS requer duas moléculas de NADPH. A

síntese de 1 mol do AG 18C requer 16 mols de NADPH. Todas as reações de síntese de AG são resultado de inúmeras atividades enzimáticas das enzimas FAS. O fornecimento de NADPH para a síntese de AG é feito principalmente pela enzima málica (ME) pela reação (Eq. 2.5), (RATLEDGE, 2004) e pela via das pentoses fosfato:

$$Malato + NADP^{+} \rightarrow piruvato + NADPH \qquad \text{Eq. (2.5)}$$

A principal via metabólica conhecida para a síntese de TAG ocorre pelo glicerol-3-fosfato (G3P) (HEIER *et al.*, 2010), seguindo a via de Kennedy (TAI e STEPHANOPOULOS, 2013) que ocorre no retículo endoplasmático e nos corpos lipídicos. Na primeira etapa, a G3P-desidrogenase (GPD) converte dihidroxiacetona fosfato (DHAP) em G3P por uma reação reversível. Acil-CoA é o precursor utilizado para a acilação do esqueleto de G3P através da G3P-aciltransferase (GPAT) para formar o ácido lisofosfatídico (LPA). O LPA é acilado pela ALP-aciltransferase (LPAT) para formar o ácido fosfatídico (PA). O PA é então desfosforilado em DAG pela fosfatidato fosfatase (PAP) e a acilação final ocorre pela DGA-aciltransferase (DGA) para a produção de TAG (ROSSI *et al.*, 2011).

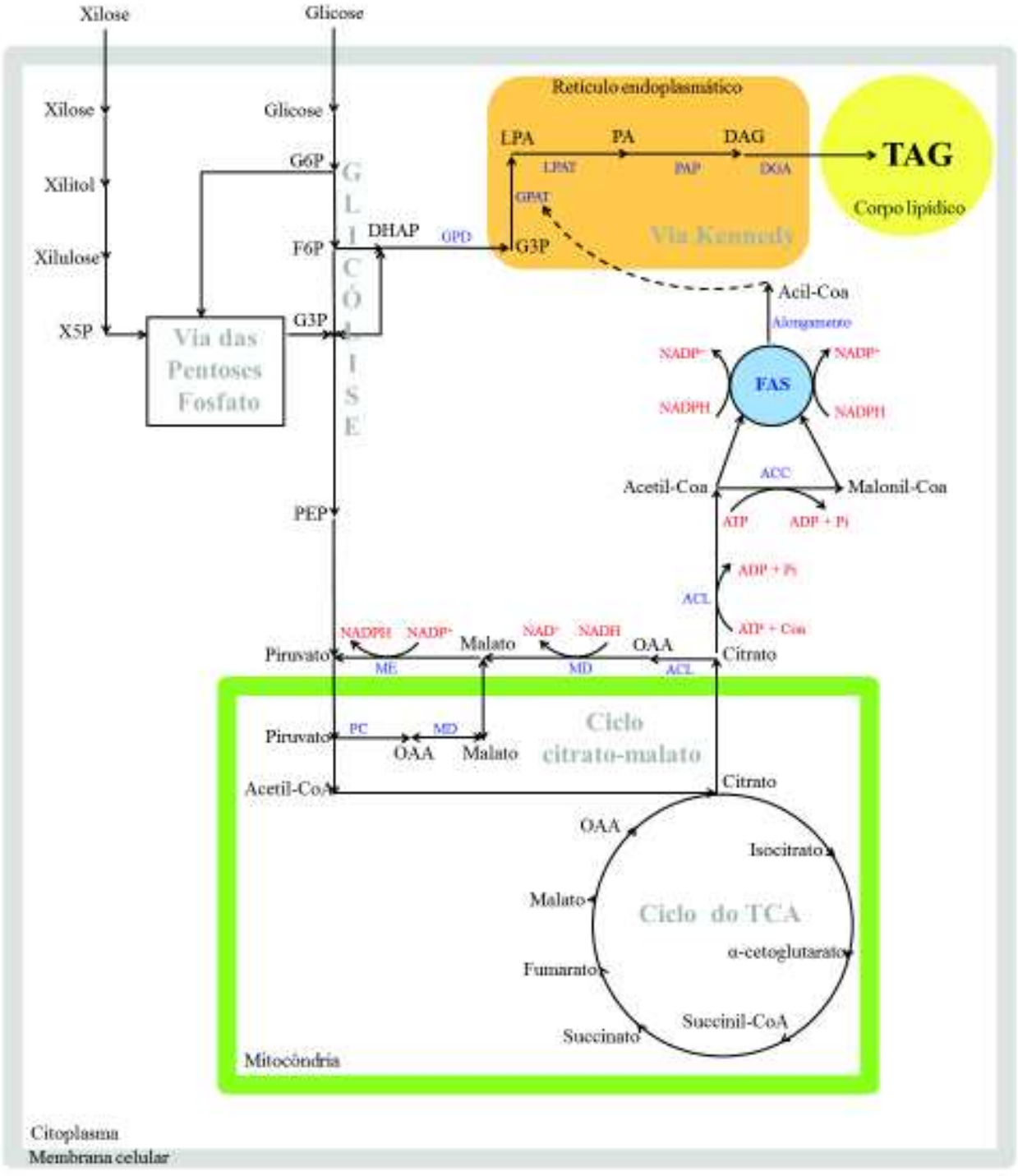

Figura 2. 1. Visão geral das principais vias metabólicas para a síntese lipídica em *Lipomyces starkeyi* a partir de xilose e glicose. As principais enzimas envolvidas na síntese de ácidos graxos estão representadas em azul. F6P: frutose-6-fosfato, G6P: glicose-6-fosfato, MD: malato desidrogenase, OAA: oxaloacetato, PC: piruvato carboxilase, PEP: fosfoenolpiruvato, X5P: xilulose-5-fosfato. Ciclo do TCA: ciclo do ácido tricarboxílico; FAS: complexo enxzimático ácido sintase; DAG: diacilglicerol; TAG: triacilglicerol. Adaptado de Beopoulos *et al.* (2011), Ratledge (2006), Tai e Stephanopoulos (2013).

A etapa final de formação de TAG pode ser catalisada tanto por uma enzima acil-CoA dependente (acil-CoA:diacilglicerol aciltransferase (DGAT)), quanto por uma enzima acil-CoA independente (fosfolipideo:diacilglicerol aciltransferase (PDAT)) (ATHENSTAEDT, 2011), conforme Figura 2.6.

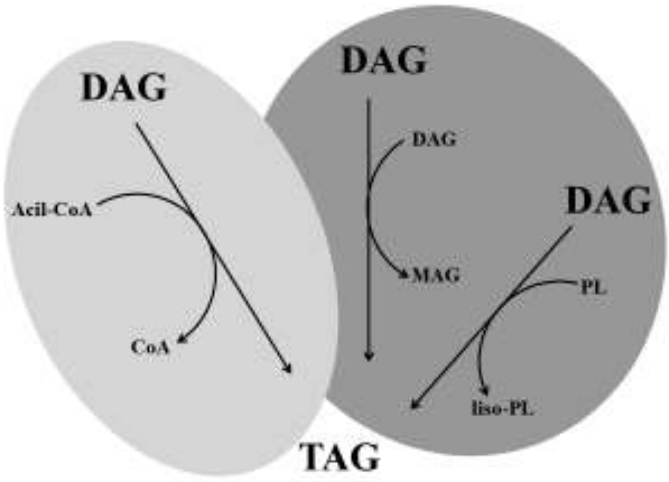

Figura 2. 2. Mecanismos de síntese de Triacilglicerol (TAG): formado via acil-CoA dependente (cinza claro) ou via acil-CoA independente (cinza escuro). DAG: diacilglicerol; MAG: monoacilglicerol; PL: fosfolipídeo.

2.4. Produção de lipídeos por diferentes modos de cultivo

Processos de batelada, batelada alimentada, batelada alimentada repetida e cultivo contínuo tem sido usados para a produção microbiana de lipídeos em escala laboratorial (SUBRAMANIAM *et al.*, 2010). Os diferentes modos de alimentação apresentam grande impacto na produtividade de lipídeos (ZHAO *et al.*, 2011).

2.4.1. Cultivo em batelada alimentada

Cultivos em batelada alimenta têm se mostrado efetivos no incremento da densidade celular e do conteúdo de lipídeos em leveduras oleaginosas. Li *et al.* (2007) realizaram cultivo em batelada com baixa razão C/N e solução de alimentação somente com glicose na etapa de batelada alimentada. Os autores obtiveram 106 g/L de massa celular e produtividade de lipídeos de 0.5 g/L.h com *Rhodosporidium toruloides* Y4. Segundo Xue *et al.* (2008), o processo de batelada alimentada pode prevenir alguns efeitos inibitórios. A combinação de baixa

concentração inicial de glicose (2%) e batelada alimentada durante o cultivo impediram os efeitos inibitórios da glicose no crescimento celular e o consumo de glicose aumentou exponencialmente. Qu *et al.* (2013) também demonstraram que o processo de batelada alimentada foi eficaz na redução da inibição de crescimento causada por altas concentrações de substrato.

Tapia *et al.* (2012) estudaram a produção de lipídeos por *L. starkeyi* A1 utilizando xilose e glicose como substratos. Cultivo em batelada alimentada com pulsos de alimentação resultou em 86 g/L de massa celular (58 % de lipídeos) e produtividade de lipídeos de 0.35 g/L.h. Outros estudos reportaram altas produtividades de lipídeos utilizando *Zygomycets, C. curvatus*, e *L. starkeyi* em cultivos de batelada alimentada (YAMAUCHI *et al.*, 1983; MEESTERS, HUIJBERTS e EGGINK, 1996).

2.4.2. Cultivo em batelada alimentada repetida

O processo de batelada alimentada repetida, durante o qual parte do meio de cultivo é periodicamente retirado e substituído por meio fresco, é conhecido por aumentar a produtividade das fermentações microbianas, reduzindo o tempo de limpeza, esterilização, inoculação e de alimentação (BAE *et al.*, 2004; SHAKERI, SUGANO e SHODA, 2007; ZHAO *et al.*, 2011). Nesse processo, certo volume do meio de fermentação contendo células com alto teor de lipídeos de fermentação prévia é utilizado diretamente como inóculo para o cultivo seguinte, reduzindo o tempo de preparo de cada fermentação e de inoculação, apresentando grande interesse industrial (QU *et al.*, 2013).

Zhao *et al.* (2011) estudaram a produção de lipídeos por *Rhodosporidium toruloides* Y4 utilizando glicose. Os resultados sugeriram que os modos de alimentação de substrato apresentam grande impacto na produtividade de lipídeos e que o processo de batelada alimentada repetida é o método mais atraente para aumentar a produção de lipídeos microbianos apresentando grande potencial para produção de larga escala.

Em processo de batelada alimentada repetida de *Schizochytrium* sp., a produtividade de ácido docosahexanóico (DHA) foi superior aos encontrados em batelada alimentada. Os resultados indicaram promissores perspectivas de industrialização para a produção de lipídeos ricos em DHA (QU *et al.*, 2013). Saenge *et al.* (2011) demostraram que o processo de batelada alimentada repetida é eficaz na manutenção de lipídeos produzidos por *R. glutinis* em baixa

concentração de células utilizando efluente de óleo de palma. Hsieh e Wu (2009) compararam a produção celular e produtividade de lipídeos na microalga *Chlorella* sp. em batelada alimentada intermitente e batelada alimentada repetida. Os autores encontraram altas produtividades em processo de batelada alimentada repetida.

2.4.3. Cultivo contínuo

Em geral, elevados rendimentos celulares ocorrem quando células são cultivadas sob condições de estado estacionário e quando o carbono é usado com a mesma eficiência em cada etapa do ciclo de crescimento. Brown *et al.* (1989) estudaram o crescimento celular e acúmulo de lipídeos em *Candida curvata* em batelada e modo contínuo. Observou-se que a taxa de acúmulo específico de lipídeos aumentou durante o tempo de fermentação em batelada e com o aumento da vazão específica de alimentação (D) em modo contínuo (BROWN *et al.*, 1989).

A levedura *Yarrowia lipolytica* é capaz de produzir grandes quantidades de lipídeos utilizando glicerol em condições limitantes de nitrogênio em cultivo contínuo. A produção de lipídeos foi favorecida a baixas vazões específicas de alimentação e a maior produtividade de lipídeos (0.12 g/L.h) foi obtida na D de 0,03h^{-1}. Incremento na D resultou no incremento do rendimento celular, mas com declínio da fração lipídica. A composição dos AG não foi afetada pela D (PAPANIKOLAOU e AGGELIS, 2002). Shen *et al.* (2013) estabeleceram um modelo cinético para cultivo contínuo com *Rhodosporidium toruloides* Y4 em glicose. Sugeriu-se produtividade lipídica específica máxima em D entre 0,05-0,09 h^{-1}.

Em cultivo contínuo, observou-se que meio de cultivo com limitação de nitrogênio e vazão específica de diluição de cerca de um terço da taxa específica de crescimento máximo é recomendado para alcançar máxima percentagem de lipídeos em microrganismos oleaginosos (RATLEDGE e HALL, 1977).

Rhodotorula glutinis foi estudada por sua habilidade de acumular lipídeos em cultivo contínuo utilizando melaço em condições de nitrogênio limitante (ALVAREZ *et al.*, 1992). O conteúdo máximo de lipídeos foi de 39% em D de 0.04 h^{-1}.

Durante cultivo em batelada, a concentração de nutrientes no meio de cultivo e a taxa de crescimento do microrganismo varia o que dificulta a compreensão do processo. Em contraste, em cultivo contínuo, em condições quimiostato, todos os parâmetros permanecem constantes,

sendo possível isolar um único parâmetro e estudar seu efeito sobre o acúmulo de lipídeos mantendo os demais parâmetros físicos e químicos constantes (GANUZA e IZQUIERDO, 2007).

O grau de insaturação e o perfil de AG em microrganismos oleaginosos pode ser manipulado durante cultivo contínuo através de vários fatores como a concentração de carbono e as fontes de nitrogênio (GILL, HALL e RATLEDGE, 1977), uso de diferentes fontes de carbono e de vazões específicas de alimentação (EVANS e RATLEDGE, 1983), oxigênio dissolvido (ROUX *et al.*, 1995) ou temperatura (KENDRICK e RATLEDGE, 1992). Em cultivo contínuo de *Candida* 107 em estado estacionário, a composição de lipídeos se manteve contínua durante várias semanas de operação (GILL, HALL e RATLEDGE, 1977).

2.5. Consistência e reconciliação de dados

Para estudos de sistemas biológicos, é de crucial importância o controle das condições ambientais para a caracterização do estado fisiológico. Por essa razão, cultivos em quimiostato são escolhidos como a melhor estratégia devido à facilidade de estudo das respostas celulares a determinadas perturbações (como efeitos de oxigenação, temperatura, pH) enquanto que outros parâmetros são mantidos constantes. Adicionalmente, cultivos em quimiostato permitem a modelagem de caixa preta do sistema experimental, devido à caracterização completa do metabolismo celular em estado estacionário (Figura 2.3). A composição elementar é necessária para descrever o modelo de caixa preta da célula microbiana, enquanto que a análise de macromoléculas deve ser considerada para cálculo de fluxos metabólicos (DUBOC *et al.*, 1995). Esses modelos levam a sistemas sobredeterminados (possuem mais equações do que incógnitas (m>n) e permitem verificar a consistência estatística dos dados a 95% de confiança, verificando possíveis erros mensuráveis (VERHEIJEN, 2009).

A reconciliação de dados consiste em ajustar as medições dos processos de forma a eliminar erros conhecidos ou ruídos medidos (WEISS, ROMAGNOLI e ISLAM, 1996). O método também pode estimar valores para variáveis não mensuráveis a partir de dados de medições de processo (MAH, STANLEY e DOWNING, 1976). É importante ter acesso a um conjunto de dados consistentes e confiáveis para um efetivo controle de bioprocessos (SCHULER, 2012).

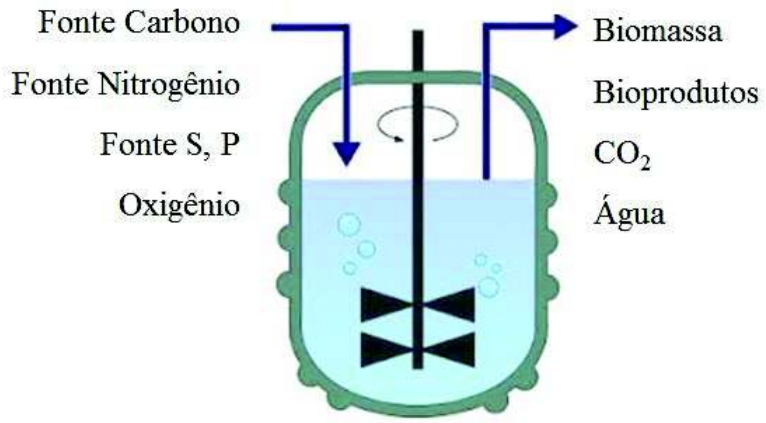

Figura 2. 3. Modelo de caixa preta para sistema experimental em quimiostato.

A metodologia utilizada para reconciliação de dados é a mesma, quer seja aplicada a uma refinaria ou ao metabolismo de uma célula microbiana. Em todos os casos, uma formulação clara do problema e uma descrição do sistema ou o modelo do processo é necessária. Porém, em bioprocessos, as equações de balanço de massa podem não ser facilmente estabelecidas devido a dificuldades na identificação de todos os componentes relevantes ou à falta de medições completas. Contudo, a reconciliação de dados tem sido aplicada com sucesso na obtenção de melhores estimativas de conversão e coeficientes de rendimento a partir de resultados macroscópicos/elementares e/ou balanços energéticos baseados em medições *on-line* de processos, como sensores de gás e controladores de fluxo de massa (DUBOC e VON STOCKAR, 1995; DABROS *et al.*, 2009). De acordo com Noorman *et al.* (1996), o objetivo geral da reconciliação de dados pode ser dividido em três pontos principais: 1) cálculo de variáveis desconhecidas a partir de grandezas mensuráveis; 2) detecção e remoção de inconsistências a partir de um conjunto de dados; 3) melhoria da exatidão dos dados medidos.

A reconciliação de dados é baseada em princípios de conservação de massa e energia e possui especial aplicação em biotecnologia para descrever processos de rotas metabólicas de processos em curso. Quando se trabalha com sistemas celulares, o requerimento de equações de conservação para reconciliação de dados pode ser derivado a partir de balanços de massa e de energia usando estequiometria metabólica.

Uma vez o sistema celular ou reacional definido e suas taxas classificadas, as leis de conservação inerentes ao modelo usadas, conduzirão à formulação de uma matriz de conservação (NOORMAN *et al.*, 1996) com certo número de limitações e certo número de taxas de conversão.

A partir desta matriz inicial de conservação, a redundância do sistema pode ser calculada e utilizada para as próximas etapas de detecção de erros e de reconciliação de dados. A explicação matemática detalhada pode ser encontrada no estudo de Noorman *et al.* (1996) e van der Heijden *et al.* (1994a; b). O algoritmo da reconciliação de dados indica que as técnicas de reconciliação de dados não só reconciliam os valores medidos, mas também estimam variáveis de processo não medidos ou parâmetros do modelo, desde que sejam observáveis, permitindo o cálculo da estimativa ótima para cada componente medido e não medido.

Técnicas como "caixa preta" e x^2 testes (LANGE e HEIJNEN, 2001) permitem a detecção e até mesmo erradicação de erros. A aplicação destes testes é simples, multidimensional e resulta em estimativas confiáveis e consistentes, permitindo a detecção de disfunções instrumentais ou distúrbios de processo (WANG e STEPHANOPOULOS, 1983).

Durante muito tempo a dificuldade de quantificar todos os componentes relevantes (especialmente a célula microbiana ou a bioatividade) em tempo real, impedia a aplicação industrial em tempo real do método de reconciliação de dados e detecção de erros. Recentes avanços no desenvolvimento de analisadores sensíveis de processo em tempo real tornaram o conjunto de dados disponíveis mais confiáveis através da utilização de dados adequados para a reconciliação. Analisadores de processo em escala de bancada como FTIR podem fornecer informações importantes em relação ao conteúdo de carbono, nitrogênio, grau de redução e balanços (JOBE *et al.*, 2003), enquanto que analisadores como biocalorímetros (DUBOC e VON STOCKAR, 1995) ou espectroscopia de fluorescência (DUBOC *et al.*, 1995) podem fornecer medições adicionais de calor (DUBOC e VON STOCKAR, 1998) ou de balanços de ATP. Quando a informação de diferentes metabólitos adicionais está disponível, o sistema descrito pode ser estendido incluindo diferentes fluxos metabólicos e suas distribuições (WISSELINK *et al.*, 2010). Atualmente, todos esses requisitos podem ser cumpridos pela reconciliação de dados, resultando em uma base excelente para o controle e tomada de decisão em processos (SCHULER, 2012).

2.6. Aspectos econômicos na produção microbiana de lipídeos

Biocombustíveis economicamente viáveis devem possuir custo competitivo com os combustíveis oriundos de petróleo. O custo de produção de SCO depende principalmente dos microrganismos escolhidos para o cultivo (BOROWITZKA, 1997), do conteúdo de lipídeos e da

concentração de células produzidas (BRENNAN and OWENDE, 2010). O custo da matéria-prima ou da fonte de carbono requerida para a produção microbiana de lipídeos representa de 60-75% (Figura 2.4) do custo total do biodiesel (AHMAD *et al.*, 2011). Assim, a escolha da matéria-prima adequada é muito importante para assegurar um custo mais baixo de produção de biodiesel (FAKAS, MAKRI*, et al.*, 2009; FAKAS, PAPANIKOLAOU*, et al.*, 2009).

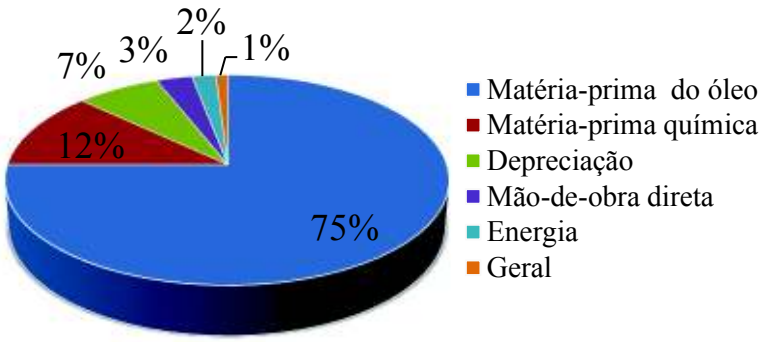

Figura 2. 4. Custos gerais para a produção de biodiesel. Adaptado de Ahmad *et al.* (2011).

Chen *et al.* (2009) cultivaram *Trichosporon cutaneum* 2.1374 em hidrolisado lignocelulósico para produção de lipídeos. Foram feitas estimativas econômicas preliminares: o preço do diesel ou biodiesel no mercado foi de cerca de US$ 1.100/ton. Como o custo de lipídeos geralmente representa 70-80% do custo total do biodiesel, o preço máximo para os lipídeos na produção de biodiesel deveria ser cerca de US$ 900/ton. Por outro lado, o custo de açúcares fermentáveis a partir de matéria-prima lignocelulósica foi de aproximadamente US$ 180/ton. O rendimento de lipídeos a partir de açúcares observado neste estudo foi de aproximadamente 10%, resultando num custo de lipídeos de aproximadamente US$ 1.800/ton. Isso significa que o custo de produção de lipídeos a partir de levedura oleaginosa foi quase o dobro do valor para produção de lipídeo na produção de biodiesel. Para ser utilizado como matéria-prima de biodiesel, lipídeos de SCO devereiam diminuir ao menos 50% dos custos de produção. Cheng *et al.* (2009) não citam o custo da matéria-prima lignocelulósica no estudo, mas segundo Dias *et al.* (2013), o custo médio da cana-de-açúcar é de 23 US$/ton, considerando dados de preços de cana de 2001 a 2010.

A Tabela 2.4 apresenta valores comparativos de uma planta de óleos vegetais (*commodities*) e lipídeos microbianos. Verifica-se que o custo da produção de lipídeos

microbianos a partir de leveduras (ou fungos filamentosos) em 2008 deveria ser superior a US$ 3,00/Kg, enquanto que a partir de microalgas seria em torno de US$ 21,00/Kg (RATLEDGE e COHEN, 2008).

Tabela 2. 4 Custo de planta de óleo de *commodities* comparada a uma planta de SCO apresentada por (RATLEDGE e COHEN, 2008).

Planta de óleo (Março/Abril 2008)	US$ por tonelada
Óleo de soja (ex USA):	1.263/1.247
Óleo de girassol (ex Rotterdam):	1.863/1.838
Óleo de canola:	1.519/1.459
Óleo de milho (ex USA):	1.842/1.919
Lipídeo microbiano	
Óleo de levedura	3.000 (excluindo custo de matéria-prima)
Óleo de alga:	5.600-7.000 a 21.000

Koutinas *et al.* (2014) fizeram uma avaliação tecno-econômica da produção de lipídeos a partir de *Rhodosporidium toruloides* como matéria-prima para biodiesel e indústria oleoquímica. Considerando recursos renováveis à base de glicose com custo zero (assumindo processo integrado de biorrefinarias nas indústrias existentes que utilizam resíduos ou subprodutos) com produção anual de 10.000 toneladas de lipídeos, o custo estimado do lipídeo purificado foi de US$ 3,40/Kg. A produção de biodiesel por transesterificação indireta (utilizando lipídeo microbiano extraído) resultou em maior custo-competitividade comparada à transesterificação direta (sem a extração dos lipídeos das células microbianas). Considerando o preço de glicose como sendo US$ 400,00 a tonelada, o custo de produção de lipídeos e de biodiesel foi estimado em US$ 5,50/Kg e US$ 5,90/Kg, respectivamente.

Quando produzidos por diferentes microrganismos oleaginosos em diferentes condições de cultivo e substratos utilizados, os lipídeos microbianos devem possuir perfil de AG diversificados, possuindo potencial de utilização com alto valor agregado como: substitutos de óleos vegetais e gorduras animais na indústria oleoquímica, incluindo, entre outros, cosméticos, produtos farmacêuticos, tintas, lubrificantes e aditivos de polímeros (KOUTINAS *et al.*, 2014). O potencial de utilização de lipídeos microbianos está relacionado com o preço de óleos e gorduras,

que pode variar de US$ 0,30/Kg a valores superiores a US$ 100,00/Kg. (WYNN e RATLEDGE, 2005; PAPANIKOLAOU e AGGELIS, 2011).

O conteúdo lipídico total contido na célula pode ser usado como fonte de produção de biodiesel enquanto que ácidos graxos poli-insaturados, como o ácido docosahexaenóico (DHA, C22:6, *n*-3), produzido por alguns microorganismos oleaginosos, possuem diversas aplicações nutracêuticas por seus efeitos benéficos para a saúde humana (SHENE *et al.*, 2013).

Outro exemplo de aplicação está nos substitutos de manteiga de cacau, constituído majoritariamente por ácido palmítico, oleico e esteárico, contendo de 55 a 67% de ácidos graxos saturados (PAPANIKOLAOU *et al.*, 2001). Atualmente o preço médio da manteiga de cacau está em $ 4,45/Kg (BURCHENAL e HART, 2013). Diversos estudos têm utilizado leveduras oleaginosas para produção de lipídeos como substitutos de manteiga de cacau (PAPANIKOLAOU e AGGELIS, 2011).

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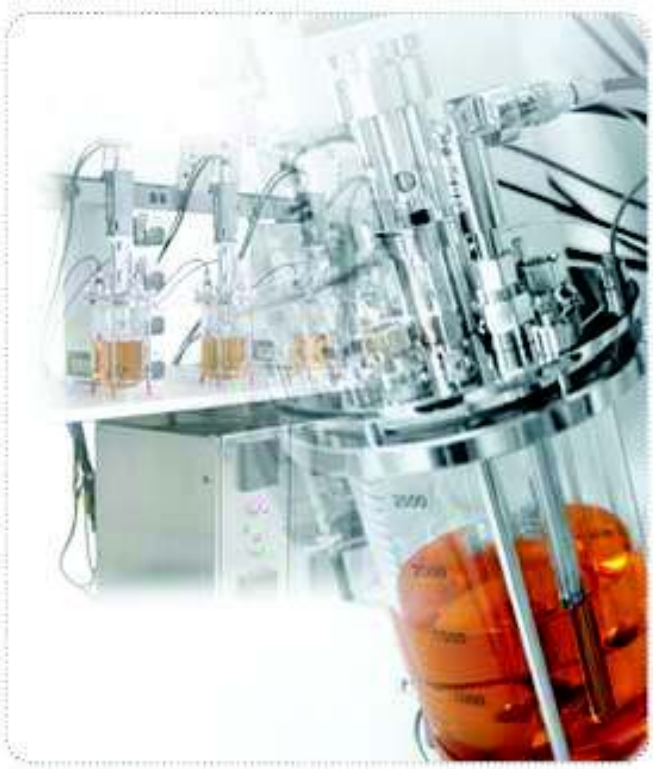
CHAPTER 3. Fed-batch experiments in bioreactor

3.1. Study of feeding pulses in fed-batch culture for lipid production by *Lipomyces starkeyi*

3.2. Lipid production by *Lipomyces starkeyi* in fed-batch culture with different nitrogen sources



"It is only because of problems that we grow mentally and spiritually."

Morgan Scott Peck

3.1. Study of feeding pulses in fed-batch culture for lipid production by *Lipomyces starkeyi*

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ABSTRACT

This work describes lipid production by the oleaginous yeast *L. starkeyi* in a bioreactor at fed−batch using xylose and glucose as carbon sources in a ratio 70:30 (w/w). In the first stage, cells were cultivated in a nutrient-rich medium (C/N 50) for propagation. In the second stage (fed−batch), the medium was fed with glucose:xylose (70:30) and C/N 900 to achieve high cellular and lipid contents. The cultivation was performed with nine feeding pulses along 672 hours (28 days). Our results demonstrated that xylose and glucose were used for cell growth and lipid accumulation processes, allowing further optimization or improvement. The use of fed−batch cultivation indicates that efficient processes to convert renewable materials into biofuel and related products are feasible, but more than six feeding pulses wouldn't be practical or economical.

3.1.1. Material and Methods

3.1.1.1. Strain and Media

The oleaginous yeast *L. starkeyi* DSM 70296 was used throughout this study. Agar slants (YPD medium) were stored in the refrigerator and propagated monthly or store by cryopreservation at −80ºC with 10% (v/v) of glycerol. The composition of the inoculum and cultivation medium was (per liter): yeast extract, 1.92 g; $(NH_4)_2SO_4$, 1.36 g; Na_2HPO_4, 1 g; KH_2PO_4, 1 g; $Mg_2SO_4{\cdot}7H_2O$, 0.4 g; $CaCl_2{\cdot}2H_2O$, 0.04 g; $ZnSO_4.7H_2O$, 0.08 g; $CuSO_4.5H_2O$, 0.001 g; $CoCL_2.6H_2O$, 0.001 g; $(NH_4)_2Mo_2O_7$, 0.005 g; pH, 5.5. The experiment was performed with a mixture of glucose and xylose as carbon sources in the cultivation medium (60 g/L) and feeding solution (600 g/L) in order to simulate the proportion of each carbohydrate in the sugar cane bagasse hydrolysate: 70% of glucose and 30% of xylose. The batch cultivation was done using C/N ratio of 50 and for fed−batch the C/N was 900. To avoid medium darkness due to sugars reactions (caramelization and Maillard), sugar solutions were sterilized in separate flasks. All chemicals and reagents used were of analytical grade.

