Universidade de Brasília
Instituto de Ciências Biológicas
Departamento de Biologia Celular
Programa de Pós-Graduação em Biologia Molecular

Engenharia metabólica em *Pichia pastoris* para produção de L-ácido lático a partir de glicerol, um resíduo da indústria de biodiesel

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Orientadora: Profª. Drª. Nádia Skorupa Parachin

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Dedicatória

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Resumo

O descarte de resíduos plásticos no meio ambiente associado à dependência de insumo petroquímico para produção dos mesmos tem gerado grande preocupação, levando à busca constante pelo desenvolvimento de tecnologias verdes renováveis. Segundo a Plastics Europe, em 2016, 25,8 milhões de toneladas de resíduos de plástico pós-consumo acabaram nos fluxos oficiais de resíduos. Destes, 69,2% foi recuperado através de processos de reciclagem e recuperação de energia, enquanto 30,8% continuaram a ser depositados em aterros sanitários. Uma alternativa a utilização de plásticos derivados de petróleo é a produção de plásticos biodegradáveis, como o PLA -poli(ácido lático)-, pois além de ter tempo de degradação diminuído, é produzido a partir de fontes renováveis em bioprocessos estabelecidos, como exemplo, a fermentação microbiana. Dentre os diferentes microrganismos utilizados no estabelecimento destes bioprocessos, a utilização de leveduras é vantajosa, por produzirem metabólitos, proteínas recombinantes e permitir manipulação gênica para otimização. A levedura metilotrófica *Pichia pastoris* tem sido bastante utilizada em processos fermentativos por atingir altas densidades celulares e produzir poucos co-produtos. Além disso, esta é capaz de utilizar glicerol como única fonte de carbono, principal resíduo da indústria do biodiesel. Porém, não é capaz de produzir ácido lático, monômero utilizado na síntese de PLA. Assim o presente estudo teve como objetivo a construção de cepas geneticamente modificadas de *P. pastoris* capazes de produzir ácido lático utilizando glicerol como substrato. Para isto, o gene codificador da enzima lactato desidrogenase bovina (*Bos taurus*) foi inserido no genoma de *P.pastoris*, permitindo que a cepa X-33 produzisse ácido lático. Embora *P. pastoris* seja conhecida por seu metabolismo respiratório, as fermentações em batelada realizadas com baixa oxigenação aumentou a produção de ácido láctico em 20%, indicando que nesta situação o metabolismo fermentativo prevaleceu. Além disso, um novo transportador putativo de lactato de *P. pastoris* denominado PAS, foi identificado por similaridade de pesquisa com o transportador de lactato de *Saccharomyces cerevisiae* Jen1p. Ambos os transportadores heterólogos e homólogos, Jen1p e PAS, foram avaliados em uma cepa que já continha atividade LDH. As fermentações em batelada das cepas de *P. pastoris* com o transportador de lactato foram realizadas em condições aeróbicas seguida por uma fase de oxigênio limitado, nesta a produção de ácido lático foi maior. Os resultados mostraram que a cepa contendo o transportador PAS apresentou o maior rendimento, 0,7 g/g (ácido lático/ glicerol).