3.1.1.2. Culture Conditions

Inoculum was prepared by two successive cell propagations in liquid media at 28 ºC, 150 rpm in an orbital shaker, pH 5.5, once for 48 h and the second time for 30 h until reaching about 10 g/L ($1x10^8$cells/mL). This inoculum preparation protocol resulted in reproducible growth in experimental shake flasks. Inoculum level used was 10 % (v/v), resulting in initial 1 g/L ($1x10^7$cells/mL) in the cultivations. The experiment was carried out in a 2.5 L bioreactor (Biostat® A plus, Sartorius BBI Systems, Melsungen, Germany). The initial working volume was 1.0 L with 1 vvm of aeration and 400 rpm of agitation. Concentrated solutions of glucose:xylose (70:30, w/w) and nutrients at C/N 900 were added into the bioreactor nine times to increase the sugar concentration to 60 g/L immediately after the residual sugar concentration decreased to 0−5 g/L. The pH was controlled at 5.5 by the automatic addition of 2 M NaOH. The dissolved oxygen (DO) was online monitoring by a DO electrode. To prevent foam formation, antifoam agent (Dow Corning FG−10) was automatically added to the vessel when needed. Aliquots were taken at various intervals and stored at –20°C to estimate sugar concentration, cell dry weight, lipid content, and nitrogen concentration.

3.1.1.3. Analytical Methods

Cell optical density was measured at 600 nm with a Nanophotometer (Implen GmbH, Munich, Germany). Cell dry weight (CDW) was determined after lyophilization of the harvested cells. Cellular concentration was determined by using the Neubauer−improved chamber. The number of non-viable cells (death number) was obtained through measurement with methylene blue (LEE, ROBINSON and WANG, 1981).

Glucose and xylose concentrations were measured using a Metrohm model 761 Compact Ion chromatography system (Metrohm, Herisau, Switzerland) (WALFORD, 2002). During the cultivations, total reducing sugars were assayed as described by Somogyi−Nelson (NELSON, 1944; SOMOGYI, 1945). Briefly, the reducing sugars when heated with alkaline copper tartrate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 620 nm. The residual nitrogen concentration in the culture medium was estimated using the Berthelot reaction (SRIENC, ARNOLD and BAILEY, 1984) and measuring the reaction product at 630 nm. A calibration curve was constructed with different ammonium sulfate concentrations (5-60 mg/L of N-NH_4).

To provide better lipid extraction efficiency, a previous acid treatment was performed by adding hydrochloric acid (4 mL of HCl 2M for each 300 mg of freeze-dried yeast cells) and incubating at 80ºC for 1 hour, after that the solution was centrifuged (6000 G, 4ºC, 15 min). The lipids were gravimetrically quantified, from this wet digested pellet, by Bligh-Dyer's method (BLIGH, E. G. and DYER, W. J., 1959; MANIRAKIZA, COVACI and SCHEPENS, 2001). Cell mass, lipid yield and lipid content were expressed as gram dry cells per liter cultivation medium, gram lipid per liter cultivation medium and gram lipid per gram dry cells (%), respectively.

3.1.2. Results and Discussion

Substrate feeding is one of the most powerful strategies to control microbial cultures in large-scale biochemical processes (SUZUKI, YAMANE and SHIMIZU, 1986; YANO, KUROKAWA and NISHIZAWA, 1991; NURMIANTO, GREENFIELD and LEE, 1994; RAU, MANZKE and WAGNER, 1996). The experiment lasted 672 hours (28 days) with nine feeding pulses (Figure 3.1A). The feeding was done with concentrated carbohydrates solution (600 g/L)

plus yeast extract and ammonium sulphate at C/N ratio 900 (SATTUR and KARANTH, 1991) to improve the lipid accumulation. We observed that the cell number after 300 hours and until the end of the cultivation remained around $3x10^8$ cells/mL (Figure 3.1A), which is lower than previous results and cell mass concentration was around 33 g/L. Up to 146 hours, cell death was below 5%, and after 430 hours the percentage of dead cells reached to 40 − 45%.

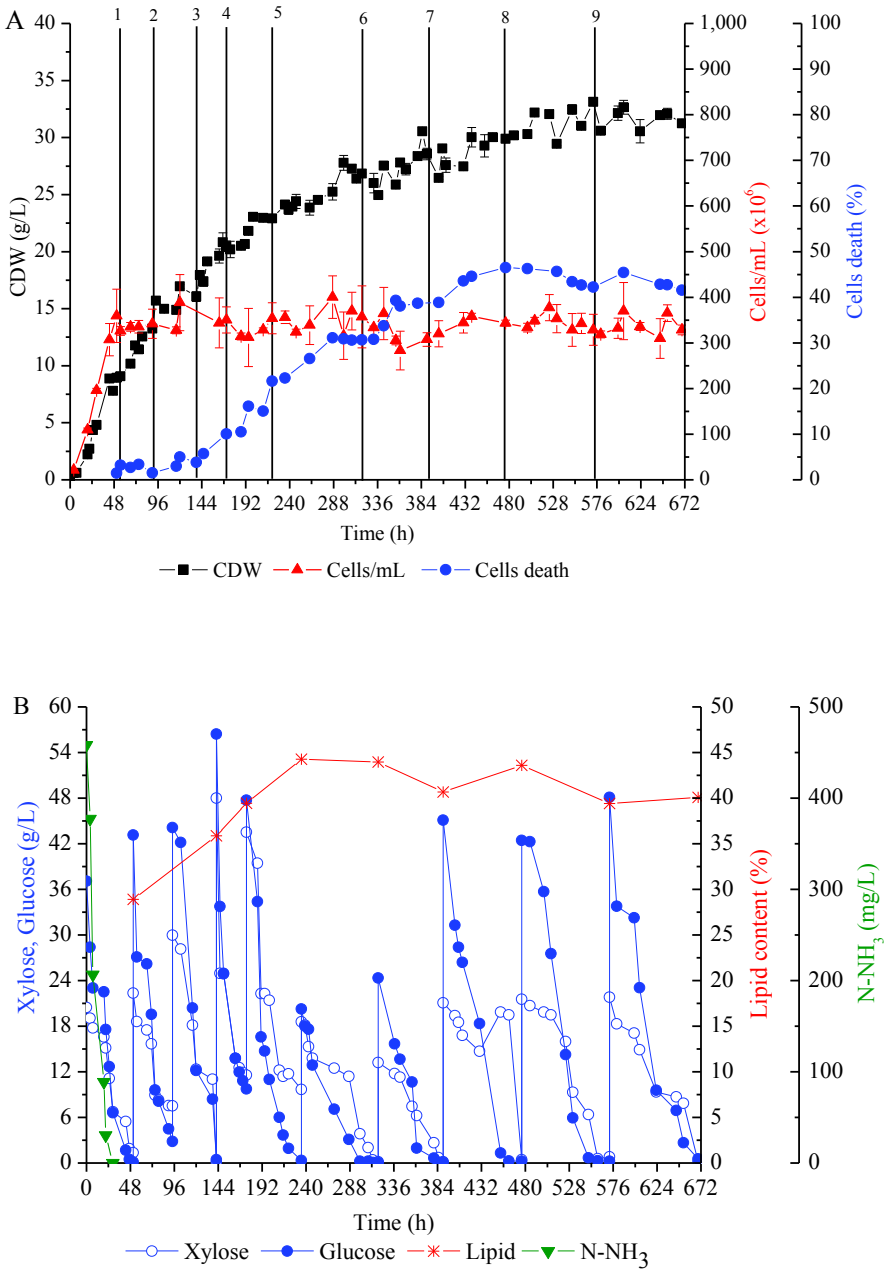

Figure 3. 1 (A) CDW (cell dry weight), cell number, cell death, (B) glucose, xylose, lipids and nitrogen of the fed−batch cultivation. The vertical lines represents the time of the feeding pulses.

Cell lipid content reached 44% (w/w) at 235 hours (Figure 3.1B) and remained constant after the 4th sugar feed, showing that the continuation of the feeding process wouldn't be practical or economical. Those results were similar to previous results, but the cultivation time was longer and more substrate was used to achieve similar results. Inorganic nitrogen was completely depleted at thirty hours and due the high C/N ratio used in the feeding along the fermentation, no nitrogen was detected until the end of the cultivation.

Problems with the feeding pump were observed for the 4th and 5th feeding, which were fed lower sugar concentrations (up to 30 g/L) (Figure 3.1B). It could also observe that glucose was always consumed before xylose. If we consider the high sugar consumption (Table 3.1) along the cultivation, the final cell mass and lipid concentrations were quite low and a part of this sugar could be converted to some organic components.

The lipid concentration reached from 2.6 to 13.1 g/L along this experiment. In addition, by Table 3.1, the overall yields (in cells and lipids) were higher at the beginning of the cultivation ($Y_{X/Sde\text{-}fatted}$= 0.110, $Y_{L/S}$= 0.045) and further decreased up to 672h ($Y_{X/Sde\text{-}fatted}$= 0.033, $Y_{L/S}$= 0.022).

Table 3. 1 Overall yields along the fed−batch cultivation.

Time (h)	**CDW (g/L)**	**CDWde-fatted (g/L)**	**Lipids (%)**	**Lipids (g/L)**	**$Y_{X/S}$ (g/g)**	**$Y_{X/S}$ de-fatted (g/g)**	**$Y_{L/S}$ (g/g)**
51	8.9	6.3	28.9	2.6	0.155	0.110	0.045
94	15.7	13.1	28.9	2.6	0.128	0.107	0.021
142	18.0	11.5	35.9	6.4	0.091	0.058	0.033
175	20.2	12.2	39.4	8.0	0.067	0.041	0.026
235	33.1	18.5	44.3	14.7	0.084	0.047	0.037
319	26.8	15.0	44.0	11.8	0.062	0.035	0.027
390	28.6	16.9	40.7	11.6	0.061	0.036	0.025
476	29.9	16.9	43.6	13.0	0.056	0.031	0.024
576	33.1	20.1	39.4	13.1	0.055	0.033	0.022
672	31.3	18.7	40.1	12.5	0.047	0.028	0.019

When *Rhodosporidium toruloides* Y4 was cultured by fed−batch mode, the lipid concentration and productivity achieved were 71.9 g/L and 0.54 g/L.h, respectively, but the initial concentration of organic nitrogen sources was 31.4 g/L and glucose, 60 g/L (LI, Y., ZHAO, Z. and BAI, F., 2007). Because yeast extract and peptone are relatively expensive, the present study applied yeast extract supplemented with ammonia sulfate resulting in 0.48 g/L of total nitrogen source. In this way, the costs for nitrogen sources were substantially reduced.

There are examples in the literature indicating that productivity may be improved by controlling the substrate concentration (CAYUELA *et al.*, 1993; SHANG, JIANG and CHANG, 2003). No nitrogen or additional nutrients, except a concentrated glucose solution, was supplemented for fed−batch cultures by *R. toruloides* Y4. During the late stage of the fed−batch process, cell mass production was reduced. This was mainly due to the fact that nitrogen sources were exhausted, and the carbon flux was channeled into lipid biosynthesis (ZHAO *et al.*, 2011). In our experiment, lipid accumulation increases only until the 5th feeding and remained constant until the end of the cultivation, showing that after 235 h, consumed sugar was mostly converted in cell mass maintenance or other compounds instead lipids.

Cell growth and division must be intimately linked and regulated in response to the environment and nutrient availability. Thus, cells need to constantly monitor the availability of nutrients and adjust cell growth and proliferation accordingly. In addition, these nutritional signals have to be integrated with hormonal signals derived from growth factors (FINGAR and BLENIS, 2004) which are secreted by specialized cell types in response to nutritional glues and serve to coordinate cell growth at the organism level (OLDHAM and HAFEN, 2003). However, much less is known about how nutrients are sensed, how nutrient−derived signals regulate cell growth and how these nutritional signals are integrated with growth factor pathways in higher eukaryotes. Limitation of nutrients evokes a complex change in the physiology of yeast cells, which allows them to either re−program their metabolism such that they can cope with the change in nutrient supply or activate a survival program to outlive sustained periods of starvation (WILSON and ROACH, 2002; GRAY *et al.*, 2004).

3.1.3. Conclusions

Our results indicated that the yeast *L. starkeyi* was a robust strain suitable for further development in terms of biotechnological production of lipid using xylose and glucose as carbon

sources. The next step aimed the utilization of hemicellulose hydrolysate as feedstock and the study of other feeding strategies to improve cell growth and lipid accumulation. Microbial lipids offer potential for sufficient production of renewable fuels to impact consumption of fossil fuels. In order to be cost effective, it will be necessary to use innovative combinations of cultivation systems involving all the carbonaceous materials including wastes.

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3.2. Lipid production by *Lipomyces starkeyi* in fed-batch culture with different nitrogen sources

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ABSTRACT

In order to investigate the effect of nitrogen sources on the growth and lipid production in *L. starkeyi* DSM 70296, ammonium sulphate, yeast extract and urea were tested as nitrogen sources with xylose as sole carbon source at C/N ratio of 50. Fed−batch cultures were conducted with three feeding pulses containing all the nutrients. The cell mass concentration and lipid content were affected by the nitrogen sources. When culture medium with urea was used, cell growth reached 94.2 g/L. Culture medium containing yeast extract plus ammonium sulphate and culture medium with only ammonium sulphate, reached 80.7 g/L and 55.6 g/L of cells, respectively. Maximum specific growth rate of 0.087 was observed with yeast extract plus ammonium sulphate. Lipid content was quite similar at the end of the fed−batch culture (148h), reaching from 35 to 38%, even varies along the cultivation time by the different nitrogen sources. Intracellular concentration of NH_4^+ is probably of considerable regulatory significance during the initial stages of lipid biosynthesis. The mechanisms by which organic and inorganic nitrogen metabolism can regulate lipid production will be reported in subsequent studies.

3.2.1. Materials and Methods

Fed-batch cultures were conducted with 3 feeding pulses containing xylose, nitrogen sources (C/N 50) and salts, added immediately after the residual xylose concentration was dropped to 0−5 g/L. Concentrated feeding solutions were used: 600 g/L of xylose solution and nutrient solution (with nitrogen sources and salts) ten times more concentrated than the culture medium. Initial working volume was 1L. More details about culture conditions and analytical methods as described in section 3.1.1.

3.2.2. Results and discussion

3.2.2.1 Fed-batch cultivations

One of the main challenges associated with the production of second-generation biodiesel is increasing the microbial conversion of low-cost substrates, such as agricultural residues, lignocellulosic materials, and wastes, into lipids. Cell growth and lipid content were affected by the nitrogen sources (Table 3.2). Maximum specific growth rate of 0.077 was observed using urea and yeast extract (YE) plus $(NH_4)_2SO_4$. Highest cell mass concentration reached 94.2 g/L using urea (Fig. 3.2). Lipid content was quite similar at the end of the fed batch culture, reaching from 35 to 38%, even varies along the cultivation time by the different nitrogen sources. Higher cell mass and lipid yields were obtained using urea as nitrogen source. Among the organic nitrogen sources, urea has received extensive attention due to its low cost and growth promotion ability (Hsieh *et al.,* 2009).

High cell density and lipid accumulation occurred when urea was used as nitrogen source and did not required addition of expensive complex ingredients such as yeast extract. Lipid produced with urea and YE plus $(NH_4)_2SO_4$ showed similar tendencies in which the microorganisms did not accumulate more lipids, but produced more cells compared to $(NH_4)_2SO_4$. Nitrogen is essential for the synthesis of proteins and nucleic acids which are required for cellular proliferation. Tsigie *et al.* (2012) studied the effect of urea and peptone on cell mass concentration and lipid production by *Y. lipolytica.* The addition of urea did not significantly affect cell mass production but resulted in lower cellular lipid accumulation than with peptone.

Table 3. 2 CDW, lipid content and yields by *L. starkeyi* fed-batch cultivations by different nitrogen sources at 148h.

Response	Urea	YE + $(NH_4)_2SO_4$	$(NH_4)_2SO_4$
CDW (g/L)	94.2	80.7	55.6
CDW de-fatted (g/L)	60.7	49.6	35.5
Cell productivity (g/L.h)	0.638	0568	0.376
Lipid (g/L)	33.7	31.1	20.1
Lipid productivity (g/L.h)	0.228	0.219	0.136
Lipid (%)	35.7	38.5	36.2
$Y_{X/S}$ (g CDW/g sugar)	0.491	0.219	0.182
$Y_{X/S}$ de-fatted (g CDW/g sugar)	0.316	0.135	0.116
$Y_{L/S}$ (g lipid/g sugar)	0.175	0.085	0.066
μ_{max}	0.077	0.077	0.035

Gao *et al.* (2013) cultivated *M. isabellina* using xylose. Different organic and inorganic nitrogen sources were used as single nitrogen sources including yeast extract, urea, peptone, glycine, KNO_3, NH_4NO_3 and $(NH4)_2SO_4$. Results showed that all the nitrogen sources could support cell growth except KNO_3. Different nitrogen sources resulted in different level of cell mass and lipid produced. For organic nitrogen sources, the highest cell mass and lipid concentrations were achieved when yeast extract was used as sole nitrogen source. Urea and $(NH4)_2SO_4$ resulted in comparable amount of cell biomass, but $(NH4)_2SO_4$ yielded less lipid.

Evans and Ratledge (1984) investigated the effects of different nitrogen sources on lipid accumulation by oleaginous yeasts, and suggested that different metabolisms of nitrogen sources resulted in different concentration of metabolites such as NH_4^+, 2-oxoglutarate or glutamate, which induced different levels of lipid accumulation.

Chen *et al.* (2013) studied many kinds of nitrogen sources (urea, peptone, ammonium chloride, ammonium sulfate and tryptone) added in the corncob acid hydrolysate to evaluate their effect on the lipid production by *T. cutaneum*. Adding all of them can hardly improve the cell growth of *T. cutaneum*. Even worse, the lipid accumulation of *T. cutaneum* was seriously inhibited: the lipid content were 19.4%, 20.0%, 20.4%, 21.6% and 23.1% on the medium using urea, peptone, ammonium chloride, ammonium sulfate and tryptone as nitrogen sources

respectively. Control experiment, wihout additional nitrogen sources reached aroun 35% of lipids. Adding other nitrogen sources into the hydrolysate was not beneficial for the lipid production possibly due to the existence of other nitrogen sources in it.

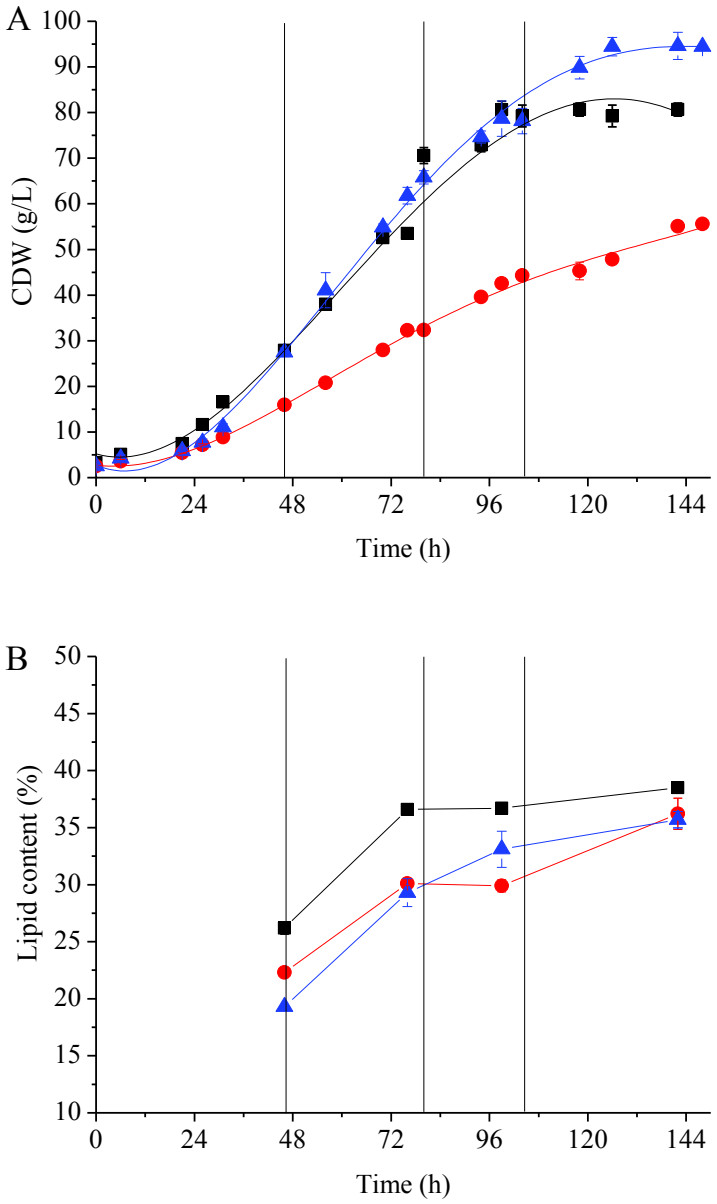

Figure 3. 2 Results of (A) CDW and (B) lipid content from fed batch experiments using urea (▲), $(NH_4)_2SO_4$ (●) and yeast extract plus $(NH_4)_2SO_4$ (■) as nitrogen sources. Vertical lines indicate the feeding pulses.

The ability of *Y. lipolytica* strains to use ammonium nitrate or urea as nitrogen sources was investigated and verified that the strains grew better with urea (SRIWONGCHAI *et al.*, 2013). At same study, when urea was supplemented with yeast extract, the cell mass yield increased with the addition yeast extract in media, however, the amount of lipids accumulation was not affected. Other researchers have also reported urea as an excellent nitrogen source for lipid production in yeasts, such as *Rhodotorula glutinis* (SAENGE *et al.*, 2011) and *Trichosporon fermentans* (ZHU, ZONG and WU, 2008). Xylose was used as sole carbon source and urea was selected for further used as nitrogen source in chemostat cultivations in order to have only one carbon source and on nitrogen source in data reconciliation method (See Chapter 6).

3.2.2.1 Maintenance coefficient

As the sole carbon source, xylose was consumed for cell growth and the maintenance of cell viability at these fed-batch cultivations. In general, consumption of materials for maintenance is small with regard to the amount of materials used in the synthesis of new biomass. Generally it is assumed that the use of materials for maintenance is proportional to the amount of cells present.

In 1965, Pirt introduced a linear correlation between the specific substrate uptake rate and the specific growth rate, and he suggested use of the term maintenance, which now is the most commonly used term to describe endogenous metabolism (STEPHANOPOULOS, ARISTIDOU, and NIELSEN, 1998).

In order to calculate the maintenance coefficient, it is first necessary to obtain an estimate of the true cell growth yield and the specific substrate uptake rate. The linear correlation of Pirt takes the following form:

$$q_s = Y_{X/S}^{true}.\mu + m_s \qquad (4.1)$$

Where q_s is the specific substrate consumption rate ($g/g_{CDW}.h$), $Y_{X/S}^{true}$ (g/g) is referred to the true growth yield coefficient and m_s is the maintenance coefficient ($g/g_{CDW}.h$). The actual yield ($Y_{X/S}$) can be smaller than the true growth yield ($Y_{X/S}^{true}$) when an increasing fraction of the substrate is used to meet the maintenance requirements of the cell, as is the case at low specific growth rates. The m_s indicates the amount of substrate needed per unit cell mass per hour to maintain cell in an active and properphysiological condition.

Jahic *et al.* (2002) describes that under constant feed conditions, the maximum cell density could be described asa function of the substrate feed rate and the maintenance coefficient. This is obtained from the mass balance on the substrate (Eq. 4.2) that can be written

$$\frac{dS}{dt} = \frac{F}{V}(S_i - S) - \frac{\mu}{Y_{X/S}}X - q_m X \qquad (4.2)$$

Where $Y_{X/S}$ (g/g) is the cell mass yield coefficient, q_m (g/g.h) is the maintenance coefficient, F is the substrate feed rate (L/h), V is the medium volume (L), S is the limiting substrate concentration (g/L) and S_i is the inlet substrate concentration (g/L).

In the present study, the calculation of kinetc parameters of fed-batch with feeding pulses culture was performed by resetting the initial values of the differential equations to the step changed concentrations at the start of the feeding pulse and solve the equations from this time point until the start of the next pulse feeding, like a new batch culture after another. This method made the calculation of kinetic parameters of this fed-batch culture easier.

The calculations of cell mass yield and m_s have been included lipids in the membranes of cell. Maintenance coefficient was calculated using Eq. 3.1 and results are shown in Table 3.3. Fed-batch cultivions with urea and with YE + $(NH_4)_2SO_4$ indicated a m_s of 0.03g/g_{CDW}.h at a batch phase and cultivation with $(NH_4)_2SO_4$ resulted in twice the value. For the intervals between the feeding pulses, m_s estimated was zero. The reason may be attributed to the high cell activity and proliferation which requires more energy for motility, active transport of molecules and synthesis of macromolecules such as lipids.

Meeuwse, Tramper and Rinzema (2011) validated a model to predict cell growth, lipid accumulation and substrate consumption of 12 oleaginous fungi in chemostat cultures. One study showed a m_s of 0.05 C_{mol}/C_{mol}.h, whereas all the other studies presented zero value of m_s. Roels (1983), presented values around 0.02 C_{mol}/C_{mol}.h for different fungal species on glucose.

When urea was used as nitrogen source, high $Y_{X/S}$ were observed at each feeding interval, unlike for fed-batch cultivations with YE and $(NH_4)_2SO_4$, $Y_{X/S}$ sharply decreased after the third feeding pulse. The μmax decreased after each feeding pulse for the three fed-batch cultivations, highlighting the stationary growth phase after the third feeding pulse. Volumetric xylose consumption rate (Qs) was higher at first and second feeding pulse intervals for cultivations with urea and with YE + $(NH_4)_2SO_4$. Fed-batch cultivation with urea presented the highest specific

lipid accumulation of 0.018 g/gCDW.h. Meeuwse, Tramper and Rinzema (2011) presented q_p values for oleaginous yeasts ranging from 0.023 to 0.069 C_{mol}/C_{mol}.h.

A kinetic model study by Jahic *et al.* (2002) describes that the lower the maintenance, the higher is the maximum cell density achievable in a constant feed fed-batch process. Shen *et al.* (2013) obtained maximum m_s of 5.7 $g_{glucose}/g_{CDW}$.h at continuous cultivation by *R. toruloides* using glucose as sole carbon source.

These data are informative to understand the growth and lipid accumulation by *L. starkeyi* and should be very helpful to develop and design a more efficient bioprocess for microbial lipid production.

Table 3. 3 Kinetic parameters of fed-batch cultivations by different nitrogen sources.

Fed-batch		$Y_{X/S}^{true}$ (g/g)	μ_{max} (h^{-1})	Q_s (g/L.h)	q_s (g/g_{CDW}.h)	q_p (g/g_{CDW}.h)	m_s (g/g_{CDW}.h)
Urea	Batch	0.507	0.077	1.07	0.011	0.005	0.03
	1st FP	0.530	0.024	1.95	0.016	0.012	0
	2nd FP	0.371	0.012	1.73	0.015	0.018	0
	3nd FP	0.416	0.005	0.89	0.009	0.011	0
YE + $(NH_4)_2SO_4$	Batch	0.486	0.077	1.10	0.011	0.006	0.03
	1st FP	0.459	0.022	1.86	0.015	0.016	0
	2nd FP	0.411	0.014	1.97	0.017	0.015	0
	3nd FP	0.245	0.002	0.66	0.005	0.012	0
$(NH_4)_2SO_4$	Batch	0.298	0.035	0.98	0.006	0.006	0.06
	1st FP	0.462	0.021	1.02	0.013	0.011	0
	2nd FP	0.397	0.013	1.31	0.016	0.012	0
	3nd FP	0.223	0.006	1.18	0.005	0.014	0

*FP: Results obtained after each feeding pulse; Q_s: volumetric substrate consumption rate; q_p: specific lipid accumulation; $Y_{X/S}$, μ_{max}, Q_s, q_s and q_p were calculated according to Hiss (2001).

3.2.3. Conclusions

It was demonstrated that nitrogen source is an important factor for improving biomass and lipid production. Fast capture and catabolism of urea could lead to the release of intracellular

NH_4^+ in sufficient amounts to permit lipid accumulation. This may be due to the increase in activity of urease over this period. Intracellular concentration of NH_4^+ probably is of considerable regulatory significance during the initial stages of lipid biosynthesis. As a conclusion, microbial metabolism could be directed by using xylose (that can be obtained from lignocellulose.) and urea as co-substrates, in order to accumulate lipids with predetermined composition, e.g., palm and cocoa-butter equivalents.

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CHAPTER 4. Effect of feeding strategies on lipid production by *Lipomyces starkeyi*

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Effect of feeding strategies on lipid production by *Lipomyces starkeyi*

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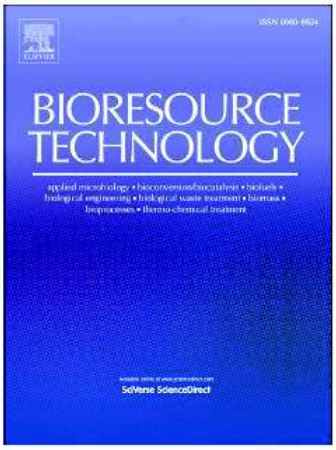

"Happiness is neither virtue nor pleasure nor this thing nor that but simply growth, we are happy when we are growing."

William Butler Yeats

Effect on feeding strategies on lipid production by *Lipomyces starkeyi*

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Abstract

The aim of this study was to produce microbial oil from *Lipomyces starkeyi* DSM 70296 grown in hemicellulose hydrolysate (H-H). Glucose and xylose were used for batch, fed-batch, repeated fed-batch, and continuous cultures, and H-H was tested at continuous culture. The highest cell mass and lipid concentrations of 85.4 and 41.8 g/L, respectively, were obtained using repeated fed-batch strategy. Continuous culture with dilution rate of 0.03 h^{-1} presented the highest overall cell mass (0.443 g/g) and lipid yields (0.236 g/g). At 0.06 h^{-1} were obtained the highest cell mass and lipid productivities. Continuous cultivation using H-H at 0.03 h^{-1} resulted in higher cell mass productivity than that obtained using glucose:xylose. Gas chromatography analysis of the esterified lipids indicated that the major constituents of this complex are palmitic acid, stearic acid, oleic acid, and linoleic acid with an estimated cetane number (approximately 61) similar to that of palm biodiesel, which is important for biofuel production.

Keywords: biodiesel, oleaginous yeast, sugarcane bagasse hydrolysate, fed−batch, continuous culture.

4.1. Introduction

The oleaginous yeast *Lipomyces starkeyi* is a species belonging to the Saccharomycetales order that was originally isolated from soil. The available information regarding how nutrient-derived signals regulate cell growth and how these nutritional signals are integrated with growth factor pathways in eukaryotes is limited. Nutrient limitation evokes a complex change in the physiology of yeast cells, which allows them either to re-program their metabolism such that they can cope with the change in nutrient supply or to activate a survival program to outlive sustained periods of starvation (GRAY *et al.*, 2004).

In general, lipid production by oleaginous microorganisms is classified into two steps: the growth phase and the lipid production phase (RATLEDGE and COHEN, 2008). The fed-batch process is an important strategy because it makes possible to control the growth and lipid accumulation phases by modifying the feed throughout the fermentation process. In theory, during the late stage of a fed-batch process, nitrogen sources are exhausted, which leads to a reduction in the cell mass production rate and the channeling of the flux of carbon toward lipid biosynthesis. However, in the repeated fed-batch culture process, fresh medium containing nitrogen is fed to restart exponential cell propagation (ZHAO *et al.*, 2011).

In addition to glucose, pentoses (mainly xylose) are the main carbon components of hemicellulose hydrolysate (H-H) derived from agricultural wastes (lignocellulose-rich material), and microbial lipid production from xylose has recently been explored (HE *et al.*, 2010). Because it can be used directly for hydrolysis following fermentation in sugar plants, sugar cane bagasse appears to be more competitive for lipid production than other lignocellulosic biomasses (HUANG *et al.*, 2012).

Because the lipid contents of oleaginous yeast are generally influenced by the media components, particularly the carbon source, nitrogen source, and minerals, it is logical to identify good conditions for lipid production from glucose and xylose. Some oleaginous

yeasts can assimilate glucose and xylose simultaneously to accumulate intracellularly a considerable amount of lipid with a good lipid coefficient, in both artificial and real hydrolysates (HU *et al.*, 2011). The fermentation of mixed sugars is not only significant for the improvement of the overall economics associated with microbial lipid production but also holds an interesting route for effective cell mass conversion (ZHAO *et al.*, 2008). However, few studies have investigated process analysis with the aim of evaluating different feeding strategies. Therefore, the present study focused on evaluating the effect of alternative feeding strategies using a mixture of glucose and xylose, as well as H-H, as carbon sources on cell mass and lipid production.

4.2. Materials and Methods

4.2.1. Strain and media

The oleaginous yeast *L. starkeyi* DSM 70296 was used throughout this study. Agar slants (YPD medium) were stored in a refrigerator and propagated monthly or stored through cryopreservation at -80ºC with 10% (v/v) glycerol. The composition of the inoculum and cultivation medium was (per liter): 1.92 g of yeast extract (0.192 g of nitrogen), 1.36 g of $(NH_4)_2SO_4$ (0.288 g of nitrogen), 1 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.4 g of $Mg_2SO_4 \cdot 7H_2O$, 0.04 g of $CaCl_2 \cdot 2H_2O$, 0.08 g of $ZnSO_4.7H_2O$, 0.001 g of $CuSO_4.5H_2O$, 0.001 g of $CoCL_2.6H_2O$, and 0.005 g of $(NH_4)_2Mo_2O_7$ (pH 5.5).

For the carbon and energy sources, sugars (glucose:xylose) at 60 g/L (24 g of carbon) were mixed at different proportions to produce an artificial lignocellulosic (70:30) or hemicellulose hydrolysate (30:70). The initial carbon-to-nitrogen (C/N) ratio was fixed at 50 for all operation modes. To avoid medium darkening due to the reaction of the sugars (caramelization and Maillard reactions), the sugar solutions were sterilized in separate flasks. All of the chemicals and reagents used were of analytical grade.

4.2.2. Raw material

Sugarcane bagasse was kindly provided by CTBE (National Laboratory of Bioethanol Science and Technology, Campinas-SP, Brazil), and its chemical composition on a dry basis 49.5% cellulose, 24.3% hemicellulose, 22.7% lignin, and 0.3% ash. The sugarcane bagasse was hydrolyzed with 1.5% sulfuric acid (w/v) at a solid-to-liquid ratio of 1:10 in an autoclave at 120°C for 20 min. After hydrolysis, the mixture was separated by centrifugation at 2980 g and vacuum filtered, and the liquid fraction (H-H) was stored at -18°C until its use in the continuous cultivation experiments. The composition of H-H obtained was 13.1 g/L xylose, 2.9 g/L glucose, 2.6 g/L acetic acid, 2.1 g/L arabinose, 0.04 g/L furfural, and 0.02 g/L hydroxymethylfurfural (HMF).

4.2.3. Culture conditions

The inoculum was prepared through two successive cell propagations in liquid media at 28ºC and 150 rpm in an orbital shaker. The first was incubated for 48 h, and the second was incubated for 30 h until a CDW of 10 g/L (equivalent to $1x10^8$ cells/mL) was obtained. The experiments using glucose:xylose were conducted in a 3.0-L bioreactor (New Brunswick, USA) with an initial working volume of 1.0 L, and the experiments using H-H medium were conducted in a 1.3-L bioreactor (New Brunswick, USA) with working volume of 0.5 L. For all cultivations, the aeration rate, agitation, and temperature were set to 1 vvm, 400 rpm, and 28ºC, respectively. The pH was maintained at 5.5 through the automatic addition of 2 M NaOH. Aliquots were collected at various intervals and stored at -20°C until their analysis for substrate concentrations, cell dry weight, and lipid content. Concentrated sugar solutions (600 g/L) were used for the fed-batch modes, and the concentration of the nutrient solution (with nitrogen sources and salts) was tenfold higher than that of the culture medium. The fed-batch cultures were started with an initial sugar concentration of 60 g/L in the bioreactor, which was

fed using a peristaltic pump. To determine an appropriate sugar and nutrient feeding strategy, different modes were studied, as described in Table 4.1.

In Study 1, concentrated solutions of glucose:xylose and nutrients were added into the bioreactor three times to increase the sugar concentration to 60 g/L immediately after the residual sugar concentration decreased to 0−5 g/L. The main goal of Study 2 was to evaluate the relationship between the cell growth and the lipid content at a constant C/N ratio throughout the cultivation. For this reason, different volumes of both feeding solutions (sugar and nutrients) were added based on the analysis of the sugar and nitrogen contents in the broth during the cultivation. After the analysis of each sample, the sugar concentration was adjusted to 60 g/L, and nitrogen and salts were also added to reach a C/N of 50 or 900 to induce lipid accumulation, as suggested by Sattur and Karanth (SATTUR and KARANTH, 1989). We previously found that this C//N value allows cell growth and high lipid storage. The C/N ratio was maintained constant (at 50) during the first 48 h of cultivation and was then increased to 900. For Study 3, the first three feeding pulses contained sugar and nutrient solutions, and the last three pulses only included sugar. The single-batch stage of this study represents Study 4. A batch experiment (Study 5) was also conducted with an inoculum concentration of 3 g/L to reduce the lag phase of growth in the bioreactor. This inoculum concentration was used for the next cultivations. Another fed-batch mode (Study 6), in which the first three feeding pulses contained sugar and nutrient solutions and the last pulse involved only sugars, was also investigated.

Table 4. 1 Cultivations of *L. starkeyi* in a 2-L bioreactor under the different culture conditions and feeding strategies studied.

Study	Process	Inoculum (g/L)	Sugar ratio (G:X)	Feeding pulses with sugar + nutrients	Feeding pulses with only sugar
1	Fed-batch	1	70:30	3	
2	Fed-batch	1	70:30	8	
3	Fed-batch	1	30:70	3	3
4	Batch	1	30:70		
5	Batch	3	30:70		
6	Fed-batch	3	30:70	3	1
7	Repeated Fed-batch	3	30:70	3[a]	1[a]
8	Continuous	3	30:70		
9	Continuous	3	30:70		
10	Continuous	3	H-H		

[a] For each cycle; G:X: glucose:xylose; H-H: hemicellulose hydrolysate.

A repeated fed-batch culture (Study 7) with two cycles was performed after study 6. At the end of the first fed-batch cycle, half of the fermentation broth was removed such that only 500 mL remained in the fermenter. The fermenter was then filled with 500 mL of fresh medium to accelerate the cell mass growth phase of the second cycle. In the first cycle, the feeding pulses were the same as those used in study 6, but the feeding pulses used in the second cycle contained only sugar solutions.

For continuous culture with glucose:xylose, a sugar solution containing 60 g/L (30:70, w/w) was used, and the C/N ratio was adjusted to 50. For continuous culture with H-H medium, a C/N of 50 was used, and yeast extract (10% of N) and $(NH_4)_2SO_4$ (21% of N) were added into the medium according to the concentration of the carbon sources (xylose, glucose, arabinose, and acetic acid) present in the H-H. The dilution rate (D, volume of incoming medium per unit time/volume of medium in the culture vessel) was attained by varying the medium flow. According to Gill et al. (1977), maximum lipid accumulation requires the yeast to be grown at a dilution rate equal to one-third the value of the maximum growth rate. In this order, dilution rates of 0.03 h^{-1} (Study 8) and 0.06 h^{-1} (Study 9) were studied with glucose:xylose, and the continuous culture was started after 24 h of batch cultivation. Study 10 was performed at a D value of 0.03 h^{-1}, and the continuous culture was started after 15 h of batch cultivation. Steady-state conditions were obtained after a continuous flow of at least four working volumes of the culture medium.

4.2.4. Analytical methods

The cell optical density was measured at 600 nm with a nanophotometer (Implen GmbH, Germany). The cell dry weight (CDW) was determined after lyophilization of the harvested cells. The cellular concentration was determined using the Neubauer-improved chamber, and the number of non-viable cells (death number) was obtained through measurement with methylene blue. The glucose, xylose, and arabinose concentrations were

measured by ion chromatography (Metrohm, Switzerland) (TAPIA *et al.*, 2012); however, during the cultivations, the total reducing sugars was assayed as described by Somogyi-Nelson. The quantitative analysis of acetic acid was also performed by ion chromatography (Metrohm, Switzerland). A Metrosep organic acid column (250 x 7.8 mm Metrohm AG CH 9101) was used with 0.5 mM H_2SO_4 as the elution solvent at a flow rate of 0.5 mL/min at 30ºC and an injection volume of 196 µL. Furfural and hydroxymethylfurfural (HMF) were determined by HPLC (Waters, USA) using a UV detector (Waters, 486) at 280 nm. A Delta-Pak C18 column (150x3.9 mm, 5 µm, 300 Å) was used with acetonitrile (2.5%) and H_3PO_4 2 mM (1:1) as the eluents at a flow rate of 0.5 mL/min at 25ºC and an injection volume of 10 µL. The residual nitrogen concentration in the culture medium was estimated using the Berthelot reaction and measuring the reaction product at 630 nm. A calibration curve was constructed with different ammonium sulfate concentrations (5-60 mg/L of N-NH_4) according to Tapia *et al.* (2012). The cells were treated with hydrochloric acid (2 M), incubated at 80ºC for 1 h, and centrifuged at 6000 g and 4ºC for 15 min, and the lipids were then extracted and quantified using Bligh-Dyer's method. The crude lipid was initially converted to fatty acid methyl ester (FAME) (LEWIS, NICHOLS and MCMEEKIN, 2000) and then analyzed using a GC/MS gas chromatography (Shimadzu, Japan) system according to Tapia *et al.* (2012). All the analysis were performed in triplicate and error bars denote the standard deviation.

4.3. Results and discussion

One of the main challenges associated with the production of second-generation biodiesel is increasing the microbial conversion of low-cost substrates, such as agricultural residues, lignocellulosic materials, and wastes, into lipids. Substrate feeding is one of the most powerful strategies for the control of microbial cultures in biochemical processes. To compare the effects of carbon source feeding, two different proportions of sugar mixtures were tested. The first one simulated the composition of the hydrolysate of whole sugarcane

bagasse, which contains more glucose than xylose (70:30, w/w) due to the hydrolysis of cellulose and hemicellulose. The second sugar mixture corresponds to the composition of H-H, which contains more xylose than glucose (30:70, w/w) because the cellulose is not hydrolyzed.

4.3.1. Study 1

Sugar and nutrient solutions were used in this fed-batch process because the effect of the C/N ratio is important for lipid accumulation. Our results (Table 4.2) show that the cell concentration increased with the feeding pulses until the end of the process (144 h) and reached 42 g/L (approximately 8 x10^8 cells/mL). The lipid content increased from 30 to 36% (w/w), indicating that the yeast used the nutrients mostly for growth and maintenance and thus favored cellular growth over lipid accumulation. The highest $Y_{X/S}$ was achieved at the end of the batch step (0.258 g/g), whereas the highest $Y_{L/S}$ was obtained at the end of the cultivation (0.08 g/g).

Although microorganisms generally metabolize sugars sequentially when exposed to a mixture of glucose and xylose because glucose can repress the utilization of other sugars via a catabolic repression mechanism or allosteric competition for sugar transporters (KAWAGUCHI *et al.*, 2006), both sugars were assimilated simultaneously rather than sequentially, in a rate of 0.46 and 0.55 g/L.h for xylose and glucose, respectively. The simultaneous utilization of glucose and xylose is an important process in the use of lignocellulosic biomass for the reduction of the costs associated with microbial lipid production.

Table 4. 2 Cultivations of *L. starkeyi* using an inoculum concentration of 1 g/L and glucose:xylose at a ratio of 70:30.

Response	Study 1				Study 2		
	Feeding pulse				Feeding pulse		
	Batch	1st	2nd	3rd	5th	6th	8th
CDW (g/L)	22.1	30.2	36.1	42.1	45.5	61.9	51.6
Cell prod. (g/L·h)	0.325	0.328	0.314	0.292	0.623	0.659	0.423
Lipid (g/L)	6.1	8.9	10.3	15.5	8.0	18.3	14.0
Lipid prod. (g/L·h)	0.09	0.097	0.089	0.107	0.109	0.194	0.115
Lipid (%, w/w)	27.7	29.6	28.4	36.7	17.5	29.3	25.9
10^8 cells/mL	5.0	9.5	8.3	7.7	5.6	5.9	6.1
$Y_{X/S}$ (g CDW/g sugar)	0.258	0.235	0.23	0.219	0.727	0.573	0.328
$Y_{L/S}$ (g lipid/g sugar)	0.072	0.07	0.066	0.08	0.128	0.169	0.089
Time (h)	68	92	115	144	73	94	122

4.3.2. Study 2

The duration of this experiment was 122 hours, and eight feeding pulses was used (Table 4.2). The cell number remained equal to approximately $6x10^8$ cells/mL from 73 h to 122 h. The C/N ratio was approximately 800 from 68 h to 111 h. The highest cell and lipid concentrations (62 g/L and 18.3 g/L, respectively) were achieved at 94 h (6^{th} feeding pulse). The maximum $Y_{X/S}$ (0.444 g/g) and $Y_{L/S}$ (0.134 g/g) were also obtained at 94 h. Higher cell mass concentration and yields were obtained in this study compared with study 1, but a lower lipid concentration was observed. Sattur and Karanth (1989) highlighted the significance of the C/N ratio as a critical parameter for obtaining high cell mass and lipid coefficients.

4.3.3. Study 3

The results of our fed-batch process with six feeding pulses showed that the cell concentration and number reached their maximum values at 167 h (86.6 g/L and $8.7x10^8$

cells/mL, respectively) and remained constant from that time until the end of the cultivation at 216 h (Table 4.3). These results showed that long cultivation times are not interesting in these modes of cultivation. The lipid content reached 36% (31.3 g/L) before the third feeding (115 h), and this value did not change after this time point. Lower cell mass and lipid yields (0.331 and 0.112 g/g, respectively) were obtained compared with Study 2. This study showed higher xylose consumption rate of 0.72 g/L.h in relation to glucose with 0.45 g/L.h. The subsequent studies utilized a lower number of feeding pulses because no increases in the cell and lipid concentrations were achieved after the fourth feeding pulse.

Table 4. 3 Cultivation of *L. starkeyi* (study 3) using an inoculum concentration of 1 g/L and glucose:xylose at a ratio of 30:70.

Response	**Feeding pulse**						
	Batch	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**
CDW (g/L)	17.3	46.4	66.6	74.6	86.4	85.7	85.5
Cell prod. (g/L·h)	0.377	0.54	0.579	0.525	0.518	0.448	0.396
Lipid (g/L)	4.4	13.0	24.0	27.2	31.3	29.9	30.1
Lipid prod. (g/L·h)	0.095	0.152	0.208	0.192	0.187	0.156	0.139
Lipid (%, w/w)	25.3	28.1	36	36.5	36.2	34.9	35.2
10^8 cells/mL	4.4	8.5	8.7	8.5	8.8	8.5	8.5
$Y_{X/S}$ (g CDW/g sugar)	0.207	0.331	0.311	0.244	0.235	0.213	0.184
$Y_{L/S}$ (g lipid/g sugar)	0.052	0.093	0.112	0.089	0.085	0.074	0.065
Time (h)	46	86	115	142	167	191	216

4.3.4. Studies 4 and 5

High inoculum concentrations would dilute the rate-limiting substrate concentration in the medium, resulting in a lower specific growth rate. In contrast, a low inoculum

concentration would result in a long lag phase (KUN, 2006). To obtain a higher cell concentration and reduce the lag phase of growth, a batch culture using an inoculum concentration of 3 g/L (Study 5) instead of 1 g/L was studied. After 46 h, the cell concentration reached 32.4 g/L with a lipid content of 31.7% (10.3 g/L). The maximum specific growth rate (μ_{max}) was 0.06 h^{-1}. These results were much higher than those obtained for the batch stage (study 4, 1 g/L of inoculum, CDW = 17.3 g/L, 25.3% lipid content, μ_{max} = 0.04 h^{-1}). In addition, the yields of cell mass (0.600 g/g) and lipids (0.187 g/g) resulted three times higher compared with those obtained with study 4. These results demonstrate that the inoculum size has a high effect on the lipid biosynthesis and cell mass concentration (Fig. 4.1). The results obtained with an inoculum concentration of 3 g/L are markedly more attractive; therefore, the next cultivation experiments were performed using this inoculum concentration. Liu et al. (LIU *et al.*, 2012) studied the inoculum concentration of *L. starkeyi* and found that the microbial oil production is profoundly related to the this parameter. High inoculum concentrations increase the cell mass productivity.

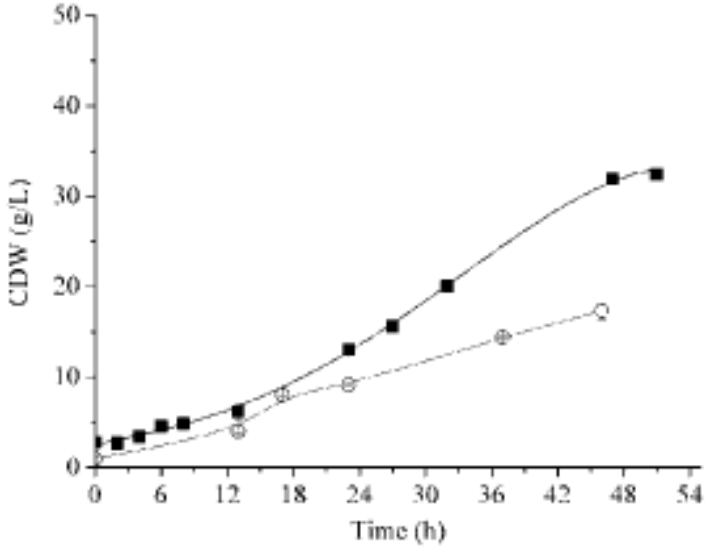

Figure 4. 1 Batch cultivations of Study 4 using inoculum concentration of 1 g/L (○) and Study 5 using 3 g/L (■).

4.3.5. Study 6

In this study, we investigated a fed-batch culture process with an inoculum concentration of 3 g/L using the same conditions as those used in Study 3. Three feeding pulses were performed with carbon and nitrogen sources, and the last pulse contained only sugars. After 138 h of cultivation, we obtained a CDW of 82.4 g/L and a lipid content of 38.6 g/L (46.9%). The cell mass and lipid yields reached 0.432 and 0.159 g/g, respectively (Table 4.4). The comparison of these results with those obtained in Study 3 revealed that higher cell mass and lipid yields and also higher productivities were obtained in this study after the fourth feeding pulse. The cell concentration was similar in both cultivations, but lipid content was 11% higher in Study 6. Taking into account the high lipid content, yields, and productivities, the feeding strategy used in Study 6 with an inoculum concentration of 3 g/L was selected for the subsequent studies.

4.3.7. Study 7

The repeat mode differs from the traditional fed-batch process because a portion of the culture containing lipid-rich cells from a cultivation is used to seed the next cultivation (ZHAO *et al.*, 2011). In our repeated fed-batch culture process, fresh medium with nitrogen sources and additional nutrients were supplied to restart the exponential cell propagation process, and the feeding pulses in the second cycle included only sugars. The conditions of the first cycle of this study were the same as those used in Study 6 and thus present similar results (Table 4.4). After the first cycle, i.e., at 138 h (Table 4.4), the bioreactor was partially discharged, and 50% of the cell broth was left in the bioreactor to serve as the inoculum. The second cycle was initiated through the addition of 500 mL of fresh medium. Overall, the cultivation lasted 237 h. The cell concentrations at the end of the first and second cycles were 80.4 and 85.4 g/L, respectively. The lipid content increased from 46.5% (37.4 g/L) to 49%

(41.8 g/L) in the second cycle. The process used in Study 7 achieved the highest lipid concentration of the different feeding strategies studied. The cell mass and lipid yields remained equal to approximately 0.210 g/g and 0.100 g/g, respectively, throughout the second cycle. The second cycle of this study produced the highest lipid concentrations compared with overall studies with a high cell concentration, but the cell and lipid productivities decreased after each cycle (Table 4.5). This cultivation mode is very interesting due to the decreased lag phase of the microorganism and the reduced preparation times between the cultivaions.

Table 4. 4 Cultivations of *L. starkeyi* using an inoculum concentration of 3 g/L and glucose:xylose at a ratio of 30:70.

Response	Study 6					Study 7								
	Feeding pulse					First cycle					Second cycle			
	Batch	1st	2nd	3rd	4th	Batch	1st	2nd	3rd	4th	5th	6th	7th	8th
CDW (g/L)	28.0	46.4	59.5	64.3	82.4	27.5	49.2	60.0	77.4	80.4	63.5	73.2	79.8	85.4
Cell prod. (g/L·h)	0.609	0.703	0.661	0.564	0.597	0.654	0.746	0.667	0.679	0.583	0.376	0.379	0.368	0.360
Lipid (g/L)	9.2	17.5	25.8	31.5	38.6	9.0	18.0	24.2	36.7	37.4	28.8	34.2	36.8	41.8
Lipid prod. (g/L·h)	0.200	0.265	0.286	0.276	0.280	0.214	0.273	0.269	0.322	0.271	0.170	0.177	0.170	0.176
Lipid (%, w/w)	32.8	37.7	43.3	49.0	46.9	32.8	36.6	40.3	49.9	46.5	45.3	46.8	46.1	49.0
10^8 cells/mL	6.1	7.5	7.2	9.8	10.1	6.5	3.2	8.3	10.9	10.2	8.6	9.9	10.1	10.2
$Y_{X/S}$ (g CDW/g sugar)	0.437	0.422	0.359	0.291	0.322	0.421	0.458	0.378	0.370	0.325	0.211	0.214	0.210	0.205
$Y_{L/S}$ (g lipid/g sugar)	0.143	0.159	0.156	0.143	0.151	0.138	0.168	0.152	0.176	0.151	0.096	0.100	0.097	0.100
Time (h)	46	66	90	114	138	42	66	90	114	138	169	193	217	237

4.3.7. Effects of dilution rate of cell growth and lipid production

The chemostat culture of *L. starkeyi* at a D of 0.03 h^{-1} (Study 8) resulted in cell mass and lipid concentrations of 13.3 g/L and 7.1 g/L (48.4%), respectively. The concentration of unconsumed sugars was approximately 30 g/L (Fig. 4.2A), which is equal 50% of the initial concentration. The cell mass yield was 0.443 g/g, whereas the lipid yield reached 0.236 g/g. In continuous culture, cell mass, lipid content, and lipid yield increase with decreasing growth rate. At a D value of 0.06 h^{-1} (Study 9), the cell mass reached 10 g/L (Fig. 4.2B) with a lipid content of 43.3% (4.33 g/L). The yields of cell mass and lipids were also lower than those obtained with a chemostat culture at 0.03 h^{-1} (0.333 g/g and 0.161 g/g, respectively). Shen et al. (2013) studied chemostat cultures ranged from 0.02 h^{-1} to 0.20 h^{-1} by *R. toruloides*. At the lowest D, 77% of glucose was consumed resulting in a cell growth of 8.67 g/L with 61.8% of lipids. At D of 0.20 h^{-1}, only 4% of glucose was consumed reaching a cell mass of 1.63 g/L and 13.2 % of lipids.

The kinetic profile obtained from a continuous cultivation with H-H was similar to that obtained from cultivation in a synthetic medium. At a D of 0.03 h^{-1} (Study 10), the cultivation yielded cell mass and lipid concentrations of 13.9 g/L (Fig. 4.3A) and 3.7 g/L (26.7%). The highest overall cell mass and lipid yields were 0.666 g/g and 0.215 g/g, respectively. Batch stage of H-H continuous cultivation resulted in a xylose and glucose consumption rates of 0.58 g/L.h and 0.16 g/L.h, respectively, highlighting the xylose assimilation ability from renewable sources of this oleaginous yeast.

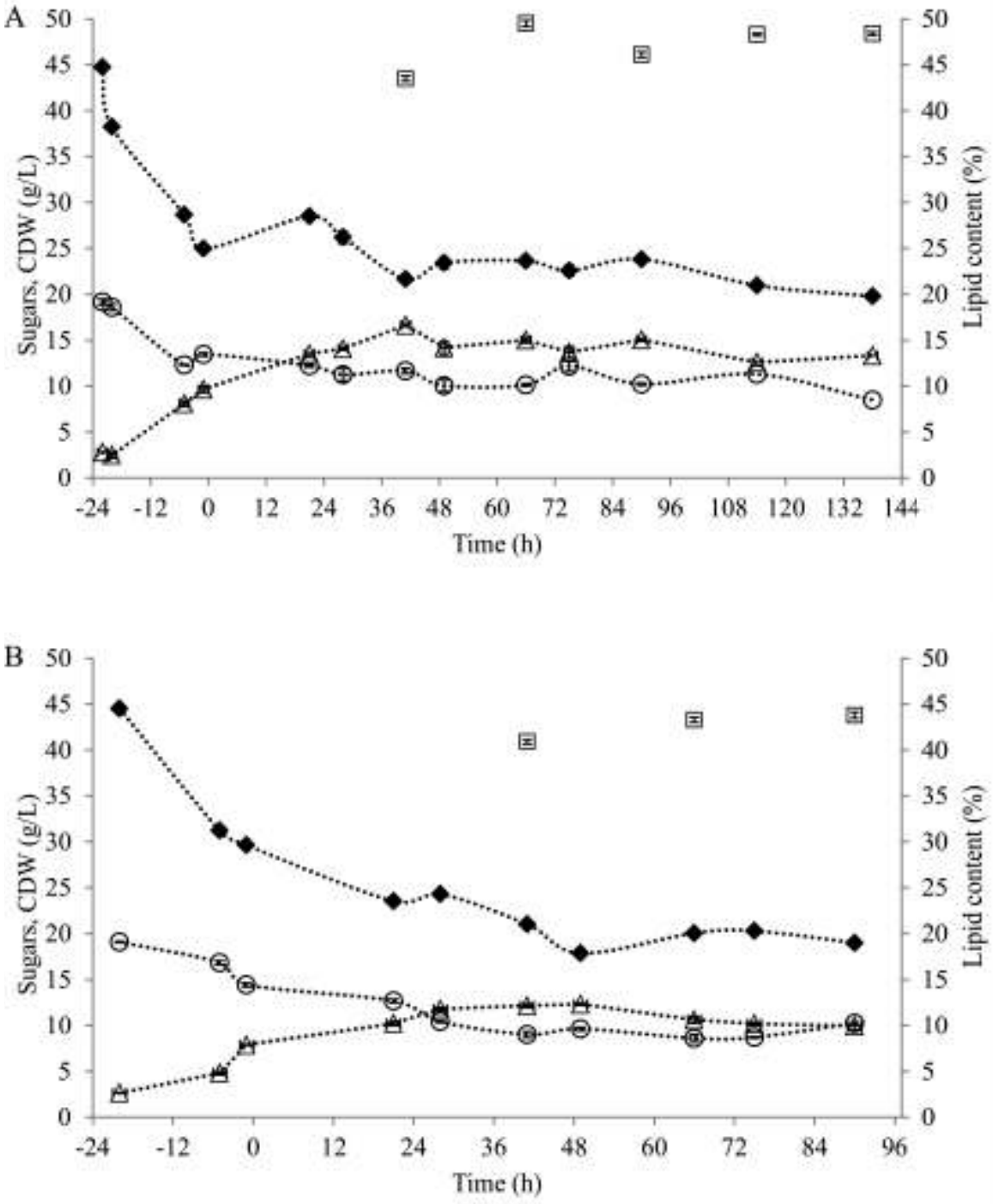

Figure 4. 2 Continuous cultivations at (A) 0.03 h^{-1} (Study 8) and (B) 0.06 h^{-1} (Study 9). The results show CDW (△), lipid content (□), xylose (♦) and glucose (○) concentrations. The continuous cultivation was started at 0 h after a batch stage (negative scale).