Abstract

The disposal of plastic waste in the environment associated with the dependence of petrochemical input for its production has generated great environmental concern, leading to the constant search for the development of green technologies. According to Plastics Europe, in 2016, 25.8 million tonnes of post-consumer plastic waste ended up in official waste streams. Of these, 69.2% was recovered through recycling and energy recovery processes, while 30.8% continued to be deposited in landfills. An alternative to the use of petroleum-based plastics is the production of biodegradable plastics, such as PLA –poly (lactic acid)-, in addition to having decreased degradation time, it is produced from renewable sources in established bioprocesses, such as microbial fermentation. Among the different microorganisms used in the establishment of these bioprocesses, the use of yeasts is advantageous, because they produce metabolites, recombinant proteins and allow gene manipulation for optimization. The methylotrophic yeast *Pichia pastoris* has been widely used in fermentative processes because it reaches high cell densities and produces few co-products. In addition, it is able to use glycerol as a unique carbon source, the main residue of the biodiesel industry. However, it is not capable of producing lactic acid, the monomer used in PLA synthesis. Thus the present study had as objective the construction of genetically modified strains of *P. pastoris* capable of producing lactic acid using glycerol as substrate. For this, the gene encoding bovine (*Bos taurus*) lactate dehydrogenase enzyme was inserted into the genome of *P.pastoris*, allowing strain X-33 to produce lactic acid. Batch fermentation of the *P. pastoris* X-33 strain producing *LDHb* allowed for lactic acid production in this yeast. Although *P. pastoris* is known for its respiratory metabolism, batch fermentations were performed with different oxygenation levels, indicating that lower oxygen availability increased lactic acid production by 20 %, pushing the yeast towards a fermentative metabolism. Furthermore, a newly putative lactate transporter from *P. pastoris* named PAS has been identified by search similarity with the lactate transporter from *Saccharomyces cerevisiae* Jen1p. Both heterologous and homologous transporters, Jen1p and PAS, were evaluated in one strain already containing LDH activity. Fed-batch experiments of *P. pastoris* strains carrying the lactate transporter were performed with the batch phase at aerobic conditions followed by an aerobic oxygen-limited phase where production of lactic acid was favored. The results showed that the strain containing PAS presented the highest lactic acid titer, reaching a yield of approximately 0.7 g/g (lactic acid/ glycerol).

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ANP	Agência Nacional de Petróleo, Gás Natural e Biocombustíveis
AOX	Álcool oxidase
ATP	Adenosina trifosfato
Bio-PE	Bio – polietileno
Bo-PET	Bio – poli (Tereftalato de Etileno)
BLAST	Basic Local Alignment Search Tool
D-LA	D(-) ácido lático
DNA	Ácido desoxirribonucleico
EDTA	Ácido etileno-diamino-tetra-acético
g	Grama
g	Força gravitacional
FDA	Food and drug administration
GAP	Gliceraldeído-3-fosfato
h	Hora
kb	Quilobase
Km	Constante de Michaelis–Menten
KOH	Hidróxido de potássio
L	Litro
LB	Luria-Bertani
LBLS	Luria-Bertani Low Salt
LDH	Lactato desidrogenase
L-LA	L(+) ácido lático
M	Molar
mg	Miligrama
min	Minuto
mL	Mililitro
mM	Milimolar
NCBI	National Center for Biotechnology Information
ng	Nanograma
NaAc	Acetato de sódio
NH_4OH	Hidróxido de amônio
OD_{600}	Densidade óptica medida a 600 nm

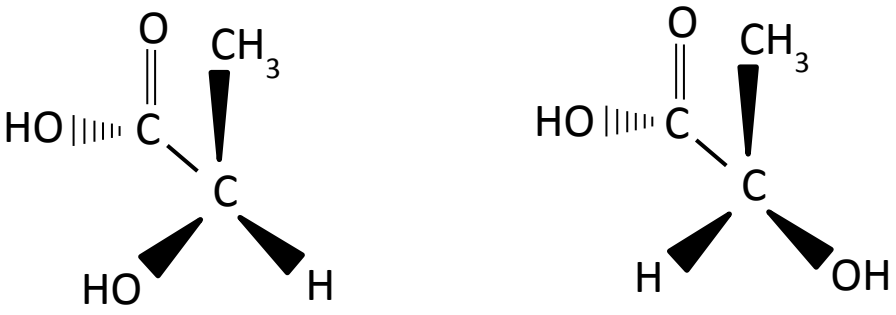
ORF	Open reading frame
pb	Par de base
PBS	Polibutadieno-Estireno
PBT	Poli (tereftalato de butileno)
PCR	Reação em cadeia de polimerase
PET	Poli (Tereftalato de Etileno)
PGK	Fosfoglicerato quinase
pH	Potencial hidrogeniônico
PHA	Polihidroxialcanoatos
pKa	Constante de dissociação ácida
PLA	Poli (ácido-lático)PP Polipropileno
PPE	Poli (p-fenileno éter)
PVC	Policloreto de Vinila
p/v	Peso por volume
q	Taxa de produção específica (g/g/h)
r	Taxa de produção volumética (g/L/h)
RNAse A	Ribonuclease A
rpm	Rotações por minuto
s	Segundo
SDS	Dodecilsulfato de sódio
TAE	Tampão TRIS-acetato-EDTA
U	Unidade
Vmáx	Velocidade máxima da reação
v	Volume
V	Volts
v/v	Volume por volume
Y	Rendimento (g/g)
YNB	Yeast nitrogen base
Y-PER	Yeast protein extraction reagent
^{o}C	Graus Celsius
µg	Micrograma
µL	Microlitro
Ω	ohm

Sumário

1. Introdução

1.1 Ácido lático

O ácido lático recebeu esta nomenclatura por ter sido descoberto e isolado a partir de leite fermentado em 1780 pelo químico sueco Carl Wilhelm Scheele (BENNINGA, 1990). Este ácido, também conhecido como ácido 2- hidroxi propanóico ($C_3H_6O_3$), possui diversas propriedades multifuncionais, como (I) acidulante; (II) umectante; (III) reatividade bifuncional associada com a presença de um grupo carboxi e um grupo hidroxi, e; (IV) atividade óptica assimétrica do carbono 2 (Martinez et al., 2013). Além disso, o ácido lático possui duas estereoformas L(+) e D(-) ácido lático (Figura 1).



D(-) ácido lático L(+) ácido lático

Figura 1 - Estereoformas do ácido lático

As propriedades físico-químicas do acido lático permitem que este tenha vasta aplicação na indústria alimentícia, em bebidas, doces, carnes e molhos; em curtimento de couros e tratamentos têxteis, bem como na indústria farmacêutica e na preparação de cosméticos (DUSSELIER et al., 2013). Em particular, as indústrias de alimentos e farmacêuticas têm uma preferência pelo isómero L (+), pois somente esta estereoforma pode ser metabolizada pelo corpo humano. Contudo, a indústria química utiliza tanto os isômeros puros como a mistura recêmica, dependendo da aplicação (MARTINEZ et al., 2013).

De acordo com Nampoothiri e colaboradores (2010), baseado nas aplicações do ácido lático, custo de matéria-prima e investimento de capital, o custo de produção da mistura racêmica deste ácido foi calculado em $0,55/kg, sendo que as formas enantioméricas puras possuem preço de mercado superiores. Estima-se que o mercado global para o ácido lático alcance 1.960,1 kilo toneladas até 2020. Em termos de receita, o mercado apresentou valor superior a USD 1,2 milhões em 2013, com expectativa de

atingir USD 4,3 milhões até 2020, representando um crescimento de 18,3% neste período (Grand View Research, 2014).

Apesar de ser amplamente encontrado na natureza, o ácido lático, um dos mais antigos ácidos orgânicos conhecidos, é atualmente produzido na sua maioria por síntese química ou fermentação lática (Figura 2) (JOHN; NAMPOOTHIRI, 2007). A síntese química de ácido lático produz mistura racêmica, enquanto que a produção fermentativa permite a produção de L- ou D- ácido lático opticamente puro, dependendo do microrganismo utilizado (LI; CUI, 2010). A eficiência dos processos de fermentação depende principalmente do microrganismo produtor, podendo ser de diversas espécies microbianas, incluindo bactérias, fungos, leveduras, algas e cianobactérias (DATTA; HENRY, 2006).