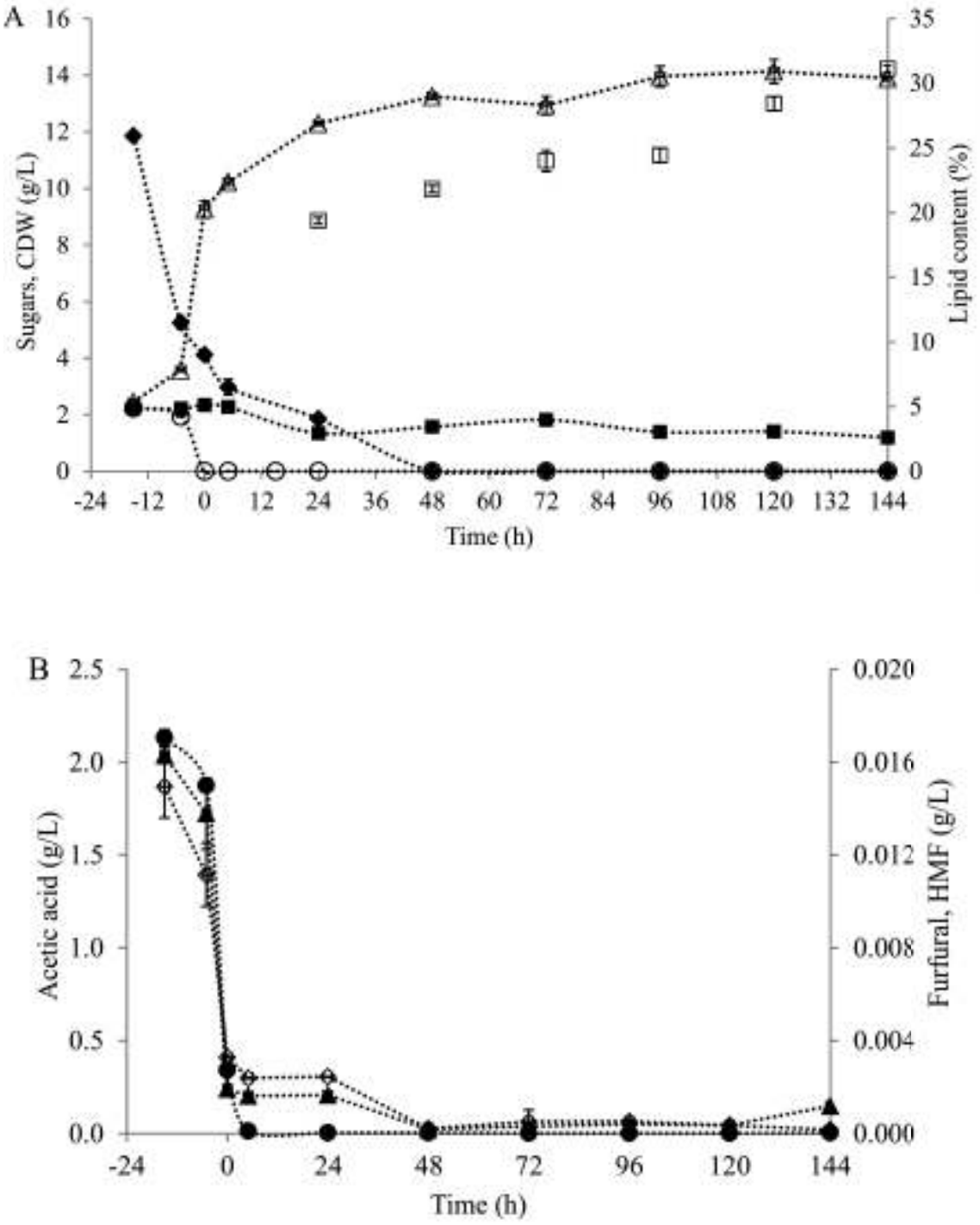

Figure 4. 3 Study 10: (A) CDW (△), lipid content (□), xylose (♦), glucose (○), arabinose (■), (B) acetic acid (●), furfural (▲) and HMF (◊) concentrations during continuous cultivation at 0.03 h^{-1} with H-H. The continuous cultivation was started at 0 h after a batch stage (negative scale).

Arabinose was not consumed throughout the cultivation. The inhibitor concentrations (acetic acid, furfural, and HMF) were reduced during the batch stage and the first residence times of the continuous cultivation, indicating that this yeast can consume these compounds (Fig. 4.3B). This is of particular interest because it is possible that *L. starkeyi* is highly tolerant to inhibitors, which may allow a broader study of H-H from sugarcane bagasse in fermentation processes.

The release of monosaccharides during hydrolysis is routinely accompanied by the generation of non-carbohydrate compounds, such as furfural and 5-hydroxymethylfurfural (HMF) from the dehydration of pentoses and hexoses, acetic acid from the acetyl group in hemicellulose, and phenolic compounds including syringaldehyde, p-hydroxybenzaldehyde, vanillin, etc. derived from lignin (HU *et al.*, 2009). These inhibitors affect the microbial metabolism by hindering the fermentative process, and their toxicity is the major limiting factor for the bioconversion of lignocelluloses materials (PALMQVIST and HAHN-HÄGERDAL, 2000).

At a D of 0.06 h^{-1}, xylose and glucose were directed to non-lipid cell mass production because this condition favored cell growth using consumed nitrogen. A D value lower than 0.06 h^{-1} is normally required for optimum conversions (HUANG *et al.*, 2009) because the microbial cells need to remain within the chemostat for at least 12–24 h to consume the available nitrogen and convert the remaining sugars to oil. Papanikolaou *et al.* (2002) described that higher concentrations of sugars are detected in the culture fluid at high dilution rates, which results in an increased cell mass yield but a decreased lipid fraction. At both dilution rates investigated in our study, the residual sugar remained at the same concentration (approximately 30 g/L), and the cell mass yield and lipid fractions were slightly higher at the lower dilution rate. Lipid-free material and lipid yields are influenced by the value of D. At higher D, sugars are consumed mainly for non-lipid material synthesis. Changes in these

yields as a function of D have been observed with other oleaginous yeasts grown in continuous cultures (see Table 5.5). The cell growth and the lipid and nutrient concentrations vary during batch and fed-batch cultivations. In continuous culture, under chemostat conditions, all of these parameters remain constant, and it is possible to isolate a single parameter and study its effect on lipid accumulation while maintaining the other physical and chemical parameters constant. Therefore, the results obtained with the continuous culture will be used further for metabolic flux analysis (MFA), which provides a highly informative view of the physiological cell status under a given environmental condition or genetic background.

4.3.8. Cell mass and lipid productivities

The production cost is the major limiting factor for a broader use of microbial lipids and can be significantly reduced by improvements in the productivity (ZHAO *et al.*, 2011). The optimization of various parameters, such as feeding strategy and media composition, in a process that uses a low-cost substrate, such as H-H, should lead to advancements in lipid productivity. Table 4.5 shows the results of the present and previous studies, allowing a comparison of the cell mass and lipid productivities of yeasts grown on various substrates under different cultivation conditions. Our batch studies led to higher cell mass and lipid productivities than those obtained by others authors using glucose and/or xylose as the carbon sources and different inoculum concentrations (WILD *et al.*, 2010; HU *et al.*, 2011; GALAFASSI *et al.*, 2012). Our fed-batch process using glucose:xylose (70:30) resulted in lower cell mass and lipid productivities than those obtained using only glucose for the growth of *L. starkeyi* (WILD *et al.*, 2010) and other oleaginous yeasts (LI, ZHAO and BAI, 2007; ZHANG *et al.*, 2011). However, the productivities obtained with a glucose:xylose ratio of 30:70 were similar to those obtained in previous study with a mutant strain of *L. starkeyi* (TAPIA *et al.*, 2012). Study 6 reached similar productivities compared with a fed-batch study that used glucose as the sole carbon source and an inoculum concentration of 1 g/L (ZHANG

et al., 2011). Study 7 also resulted in very satisfactory productivities with overall lipid productivities for the first and second cycle of 0.271 and 0.176 g/L·h, respectively.

The cell mass and lipid productivities remained constant during the second cycle. The lipid productivity decreased after each cycle, in accordance with the results of a repeated fed-batch study of lipid production by *Rhodosporidium toruloides* Y4 (ZHAO *et al.*, 2011). For continuous culture using glucose:xylose, the cell mass and lipid productivities were estimated to be 0.399 and 0.213 g/L·h, respectively, at a D of 0.03 h^{-1} (study 8), and 0.600 and 0.288 g/L·h, respectively, at a D of 0.06 h^{-1} (study 9). The continuous culture at a D of 0.03 h^{-1} using H-H obtained a higher cell mass productivity (0.417 g/L·h) but a lower lipid productivity (0.111 g/L·h) compared with study 8. Our studies resulted in higher productivities than those reported in the literature for other oleaginous yeasts using glucose as the carbon source (AGGELIS and KOMAITIS, 1999; SHEN *et al.*, 2013) (Table 4.5). Our results indicate that the feeding strategy has a high impact on lipid productivity and that continuous culture is the most appealing method for the enhancement of microbial lipid production using H-H as the substrate.

Table 4. 5 Productivity of oleaginous yeasts grown on xylose and/or glucose with different cultivation modes.

Cultivation mode and yeast	Carbon source (g/L)[a]	Working volume (L)	Inoculum concentration	Cell mass productivity[b] (g/L·h)	Lipid productivity[b] (g/L·h)	Reference
Batch						
R. graminis	G^{50}	0.8	0.2 OD_{660}	0.219	0.098	(GALAFASSI *et al.*, 2012)
R. graminis	$G^{25}X^{25}$	0.8	0.2 OD_{660}	0.215	0.101	(GALAFASSI *et al.*, 2012)
T. cutaneum	$G^{47}X^{23}$	2.0	10% (v/v)	0.275	0.160	(HU *et al.*, 2011)
L. starkeyi	G^{30}	1.0	4% (v/v)	0.560	0.133	(WILD *et al.*, 2010)
L. starkeyi	$G^{42}X^{18}$	1.0	1 g/L	0.376	0.095	Study 4
L. starkeyi	$G^{42}X^{18}$	1.0	3 g/L	0.704	0.224	Study 5
Fed-batch						
R. toruloides	G^{60}	7.0	10% (v/v)	0.794	0.540	(LI, ZHAO, and BAI, 2007)
C. curvatus	G^{60}	14.0	10% (v/v)	0.563	0.470	(ZHANG *et al.*, 2011)
L. starkeyi	G^{30}	1.0	4% (v/v)	0.560	0.151	(WILD *et al.*, 2010)
L. starkeyi	$G^{18}X^{42}$	1.0	1 g/L	0.600	0.350	(TAPIA *et al.*, 2012)
L. starkeyi	$G^{42}X^{18}$	1.0	1 g/L	0.292	0.107	Study 1
L. starkeyi	$G^{42}X^{18}$	1.0	1 g/L	0.423	0.115	Study 2
L. starkeyi	$G^{18}X^{42}$	1.0	1 g/L	0.396	0.139	Study 3
L. starkeyi	$G^{18}X^{42}$	1.2	3 g/L	0.597	0.280	Study 6

Table 4.5 (Continued)

Cultivation mode and yeast	Carbon source (g/L)[a]	Working volume (L)	Inoculum concentration	Cell mass productivity[b] (g/L·h)	Lipid productivity[b] (g/L·h)	Reference
Repeated fed-batch						
R. toruloides	G^{55}	9.0	10% (v/v)	0.283	0.172	(ZHAO *et al.*, 2011)
L. starkeyi	$G^{18}X^{42}$	1.2	3 g/L	0.360	0.176	Study 7
Continuous						
Y. lipolytica (0.032 h^{-1}[c])	G^{30}	1.3		0.297	0.074	(AGGELIS and KOMAITIS, 1999)
R. toruloides (0.06 h^{-1}[c])	G^{36}	1.85		0.286	0.135	(SHEN *et al.*, 2013)
C. curvata D (0.05 h^{-1})	X^{30}		2% (v/v)	0.750	0.280	(EVANS and RATLEDGE, 1983)
L. starkeyi (0.03 h^{-1})	$G^{18}X^{42}$	1.2	3 g/L	0.399	0.213	Study 8
L. starkeyi (0.06 h^{-1})	$G^{18}X^{42}$	1.2	3 g/L	0.600	0.288	Study 9
L. starkeyi (0.03 h^{-1})	H-H	0.5	3 g/L	0.417	0.111	Study 10

[a] Superscript numbers refers the concentration (g/L) of each sugar. Glucose, xylose, and arabinose are abbreviated as G, X, and A, respectively. The initial sugar concentrations are indicated by superscripts after the sugar abbreviations; [b] Productivity values from the end of the cultivations; [c] Unknown inoculum concentration.

4.3.9. Fatty acid profile and estimated cetane number

The lipids produced by different cultivations were transmethylated and analyzed by gas chromatography. The fatty acid profile showed slight changes between the different culture conditions (Table 4.6). The microbial lipids chains from L. starkeyi contain 14 to 18 carbons and exhibit low degrees of unsaturation, which is desirable for their application in biodiesel production. The lipids produced mainly include palmitic acid and oleic acid. Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) are the main fatty acids in the lipids produced by yeasts (LI, ZHAO and BAI, 2007; LIU and ZHAO, 2007; LIN et al., 2011; TAPIA *et al*., 2012). The fatty acid composition profile was quite similar to that of palm oil (LIU *et al.,* 2010).

Soap, hand and body lotions, fatty acid methyl esters, and epoxidized palm oil are the main industrial products that are produced from palm oil. This oil is also an excellent frying oil due to its non-sticky and non-foaming characteristics (NORHAIZAN *et al.*, 2013), indicating that the lipids produced by *L. starkeyi* have great potential as a feedstock for biodiesel production and other oleochemical industry applications. Table 6 shows the lipid profiles produced by *L. starkeyi* in the present studies (LIU and ZHAO, 2007; LIN *et al.*, 2011; TAPIA *et al.*, 2012).

Table 4. 6 Comparison of the fatty acid profiles obtained in the present and previous studies.

***Lipomyces starkeyi* lipid profiles (%, weight)[a]**								
Substrate	**Reference**	**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**	**Estimated Cetane Number**
Glucose	(LIU and ZHAO, 2007)	0.4	33.0	4.7	4.8	55.1	1.6	60.7
Glucose	(LIN *et al.*, 2011)	0.7	36.3	5.0	4.2	50.1	3.7	61.1
Gluc:xyl[b]	(TAPIA *et al.*, 2012)	0.4	33.9	7.5	3.4	50.6	3.9	61.0
Gluc:xyl[b]	Study 1	0.6	35.1	9.5	2.7	48.7	3.3	61.7
Gluc:xyl[b]	Study 2	0.3	33.9	8.9	2.6	51.1	2.7	61.3
Gluc:xyl[b]	Study 3	0.5	33.2	8.0	2.6	54.4	1.3	61.6
Gluc:xyl[b]	Study 6	n.d.	36.2	9.7	2.8	47.8	3.5	61.8
Gluc:xyl[b]	Study 7	0.4	38.9	14.0	2.1	42.5	2.0	63.1
Gluc:xyl[b]	Study 8	n.d.	33.5	16.1	n.d.	48.3	2.0	62.8
Gluc:xyl[b]	Study 9	n.d.	33.1	16.2	1.0	46.7	3.0	62.6
H-H	Study 10	n.d.	30.3	12.6	0.7	46.7	7.0	62.9
Palm oil lipid profile (%, weight)[a]								
Reference		**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**	**Estimated Cetane Number**
(LIU *et al.*, 2010)		1.3	50.4	4.7	0.1	33.5	8.2	61.4
(MALEKI, AROUA and SULAIMAN, 2013)		1.2	43.6	4.6	n.d.	40.5	10.1	61.2

[a]Peak areas less than 0.1% were considered insignificant and are presented as not detected (n.d.); [b] Gluc:xyl represents glucose:xylose; H-H represents hemicellulose hydrolysate.

The estimated cetane number of diesel, which is specified by ASTM D613, is a measure of its ignition delay time, and compared with fossil diesel, biodiesel has higher values. In Europe, the minimum acceptable cetane number for both diesel and biodiesel is set at 51 (EM 14214). The American standard quality (ASTMD 6751) sets the cetane numbers for diesel and biodiesel at 40 and 47, respectively. Brazil does not have a standard minimum cetane number for biodiesel (LOBO, FERREIRA and CRUZ, 2009). This number is relatively difficult to measure and has rarely been determined for vegetable oils and fatty acid esters (KRISNANGKURA, 1986). The cetane number of biodiesel increases with an increase in the weight percentage of saturated FAMEs and longer-chain FAMEs (KNOTHE, 2008; SHARMA, SINGH and UPADHYAY, 2008). Using the equation developed by Klopfenstein (KLOPFENSTEIN, 1985), we estimated the cetane index of fatty acid esters produced in this study to be approximately 61, which is similar to the value obtained for palm oil (Table 4.6). Thus, the biodiesel produced from microbial lipids by *L. starkeyi* DSM 70296 meets international specifications for fuel.

The development of inexpensive triacylglycerol lipids will release the pressure on the ever-increasing prices of vegetable oils (first-generation biodiesel) in the future (HE *et al.*, 2010). One of the main challenges in the production of second-generation biodiesel is the improvement in the use of non-food materials as nutrients that can be converted by microorganisms into lipids. The use of lignocellulosic biomass appears to be a better strategy for the cost-effective preparation of lipids on a large scale due to its low cost and wide availability in nature. This work demonstrates the possibility of using *L. starkeyi* DSM 70296 for the production of cell mass rich in lipids from xylose and glucose (30:70, w/w), which simulates the hemicellulose hydrolysate of sugarcane bagasse, and from H-H, which contains xylose, glucose, and inhibitors in the cultivation medium. The identification of microorganisms that can utilize both glucose and xylose simultaneously and efficiently

appears to be a key aspect of the utilization of a lignocellulosic feedstock, and the elucidation of the mechanism underlying the assimilation of these sugars is crucial for the further development of this process. This extensive preliminary information on the TAG accumulation and fatty acid profiles using H-H by *L. starkeyi*, indicates that it can be used for oleochemicals including biofuels, platform chemicals, and nutritional oils. Further investigations are needed to study the ability to accumulate lipids under industrially relevant conditions.

4.4. Conclusion

This study investigated the cell mass lipid-rich production by *L. starkeyi* using different feeding strategies. High cell mass (~85 g/L) and lipid (~40 g/L) concentrations were achieved through both fed-batch and repeated fed-batch cultivations using glucose:xylose (30:70). Continuous cultivations at 0.03 h^{-1} using glucose:xylose and H-H presented the highest yields and productivities, indicating that this approach is the most appropriate. To the best of our knowledge, this study provides the first demonstration of the use of *L. starkeyi* for microbial oil production in continuous culture with H-H, indicating the possibility of transformation of lignocellulosic materials into biodiesel in the future.

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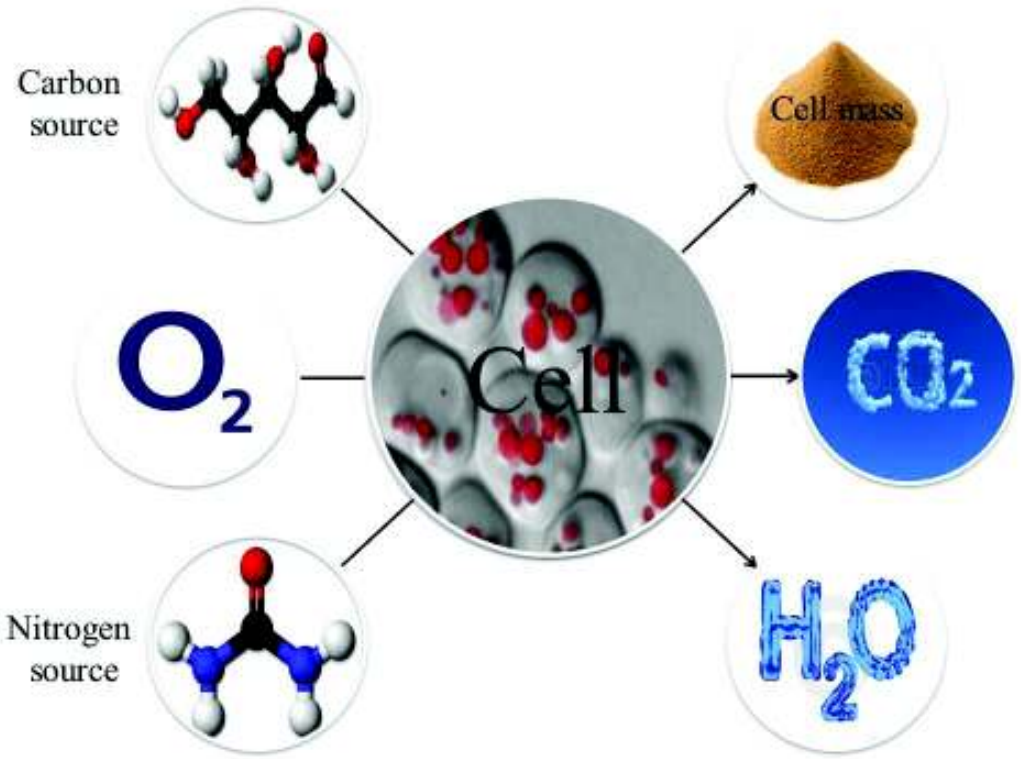
CHAPTER 5. Mass and energy balances for analysis of oleaginous yeast growth



"It is only because of problems that we grow mentally and spiritually."

Morgan Scott Peck

Mass and energy balances for analysis of oleaginous yeast growth

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SUMMARY

Energy and mass efficiency studies are very important for microbial biodiesel production. The estimation of energy capacity of microbial cell mass on the basis of its lipid content and elemental composition can be applied for the comparative evaluation of different microbial sources of biodiesel. Generally, the energy contents of microbial cells could be assumed to be constant, however, not in the case of oleaginous yeasts. The approach used in this work makes it possible to determine the energy value of cell mass by its elemental composition without application of laborious and expensive calorimetric measurements of combustion heats (Q_c). A Q_c value 25.7 (kJ/g) obtained after 142 h of fed-batch cultivation, which represents approximately 56% of the energy content of diesel oil (45.4 kJ/g), indicating the potential of *L. starkeyi* for biodiesel. *L. starkeyi* is an efficient "energy-converter" in lipid production with xylose as substrate. Our estimated results for cell mass energy yield (η) and Q_c of the yeast oil should be valuable to address the overall techno-economic analysis of bioenergy production.

5.1. Introduction

The application of mass and energy balances have not been fully explored in fermentation processing due to the complexity of cell growth and product formation. In some cases, material and energy balances were only roughly estimated in growth processes (ZHOU *et al.*, 2012). Precise energy characteristics for growth efficiency and product formation are important, including cell mass energy yield (η) and combustion heat (Q_c) of cell mass. From the viewpoint of applications, the biomass of oleaginous yeasts can be considered as a possible king of biodiesel fuel (MINKEVICH, DEDYUKHINA and CHISTYAKOVA, 2010).

Material and energy balances for fermentation processes have been developed based on that the reaction heat per electron transferred to oxygen, the number of available electrons per carbon atom in the cell, and the carbon weight fraction were relatively constant (ERICKSON, MINKEVICH and EROSHIN, 1978).

In order to calculate η, a precise elemental composition of the microbial cell is necessary. Combustion heat of cell mass (Q_c) indicates the energy content of the microbial cells intuitively and accurately, which is the most essential factor for the selection of promising biodiesel sources (MINKEVICH, DEDYUKHINA and CHISTYAKOVA, 2010). Nevertheless, a bomb-calorimeter is needed for the analysis of Q_c of the cell mass, which is not available in many laboratories (DI *et al.*, 2004). Another method to obtain Q_c is based on an equation involving elemental composition of cell mass as variables (CORDIER *et al.*, 1987; AKDENIZ and GUNDOGDU, 2007), which makes energy analysis of fermentation more convenient.

Mass and energy balances of lipid cultivation by the oleaginous yeast *Lipomyces starkeyi* were investigated with xylose as sole carbon source. The elemental composition of freeze-dried cells was determined in order to estimate the cel mass energetic yield (η). The reductance degree (γ) of xylose was calculated and the cell mass energetic yield (η) is the ratio of the heat produced by oxidation of the cell mass to that of the substrate used, where the oxidation results in the production of CO_2, H_2O and NH_3. The present work examined the elemental composition and reductivity of dry cell mass of *Lipomyces starkeyi* with the aim of finding possible constancies and strict interrelations between these characteristics and the measured lipid content in the cell mass. Correlation between the lipid content and the combustion heat of dry biomass is also the subject of this work.

5.2. Fundamentals of mass and energy balances

Elemental balance equation of microbial growth may be written,

$$C_W H_x O_Y N_Z + aO_2 + bH_g O_h N_i \rightarrow cCH_\alpha O_\beta N_\delta + dCO_2 + eH_2O + fC_j H_K O_l N_m \quad (5.1)$$

where $C_WH_xO_YN_Z$, $H_gO_hN_i$, $CH_\alpha O_\beta N_\delta$ and $fC_jH_KO_lN_m$ denote the elemental composition of substrate, nitrogen, cell mass and product, respectively. For oleaginous yeast, the product (lipids) is inside the cells, therefore $CH_\alpha O_\beta N_\delta$ is related to the cell mass plus lipis and $fC_jH_KO_lN_m$ is not used in this study. A carbon balance in Eq. (5.1) may be written

$$1 = c + d + f \quad (5.2)$$

where "c" is cell mass carbon yield and "f" is product carbon yield.

The balance of both parts of equation (5.1) with respect to the available electrons results in

$$\gamma_S + a(-4) = c\,\gamma_B + f\,\gamma_P \quad (5.3)$$

where $\gamma_S = 4 + x - 2y$

$\gamma_B = 4 + \alpha - 2\beta - 3\delta$

$\gamma_P = 4j + K - 2J - 3m$

Reductance degree (γ), is the number of equivalents of available electrons/g atom carbon based on carbon (4), hydrogen (1), oxygen (–2), and nitrogen (–3). A value of "–3" is used for nitrogen due the reductance degree of nitrogen in cell mass and ammonia is "–3". Eq. (5.3) may be written in another form

$$4a/\gamma_S = c\,(\gamma_B/\gamma_S) + f\,(\gamma_P/\gamma_S) = 1 \quad (5.4)$$

Due the heat evolved per equivalent of available equivalent (PAN and RHEE, 1985), eq. (5.4) may be used as an energy balance which leads to

$$\varepsilon + \eta + \xi_P = 1 \quad (5.5)$$

where

$$\varepsilon = 4a/\gamma_S \quad (5.6)$$

$$\eta = c(\gamma_B/\gamma_S) \quad (5.7)$$

$$\xi_P = f(\gamma_P/\gamma_S) \quad (5.8)$$

The term ε, is a fraction of the available electrons in substrate transferred to oxygen and then evolved as heat, η is a fraction of the available electrons in substrate (i.e. energy) transferred to cell mass, and ξ_P gives a fraction of the substrate energy incorporated into extracellular product(s). Equation (5.2) and (5.5) may be used to check the data consistency in continuous

culture in a way to see whether sum of three terms on the left hand side of the equations gives unity. η is termed as energetic yield for cell mass formation (MINKEVICH and EROSHIN, 1973). η may be correlated with cell mass yield, $Y_{X/S}$ as follows:

$$\eta = (\sigma_B \gamma_B / \sigma_S \gamma_S).Y_{X/S} \qquad (5.9)$$

where σ_S and σ_B are the weight fractions of carbon in the organic substrate and cell mass, respectively. Heijnen and Roels (1981) reported that the average values of γ_B and σ_B calculated from the data available in the literature were 4.17 and 0.489, respectively and the coefficient of variation is less than 5% for both γ_B, and σ_B. Therefore, γ_B and σ_B may be assumed to be constant for non-oleaginous microorganisms, whereas the assumption is not valid for oleaginous microorganisms, due thermodynamic energy content of the oleaginous microorganisms increases with accumulation of the intracellular lipids (EROSHIN and KRYLOVA, 1983). For most of the microorganisms, Eq. (5.9) becomes

$$\eta = (2.0/ \sigma_S \gamma_S).Y_{X/S} \qquad (5.10)$$

According to the thermodynamic analysis of microbial growth (PATEL and ERICKSON, 1981), phosphorous was not considered in this work. The reductance degree (γ) of organic substance with a chemical formula $C_5H_{10}O_5$ (xylose) was calculated based on Eq. (5.3). The biomass energetic yield (η) was the ratio of the heat produced by oxidation of the biomass to that of the substrate utilized in the fermentation process, where the oxidation results in the production of CO_2, H_2O and NH_3. According to Erickson et al. (1978) microbial growth energetic yield was defined in Eq. (5.9).

Combustion heat (kJ/g) of cell mass could be determined not only by bomb-calorimetric, but also by calculation based on the elements composition of the cells according to Eq. (5.11), based on Dulong's formula and usually gives the theorical higher heating values within 5% error (MEIER, LARIMER and FAIX, 1986):

$$Q_c = 33.5\ (C\%) + 142.3\ (H\%) - 15.4\ (O\%) - 14.5\ (N\%) \qquad (5.11)$$

where C, H, O, N are the weight of corresponding element in per g cell mass. The relationship between lipid content and Qc of the cells could also be simulated. This approach is applied here for the determination of the energy value of cell biomass by its elemental composition without utilization of laborious and expensive calorimetric measurement of combustion heats.

5.3. Experimental Study

5.3.1. Organism, media and chemicals

The oleaginous yeast *L. starkeyi* DSM 70296 was used throughout this study. Agar slants (YPD medium) were stored in a refrigerator and propagated monthly or stored through cryopreservation at -80ºC with 10% (v/v) glycerol. The composition of the inoculum and cultivation medium was (per liter): 60 g of xylose, 1.03 g of urea, 1 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.4 g of $Mg_2SO_4{\cdot}7H_2O$, 0.04 g of $CaCl_2{\cdot}2H_2O$, 0.08 g of $ZnSO_4.7H_2O$, 0.001 g of $CuSO_4.5H_2O$, 0.001 g of $CoCL_2.6H_2O$, and 0.005 g of $(NH_4)_2Mo_2O_7$ (pH 5.5). The carbon-to-nitrogen (C/N) ratio was fixed at 50. To avoid medium darkening due to the reaction of the sugars (caramelization and Maillard reactions), the xylose solution was sterilized in separate flask. All of the chemicals and reagents used were of analytical grade. The inoculum was prepared through two successive cell propagations in liquid media at 28ºC and 150 rpm in an orbital shaker. The first was incubated for 48 h, and the second was incubated for 30 h until a CDW of 10 g/L (equivalent to $1x10^8$ cells/mL) was obtained.

Experiments were carried out in a 3.0 L bioreactor (BioFlo/CelliGen 115, New Brunswick Scientific, USA) with an initial working volume of 1.2 L, 1 vvm of aeration, 28ºC and 400 rpm of agitation. The pH was maintained at 5.5 through the automatic addition of 2 M NaOH. Aliquots were collected at various intervals and stored at -20°C until their analysis. Concentrated xylose solution (600 g/L) was used for the fed-batch mode, and the concentration of the nutrient solution (urea and salts) was tenfold higher than that of the culture medium. Fed−batch culture was carried out with three feeding pulses, using a peristaltic pump, to increase the sugar concentration to 60 g/L immediately after the residual sugar concentration was dropped to 0-5 g/L.

5.3.1. Analytical Methods

Cell optical density was measured at 600 nm with a Nanophotometer (Implen GmbH, Munich, Germany). Cell dry weight (CDW) was determined after lyophiliation of the harvest cells (KAVADIA *et al.*, 2001). The freeze-dried cells were quantified using broth samples in triplicates for each time. Cellular concentration was determined by using the Neubauer−improved

chamber. The number of non-viable cells (death number) was obtained through measurement with methylene blue (LEE, ROBINSON and WANG, 1981).

Xylose concentration was measured using an Ion chromatography system (Metrohm, Herisau, Switzerland) (WALFORD, 2002). During the cultivations, total reducing sugars were assayed as described by Somogyi−Nelson (NELSON, 1944; SOMOGYI, 1945).

The nitrogen content was derived through balancing the urea feed and its outflow in the supernatant of the broth. Urea measurements were performed using an enzymatic colorimetric method. In short, urea in the sample was hydrolyzed enzymatically into ammonia (NH_4^+) and carbon dioxide (CO_2). Ammonia ions formed reacts with salicylate and hypochloride (NaClO), in presence of nitroprusside, to form a green indophenol. The absorbance (580 nm) of samples and calibrator were reading against a blank.