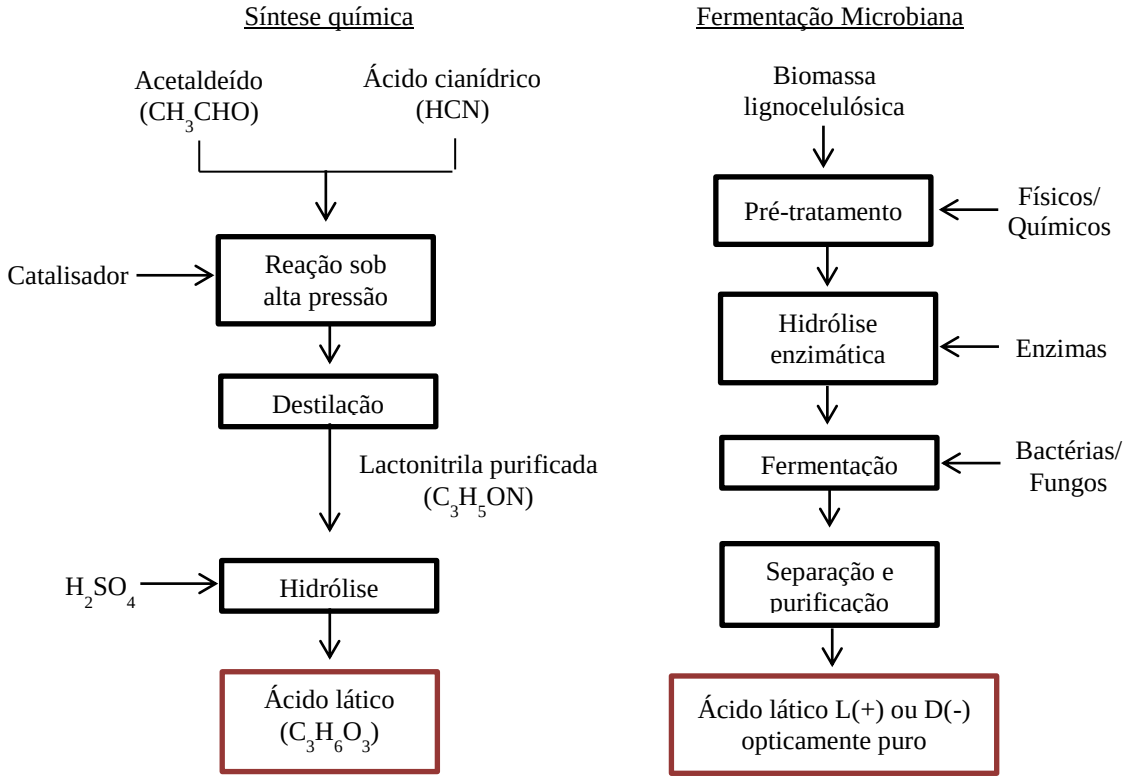


Figura 2 - Esquema das estapas da síntese química e fermentação microbiana para obtenção de ácido lático (Adaptado de Li e Cui, 2010).

Vários microrganismos já foram modificados geneticamente para produção eficiente de ácido lático. Dentre eles destacam-se as bactérias *Lactococcus lactis* (OKANO et al., 2007), *Lactobacillus helveticus* (KYLÄ-NIKKILÄ et al., 2000),

Lactobacillus plantarum (OKANO et al., 2009), *Corynebacterium glutamicum* (KAWAGUCHI et al., 2008; KAWAGUCHI et al., 2006; SASAKI et al., 2008) e *Escherichia coli* (MAZUMDAR et al., 2010; MAZUMDAR et al., 2013) as leveduras *Candida sonorensis* (KOIVURANTA et al., 2014), *Pichia stipiti* e *Saccharomyces cerevisiae* (ILMÉM et al., 2007) e *Pichia pastoris* (LIMA et al., 2016); o fungo *Rhizopus oryzae* (SOCCOL et al., 1994; YIN et al., 1997; TAY e YANG, 2002) e o fungo filamentoso *Aspergillus oryzae* (WAKAI et al., 2014).

O interesse em ácido lático está relacionado com muitos aspectos, entre os quais o seu alto valor agregado, principalmente as formas enantioméricas puras L- e D- ácido lático. Além disso, o ácido lático é a unidade monomérica do poli (ácido lático), um bioplástico empregado na produção de diversas embalagens, obtido a partir da polimerização química do L-ácido lático(JAMSHIDIAN et al., 2010).

1.2 O bioplástico Poli-(ácido lático)

Os polímeros biodegradáveis podem ser agrupados em duas classes: polímeros naturais, obtidos a partir de fontes naturais, e sintéticos, os quais exigem síntese química na sua produção. Além disso, são classificados quanto à degradabilidade, por ser ou não biodegradáveis (Figura 3) (KOLYBABA et al., 2003).

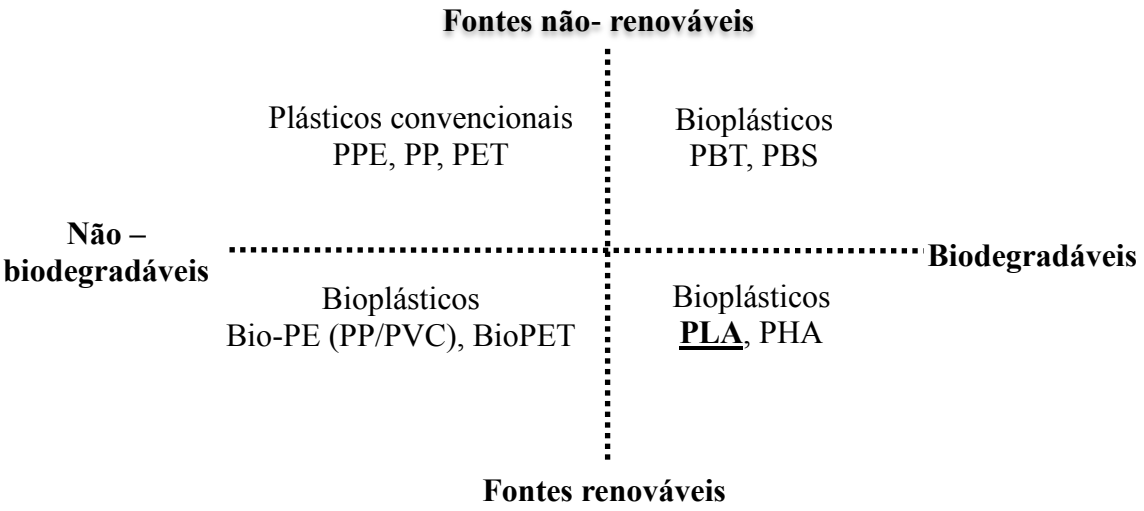


Figura 3 - Classificação dos plásticos convencionais (PPE, PP, PET) e bioplásticos (PBT, PBS, Bio-PE, Bio-PET, PLA e PHA) de acordo com o substrato e a degradabilidade

Dentre os bioplásticos biodegradáveis e produzidos a partir de fontes renováveis, tais como milho, batata, melaço de cana e de beterraba encontra-se o poli (ácido lático), PLA (WILLIAMS; HILLMYER, 2008). Poliésteres alifáticos tais como PLA, têm

promissoras aplicações em embalagens, bens de consumo, fibras e na biomedicina devido à sua excelente propriedade mecânica, transparência, compostagem e biossegurança. Estima-se que com o aumento da utilização de PLA a indústria de polímeros verdes será capaz de fechar o ciclo do carbono com concomitante redução na dependência de recursos fósseis não renováveis (MAHARANA et al., 2009).

O PLA tem boa biodegradabilidade e propriedades mecânicas, por exemplo, temperatura de transição vítrea entre 53 e 60^{o}C, temperatura de fusão entre 145 e 168^{o}C e resistência à tração entre 28 e 50 Mpa, sendo o bioplástico mais utilizado quando se busca elevada resistência mecânica e tenacidade (SUZUKI et al., 2014).

As vantagens de PLAs são numerosas e incluem: (a) produção de monômero de ácido lático por fermentação de fonte renovável, em grande maioria amido; (b) Fixação de quantidades significativas de dióxido de carbono, o principal gás de efeito estufa; (c) economia significativa de energia; (d) a capacidade de reciclar de volta ao ácido lático por hidrólise ou alcóolise; (e) capacidade de produzir híbrido papel-plástico em embalagens de consumo, sendo esta compostável, e; (f) redução do volume de depósitos no meio ambiente (KE e SUN, 2000).

Com o desenvolvimento de uma sociedade mais sustentável é esperada a substituição gradativa de plásticos originados do petróleo por bioplásticos. Assim, prevê-se que o mercado mundial PLA chegue a USD 5,16 bilhões até 2020, crescendo a uma taxa de crescimento anual composta (CAGR) de 20,9% entre 2015 e 2020 (Plastics Europe, 2016). Alguns dos maiores produtores de PLA são *Nature Works* (EUA), *Cargill Dow* (EUA), *Galatic* (Bélgica), *Mitsui Chemicals* e *Shimadzu* (Japão), *PURAC Biomaterials* (Holanda) e *Inventa-Fischer* (Alemanha). Enquanto que no Brasil não há nenhuma produção de PLA. Além disso, a crescente necessidade de ácido lático, tem mantido uma busca constante por processos de maior eficiência, considerando melhorias no processo fermentativo e melhores microrganismos produtores (ABDEL-RAHMAN et al., 2013).