To provide better lipid extraction efficiency, a previous acid treatment was performed by adding hydrochloric acid (2M) and incubating at 80ºC for 1 hour, after that the solution was centrifuged (6000 G, 4ºC, 15 min) (GARZÓN, 2009). The lipids were gravimetrically quantified, from this wet digested pellet, by Bligh-Dyer's method (BLIGH and DYER 1959; MANIRAKIZA, COVACI and SCHEPENS, 2001). The elemental composition of the freeze-dried cells was determined using a Perkin Elmer – Series II 2400 analyzer (PerkinElmer, Inc., USA). Results for Q_c and the elemental composition were all present in terms of ash and water free cells. The ash content was determined by heating to 600ºC for 6h (ZHOU *et al.*, 2012).

5.4. Data Analysis and Discussion

5.4.1. Cell mass energetic yield of lipid cultivation process

Figure 5.1 shows the results of xylose and urea consumption, cell growth and lipid content. At the end of the fcultivation, cell mass concentration reached to 94.6 g/L with 37.4% of lipids. Maximum cell mass yield ($Y_{X/S}$) and lipid yield ($Y_{L/S}$) were achieved at 70 h of cultivation, 0.644 and 0.173 g/g, respectively, highlighting the potential lipid production by *L. starkeyi* (Table 5.1).

Results of elemental analysis of cell mass from the fed−batch culture were summarized in Table 5.1. Generally, N and O contents continuously declined, while C and H contents increased upon lipid accumulation (ZHOU *et al.*, 2012). This was in accordance with the evaluation profile of cellular lipid content. At 31 h, C and N content were 45.4 and 4.4%, respectively, similar to

those of conventional microbial cell mass (CORDIER *et al.*, 1987). Figure 5.2 shows the increase of weight fraction of carbon in the cell mass (from 0.454 to 0.55) as a function of lipid content.

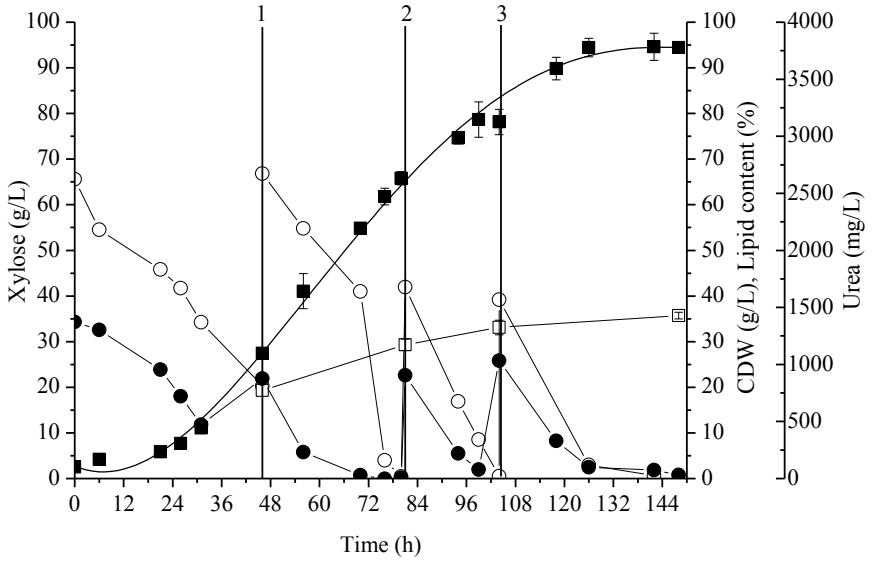

Figure 5. 1 Fed−batch cultivation by *L. starkeyi*: (■) CDW, (□) lipids, (○) xylose, (●) urea. Vertical lines represents the time of the feeding pulses.

Cell growth energetic yield (η) was estimated according to Eq. (5.9) and presented in Table 5.1. Compared with $Y_{X/S}$, η helped to distinguish microbial growth on substrates with different energy contents by considering the energy content of substrates in calculation. Our data showed (Table 5.1) that at 70 h the η value of *L. starkeyi* was higher (0.85) than the maximum value of 0.7 proposed for heterotrophic microbial growth (ERICKSON, MINKEVICH and EROSHIN, 1978). That probably resulted from additional energy produced and stored as lipid. Pan and Rhee (1986) collected data from 21 sources and showed that the energetic yield (η) reached mostly from 0.6 to 0.8. Eroshin and Krylova (1983) reported η values for *L. Starkeyi* strains from 0.15 to 0.31 grown on ethanol.

Table 5. 1 Results of fed-batch cultivation by *L. starkeyi* in bioreactor.

Time	$Y_{L/X}$	$Y_{X/S}$	Cell mass Elemental Composition				Chemical	γ_S	γ_B	η^a	η^b	Re[c]	Q_c^d	Q_c^e	Re[f]
(h)	(g/g)	(g/g)	C (%)	H (%)	O (%)	N (%)	formula					(%)	(kJ/Kg)	(kJ/Kg)	(%)
31	0.179	0.354	45.4	7.0	38.0	4.4	$CH_{1.85}O_{0.63}N_{0.08}$	4	4.35	0.44	0.43	2.3	18.7	18.1	3.2
46	0.193	0.607	47.1	7.0	37.8	2.9	$CH_{1.78}O_{0.60}N_{0.05}$	4	4.42	0.73	0.69	5.5	19.5	18.6	4.6
70	0.268	0.771	47.5	7.0	38.3	2.0	$CH_{1.77}O_{0.60}N_{0.04}$	4	4.45	0.85	0.91	-5.1	19.7	21.1	-5.1
80	0.293	0.478	49.5	7.2	36.2	1.9	$CH_{1.75}O_{0.55}N_{0.03}$	4	4.55	0.61	0.64	-4.9	21.0	22.0	-4.8
94	0.314	0.459	51.0	7.4	34.4	2.0	$CH_{1.74}O_{0.51}N_{0.03}$	4	4.63	0.62	0.64	-3.2	22.0	22.7	-3.2
104	0.331	0.383	51.0	7.8	34.2	1.8	$CH_{1.84}O_{0.50}N_{0.03}$	4	4.74	0.54	0.56	-3.7	22.7	23.3	-2.6
126	0.357	0.393	53.3	8.8	31.0	1.7	$CH_{1.98}O_{0.44}N_{0.03}$	4	5.03	0.62	0.60	3.2	25.4	24.1	5.1
148	0.374	0.339	55.0	8.5	29.7	1.6	$CH_{1.85}O_{0.41}N_{0.02}$	4	4.97	0.55	0.53	3.6	25.7	24.7	3.9

[a] Obtained from Eq. 5.9; [b] Obtained from Eq. 5.13; [c] Relative error between η^a and η^b; [d] Obtained from Eq. 5.11; [e] Obtained from Eq. 5.14; [f] Relative error between Q_c^d e and Q_c^e.

Through the above analysis, it was observed that *L. starkeyi* convert energy from xylose into biomass with high efficiency. Cell mass yields of the oleaginous yeasts were consistently lower than that of non-oleaginous microorganisms, whereas their energetic yields were higher (ZHOU *et al.*, 2012). Although Eq. (5.9) is very useful, a reliable elemental data was very important to calculate $\sigma_B\gamma_B$, and thus to acquire η value.

Lipids contain more energy than proteins or carbohydrates. In addition, oleaginous microorganisms can store more lipids than other compound. Energy content ($\sigma_B\gamma_B$), that requires the elemental composition data, can be estimated by lipid content. When $\sigma_B\gamma_B$ is plotted with lipid content data from fed-batch cultivation, a straight line was obtained. Figure 5.2 shows that the energy content ($\sigma_B\gamma_B$) of our oleaginous yeast increases with the accumulation of intracellular lipids. Square correlation between lipid content, L (%), and $\sigma_B\gamma_B$ gives

$$\sigma_B\gamma_B = 0.036L + 1.2868 \qquad (5.12)$$

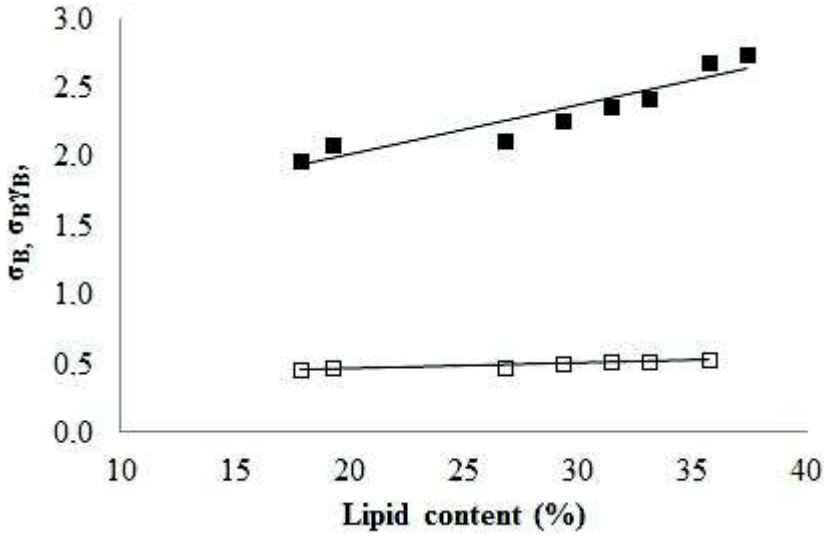

Figure 5. 2 Change of energy content (■) and weight fraction of carbon in the yeast cell (□) as a function of lipid content.

According to the linear relationship in Figure 5.2 and Eq. 5.9, cell growth energetic yield (η) could also be written as equation 5.13. The relative error between η obtained from Eq. 5.9 or Eq. 5.13 was less than 6%. Thus, energy efficiency could be obtained based on these experimental data, while elemental composition of cell mass was unnecessary. If the energy content ($\sigma_B\gamma_B$) of substrates utilized by oleaginous microorganisms is determined, η of the

biomass could also be estimated, which helped to choose the optimum cultural media for economic production of lipid (ZHOU *et al.*, 2012). According Eq. 5.13, overall energy efficiency of the lipid production shown in Figure 5.1 was 0.53, similar to that based on Eq. 5.2.

$$\eta = \frac{0.036L + 1.2868}{\gamma_S \sigma_S} Y_{X/S} \qquad (5.13)$$

Eroshin and Krylova (1983) demonstrated that there was a good linear relationship between $\sigma_B\gamma_B$ and cellular lipid content for oleaginous yeast growth on ethanol, with the equation $\sigma_B\gamma_B = 0.0278L + 1.5655$. Pan and Rhee (1985) achieved a linear relationship ($\sigma_B\gamma_B = 0.0308L + 1.6$) valid for glucose grown oleaginous yeasts, although it was derived from ethanol grown oleaginous yeasts.

5.4.2. Combustion heat of cell mass

Using elemental data, the combustion heat (Q_c) of yeast cells was estimated according to Eq. 5.11 (Table 5.1). Equation 5.11 was based on Dulong's formula and usually gives the Q_c within 5% error (MEIER, LARIMER and FAIX, 1986). Akdeniz and Gündoğdu (2007) obtained experimentally and calculated Q_c of 17.89 kJ/g and 17.09 kJ/g, respectively using equation 5.11, resulting in 4.47% of difference between these two values.

Alternatively, the combustion energy of cell mass could be determined using oxygen-bomb calorimeter. The Q_c value 25.7 kJ/g (using cell mass elemental data), obtained after 142 h of cultivation, up to 56% of the energy content of diesel oil (45.4 kJ/g) indicated the potential of cell mass of oleaginous yeasts used as biodiesel fuel. Q_c obtained from Eq. 11 or 14, resulted in less than 5% of difference, highlighting the linear relationship of Q_c with the lipid content. Thus, Q_c could be obtained based on lipid experimental data. Our result was lower than that obtained in Minkevich et al. (MINKEVICH, DEDYUKHINA and CHISTYAKOVA, 2010) for *L. starkeyi*. These authors achieved 39 kJ/g using ethanol as sole carbon source. When Q_c was plotted with lipid content, Eq. 5.14 was developed to estimate Q_c of cell mass based on lipid content.

$$Q_c = 0.3393L + 12.032 \qquad (5.14)$$

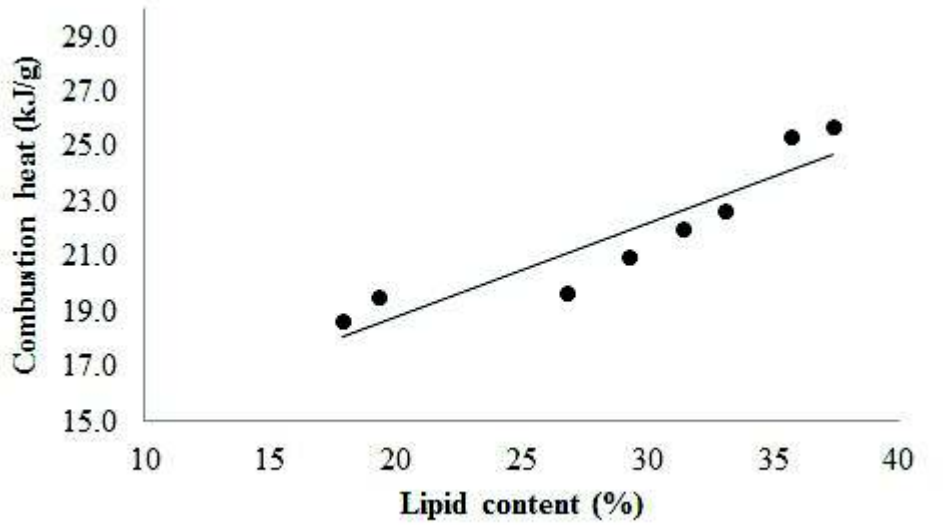

Figure 5. 3 Correlation between the energy content of cell mass (σBγB) and lipid content (%). Data were obtained from lipid cultivation results shown in Figure 5.1 and Table 5.1.

The Qc calculated by Eq. 5.14 fit the experimental data quite well according to the Figure 5.3. Nevertheless, when Eq. 5.13 established by our data was utilized to estimate the η of *L. starkeyi*, similar values were obtained with those calculated based on elemental composition with the relative errors less than 5.5%. Similar situation happened for comparing the Qc estimated by Eq. 5.14 and that based on Eq. 5.11 (5.1%).

These data are informative to understand the growth and lipid accumulation by *L. starkeyi* using xylose and urea as substrates and should be very helpful to develop and design a more efficient bioprocess for microbial lipid production. As a result, Eqs. 5.13 and 5.14 could be exploited to investigate efficiency of energy conversion of other microbial growth processes cultured in media with different energy content, such as fungus and microalgaes for lipid production. It would be useful for understanding the process for lipid fermentation.

5.5. Conclusions

This preliminary study provided the analysis on cell mass yields and energetic yields by *L. starkeyi* studying the relationship between cell mass energetic yield coefficient and combustion heat with the lipid content. Mass and energy balance method could be applied to predict the cell mass yield from xylose in fed-batch cultivation. Our estimated results for η and Q_c give simple

and efficient tools of the cell mass characteristics to address it as feedstock for bioenergy and oleochemicals production.

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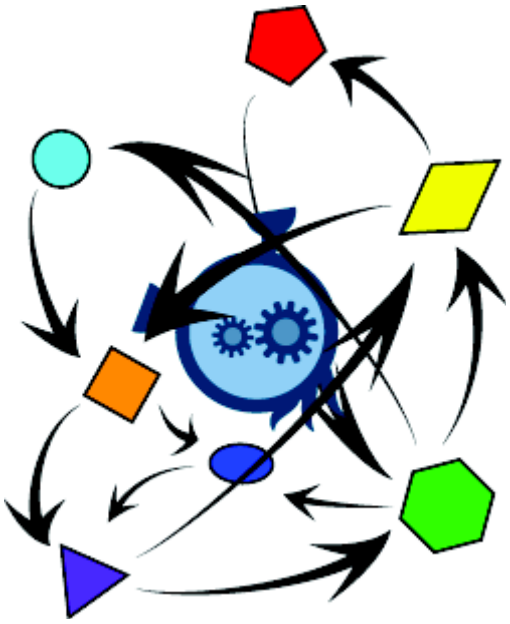
CHAPTER 6. Continuous cultivation of *Lipomyces starkeyi*: cell mass data reconciliation and extracellular metabolite balances



"You have to do your own growing no matter how tall your grandfather was."

Abraham Lincoln

Continuous cultivation of *Lipomyces starkeyi*: cell mass data reconciliation and extracellular metabolite balances

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Article to be submitted.

Abstract:

Continuous cultivations of *L. starkeyi* were carried out to attain parameters for understanding the process of lipid accumulation. Cell mass composition of *L. starkeyi* grown in chemostat under glucose, xylose and glucose + xylose limitation was analyzed for elemental and macromolecular composition. Statistical reconciliation method, based on elemental balances and equality relations, was used to obtain a consistent cell masscomposition and also a carbon balance of the cultivations. The results showed that continuous culture is a powerful tool to investigate the cell growth kinetics and physiological behaviors of this oleaginous yeast, providing consistent basis for metabolic flux analysis modeling approaches. *L. starkeyi* could also convert energy from xylose:glucose into cell mass with high efficiency, which is an important aspect for the utilization of lignocellulosic biomass for microbial lipid production and its conversion to biofuel.

Keywords: chemostat cultures, glucose + xylose utilization, statistical reconciliation method, mass balances, cell mass energetic yield.

6.1. Introduction

To make microbial lipid-based biofuel production sustainable, lignocellulosic material has to be used as inexpensive and abundant carbon source. Economically viable production of microbial lipid requires efficient utilization of glucose and xylose, the most two abundant sugars in lignocellulose (TILMAN *et al.*, 2009). *Lipomyces starkeyi* is an attractive yeast interesting because it can accumulate over 50% of its dry cell mass as lipid and utilizes both five and six carbon carbohydrates, which are present in plant biomass hydrolysates (TAPIA *et al.*, 2012; ANSCHAU *et al.*, 2014).

Understanding the growth characteristics of microorganisms is an essential step in bioprocessing, not only because product formation may be growth associated but also because they might influence cell physiology and thereby product quality (SCHULER and MARISON, 2012). In continuous culture, under chemostat conditions, all parameters remain constant, and it is possible to isolate a single parameter effect on lipid accumulation while maintaining constant other physical and chemical parameters (GANUZA and IZQUIERDO, 2007).

Knowledge about the biopolymers and macromolecules composition of the cells is essential for a metabolic and energetic analysis of the cell mass growth (DUBOC, PH. *et al.*, 1995). Cell mass is commonly described as consisting of five groups of macromolecules: proteins, carbohydrates, lipids, RNA, and DNA. Together with water and metals these components give the molecular composition of the cell mass (LANGE and HEIJNEN, 2001). Determination of cell mass molecular composition usually relies on application of different analytical techniques for the range of biochemical compounds considered, each one with its own sensitivity, interferences and confidence level (CARNICER *et al.*, 2009).

When working with cellular systems, the conservation equations required for data reconciliation can be derived from mass and energy balances using metabolic stoichiometry

(SCHULER, 2012). The statistical reconciliation uses statistical tools to improve the experimental data adapting them better to the mass balances and reducing the experimental errors (STEPHANOPOULOS, ARISTIDOU and NIELSEN, 1998). Application of numerical techniques for metabolic flux analysis requires the quantitative determination of a consistent cell mass composition and confidence intervals for all the elements and components have to be calculated (LANGE and HEIJNEN, 2001). Applications of data reconciliation to steady state processes have been widely recognized (COONEY, WANG and WANG, 1977; SODERSTROM *et al.*, 2000; COONEY, WANG and WANG, 2006), improving confidence in measurements, fault identification, higher level production planning and optimization, and steady-state identification (ALBERS, 1997).

Examples of applications of data reconciliation in industrial bioprocesses are rather sparse. Perhaps the required pre-conditioning of the data or the need of appropriate computing tools for data acquisition, computation and data distribution are lacking or implementation is hindered by the difficulties in choosing a suitable model and adequate simplifications for the system (SODERSTROM *et al.*, 2000). However, when used appropriately, data reconciliation can turn out to be an excellent basis for enhanced process understanding and for the development of control strategies and a powerful tool for decision-based bioprocessing through the creation of a consistent and reliable data set (HERWIG, 2010; SELVARASU *et al.*, 2010).

In this study, a consistent description of dry cell mass composition of *L. starkeyi* has been obtained at continuous cultivation with xylose and glucose at different dilution rates. The reconciled measurements give a consistent description of the cell mass with greatly reduced error margins for its elemental and macromolecular content.

6.2. Materials and methods

6.2.1. Strain and media

The oleaginous yeast *L. starkeyi* DSM 70296 was used throughout this study. Agar slants (YPD medium) were stored in refrigeration and propagated monthly or store by cryopreservation at −80ºC with 10% (v/v) of glycerol. The composition of the inoculum and cultivation medium was (per liter): CH_4N_2O, 0.52 g; Na_2HPO_4, 1 g; KH_2PO_4, 1 g; $Mg_2SO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; $ZnSO_4.7H_2O$, 0.08 g; $CuSO_4.5H_2O$, 0.001 g; $CoCL_2.6H_2O$, 0.001 g; $(NH_4)_2Mo_2O_7$, 0.005 g.

Concentration of 30 g/L of xylose or glucose was used for cultivations with these sugars as sole carbon sources. For a mixture of xylose:glucose (70:30, w/w), a concentration of 21 g/L and 9 g/L, respectively, was used to produce an artificial hemicellulose hydrolysate. To avoid medium darkness due to sugars reactions (caramelization and Maillard reactions), sugar solutions were sterilized in separate flasks. All chemicals and reagents used were of analytical grade.

6.2.2. Culture conditions

Inoculum was prepared in liquid media at 28 ºC, 150 rpm in orbital shaker, until reaching dry cell mass (CDW) of 10 g/L (equivalent to $1x10^8$ cells/mL). Chemostat cultivations were performed in a 2L benchtop bioreactor (B. Braun Biotech International, Biostat B) according Figure 6.1. Cells were grown at dilution rates (D) of $0.03h^{-1}$ and $0.06\ h^{-1}$. The total inlet gas flow was controlled by mass flowmeters at 1.0 vvm. The outlet gas flow was dried through a silica gel column to remove the humidity and analyzed to determine its CO_2 and O_2 content (BCP-CO_2 and BCP-O_2 Sensors, Blue-Sens). A multi-channel peristaltic pump was used for feeding the fermentor and also for removal the culture mediun into the waste bottle. The pressure in the culture vessel was maintained at 1.2 bars using a pressure valve (GO Inc). The pH, stirring speed and temperature were maintained at 5.5 (NaOH, 2M),

400 rpm and 28°C, respectively. Dissolved oxygen (DO) was online monitored by a DO electrode. To prevent foam formation, antifoam agent was automatically added to the vessel when needed. During the experiments the data acquisition and control of the different variables were done using UAB proprietary software (O. Cos, Universitat Autònoma de Barcelona).

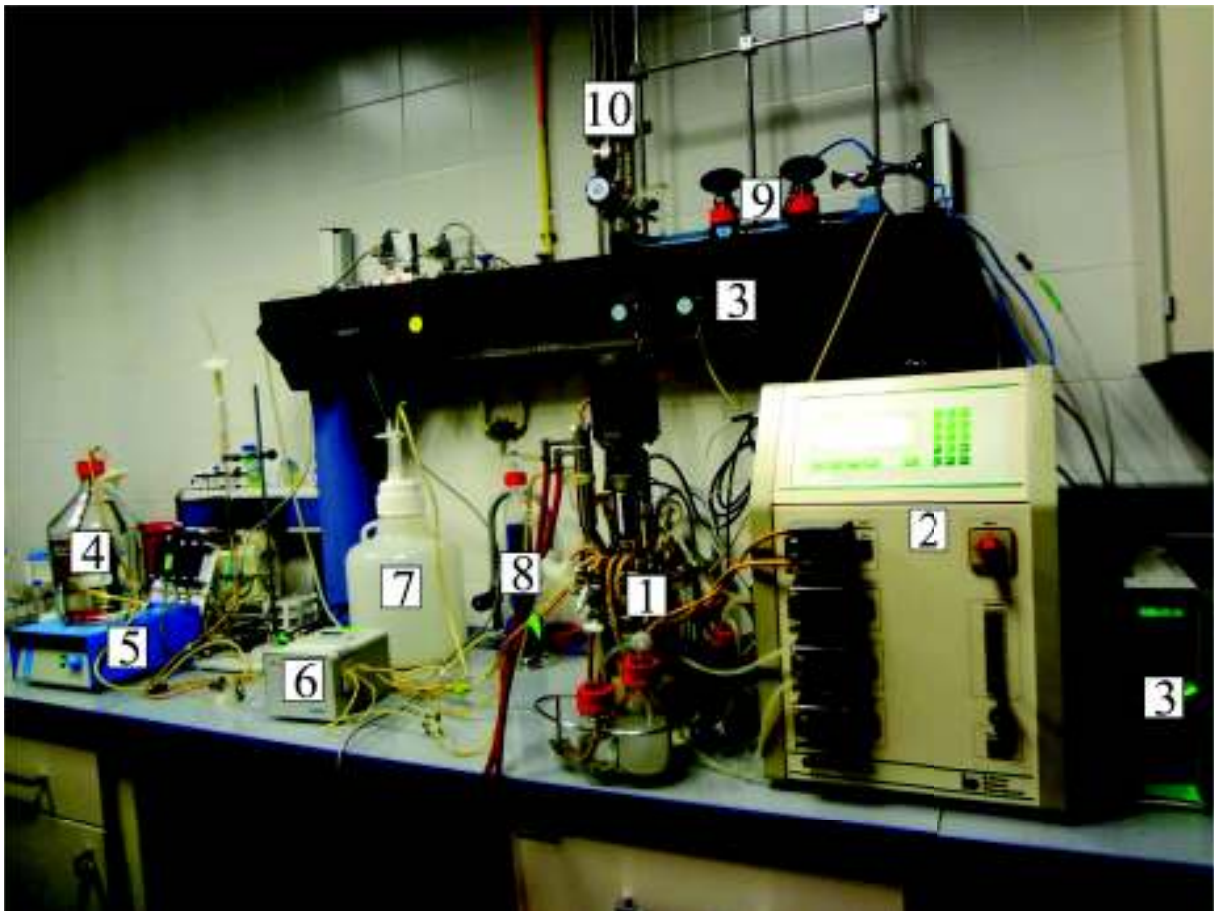

Figure 6. 1 Experimental apparatus: (1): 3L fermentor; (2): operation tower; (3): mass flowmeter; (4): feeding bottle; (5): homogenizer; (6): multi-channel peristaltic pump; (7): waste bottle; (8): silica gel column; (9): CO_2 and O_2 sensors; (10): pressure valve.

For each independent experiment, chemostat culture conditions were maintained constant for at least 3 residence times to allow reaching a metabolic steady state. At least three independent samples were analyzed for each tested condition in order to treat the data

by ANOVA and Tukey test for determining the significant differences between the culture conditions, at 95% of confidence ($p \leq 0.05$) using the software Statistica 7.0 (Statsoft).

6. 2. 3. Cell mass Analysis

Cell mass was harvested after reaching steady state and lyophilized for further analysis. A culture was considered to be in steady state when the cell mass concentration remainend constant for at least three residence times. To lyophilize the cell mass, samples of cultivation broth were centrifuged (5,000 rpm, 4ºC, 5min) and the cell pellet was washed twice with 1 ml of 20 mM Tris· HCl, pH 7.6 to eliminate traces of culture medium. The recovered pellet was immediately frozen and lyophilized under vacuum (Virtys Sentry).

The elemental composition of the cell mass was determined taking 1 mg of a lyophilized cells, adding 1 mg of V_2O_5 and introducing the samples in an oven at 1000°C. The volatile compounds were measured with an Elemental Analyzer (Flash 2000, ThermoFisher).

The ash content of lyophilized cells was determined using pre-dried ceramic cups in an oven at 550°C for 12 h.

Total protein content was determined by Lowry method (HERBERT, PHIPPS and STRANGE, 1971) from a solution of lyophilized cells at 0.5 g/L dry weight. Protein concentration was calculated using bovine serum albumin (BSA) as standard.

Total carbohydrates were determined by phenol method (HERBERT, PHIPPS and STRANGE, 1971). A 1 mL sample of lyophilized cells (0.1 mg dry biomass/mL) was mixed with 1 mL phenol (5%) and 5 ml sulphuric acid (96%). After 10 min, the tubes were cooled (15 min, 25°C). Absorbance at 488 nm was measured using glucose solutions as standard.

The lipids were gravimetrically quantified, from this wet digested pellet, by Bligh-Dyer's adapted method (BLIGH and DYER, 1959; MANIRAKIZA, COVACI and SCHEPENS, 2001). For lipid extraction, 5 mL of hydrochloric acid (2M) were added to 300 mg of lyophilized cells in a tube and incubated at 80ºC for 1 hour. The solution was then

centrifuged (5000 rpm, 4ºC, 15 min). After acid digestion, the HCl was discarded and 6 mL of a methanol:chloroform (2:1, v/v) was added to the solid pellet. The suspension was shaken in vortex for 2 minutes. Then, 2 mL of chloroform were added to the tube and stirred for 2 minutes. To generate a system with two liquid phases, 3.6 mL of distilled water were added and the system was shaken again for 2 minutes. The phases were separated by centrifugation for 10 minutes at 2000 rpm. The lower phase (first extract) was transferred to a flask using Pasteur pipette. A second extraction was performed using 4 mL of 10% (v/v) methanol in chloroform in a vortex for 2 minutes. After centrifugation, the chloroform phase was added to the first extract. The chloroform was evaporated in a rotary evaporator and the residue dried in oven at 60 ºC for 24 hours. The final lipid was quantified gravimetrically.

DNA content of biomass was determined by means of the Hoechst fluorescent dye method (HERBERT, PHIPPS and STRANGE, 1971). In short, lyophilized cells samples were dissolved in TNE buffer (1 M NaCl, 10 mM EDTA, 0.1 M Tris·HCl, pH 7.4) at a concentration of 25 mg/mL. Sample solution was mixed with 2 mL of the Hoechst dye solution (Hoechst 33258, 0.5 μg/ml in TNE buffer). Fluorescence was measured using the excitation/emission wavelengths of 356/468. DNA content was calculated by interpolation in a calibration curve performed using standard DNA (DNA sodium salt from calf thymus from Sigma-Aldrich).

RNA content of biomass was determined according to Benthin et al. (BENTHIN, NIELSEN and VILLADSEN, 1991). Briefly 5 mg of lyophilized cells were ressuspended in 10 ml of cold 0.7 M $HClO_4$ and incubated for 5 min. After incubation biomass was centrifuged (8,000 rpm, 10 min, 4°C), washed twice and ressuspended in 10 ml 0.3 M KOH. Two 5 ml aliquots of the ressuspended biomass were incubated at 37°C for 1 h. After cooling 1 ml cold 3 M $HClO_4$ was added and samples were centrifuged in the same conditions. Supernatant was collected and the pellet washed twice with 1 ml 0.5 M $HClO_4$. The 3

supernatants collected were mixed and absorbance measured at 260 nm in a quartz cuvette. The percentage (w/w) was calculated using 1 A_{260} unit (1 cm path length): 0.038 mgRNA/mL and taking into account the sample dilution.