1.3 Engenharia metabólica aplicada à produção de ácido lático

Os gêneros bacterianos *Lactobacillus* e *Lactococcus* são os mais comumente usados na obtenção de ácido lático por fermentação, principalmente na indústria alimentícia para produção de derivados lácteos (LEROY; VUYST, 2004). Além destas, outros microrganismos são utilizados na produção de ácido lático, tais como fungos, leveduras, cianobactérias e algas.

A utilização das bactérias ácido lático (LAB) tem sido avaliada, já que as mesmas produzem forma racêmica de ácido lático, além de necessitarem de nutrientes complexos, aumentando o custo do processo de produção e, consequentemente, da purificação do produto da fermentação. O microrganismo ideal para a produção de ácido lático é aquele que atinja alto rendimento e seja capaz de utilizar um substrato de baixo custo (ZHOU et al., 2003).

A engenharia genética tem possibilitado o desenvolvimento de microrganismos com características ideais para que químicos de alto valor agregado sejam produzidos, a partir da modificação, deleção ou adição de vias metabólicas. Recentemente, a engenharia metabólica já foi utilizada, por exemplo, para produção de moléculas precursoras de taxol (PARACHIN; CARLQUIST; GORWA-GRAUSLUND, 2009) succinato a partir de sucrose (WANG; BENNETT, 2011) etanol a partir de xilose (PARACHIN et al., 2010) e de antibióticos (PLANSON et al., 2011).

Cerca de noventa por cento da produção comercial de ácido lático é obtida por fermentação. A via metabólica de obtenção do ácido lático é um dos destinos do piruvato que pode ser reduzido a ácido lático pela enzima lactato desidrogenase (LDH), a qual sintetiza ácido lático a partir de piruvato com concomitante oxidação do cofator NADH (DUSSELIER et al., 2013).Diversos microrganismos já foram modificados geneticamente para produção de ácido lático, destes os mais comumente utilizados são bactérias, tais como *Lactobacillus helveticus*, *Lactobacillus lactis e Lactobacillus plantarum*, naturalmente produtoras de ácido lático, que tiveram o rendimento aumentado a partir da superexpressão do gene da L- lactato desidrogenase, assim como deleção do gene D- lactato desidrogenase (Tabela 1).

O uso de leveduras em fermentações para produção de ácido lático pode ser vantajoso já que estes microrganismos têm maior tolerância a baixo pH. A levedura *Saccharomyces cerevisiae* (Tabela 1) é a mais utilizada, já que possui genética, fisiologia e bioquímica bem conhecida, além de ser utilizada tradicionalmente na produção de pães e vinhos. As principais modificações que foram realizadas em *S. cerevisiae* são: (I) deleção do gene codificador da piruvato descarboxilase, aumentando a disponibilidade de piruvato, o qual é convertido em ácido lático a partir da (II) superexpressão do gene codificador da lactato desidrogenase (Tabela 1). Apesar das modificações no metabolismo, o rendimento da fermentação utilizando *S. cerevisiae* ainda é inferior quando comparado a bactérias (ABDEL-RAHMAN; TASHIRO; SONOMOTO, 2013).

Além do rendimento abaixo do esperado, *S. cerevisiae* utiliza, preferencialmente, glicose como fonte de carbono, sendo uma desvantagem já que este substrato compete com a indústria alimentícia, aumentando o seu valor de mercado (DA SILVA et al., 2009). Testes realizados com glicerol bruto fornecem subsídios sólidos para aplicação industrial. Neste âmbito, a levedura metilotrófica *Pichia pastoris* é uma alternativa no desenvolvimento de processos de baixo custo e maior rendimento, pois se destaca pela capacidade de utilizar uma fonte de carbono barata como glicerol, exibindo altos níveis de crescimento celular (130 g/L)(MACAULEY-PATRICK et al., 2005).