Reductance degree (γ), is the number of equivalents of available electrons/g atom carbon based on carbon (4), hydrogen (1), oxygen (–2), and nitrogen (–3). A value of "–3" is used for nitrogen due the reductance degree of nitrogen in cell mass and ammonia is "–3". The cell mass energetic yield (η) was the ratio of the heat produced by oxidation of the cell mass to that of the substrate utilized in the fermentation process, where the oxidation results in the production of CO_2, H_2O and NH_3. According to Eroshin and Kryola (1983), microbial growth energetic yield was defined in Eq. (6.1).

$$\eta = (\sigma_X\gamma_X/ \sigma_S\gamma_S).Y_{X/S} \quad (6.1)$$

where σ_X and σ_S are the weight fractions of carbon in the cell mass and organic substrate, respectively.; γ_X and γ_S are the reductance degree of cell mass and organic substrate, respectively and $Y_{X/S}$ represents the cell mass yield (g/g substrate).

6.2.4. Quantification of extracellular metabolites

Cell mass was monitored by measuring the optical density at 600 nm (OD_{600}). For cellular dry weight, a known volume of cultivation broth was filtered using pre-weighted filters; these were washed with two volumes of distilled water and dried to constant weight at 105°C for 24 h. Triplicate samples (5 ml) for extracellular metabolite analyses were centrifuged at 10,000 rpm for 2 min in a microcentrifuge to remove the cells and subsequently filtered through 0.45 μm filters (Millipore type HAWP).

The nitrogen content was derived through balancing the urea feed and its outflow in the supernatant of the broth. Urea measurements were performed using an enzymatic colorimetric method. In short, urea in the sample was hydrolyzed enzymatically into ammonia (NH_4^+) and carbon dioxide (CO_2). Ammonia ions formed reacts with salicylate and

hypochloride (NaClO), in presence of nitroprusside, to form a green indophenol. The absorbance (580 nm) of samples and calibrator were reading against a blank.

For the identification of unknown extracellular metabolites, a High Performance Liquid Chromatography with detection by Light Scattering plus Mass Spectrometry (HPLC-ELSD +MS) system was used. A Prevail Carbohydrate ES column (Alltech Associates, Inc., Deerfield, IL) was used with MeCN:H_2O (72:28) as mobile phase in isocratic mode at 30ºC and 4µL of injection volume. This column was used with evaporative light scattering detection (ELSD), nebulization temperature of 81.3ºC, gas cabal (N_2): 2.1 L/min, impactor: OFF. Mass spectrometry experiments were performed on a Bruker micrOTOF-Q mass spectrometer (Bremen, Germany) with electrospray ionization, using 10 mM sodium formate for calibration.

After identification of the extracellular metabolites, xylose, glucose, xylitol and mannitol were analyzed by HPLC (Series 1050, Hewlett Packard) with a Rezex RPM-monosaccharide Pb+2 column (Phenomenex, Torrance, CA) using deionized water as mobile phase in isocratic mode. The analyses were performed at a flow rate of 0.6 mL/min at 75ºC using a 20 µL injection volume.

6.3. Results and discussion

6.3.1. Identification of unknown extracellular metabolites produced

To identify unknown extracellular compounds produced during cultivations, samples were submitted to HPLC-ELSD/MS analysis. According to the nature of the samples injected and to chemical formulas proposed in the simulation (Additional file 1), were considered xylitol ($C_5H_{12}O_5$) and sorbitol ($C_6H_{14}O_6$) as possible unidentified compounds. In order to evaluate this hypothesis, were injected standards of these components at the same analysis conditions as the samples. Also a standard solution containing the four analytics (xylose, glucose, xylitol and sorbitol) was injected. The retention times of xylose, xylitol, sorbitol and

glucose were consistent between the standards and samples and the HPLC-ELSD method was adequate to separate the four components (BHANDARI *et al.*, 2008). The retention times of the chromatographic peaks and the mass spectra of the standards and the samples were compared. The unknown metabolites were identified as xylitol (MW 152) and sorbitol (MW 182). In order to use a more friendly, simple and rapid method, further analysis of extracellular metabolites were performed using a HPLC system with Rezex RPM column which uses only deionized water as mobile phase to separate the four components identificated. During the sample analysis, glucose, xylose and xylitol were identificated and quantified according to the retention times of the calibration curve (12.9, 13.8 and 32.4 min, respectively). But, for our surprise, there wasn't a chromatographic peak at sorbitol retention time (35.9 min), but at retention time of 24.9 min. This component was identificated as mannitol (MW 182). Mannitol and sorbitol are isomers, the only difference being the orientation of the hydroxyl group on carbon two.

Xylitol is a five-carbon sugar alcohol and can be produced by biotechnological conversion of xylose by some yeast strains (SIRISANSANEEYAKUL, WANNAWILAI and CHISTI, 2013). D-xylose is first reduced to D-xylitol by either NADH- or NADPH-dependent xylose reductase (XR); the resulting D-xylitol is either secreted or further oxidized to D-xylulose by NAD- or NADP-dependent xylitol dehydrogenase (XDH). These two reactions are considered to be the rate-limiting steps in D-xylose fermentation and D-xylitol production. Some yeast strains could metabolize D-xylulose to xylulose-5-phosphate by xylulokinase (XK). Xylulose-5-phosphate can subsequently enter the pentose phosphate pathway (CHEN *et al.*, 2010) as shown in Figure 6.2.

Xylitol secretion also occurred when xylose was utilized as carbon source, solely or in mixtures with glucose for lipid production by *Thamnidium elegans* (ZIKOU *et al.*, 2013). The cultivation in shake flask using xylose (100 g/L), resulted in a xylitol yield ($Y_{P/S}$) of 0.40 g/g

(31.3 g/L) with a remained xylose of 22 g/L. Additionally, when *T. elegans* was cultivated on glucose:xylose (25:75) mixture, a xylitol yield of 0.25 g/g was found. The amount of accumulated xylitol remained lower than that achieved on the substrate with xylose as the sole carbon source, in accordance with the literature (KIM *et al.*, 2010) that xylitol production could be significantly altered by the presence of glucose in the fermentation medium.

Mannitol is a six-carbon sugar alcohol produced by glucose or fructose (RACINE and SAHA, 2007) and some microorganisms can specifically produce mannitol from glucose (Figure 6.2) without producing sorbitol (optical isomer) as byproduct (SONG *et al.*, 2002). The supplementation of $CaCl_2.2H_2O$ and $CuSO_4.5H_2O$ in the culture medium could promote mannitol production by *Candida magnolia*. Mannitol resulted from the synergistic effect of Ca^{2+} on cell permeability and Cu^{2+} on the biosynthetic mannitol dehydrogenase activity for mannitol production (LEE *et al.*, 2007). These could explain the mannitol production in present continuous cultivations, since $CaCl_2.2H_2O$ and $CuSO_4.5H_2O$ are present as trace elements in the culture medium. It is known that osmophilic yeast and some bacteria can produce sugar alcohols or their derivatives in response to increased external osmotic pressure. Mannitol production was also reported by the oleaginous yeast *Y. lipolytica* (RYWIŃSKA, RYMOWICZ and MARCINKIEWICZ, 2010).

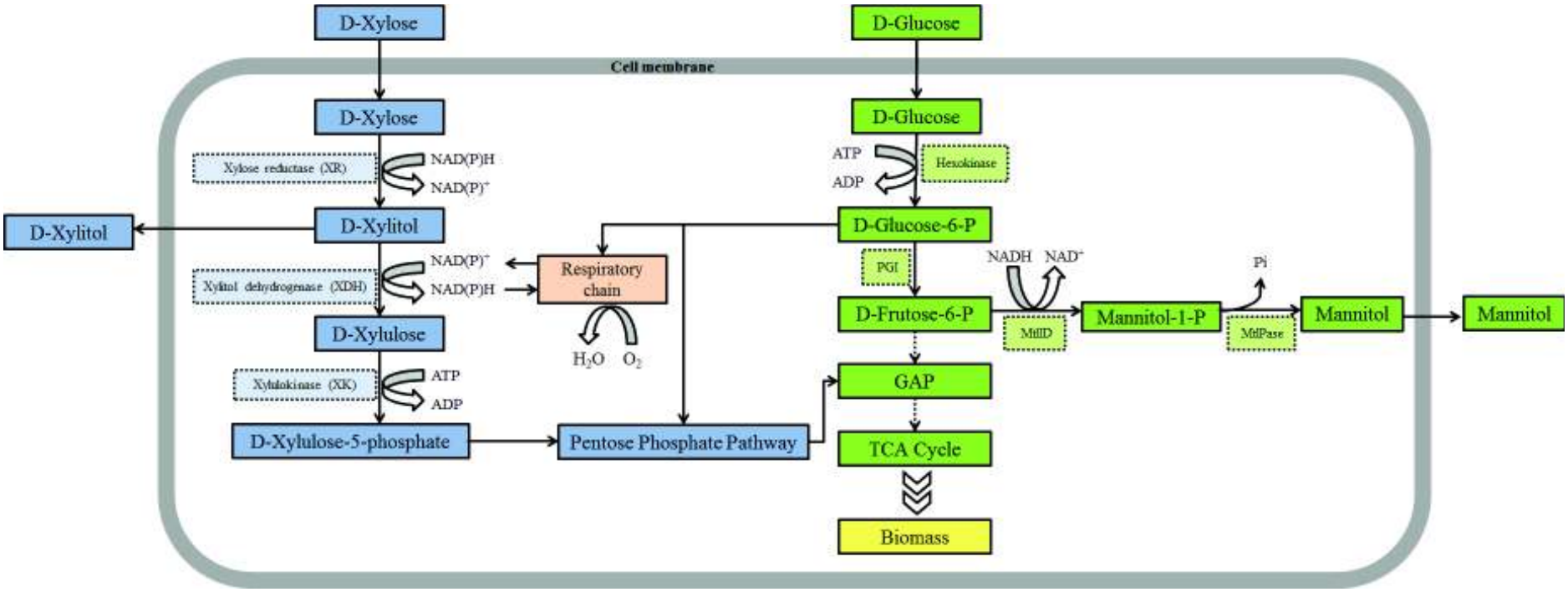

Figure 6. 2 Simplified representation of xylose, glucose, xylitol and mannitol metabolism in yeasts. GAP: glyceraldehyde-3-phosphate; TCA cycle: tricarboxylic acid cycle; MtlD: mannitol 1-phosphate dehydrogenase; MtlPase: Mannitol-1-phosphatase; PGI: phosphoglucose isomerase. Dotted arrows represent multiple consecutive steps in the pathways.

6.3.2. Effect of dilution rate and carbon source in continuous culture

Continuous cultivation with xylose:glucose showed similar kinetic profile as using only glucose. With xylose as sole carbon source, lower cell mass and lipid concentrations were obtained at chemostat culture for both D. Lipid content decreased 5% at 0.06 h^{-1} independently of the carbon source studied and cell mass concentration presented more oscillation in relation to the D and carbon sources tested (Table 6.1). When *L. starkeyi* was growing at D of 0.03 h^{-1}, highest cell mass (16.1 g/L) concentration and lipid content (34.9%) were achieved using xylose:glucose. At D of 0.06 h^{-1}, were obtained the lowest responses in comparison to 0.03 h^{-1} at all carbon sources tested, only lipid yield ($Y_{P/S}$) was around 0.20 g/g independently of the condition studied. Cell mmass yield ($Y_{X/S}$) was influenced by the D and carbon source: $Y_{X/S}$ increased with the increasing of D when was used glucose and the mixture xylose:glucose. Continuous cultivation with xylose resulted in the lowest $Y_{X/S}$ of the cultures studied and decreased with the increase of D. Shen et al. (2013) studied the effect of D from 0.02 to 0.20 h^{-1} by *R. toruloides* using 36 g/L of glucose as carbon source. Present studies resulted in higher cell massconcentration and lower lipid content at all conditions.

The highest lipid productivity was obtained with xylose:glucose at 0.06 h^{-1}, same D which was resulted in the highest unconsumed sugars and urea. Our studies resulted in higher lipid productivities using xylose:glucose and glucose in comparison with continuous cultivations reported in literature which used glucose as sole carbon source at 0.03 h^{-1} (AGGELIS and KOMAITIS, 1999) and 0.06 h^{-1} (SHEN *et al.*, 2013), resulting in 0.073 and 0.135 g/L.h, respectively. It was interesting to observe that the concentration of extracellular metabolites produced was lower at D of 0.06 h^{-1} at all sugars studied, this may occur due the assimilation of these compounds previous produced at D of 0.03 h^{-1}. *R. toruloides* was reported as assimilating a mixture of glucose, xylose, xylulose and xylitol during cultivation (HSIAO *et al.*, 1982).

Table 6. 1 Results of chemostat cultivations.

Response	Xylose		Glucose		Xylose:Glucose (70:30)	
	0.03 h^{-1}	**0.06 h^{-1}**	**0.03 h^{-1}**	**0.06 h^{-1}**	**0.03 h^{-1}**	**0.06 h^{-1}**
CDW (g/L)	9.4 ± 0.16	7.2 ± 0.27	18.7 ± 0.2	10.4 ± 0.2	16.1 ± 0.27	11.5 ± 0.52
Lipid (g/L)	3.2 ± 0.18	2.1 ± 0.13	5.8 ± 0.18	2.7 ± 0.15	5.6 ± 0.21	3.5 ± 0.21
$Y_{L/S}$ (g/g)	0.203	0.192	0.205	0.201	0.193	0.193
$Y_{X/S}$ (g/g)	0.323	0.241	0.657	0.708	0.557	0.628
Lipid productivity (g/L.h)	0.096	0.126	0.174	0.162	0.168	0.210
Input glucose (g/L)	0	0	29.5 ± 0.09	30.2 ± 0.17	9.2 ± 0.22	8.2 ± 0.16
Output glucose (g/L)	0	0	0.03 ± 0.01	17.2 ± 0.11	0	0.3 ± 0.10
Input xylose (g/L)	29.4 ± 0.24	30.1 ± 0.27	0	0	21.7 ± 0.17	20.1 ± 0.14
Output xylose (g/L)	16.4 ± 0.16	20.5 ± 0.10	0	0	0.7 ± 0.04	9.2 ± 0.94
Output xylitol (g/L)	0.3 ± 0.12	0.2 ± 0.07	0	0	0.6 ± 0.34	0.2 ± 0.13
Output mannitol (g/L)	0	0	1.2 ± 0.05	0.2 ± 0.03	0.7 ± 0.02	0.5 ± 0.11
Input urea (g/L)	1.0 ± 0.18	1.1 ± 0.12	1.1 ± 0.09	1.1 ± 0.11	1.1 ± 0.12	1.1 ± 0.10
Output Urea (g/L)	0.3 ± 0.09	0.6 ± 0.11	0.2 ± 0.04	0.5 ± 0.07	0.3 ± 0.09	0.53 ± 0.12
CER (mol/L.h)	0.049 ± 0.005	0.045 ± 0.001	0.086 ± 0.005	0.030 ± 0.001	0.055 ± 0.006	0.052 ± 0.001
OUR (mol/L.h)	0.043 ± 0.0005	0.040 ± 0.0004	0.066 ± 0.004	0.010 ± 0.001	0.058 ± 0.005	0.048 ± 0.0003

Lipid-free material and lipid yields were influenced by the change of D. D less than 0.06 h^{-1} were normally required for optimum conversions. Normally, at high dilution rates, higher concentrations of sugars were detected in the culture fluid and also resulted in increased cell mass yield but with a decreasing lipid fraction (PAPANIKOLAOU and AGGELIS, 2002). Therefore, results of continuous culture will be used further for metabolic flux analysis (MFA), which provides a highly informative view of the physiological cell status under a given environmental condition or genetic background.

6.3.3. Cell mass data reconciliation and extracellular metabolite balances

6.3.3.1. Macromolecular compositions

In order to elucidate the influence of D and carbon sources on macromolecular compositions of *L. starkeyi*, were analyzed the contents of five major cell components (lipid, carbohydrate, protein, DNA and RNA) that accounted around 90% of total dry cell weight (Table 6.2). Lipids are one of the major constituents of the cell mass in oleaginous yeasts and can constitute up to 70% of the weight of the cells (RATLEDGE and COHEN, 2008). Consequently, detailed knowledge of their composition is important for any metabolic and energetic calculations. Lipid content was higher at D of 0.03 h^{-1} using xylose:glucose and resulted in 34.9% (Figure 6.3a and Table 6.2). The decrease of lipid content at 0.06 h^{-1} was coherent with the increase in the relative total protein and carbohydrate contents. The lipid content, changed significantly between the dilution rates and carbon sources according to Tukey test ($p \leq 0.05$). The lipids produced by different cultivations were transmethylated and analyzed by gas chromatography. The fatty acid profile showed little change regardless of culture conditions (Additional file 2). Microbial lipids chains from *L. starkeyi* have between 16 and 18 carbon and low degrees of unsaturation, which is desirable for their application in biodiesel production.

Table 6. 2 Measured chemical elements in the cell mass.

Component	Xylose		Glucose		Xylose:Glucose (70:30)	
	0.03 h^{-1} (% ± sd)	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**
Lipid	34.4 ± 0.9^{a}	28.6 ± 0.8^{bc}	30.9 ± 0.4^{b}	25.7 ± 0.5^{c}	34.9 ± 1.3^{a}	30.1 ± 1.8^{b}
Protein	21.8 ± 5.3^{a}	23.6 ± 5.4^{a}	23.4 ± 4.7^{a}	25.3 ± 4.2^{a}	20.1 ± 3.0^{a}	21.3 ± 3.8^{a}
Carbohydrate	35.9 ± 3.1^{a}	35.4 ± 4.3^{a}	35.7 ± 5.1^{a}	39.2 ± 4.9^{a}	36.4 ± 5.5^{a}	38.9 ± 5.0^{a}
RNA	1.9 ± 0.1^{ab}	2.1 ± 0.2^{b}	1.3 ± 0.1^{ac}	1.4 ± 0.1^{a}	1.4 ± 0.2^{a}	2.1 ± 0.3^{b}
DNA	0.5 ± 0.1^{a}	0.5 ± 0.01^{a}	0.8 ± 0.1^{b}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.06^{a}
Others	5.5	9.8	7.9	8.0	6.8	7.2
C	50.0 ± 0.9^{b}	49.5 ± 0.9^{b}	49.8 ± 0.5^{b}	49.5 ± 0.5^{b}	52.7 ± 0.4^{a}	52 ± 0.4^{a}
H	7.7 ± 0.5^{a}	7.7 ± 0.5^{a}	7.4 ± 1.8^{a}	7.6 ± 1.8^{a}	8.1 ± 0.9^{a}	8.1 ± 0.5^{a}
N	2.4 ± 0.3^{a}	2.5 ± 0.3^{a}	2.7 ± 0.3^{a}	2.7 ± 0.3^{a}	2.5 ± 0.1^{a}	2.8 ± 0.2^{a}
O	35.1 ± 1.7^{a}	35.6 ± 1.8^{a}	34.8 ± 1.7^{a}	35.3 ± 1.7^{a}	31.4 ± 1.6^{a}	32.4 ± 1.6^{a}
S	0	0	0	0	0	0.19 ± 0.07
Ashes	4.8 ± 0.8^{a}	4.7 ± 0.8^{a}	5.2 ± 0.9^{a}	4.9 ± 0.9^{a}	5.3 ± 1.0^{a}	4.6 ± 0.8^{a}

[a] Equal lower case letters indicate no significant difference between columns, for each response at 95% of confidence ($p < 0.05$); %: percentage (w/w); sd: standard deviation.

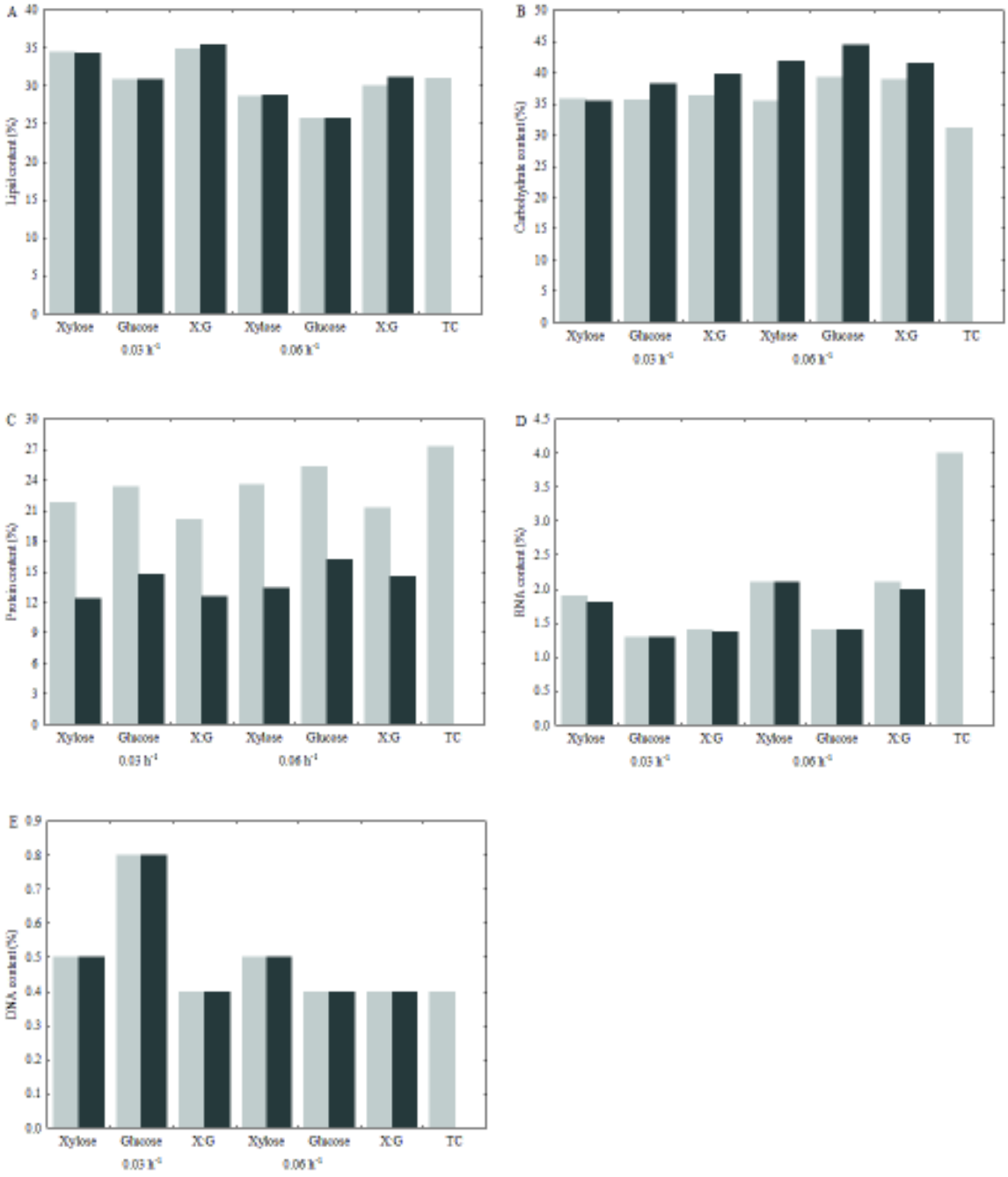

Figure 6. 3 Summary of the macromolecular cell mass composition results obtained for the different experimental conditions (■) and the reconciled value (■). X:G: xylose:glucose; TC: *Trichosporon cutaneum*, data from Liu et al. (2013) at batch culture using glucose as carbon source.

Cultivations using xylose:glucose presented more oleic acid (C18:1) and lower stearic acid (C18:0), compared with cultivations with xylose and glucose as sole carbon sources. The lipids produced are mainly composed of palmitic acid (C16:0) and oleic acid (C18:1), quite similar profile to those of palm oil (MALEKI, AROUA and SULAIMAN, 2013), indicating that lipid produced by *L. starkeyi* has great potential as a feedstock for biodiesel production and other industry applications for production of soaps and detergents, pharmaceutical products, cosmetics, oleochemical products and food applications (SALIMON, SALIH and YOUSIF, 2012; NORHAIZAN *et al.*, 2013).

Figure 6.3b, shows the cell's total carbohydrate content measured for both dilution rates at all carbon sources tested with fairly constant levels (35.4 – 39.2%). The ANOVA followed by Tukey test showed that there was no significant difference ($p < 0.05$) for the carbohydrate content at the cultures conditions studied. The carbohydrate concentrations obtained in present studies were higher than achieved by the oleaginous yeast *Trichosporon cutaneum* at batch culture (31.2%).

Total cell protein content presented in Figure 6.3c, shows no significant difference ($p < 0.05$) between the carbon sources and D studied, ranging from 20.1 to 25.3%.

RNA content ranged from 1.3 to 2.1% (Figure 6.3d) resulting in significant differences between dilution rates according to Tukey test. The only literature data about RNA content for oleaginous yeast (LIU *et al.*, 2013) present a double amount (4%) in comparison to results from present study.

As shown in Figure 6.3e, the measured DNA content of *L. starkeyi* present similar amounts than reported by *T. cutaneum* (LIU *et al.*, 2013). Only chemostat culture using glucose at D of $0.03h^{-1}$ presented significant difference ($p < 0.05$) in relation to the other conditions.

6.3.3.2. Elemental cell mmass composition

The elemental composition was quite similar at all conditions studied. The carbon amount presented no significant difference ($p < 0.05$) between glucose and xylose as sole carbon sources at the two D studied and for the mixture of xylose:glucose also didn't present significant difference at 0.03 h^{-1} or 0.06 h^{-1}. Generally, N and O contents continuously declined, while C and H contents increased upon lipid accumulation (ZHOU *et al.*, 2012). At low D, which contains higher lipid content at all carbon sources studied, only slight variations were observed at these components proportions. For the reconciliation procedure, only C, H, N, S and ash content were measured (Table 6.2) and P was included into a composite compound which also included ashes. Therefore, the reconciliation procedure calculated the oxygen content by a difference between weights of total cell mass and (C + H + N + ash).

6.3.3.3. Consistency check and data reconciliation

The particular challenge in implementing data reconciliation for further perform detailed metabolic flux analyses (MFA) calculations for bioprocesses arises from the necessity to accurately formulate elemental balances for the system. Data reconciliation also serves as a tool to gain insight into the process and to check for the validity of the balances (DABROS *et al.*, 2009). This procedure was performed in two steps: i) the cell mass compositional data available was used to obtain reconciled biomass composition; ii) the consistency check of the measured substrate consumption and cells and products generated by metabolite mass balances (WANG and STEPHANOPOULOS, 1983).

6.3.3.3.1. Reconciled cell massmass composition

The maximum likelihood method described by Lange and Heijnen (2001) allows the calculation of the best estimation of the cell mass composition by solving a linear system which takes conservation laws of elemental and mass balances into account. For the reconciliation method, was used the elemental composition of the macromolecular

compounds in terms of a C-mol formula (Additional file 3), which the lipid composition was derived from a triacylglycerol (TAG) molecule containing palmitic acid, oleic acid and linoleic acid ($C_{55}H_{98}O_6$), the main constituents of TAG produced by *L. starkeyi* in these culture conditions. The compositions of the other components (protein, carbohydrate, RNA, DNA, metals, H_2O and sulphate) were included as described for *S. cerevisiae* in Lange and Heijnen (2010) since there are no data available for oleaginous yeast to date.

Cell mass composition obtained after the reconciliation method is shown in Table 6.3. The calculated cell mass compositional values were similar than the experimental ones, with exception of protein content. The reconciled values indicated lower global protein content for all cultivations, although for *T. cutaneum* (LIU *et al.*, 2013) at batch culture was achieved similar protein content (27.3%) than our experimental results. In order to check the experimental protein content, is possible to analyse the amino acid composition of the whole protein extract, for the different experimental conditions estudied, according methodology described at Carnicer *et al.* (2009).

The reconciliation provide a better estimation of the cell mass carbon elemental formula (Table 6.4) because includes values (such O) that were not measured. It can be observed that as the D increased, the C/N ratio, H/O ratio and cell mass degree of reduction (γ) decreased for all carbon sources tested. There is no other study yet published about cell mass composition or elemental formula for oleaginous yeast in continuous cultivation. A batch cultivation by *Rhodosporidium toruloides* Y4 using glucose as carbon source (ZHOU *et al.*, 2012) resulted in a higher γ (5.26) than present studies (4.46 – 4.69), as well as batch cultures with *L. starkeyi* strains using ethanol as carbon source (4.98 to 5.08) (EROSHIN and KRYLOVA, 1983). The reconciled cell mass composition was used in the following calculations.

Table 6. 3 Cell mass composition calculated after reconciliation procedure.

Component	Xylose		Glucose		Xylose:Glucose (70:30)	
	0.03 h^{-1} (% ± sd)	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**
Lipid	34.3 ± 0.8	28.8 ± 0.7	30.9 ± 0.4	25.8 ± 0.5	35.4 ± 1.2	31.2 ± 1.5
Protein	12.4 ± 1.4	13.4 ± 1.5	14.7 ± 1.5	16.2 ± 1.5	12.6 ± 0.6	14.5 ± 1.1
Carbohydrate	35.5 ± 2.2	41.9 ± 2.4	38.2 ± 2.2	44.5 ± 2.2	39.8 ± 2.3	41.5 ± 2.8
RNA	1.8 ± 0.3	2.1 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.37 ± 0.2	2.0 ± 0.3
DNA	0.5 ± 0.07	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.40 ± 0.06	0.40± 0.06
C	50.0 ± 0.7	49.3 ± 0.6	49.8 ± 0.5	49.4 ± 0.5	52.7 ± 0.4	51.9 ± 0.4
H	7.8 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.0 ± 0.1	7.5 ± 0.3	7.3 ± 0.2
N	2.6 ± 0.2	2.8 ± 0.3	3.0 ± 0.3	3.2 ± 0.3	2.5 ± 0.1	2.9 ± 0.2
O	34.9 ± 0.9	35.8 ± 0.8	34.7 ± 0.8	35.5 ± 0.8	31.7± 0.8	33.0± 0.7
S	0	0	0.0	0	0	0.18 ± 0.08
Ashes	4.7 ± 0.7	4.6 ± 0.8	5.2 ± 0.8	4.9 ± 0.8	5.4 ± 0.9	4.5 ± 0.7
h	3.5	7.5	5.3	7.9	7.5	7.9

Table 6. 4 Cell mass C-molecular formula calculated after reconciliation procedure.

Carbon source	Dilution rate	C-molecular formula	C/N Ratio	H/O Ratio	γx	η
Xylose	0.03 h^{-1}	C $H_{1.868}$ $N_{0.044}$ $O_{0.523}$	22.5	3.57	4.69	0.47
	0.06 h^{-1}	C $H_{1.791}$ $N_{0.049}$ $O_{0.545}$	20.5	3.28	4.55	0.34
Glucose	0.03 h^{-1}	C $H_{1.789}$ $N_{0.051}$ $O_{0.523}$	19.7	3.42	4.59	0.94
	0.06 h^{-1}	C $H_{1.702}$ $N_{0.055}$ $O_{0.539}$	18.1	3.16	4.46	0.97
Xylose:Glucose	0.03 h^{-1}	C $H_{1.714}$ $N_{0.041}$ $O_{0.454}$	24.2	3.77	4.68	0.86
(70:30)	0.06 h^{-1}	C $H_{1.676}$ $N_{0.049}$ $O_{0.478}$	20.3	3.51	4.57	0.93
Glucose*	Batch	C $H_{1.81}$ $N_{0.01}$ $O_{0.26}$	100	3.84	5.26	0.77

Elementary composition of ell mass, expressed as C-molecular formula, for the carbon sources and dilution rates. C/N: carbon/nitrogen ratio; H/O: hydrogen/oxygen ratio; γ: reduction degree of the cell mass; η: cell mass energetic yield. *Data from Zhou *et al.* (2012) by *Rhodosporidium toruloides* Y4 at 123h of batch cultivation.

6.3.3.3.2. Metabolite mass balances

Mass balance equations cannot be easily established in bioprocess applications because of difficulties in identifying the presence of all relevant species and the lack of complete measurements. A statistical test using reconciled data was applied to determine whether the balance errors fall inside a normally distributed range of acceptable values. A standard way of performing such a test is to calculate the statistical function *h* based on the measurement variance–covariance matrix and to check whether *h* falls below an upper control limit defined by a x^2-distribution (WANG and STEPHANOPOULOS, 1983; VAN DER HEIJDEN *et al.*, 1994b; DUBOC and VON STOCKAR, 1995). The x^2-distributed test variable *h* was calculated for the reconciled data of all continuous cultivations, based on the cell mass C-molecular formula calculated in previous step. It was considered that cell mass, CO_2 and by-products (xylitol and mannitol) generation resulted from glucose, xylose, urea and oxygen consumption. As can be observed in Table 6.5, a carbon balance performed for

the carbon sources studied in both dilution rates, indicated that there was only a mismatch from 0.24 to 1.89 %, well in agreement with the analysis performed and there were no systematic or gross measurement errors (0.83 to 6.5 %). The value obtained for the consistency index *h* for steady state at 0.03 h^{-1} was lower (3.5) than the corresponding χ^2 (chi-square) value for a 95% confidence interval (3.84). For the other cultivations studied *h* ranged from 5.3 to 7.9, above the upper control limit of 3.84, but with a very reliable carbon balance at all conditions (Table 6.3). Figure 6.4 shows the measured values (a, c and e), as well as the reconciled values (b, d and f) of the metabolites in mmol/(gDW·h). At each D cultivations using xylose:glucose, a higher production of cell mass in mmol/(gDW·h) was observe. Chemostat cultures with xylose and glucose resulted in similar biomass production rate (mmol/(gDW·h)) at each dilution rate.

Table 6. 5 Measured substrates and products consumed/produced at steady state in each experimental condition.

mmol/gDW·h	Xylose		Glucose		Xylose:Glucose (70:30)	
	0.03 h^{-1} (% ± sd)	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**	**0.03 h^{-1} (% ± sd)**	**0.06 h^{-1} (% ± sd)**
CDW	1.250 ± 0.124	2.478 ± 0.124	1.246 ± 0.062	2.475 ± 0.124	1.318 ± 0.066	2.600 ± 0.130
Glucose	-	-	-0.263 ± 0.013	-0.418 ± 0.021	-0.077 ± 0.004	-0.137 ± 0.007
Xylose	-0.275 ± 0.027	-0.518 ± 0.026	-	-	-0.202 ± 0.010	-0.373 ± 0.019
Urea	-0.034 ± 0.007	-0.075 ± 0.008	-0.025 ± 0.003	-0.054 ± 0.002	-0.024 ± 0.002	-0.051 ± 0.005
Xylitol	0.037 ± 0.003	0.049 ± 0.002	-	-	0.016 ± 0.001	-
Mannitol	-	-	0.062 ± 0.003	0.042 ± 0.001	0.018 ± 0.001	0.045 ± 0.002
CER	0.005 ± 0.001	0.005 ± 0.001	0.005 ± <0.001	0.003 ± <0.001	0.003 ± <0.001	0.002 ± <0.001
OUR	-0.006 ± 0.001	-0.004 ± 0.001	-0.003 ± <0.001	-0.001 ± <0.001	-0.005 ± <0.001	-0.002 ± <0.001
C balance (mmol/h)	0.67	0.47	0.37	1.73	0.24	1.89
Error (%)	2.0	2.5	1.23	6.50	0.83	4.93

Substrate consumption, CDW and metabolites production rates at mmol per g of dry weight. OUR: oxygen uptake rate; CER: CO_2 exchange rate.

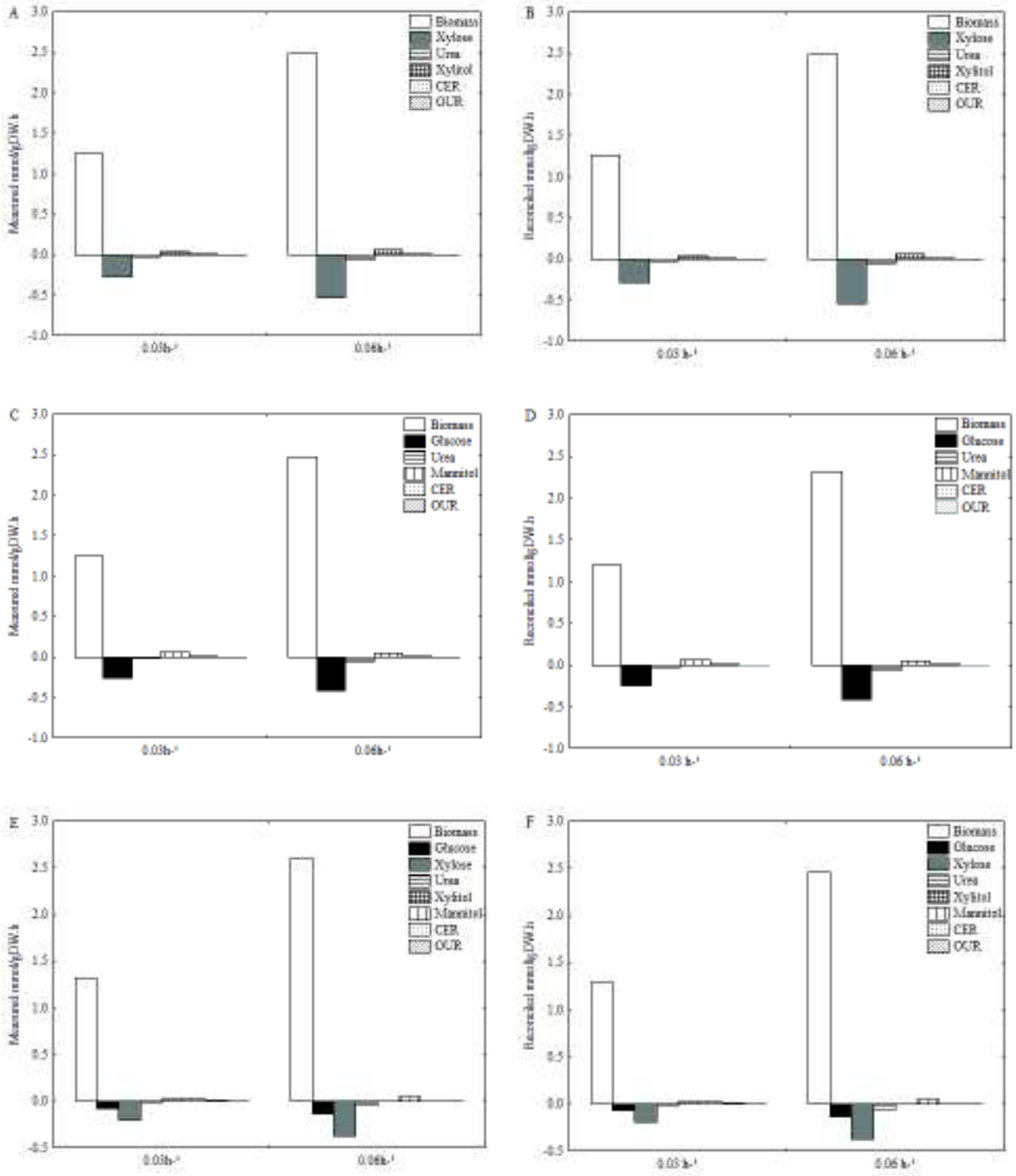

Figure 6. 4 Measured (A, C and E) and reconciled (B, D and F) values of substrates consumed and metabolites generated for the different experimental conditions. Negative values represent substrate consumed, while positive values represent products generated. OUR, oxygen uptake rate; CER, CO_2 exchange rate.

6.3.4 Energy efficiency evaluation

Cell mass energetic yield (η) was estimated according to Eq. (6.1). Compared to $Y_{X/S}$, η helped to distinguish microbial growth on substrates with different energy contents by considering the energy content of substrates in calculation. *Lipomyces starkeyi* converted more than 90% of the energy contained in xylose:glucose into cell mass energy (Table 6.4). The cell mass energetic yields of the cultivations studied are shown in Fig. 6.5. The highest yield of 97% was achieved when using glucose at D of 0.06 h^{-1}. Our data showed (Table 6.4) that η values of *L. starkeyi* were higher than the maximum value of 0.7 proposed for heterotrophic microbial growth (ERICKSON, MINKEVICH and EROSHIN, 1978), except at cultivations with xylose. That probably resulted from additional energy produced in metabolism and stored in its cells as lipid. Pan and Rhee (1986) collected data from 21 sources and showed that the energetic yield (η) reached mostly from 0.6 to 0.8. Eroshin and Krylova (1983), reported η values for *L. Starkeyi* strains from 0.15 to 0.31 grown on ethanol. Cell mass yields of oleaginous yeasts were consistently lower than that of non-oleaginous microorganisms, whereas their energetic yields were higher (ZHOU *et al.*, 2012). *Lipomyces starkeyi* could convert energy from xylose:glucose into cell mass with high efficiency. This type of energy evaluation can supply important information for strain and substrate selection and optimization of the cultivation process.

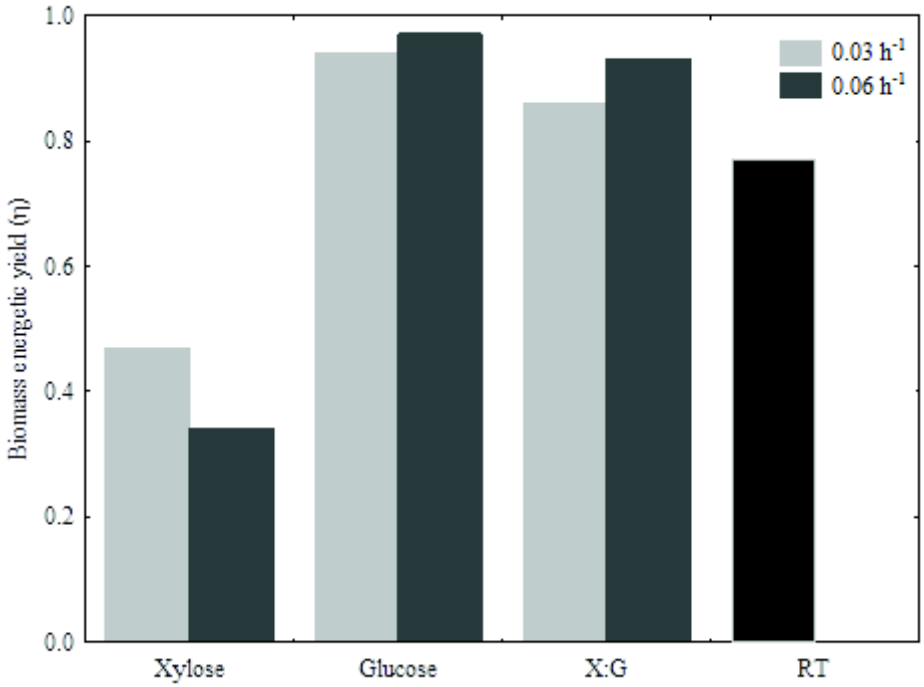

Figure 6. 5 Comparison of the cell mass energetic yield at the cultivations studied. X:G: xylose:glucose (70:30); RT: *Rhodosporidium toruloides* Y2 from Zhou et al. (2012) using glucose at batch culture.

6.4. Conclusions

In this study, continuous cultivations using xylose, glucose and xylose:glucose (70:30, w/w) at D of 0.03 and 0.06 h^{-1} were studied in order to obtain first hand data on the cell mass composition of *L. starkeyi*. Application of elemental mass balances to the input and output metabolite data allowed detecting the lack of extracellular metabolites. Unknown extracellular metabolites produced were identified by HPLC-MS as xylitol and mannitol. The experimental data obtained were used for data reconciliation method and the best estimation of the cell mass composition was obtained with a very low mismatch of carbon balance (0.24 to 1.89 %). *L. starkeyi* could also convert energy from xylose:glucose into cell mass with high efficiency (η over 0.90), which is an important aspect for the utilization of lignocellulosic biomass for microbial lipid production and its conversion to biofuel. The obtained results will be further used in metabolic flux analyses (MFA) of *L. starkeyi*.

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ADDITIONAL FILE 1

Identification of unknown extracellular compounds submitted to HPLC-ELSD/MS analysis

The possible molecular formulas have been generated by the two almost molecular ions in study considering that: the molecule consists of atoms C, H, N and O; were considered the entry of the ion Na^+; the maximum massic error is 2.5 mDa; the electronic parity is even electronics; Z = +1; the nitrogen rule was applied. The molecular formulas proposed were described in Table A1a and the consistency between experimental results and theoretical exact mass are presented in Table A1b.

Table A1a: Formulas proposed according all ions in study (ESI positive).

m/z measured		Formula proposed	m/z	Error (mDa)
175.057	1	$C_5 H_{12} Na O_5$	175.0577	0.7
	2*	$C_4 H_9 N_4 Na_2 O$	175.0566	-0.3
	3*	$C_6 H_8 N_4 Na O$	175.059	2.1
205.0683	1	$C_6 H_{14} Na O_6$	205.0683	0.0
	2*	$C_5 H_{11} N_4 Na_2 O_2$	205.0672	-1.1
	3*	$C_3 H_6 N_{10} Na$	205.0669	-1.3
	4*	$C_6 H_{13} N_2 Na_4$	205.0664	-1.9
	5*	$C_7 H_{10} N_4 Na O_2$	205.0696	1.3
	6*	$C_8 H_{12} N_2 Na_3$	205.0688	0.6

*Formulas with low probability.

Table A1b: Consistency between experimental results and theoretical exact mass

Analyte	**Chemical formula**	**$[M + Na]^+$ teoric**	**$[M + Na]^+$ standard**	**Error (mDa)**	**$[M + Na]^+$ sample**	**Error (mDa)**
Xylose	$C_5 H_{10} O_5$	173.042045	173.0412	-0.8	173.0414	-0.7
Xylitol	$C_5 H_{10} O_5$	175.057695	175.0577	0.0	175.0570	-0.7
Sorbitol	$C_6 H_{14} O_6$	205.068259	205.0691	0.8	205.0683	0.0
Glucose	$C_6 H_{12} O_6$	203.052609	203.0523	-0.3	203.0526	0.0

The identification of the ions 175 and 205 is compatible with xylitol and sorbitol, respectively according the chromatograms and mass spectra obtained and theoretical study of the analytes. For both compounds, the majority cluster formed in electrospray was [M + Na] +.

ADDITIONAL FILE 2

Table A2. Comparison of fatty acid profiles from present studies and reported in literature.

***Lipomyces starkeyi* lipid profiles (%, weight)[a]**							
Substrate	**C14:0**	**C16:0**	**C18:0**	**C16:1**	**C18:1**	**C18:2**	**Reference**
Glucose	0.4	33.0	4.7	4.8	55.1	1.6	(LIU and ZHAO, 2007)
Glucose	0.7	36.3	5.0	4.2	50.1	3.7	(LIN *et al.*, 2011)
X:G[b]	0.4	33.9	7.5	3.4	50.6	3.9	(TAPIA *et al.*, 2012)
X ($0.03h^{-1}$)[c]	n.d.	39.8	16.8	1.5	37.6	4.4	Present study
X ($0.06h^{-1}$)[c]	n.d.	42.3	8.7	2.8	38.6	7.7	Present study
G ($0.03h^{-1}$)[d]	n.d.	37.8	11.7	2.1	42.2	6.2	Present study
G ($0.06h^{-1}$)[d]	n.d.	39.2	15.2	1.5	38.9	5.2	Present study
X:G ($0.03h^{-1}$)[b]	n.d.	34.0	6.2	3.1	50.1	6.5	Present study
X:G ($0.06h^{-1}$)[b]	n.d.	37.3	8.7	2.8	47.3	3.9	Present study

[a]Peak areas less than 0.1% were considered insignificant and are presented as not detected (n.d.); [b] X:G represents xylose:glucose; X represents xylose; G represents glucose.

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ADDITIONAL FILE 3

Table A3. Cell mass constituents.

Component	Chemical formula
Lipid	$C\ H_{1.782}\ O_{0.110}$
Protein[a]	$C\ H_{0.581}\ N_{0.275}\ O_{0.318}$
Carbohydrate[a]	$C\ H_{1.667}\ O_{0.833}$
RNA[a]	$C\ H_{1.232}\ N_{0.389}\ O_{0.737}\ P_{0.105}$
DNA[a]	$C\ H_{1.255}\ N_{0.378}\ O_{0.612}\ P_{0.102}$
Phosphate	HPO_4
Sulfate	SO_4
Water	H_2O
Metals	M

[a]According to Lange and Heijnen (2001).

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CAPÍTULO 7. Conclusões e Sugestões para trabalhos futuros

"Embora ninguém possa voltar atrás e fazer um novo começo,
qualquer um pode começar agora e fazer um novo fim."

Chico Xavier

7.1. CONCLUSÕES

Foram feitos cultivos em batelada alimentada em biorreator estudando diferentes fontes de nitrogênio. Altas densidades celulares foram observadas utilizando uréia (94,2 g/L) e extrato de levedura + sulfato e amônio (80,7 g/L). Utilizando sulfato de amônio como única fonte de nitrogênio, a concentração celular resultou em somente 55,6 g/L. O conteúdo de lipídeos ao final dos cultivos foi similar entre as diferentes fontes de nitrogênio, variando de 35 a 38 % de lipídeos.

Entre as diferentes estratégias de alimentação estudadas em batelada alimentada e batelada alimentada repetida, altas concentrações celulares (~85 g/L) e de lipídeos (~40 g/L) foram encontradas quando utilizada a proporção de xilose:glicose (70:30) como fonte de carbono, simulando o hidrolisado hemicelulósico de bagaço de cana-de-açúcar.

Cultivos contínuos a baixas vazões específicas de alimentação (0,03 h^{-1}) resultaram nos maiores rendimentos de células (0,443 g/g) e lipídeos (0,236 g/g), ao passo que em D de 0,06 h^{-1} foram obtidas as maiores produtividades de células (0,600 g/L.h) e de lipídeos (0,288 g/L.h), indicando ser um processo apropriado para diversas aplicações industriais futuras.

O perfil de ácidos graxos apresentou pequenas variações de acordo com as condições de cultivo. Os lipídeos produzidos por *Lipomyces starkeyi* apresentaram perfil semelhante ao óleo de palma, sendo compostos majoritariamente por ácido palmítico (C16:0) e ácido oleico (C18:1), apresentando ainda menores proporções de ácido mirístico (C14:0), ácido esteárico (C18:0) e ácido linoleico (C18:2). Foi estimado o número de cetano dos ésteres de ácidos graxos, apresentando valores ao redor de 61, similar ao obtido para óleo de palma.

Em estudos de balanço de massa e de energia, verificou-se que o rendimento energético celular em cultivo em batelada alimentada (70 h) chegou a 0.9, valor mais alto que máximos encontrados na literatura (0,7), possivelmente devido à energia adicional produzida no metabolismo e estocada nas células como lipídeos. O calor de combustão resultou em 25,4 kJ/g, o que representa 56% do conteúdo energético do óleo diesel, indicando o potencial da levedura *L. starkeyi* para o biodiesel.

Cultivos contínuos em quimiostato (0,03 h^{-1} e 0,06 h^{-1}) foram feitos com diferentes fontes de carbono (xilose, glucose e xilose + glicose) a fim de compreender melhor o processo de acúmulo de lipídeos. Os resultados foram tratados pela ferramenta estatística de reconciliação de

dados e foi possível verificar a consistências da composição das células e o balanço de carbono dos cultivos. As medidas reconciliadas resultam em uma descrição consistente da massa celular com reduzidos erros do conteúdo elementar e macromolecular para posterior análise de fluxos metabólicos (AFM).

7.2. SUGESTÕES PARA TRABALHOS FUTUROS

Avaliar novas formas de estresse celular para maximizar o acúmulo de lipídeos sem que o crescimento celular seja influenciado, utilizando a proporção xilose:glicose (70:30).

Utilizar as condições otimizadas em meio contendo xilose:glicose (70:30) para cultivos contendo meio com hidrolisado hemicelulósico (H-H).

Implantar o processo de *scale up* em uma planta piloto, verificando a estabilidade do processo.

Estudar Análise de Fluxos Metabólicos (MFA) a partir de resultados de cultivos em quimiostato, para melhor compreensão do metabolismo de acúmulo de lipídeos na *L. starkeyi*.

Usar ferramentas de engenharia metabólica e engenharia genética para verificar a expressão de genes e direcionar o fluxo para a via metabólica de acúmulo de lipídeos da *L. starkeyi*.

Estudar o papel das principais enzimas envolvidas na via metabólica de acúmulo de lipídeos como a acetil-Coa carboxilase (ACC) presente na etapa inicial de síntese de ácidos graxos ou a diacilglicerol aciltransferase (DGA) na etapa final de síntese de triacilgliceróis (TGA).

Verificar a viabilidade econômica da produção de biodiesel a partir de leveduras oleaginosas, integrando à planta de produção de etanol, reaproveitando os efluentes líquidos e gasosos de cada unidade para diminuir os gastos com matéria-prima e maximizar o aproveitamento da biomassa.

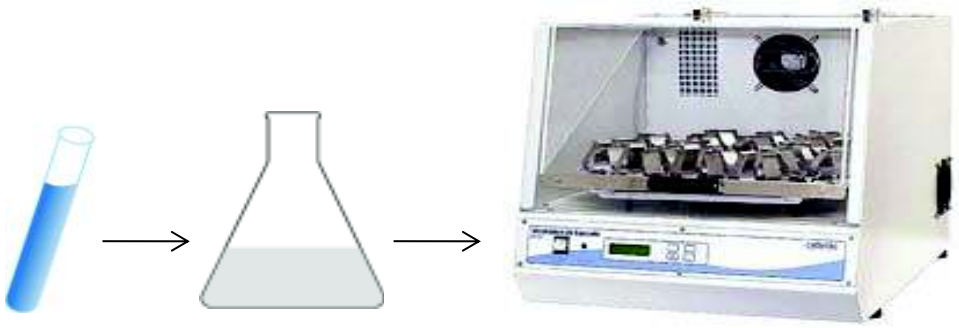
Anexo

I. Experiments in shake flasks

I.1. Effect of $FeSO_4$ and inoculum concentrations on *L. starkeyi* cultivation by Response Surface Methodology

I.2. Effect of carbohydrate concentration and C/N ratio on lipid production and cell growth by *Lipomyces starkeyi* DSM 70296 using experiment design in shake flasks

I.3. Studies of substrate inhibition for *L. starkeyi* cultivation



"You have to do your own growing no matter how tall your grandfather was."

Abraham Lincoln

I.1. Effect of $FeSO_4$ and inoculum concentrations on *L. starkeyi* cultivation by Response Surface Methodology

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ABSTRACT

In the present study, experiments were performed to optimize the medium composition for lipid production by the oleaginous yeast *Lipomyces starkeyi* DSM 70296 through co-fermentation of glucose and xylose (70:30, w/w). Optimization was carried out using a 2^2 full factorial design to study the effects of inoculum concentration (0.59 to 3.41 g/L) and $FeSO_4$ (0 to 13.8 mg/L) on lipid production and cell growth. Results indicated that the cell growth is more strongly influenced by inoculum concentration instead $FeSO_4$. The optimum conditions of cultivation promoted cell mass concentration around 25 g/L (with 3 g/L of inoculum, 2 mg/L of $FeSO_4$), while the highest lipid content was around 41% (1 g/L of inoculum, 2 mg/L of $FeSO_4$).

Keywords: Co-fermentation, *Lipomyces starkeyi DSM 70296,* Microbial oil, Oleaginous yeast, Response surface methodology.

I.1.1. Material and Methods

I.1.1.1. Microorganism and culture conditions

Lipomyces starkeyi DSM 70296 was used throughout this study. The yeast was preserved in agar slant (PDA). The yeast was inoculated in YPG medium to reactivate and was transfer to a inoculum medium with Carbohydrate (glucose + xylose) 20 g/L; $(NH_4)_2SO_4$, 1.0 g/L; KH_2PO_4, 3.5 g/L; Na_2HPO_4, 1.0 g/L; $Mg_2SO_4 \cdot 7H_2O$, 1.5 g/L; $CaCl_2 \cdot 2H_2O$, 0.2 g/L, yeast extract, 2.0 g/L. The inoculum was performed in 250 mL flasks (work volume of 50 mL) at 150 rpm, 28ºC and the pH was adjusted to 5.5.

The standard cultivation media was: 60 g/L of glucose:xylose (70:30, w/w), 1.92 g/L yeast extract, 1.36 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 1 g/L Na_2HPO_4, 0.4 g/L $MgSO_4.7H_2O$, 0.04 g/L $CaCl_2.2H_2O$, 0.08 g/L $ZnSO_4.7H_2O$, 0.001 g/L $CuSO_4.5H_2O$, 0.001 g/L $CoCL_2.6H_2O$, 0.001 g/L $(NH_4)_2Mo_2O_7$, 0.005 g/L $MnSO_4.H_2O$.

The cultivation was done using C/N ratio 50. To avoid medium darkness due to sugars reactions (caramelization and Maillard), carbohydrates solutions were sterilized (121ºC, 20 minutes) in a separate flask. All chemicals were obtained from local suppliers and were of analytical reagent grade. Shaking-flask cultures were carried out in 250-mL Erlenmeyer flasks containing 50 mL medium and incubated in a rotary shaker at 150 rpm, 28ºC, for 72 h.

I.1.1.2. Analytical Methods

For analyzing the growth pattern of the microorganisms, cell mass was assessed by turbidimetric estimation (600 nm) and with CDW using freeze-dried cells. Glucose and xylose were analyzed by ion chromatography (Metrohm system). Lipids were gravimetrically quantified by Bligh-Dyer's method (BLIGH, E. G. and DYER, W. J., 1959; MANIRAKIZA, COVACI and SCHEPENS, 2001). Inorganic nitrogen was determined by Berthelot reaction as described by Srienc (SRIENC, ARNOLD and BAILEY, 1984).

I.1.1.3 Experimental Design

A 2^2 full factorial design with four replicates at the center point (to estimate the random error for the analysis of variance), leading to a set of eight experiments were used to evaluate the effect of two variables: inoculum and $FeSO_4$ concentrations, on cell growth and lipid

accumulation in flask culture conditions. Table I.1 shows the ranges of the variables studied with the coded and real levels. RSM using the full factorial design was applied to further develop mathematical correlations between the two independent variables on the responses. The Statistica (version 7.0) software was used for regression and graphical analysis of the obtained data.

I.1.2. Results and Discussion

Table I.1 displays the design matrix and experimental results (responses). The highest values were obtained at different runs: a maximum cell mass concentration (around 25 g/L) was observed at run 2 (3 g/L of inoculum, 2 mg/L of $FeSO_4$). Highest lipid content (41%) and highest cellular productivity (0.140 g/L.h) were found at run 2 (1 g/L of inoculum, 2 mg/L of $FeSO_4$). There is also, that at runs 7 and 8, containing the same inoculum concentration (2 g/L) and varying concentrations of $FeSO_4$ (0 and 13.8 mg/L, respectively), the cell mass concentration did not change much. Also at run 8 was obtained the lowest lipid content (29 %). Already at runs 5 and 6 containing the same $FeSO_4$ concentration (6.9 mg/L) and varying the inoculum concentration (0.59 and 3.41 g/L, respectively), cell mass concentration varied considerably (17 to 23.78 g/L), indicating that the cell growth is more strongly influenced by inoculum concentration instead $FeSO_4$. Run 5 contained the lowest inoculum concentration (0.59%) justifying lower cell growth and productivities.

Results of cell mass concentration and lipid content were analyzed for regression analysis and variance (ANOVA). By the results of ANOVA, R^2 and statistic test factor (F) for the response lipid content, it was not possible to neither obtain a polynomial equation neither construct the surface responses. For cell mass concentration, a second order model was fitted to the data in order to evaluate the main effects of the two factors. The statistic test factor, F, was used to evaluate the significance of the models and factors at the 95% confidence level.

Table I. 1 Experimental design matrix with real values (in parentheses), coded levels and experimental results for the factorial design at 72 h.

Run	Variable level		Experimental results			
	x_1	x_2	CDW (g/L)	Cellular prod. (g/L.h)	Lipid content (%)	Lipid Prod. (g/L.h)
1	-1 (1)	-1 (2)	20.11	0.275	38.1	0.105
2	1 (3)	-1 (2)	24.89	0.341	41.1	0.140
3	-1 (1)	1 (11.8)	20.22	0.277	38.5	0.107
4	1 (3)	1 (11.8)	23.33	0.320	38.9	0.124
5	-1.41 (0.59)	0 (6.9)	17.00	0.233	37.4	0.087
6	1.41 (3.41)	0 (6.9)	23.78	0.326	38.0	0.124
7	0 (2)	-1.41 (0)	21.56	0.295	30.4	0.090
8	0 (2)	1.41 (13.8)	21.67	0.297	29.0	0.086
9	0 (2)	0 (6.9)	22.44	0.307	31.7	0.097
10	0 (2)	0 (6.9)	23.67	0.324	32.2	0.104
11	0 (2)	0 (6.9)	23.44	0.321	32.3	0.104
12	0 (2)	0 (6.9)	22.89	0.314	30.5	0.096

x_1: Inoculum concentration (g/L), x_2: $FeSO_4$ concentration (mg/L).

The model coefficients were calculated by regression analysis for each variable. The ANOVA (Table I.2) indicated that the model was significant and adequate to represent the actual relationship between the response and the significant variables with very small p-value (0.05). The pure error was very low (0.91), indicating good reproducibility from the cell mass data obtained. Fisher's *F*-test also demonstrates a very high significance for the regression model since the computed *F*-value (103.5) is much greater than the tabular *F*-value (4.26) at 5% level for cell mass concentration. Through the Analysis of Variance (Table I.2), the correlation coefficient obtained for the cell mass concentration and the result of F-test (24.3 times higher than F_{tab}) were good indicators for a model (coded equation) representative of the actual relationship among the selected reaction parameters (Eq. I.1).

Table I. 2 Analysis of variance for the regression model for cell mass concentration.

Source of variation	Sum of square	Degrees of freedom	Mean square	F-value[a]	*p*-Value
Regression	45.54	2	22.77	103.5	<0.0001
Residual	6.50	9	0.22		
Total	52.04	11			

R = 0.9171; $^{a}F_{2;9;0,5} = 4.26$

A direct correlation between the inoculum concentarion and cell mass was obviously found. Clearly, a higher inoculum concentration leads to higher cell mass. The R^2 value for Equation (I.1) is 0.9171, indicating that about 92% of the variations in cell mass can be explained by the quadratic polynomial. This means that Equation (I.1) is adequate for correlating the experimental results. The factors x_1 and x_2 are specified in their coded units.

$$CDW\ (g/L) = 23.11 + 2.18x_1 - 1.08x_1^2 - 0.16x_2 - 0.46x_2^2 - 0.42\ x_1x_2 \qquad (I.1)$$

According to the *t* and *p* value, the term of x_2 and x_2^2, did not have the statistical significance. In agreement with the former conclusions that the corresponding variables might be more significant if the absolute *t* value became large and the *p* value became smaller, the analysis indicated that independent variable x_2 ($FeSO_4$ concentration) in investigated range, did not have significant effect on the response variable. The regression model was used to construct the response surface and contour plot (Figure I.1).

The measured value of CDW is also shown and this figure also depicts the interactive effect of inoculum and $FeSO_4$ concentrations on cell mass concentration. The cell mass concentration showed no significant increase with increase of $FeSO_4$ concentration. The contour plot indicates that the highest cell mass concentration occurs at a higher inoculum concentration at any $FeSO_4$ concentration at the studied range.

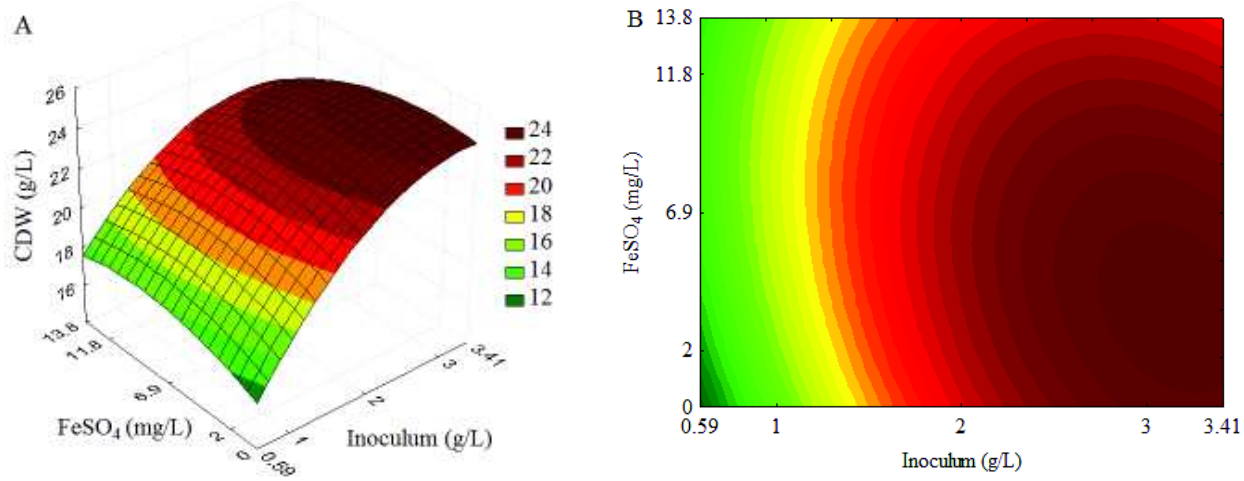

Figure I. 1 Response surface (A) and contour plot (B) obtained from Equation (I.1) showing the effect of percent of inoculum and $FeSO_4$ concentration on cell mass concentration.

The experimental cell mass versus the corresponding values were calculated by the regression model (Figure I.2). A line of perfect fit is also shown in this figure. This plot therefore visualizes the performance of the quadratic model in an obvious way. The results in Figure I.2 confirm that the regression model provide an accurate description of the experimental data.

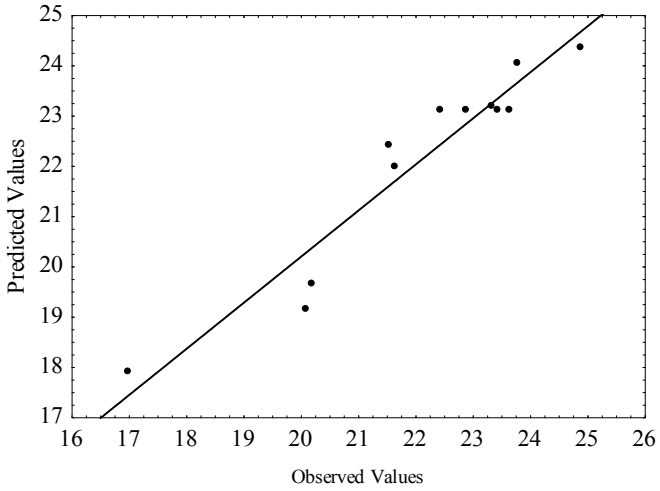

Figure I. 2 Cell mass concentration calculated from second-order regression model (Equation (I.1)) vs. the corresponding experimentally measured values.

The influence of inoculation volume (10, 15, 20, 25, and 30%) on the lipid content by *Rhodotorula glutinis* was investigated. Uniform design and single-factor experimental design were employed to investigate the effects of culture conditions on the lipid production by *R.*

glutinis. Optimal cultivation conditions were obtained as follows: glucose as carbon source 100 g/L; yeast extract and peptone as nitrogen sources at, respectively, 8 and 3 g/L; initial pH of 5.0; inoculation volume of 5%; temperature at 28ºC, shaking speed of 180 r/min. Under these conditions, *R. glutinis* accumulated lipids up to 49.25% on a cellular mass basis with cell mass yield of 29.77 g/L and lipid productivity thus reached 14.66 g/L (DAI *et al.*, 2007).

At higher levels of inoculum, *Rhodotorula gracilis* CFR-1 was found to be more tolerant to higher concentrations of sugar, and significantly increased lipid production was noticed. Through the fitted models of second order, as per RSM, carbon at 10.24%, nitrogen at 0.37 g/L, and inoculum at the 20% level resulted in maximum biosynthesis of lipids (PRAPULLA *et al.*, 1992). Others studies uses 10% of inoculum for lipid production in oleaginous yeast (ZHAO *et al.*, 2008; WU *et al.*, 2011).

Zhao et al. (2008) studied the effect of $FeSO_4$ in the range of 4 to 8 mg/L on cell growth and lipid accumulation using *L. starkeyi* AS 2.1560. The RSM model indicated that the optimal condition for the lipid content of *L. starkeyi* AS 2.1560 corresponded to $FeSO_4$ at 4.0 mg/L (ZHAO *et al.*, 2008). Other study uses 8.2 mg/L of $FeSO_4$ for lipid production using *L. starkeyi* NRRL Y-11557 and glucose (30 g/L) as carbon source in different cultivation modes (WILD *et al.*, 2010). Kurosawa *et al.* (2010) studied different glucose and NH_4SO_4 concentrations in lipid accumulation using *Rhodococcus opacus* PD630 with 0.5 g/L of $FeSO_4.7H_2O$ (KUROSAWA *et al.*, 2010). Other studies also used $FeSO_4.7H_2O$ in the media for lipid production by oleaginous yeasts with 16 mg/L (ANGERBAUER *et al.*, 2008) and 0.55 g/L (MEESTERS, HUIJBERTS and EGGINK, 1996).

I.1.3. Conclusions

Our results clearly showed that the cell mass concentration directly depends on inoculum concentration. The optimum conditions of fcultivation (3 g/L of inoculum and 2 mg/L of $FeSO_4$) promoted cell mass concentration around 25 g/L. This work provided valuable information for further investigation of higher inoculum concentrations on lipid production and cell growth in bioreactor experiments in order to decrease the lag growth phase.

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I.2. Effect of carbohydrate concentration and C/N ratio on lipid production and cell growth by *Lipomyces starkeyi* DSM 70296 using experiment design in shake flasks

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Abstract

In this study, a 2^2 Full Factorial Design was used to evaluate the effect of xylose concentration (17.7 to 102.3 g/L) and C/N ratio (8.9 to 51.2), on cell growth and lipid accumulation in flask culture conditions. By the results, there was no clear optimum response within the experimental area investigated because the highest cell mass concentration lies at the upper bound of the xylose concentration range. Nevertheless, the contour plot indicates that the hihgest cell mass concentration occurs at a higher carbohydrate concentration at any C/N ratios within the studied range.

I.2.1. Materials and Methods

Same culture conditions and analytical methods were used as Section I.1.1.

I.2.2. Results and Discussion

Table I.3 displays the design matrix and results. Four additional runs at the center point level were included in the design matrix to check reproducibility. Runs were conducted in randomized order to guard against systematic bias. Significant variation on cell mass concentration and lipid content exist within the 12 runs (Table I.3) and the highest values were obtained at different runs: a maximum cell mass concentration (around 30 g/L) was observed at run 2 (90 g/L xylose, C/N 15). Highest lipid content around 44% was found for the center points (60 g/ xylose and C/N 30), and the highest cellular productivity, 0.1574 g/L.h was found at run 7 (60 g/L and C/N 8.9). Run 8 was done with 60 g/L of xylose and C/N ratio of 51.2, resulting in 19.5 g/L cell mass concentration. Run 5 contained the lowest xylose concentration (17.7), which was depleted at 72h, justifying the lower cell growth and productivities.

The results of cell mass concentration were analyzed for regression analysis and variance (ANOVA). A first order model was fitted to the data to evaluate the main effects of the two factors. The statistic test factor, F, was used to evaluate the significance of the models and factors at the 95% confidence level. If the calculated value of F is greater than the tabular F at the specified probability level, a statistically significant model or factor is obtained. After applying ANOVA statistical test, it was found that a first-order model for cell mass was satisfactory.

Table I. 3 Experimental design matrix with values of real (in parentheses), coded levels and experimental results for the factorial design.

	Variable level		Experimental results					
Run	x_1	x_2	**Xylose depletion (h)***	**CDW (g/L)**	**Celular produtiv. (g/L.h)**	**Lipid conten (%)**	**Lipid Produtiv. (g/L.h)**	**Free CDW (g/L)**
1	-1 (30)	-1 (15)	96	11.6	0.1208	34.6	4.02	7.6
2	1 (90)	-1 (15)	216	29.9	0.1382	41.3	6.17	17.5
3	-1 (30)	1 (45)	120	11.7	0.0978	38.3	1.50	7.2
4	1 (90)	1 (45)	216	25.2	0.1306	37.2	2.62	17.7
5	-1.41 (17.7)	0 (30)	72	7.3	0.1019	35.0	0.51	4.8
6	1.41 (102.3)	0 (30)	216	25.4	0.1177	40.2	1.70	15.2
7	0 (60)	-1.41 (8.9)	144	22.7	0.1574	42.2	1.37	13.1
8	0 (60)	1.41 (51.2)	216	19.5	0.0904	34.8	0.85	12.7
9	0 (60)	0 (30)	216	20.1	0.0931	44.1	0.98	11.2
10	0 (60)	0 (30)	192	21.5	0.1141	45.6	1.00	11.9
11	0 (60)	0 (30)	192	20.9	0.1083	42.0	0.79	12.1
12	0 (60)	0 (30)	168	21.2	0.1263	44.6	0.79	11.8

* x_1: xylose concentration (g/L), x_2: C/N molar ratio. The cultivation was finished at this time for each experiment.

Clearly, higher xylose concentration leads to higher cell mass. Comparing the cell mass concentration at the low level of C/N ratio with the cell mass concentration at the high level in Table I.3 indicates that the cell mass was somewhat negatively affected by C/N ratio (22.7 vs. 19.5 g/L) since there would be less available nitrogen for biosynthesis of yeast cells.

A good fit of quadratic polynomials is expressed by the coefficient of determination, R^2. The closer the value of R^2 is to 1, the better is the correlation between the observed and predicted values. The R^2 value for Equation (3.2) was 0.9822, indicating that about 98% of the variations in cell mass can be explained by the quadratic polynomial. This means that Equation (I.2) is adequate for correlating the experimental results. The factors x_1 and x_2 are specified in their coded units.

$$CDW\ (g/L) = 20.98 + 8.92x_1 - 0.09x_1^2 - 0.01\ x_1x_2 \qquad (I.2)$$

According to the t and p value, the term of x_2 and x_2^2, did not have the statistical significance. In agreement with the former conclusions that the corresponding variables might be more significant if the absolute t value became large and the p value became smaller, the analysis indicated that independent variable x_2 (C/N ratio) in investigated range, did not have significant effect on the response variable.

The regression model was used to construct the response surface and contour plot (Figure I.3). It can be seen that there is no clear optimum within the experimental area investigated because the best cell mass concentration lies at the upper bound of the xylose concentration range. Nevertheless, the contour plot indicates that the best cell mass concentration occurs at a higher xyloseconcentration at any C/N ratios within the studied range.

The model coefficients were calculated by regression analysis for each variable. Table I.4 shows that only the regression coefficients of xylose concentration linear (L), quadratic term (Q) and two cross-products are significant at 5% level.

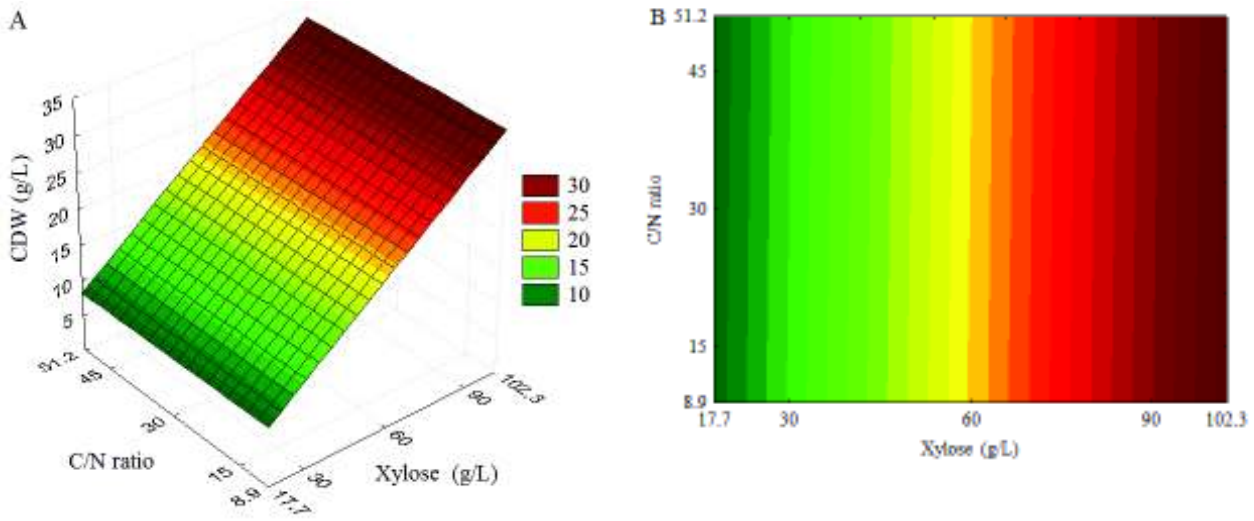

Figure I. 3 Response surface (A) and contour plot (B) obtained from Equation (I.2) showing the effect of xylose concentration, C/N ratio, and their mutual interaction on cell mass concentration.

Table I. 4 Significance of regression coefficients for the cell mass concentration.

Variables	Regression coefficients	Standard error	Computed *t* value	Significance level, *p* value
Mean *	20.98	0.56	37.09	0.0000
(1) X (L) *	8.92	0.66	13.52	0.0000
X (Q) *	-0.09	0.01	-13.40	0.0000
(2) C/N (L)	-0.08	0.66	-0.13	0.9024
C/N (Q)	0.01	0.01	0.11	0.9127
1 L x 2 L*	-0.01	0.00	-2.67	0.0368

* Significant factors at 5% level; X: xylose concentration.

According to the *t* and *p* value, the term of x_2 and x_2^2, did not have statistical significance. The analysis indicated that the independent variable x_2 (C/N ratio) in investigated range, did not have significant effect on the response variable. The ANOVA (Table 3.5) indicated that the model was significant and adequate to represent the actual relationship between the response and the significant variables with very small p-value (0.05).

The pure error was very low, indicating good reproducibility from the cell mass concentration obtained. Fisher's *F*-test also demonstrates a very high significance for the

regression model since the computed *F*-value (97.03) is much greater than the tabular *F*-value (4.12) at 5% level for the cell mass concentration.

Table I. 5 Analysis of variance for the regression model for cell mass concentration.

Source of variation	**Sum of square**	**Degrees of freedom**	**Mean square**	**F-value[a]**	***p*-Value**
Regression	434.66	4	108.67	97.03	<0.0001
Residual	7.86	7	1.12		
Total	442.52	11			

$R^2 = 0.9822$; $^{a}F_{4;7;0,5} = 4.12$

Through the Analysis of Variance (Table I.5), the correlation coefficient obtained for the cell mass concentration and the result of F-test (23.55 times higher than F_{tab}) were good indicators for a model (coded equation) representative of the actual relationship among the selected reaction parameters (Eq. I.2). The experimental cell mass versus the corresponding values were calculated by the regression model (Figure I.4). A line of perfect fit is also shown in this figure. This plot therefore visualizes the performance of the quadratic model in an obvious way. The results in Figure I.4 confirm that the regression model provide an accurate description of the experimental data.

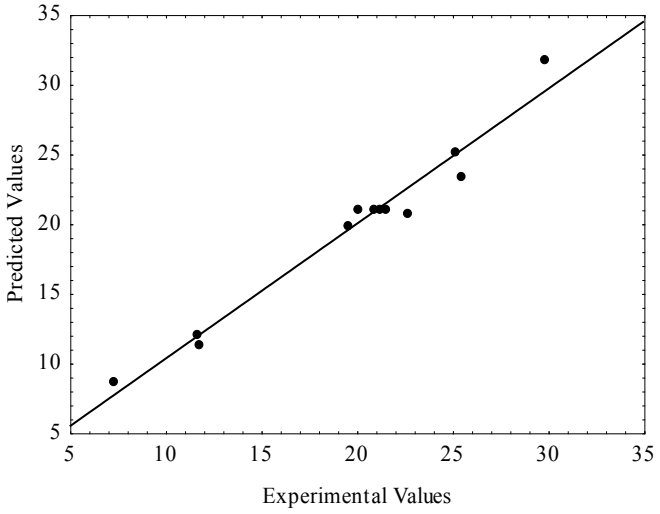

Figure I. 4 Cell mass concentration calculated from second-order regression model (Equation (I.2) vs. the corresponding experimentally measured values.

Angerbauer et al. (2008) compared the influence of C/N ratios (15, 20, 30, 60 and 150) on lipid accumulation by *L. starkeyi*. The highest lipid content was measured at a C/N ratio of 150, with 68% lipids of the dry matter. The CDW was 9.5 g/L, with a lipid concentration of 6.4 g/L.

Nutrient imbalance in the culture medium has long been known to trigger lipid accumulation by oleaginous microorganisms. When cells run out of key nutrients, usually nitrogen, excess substrate continues to be assimilated by the cells and converted into fat for storage. However, under nitrogen-limited conditions, cell propagation is drastically depressed, which in many cases restricts cell density and cell productivities (LI, ZHAO and BAI, 2007).

It was reported that when C/N ratio was increased from 25 to 70, oil content increased from 18% to 46% (HASSAN *et al.*, 1996). Different nitrogen sources also affected the oil production. Both inorganic nitrogen sources and organic nitrogen sources can be used for yeast cultivation with differences in oil accumulation (LI, DU and LIU, 2008).

Results of the study illustrated the importance of these factors on cell mass concentration. The experimental results clearly showed that the cell mass directly depends on carbohydrate concentration and secondary on C/N ratio. The optimum cultivation condition (90 g/L of xylose and C/N 15) promoted cell mass concentration of 30 g/L.

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I.3. Studies of substrate inhibition for *L. starkeyi* cultivation

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ABSTRACT

Inhibition studies are needed when the goal is to establish maximum substrate concentration that can be used in a single batch process in order to increase overall productivity. In addition, better knowledge of the kinetics of the process enables the definition of necessary parameters for the mathematical description. In this study are presented the results of xylose inhibition by *L. starkey* DSM 70296. Xylose inhibition experiments conducted from 10 g/L to 300 g/L revealed that high cell density and lipid content were achieved by keeping the sugar concentration below 90g/L. These data suggested that a wide range of substrate concentrations were suitable for lipid production and cell growth by this strain with no significant substrate inhibition. This made it possible to feed concentrated xylose solution at long time intervals in a discontinuous way during the fed-batch cultivation experiments.

Keywords: *L. starkeyi* DSM 70296, microbial oil, oleaginous yeast, substrate inhibition.

I.3.1. Material and Methods

Single isolated colonies from fresh PDA slant were used to inoculate 50 mL of YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28°C and 150 rpm in shake flasks. After the pre-culture medium reached the logarithmic growth phase, a second flask was inoculated with 10% (v/v) of seed suspension containing 50 mL of inoculum medium (xylose 20 g/L; $(NH_4)_2SO_4$, 1.0 g/L; KH_2PO_4, 3.5 g/L; Na_2HPO_4, 1.0 g/L; $Mg_2SO_4 \cdot 7H_2O$, 1.5 g/L; $CaCl_2 \cdot 2H_2O$, 0.2 g/L, yeast extract, 2.0 g/L). The pH was adjusted to 5.5 with HCl 1 M and the flasks were incubated under the same temperature and agitation conditions for a period between 24 and 36 hours.

The cultivations were done under the same conditions using a standard medium with a C/N 10 (using yeast extract and $(NH_4)_2SO_4$) containing xylose (10 to 300 g/L), 1 g/L KH_2PO_4, 1 g/L Na_2HPO_4, 0.4 g/L $MgSO_4.7H_2O$, 0.04 g/L $CaCl_2.2H_2O$, 0.08 g/L $ZnSO_4.7H_2O$, 0.001 g/L $CuSO_4.5H_2O$, 0.001 g/L, $CoCL_2.6H_2O$, 0.001 g/L, $(NH_4)_2Mo_2O_7$, 0.005 g/L $MnSO_4.H_2O$.

To avoid medium darkness due to sugars reactions (caramelization and Maillard), carbohydrates solutions were sterilized (121ºC, 20 minutes) in a separate flask. All chemicals were obtained from local suppliers and were of analytical reagent grade. Shaking-flask cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL medium and incubated in a rotary shaker at 150 rpm, 28ºC, for 192 h. Analytical methods were performed according Section I.1.1.2.

I.3.2. Results and Discussion

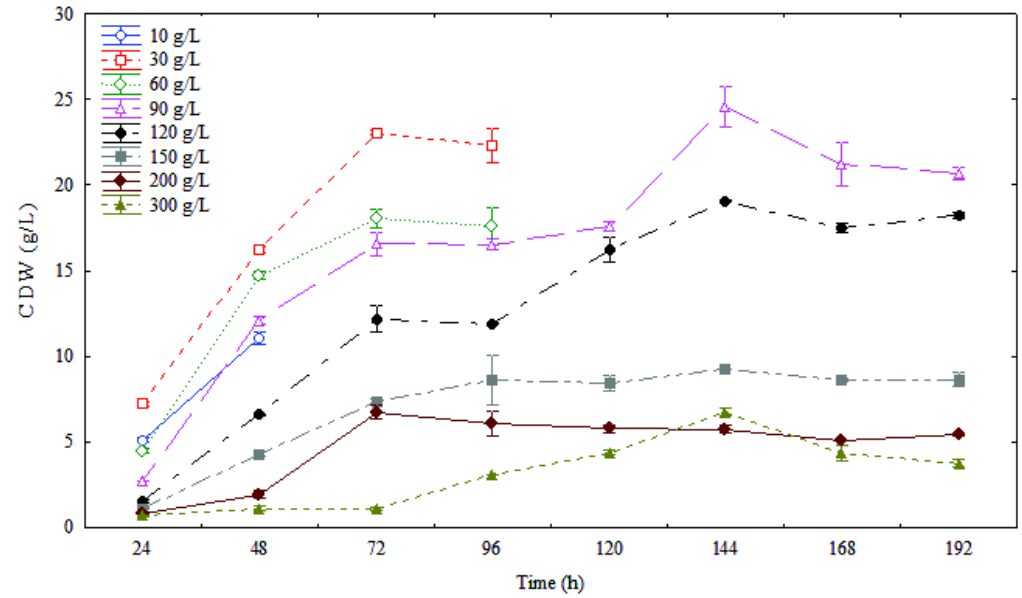
In order to investigate a possible inhibitory effect of xylose on the growth of *L. starkeyi* DSM 70296, the experiments in shaker were performed at C/N 10 to favor the growth of the oleaginous yeast. When the initial xylose concentration increased from 10 to 90 g/L, cell mass concentration and lipid content increased from 11.0 to 22.3 g/L and from 14.9 from 23.2 %, respectively (Table I.6). However, cell mass concentration and lipid content slightly dropped to 18.2 g/L and 13 % at cultures with an initial xylose concentration of 120 g/L. When the substrate concentration reached 150 g/L, cell mass and lipid concentrations were greatly decreased, suggesting that a considerable inhibitory effect had occurred.

It was observed that the lipid yield ($Y_{L/S}$) was maximum (0.164 g/g) at the lowest initial xylose concentration tested, and by using 30 g/L of xylose a good lipid yield (0.144 g/g) was also found.

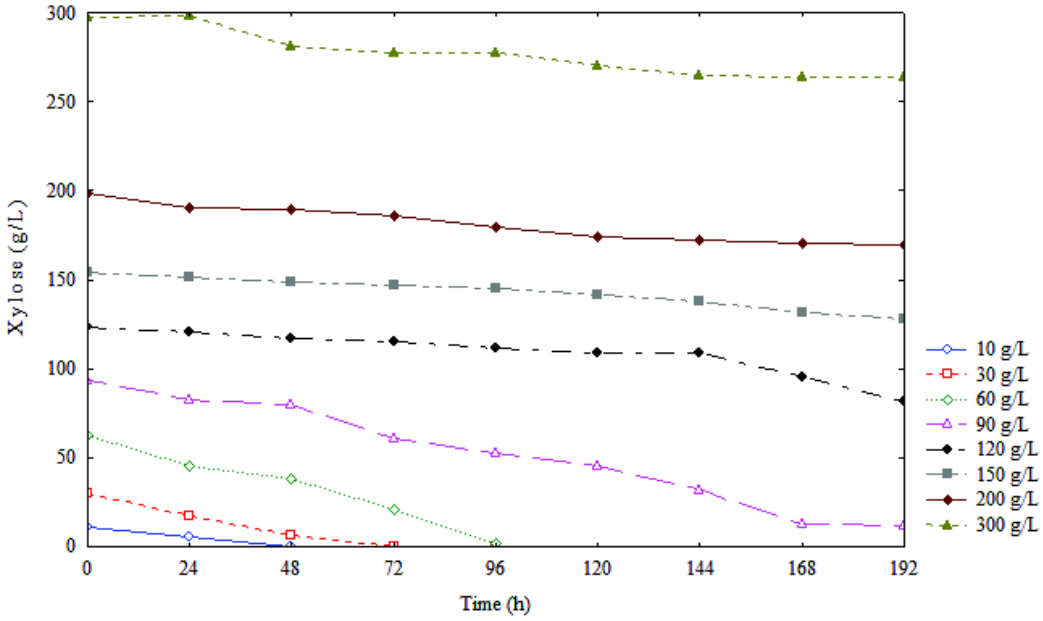
Table I. 6 Data extracted from the inhibition substrate study conducted with *L. starkeyi* cultivated at C/N 10.

Initial xylose concentration (g/L)	CDW (g/L)	Xylose conversion (%)	Lipid content (%)	$Y_{L/S}$ (g/g)
10	11.0	100	14.9	0.164
30	22.3	100	19.4	0.144
60	17.6	97.1	22.1	0.067
90	20.7	87.2	23.2	0.061
120	18.2	12.2	13.0	0.062
150	8.6	8.3	12.9	0.050
200	5.5	15.4	9.8	0.017
300	3.8	12.1	6.6	0.007

A maximum cell mass concentration of 24.6 g/L (Figure I.4a) and lipid content (23.2) were obtained at experiment with initial xylose concentration of 90 g/L (144h). When 120 g/L of xylose was used, was obtained 19 g/L of cells at 192 h, but with lower xylose conversion. Using 10 g/L of xylose, the sugar was completely consumed at 48 h and using 30 and 60 g/L of xylose, it was depleted at 96 h (Figure I.4b). At higher xylose concentrations (above 90 g/L), the carbohydrate was not completed consumed up to 192 h. Experiments were stopped when all xylose was consumed and stationary phase was observed. This data set indicates that a xylose concentration above 90 g/L inhibits the growth of *L. starkeyi*.



(a)



(b)

Figure I. 5 Effect of initial xylose concentration in the growth of L. starkeyi DSM 70296 for a) growth kinetics; b) xylose consumption.

By the results, it can be observed that this yeast is able to produce triglycerides even in low C/N ratios (10 g/g), with lipid content higher than expected due to the C/N used. Above 120 g/L, the studied parameters (xylose conversion, lipid content and lipid yield) were greatly repressed and more severe inhibitory effects were observed at even higher xylose concentrations.

Nutrient imbalance in the culture medium has long been known to trigger lipid accumulation by oleaginous microorganisms. When cells run out of key nutrients, usually nitrogen, excess substrate continues to be assimilated by the cells and converted into fat for storage. However, under nitrogen-limited conditions, cell propagation is drastically depressed, which in many cases restricts cell density. To achieve a high-density cell culture for microbial lipid fermentation, different substrates and cultivation modes have been used (LI, , ZHAO and BAI, 2007).

Studies performed by other oleaginous yeasts by Sattur and Karanth (1991) showed growth inhibition by glucose in the range of 20 to 100 g/L using *Rhodotorula gracilis* CFR-1 with a C/N ratio of 70, the maximum specific growth rate of 0.105 h^{-1} was applicable for substrate concentrations less than 60 g/L.

To determine the effect of substrate concentration on growth profile of *R. toruloides* Y4, batch experiments were performed in conical flasks with glucose concentration ranging from 10 to 400 g/L (LI, ZHAO and BAI, 2007). The results indicated that the yeast grew well on glucose as a sole source of carbon and energy at a concentration up to 150 g/L.

It is well known that sugars in various forms are the most common carbon source, easily assimilated by many oleaginous microorganisms. However, experimental and theoretical results clearly indicate that sugars could be an inhibitory factor for microbial growth. Thus, in order to achieve higher cell mass growth rates, researchers should take into account such limitations and estimate in each particular case the best conditions concerning nutrient concentrations (ECONOMOU *et al.*, 2011).

I.3.3. Conclusions

To develop an improved culture method for lipid production with *L. starkeyi* DSM 70296, batch flask cultures were carried out to determine the suitable substrate concentration of the initial medium. These data suggested that a wide range of substrate concentrations were suitable for lipid production and cell growth by this strain with no significant substrate inhibition. It was found that *L. starkeyi* DSM 70296 grew in xylose concentrations up to 120 g/L, indicating a good ability to deal with osmotic stress. However, drastic decrease in cell mass concentration was found for the culture when xylose concentrations were higher than 150 g/L suggesting that a considerable inhibitory effect had occurred. This made it possible to feed concentrated xylose

solution at long time intervals in a discontinuous way during the fed-batch cultivation experiments.

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