Tabela 1 - Microrganismos modificados geneticamente para super produção de ácido lático. * calculado a partir dos dados relatados

Microrganismo	Produto	Principais modificações genéticas	Fonte de carbono	Rendimento (g/g)	Produtividade (g/L/h)	Pureza (%)	Citação
Candida sonorensis	L-LA	Expressão de L-*ldh* de *Lactobacillus helveticus* e deleção de *PDC1/PDC2*	Glicose	0.94	3.3	99.0	(ILMÉN et al., 2013)
Candida sonorensis	L-LA	Expressão de L-*ldh* de *Bacillus megaterium* e deleção de *PDC1/PDC2*	Glicose	0.85	2.0	99.0	(ILMÉN et al., 2013)
Candida sonorensis	L-LA	Expressão de L-*ldh* de *Rhizopus oryzae* e deleção de *PDC1/PDC2*	Glicose	0.81	1.5	99.0	(ILMÉN et al., 2013)
Corynebacterium glutamicum	D-LA	Superexpressão D-*ldh*	Glicose	-	4.0	99.9	(OKINO; SUDA; FUJIKURA, 2008)
Escherichia coli	LA	Superexpressão *ldh* e deleção fosfofoenol piruvato descarboxilase	Glicose	-	-	-	(JUNG et al., 2010)
Escherichia coli	LA	Superexpressão glicerol-3-fostato desidrogenase e glicerol kinase	Glicerol	0.80	0.41	99.9	(MAZUMDAR et al., 2013b)
Escherichia coli	LA	Superexpressão *ldh*	Glicerol	0.78	3.65	99.9	(NIU et al., 2014)
Escherichia coli (pta)	D-LA	Deleção fosfotransacetilase	Glicose	0.80	1*	-	(CHANG; JUNG; RHEE, 1999)
Escherichia coli (pflB frdBC)	D-LA	Deleção ácido pirúvico liase e fumarato redutase	Glicose	0.99	0,27*	>99	(ZHOU; SHANMUGAM; INGRAM, 2003)
Escherichia coli (pflB frdBC adhE ackA)	D-LA	Deleção ácido pirúvico liase, fumarato redutase, álcool desidrogenase e acetato kinase	Glicose	0.98	0,29*	-	(ZHOU; SHANMUGAM; INGRAM, 2003)

Escherichia coli (pflB frdBC adhE ackA ldhA::ldhL)	L-LA	Deleção ácido pirúvico liase, fumarato redutase, álcool desidrogenase e acetato kinase. Substituição de D- *ldh* por L- *ldh*	Glicose	0.91	0.11*	-	(ZHOU; SHANMUGAM; INGRAM, 2003)
Kluyveromyces lactis	L-LA	Deleção PDC/PDH e expressão de *ldh*	Glicose	0.85	-	-	(BIANCHI et al., 2001)
Lactobacillus helveticus	L-LA	Superexpressão L- *ldh* e deleção D- *ldh*	-	-	3.42	-	(KYLÄ-NIKKILÄ et al., 2000)
Lactobacillus lactis	L-LA	Superexpressão α-amy	Amido solúvel	0.89	1.57	99.2	(OKANO; KIMURA; NARITA, 2007)
Lactobacillus plantarum	D-LA	Deleção L-LDH	Glicose	0.80	2.28	99.2	(OKANO et al., 2009)
Lactobacillus plantarum	D-LA	Deleção L- *ldh* e superexpressão α-amy	Amido bruto	0.85	3.86	99.6	(OKANO et al., 2009)
Pichia pastoris	L-LDH	Expressão de *ldh* (*Bos tauros)* Superexpressão de transportadores PAS e JEN1p	Glicerol	0.70	0.14		(LIMA et al., 2016)
Pichia stipitis	L-LA	Superexpressão L- *ldh*	Xilose	0.60	0.38	-	(ILMEN et al., 2007)
Saccharomyces cerevisiae	LA	Deleção PDC e superexpressão *ldh*	Glicose	0.65	0.30	-	(ISHIDA et al., 2005)
Saccharomyces cerevisiae	LA	Deleção PDC e ADH e superexpressão *ldh*	Glicose	0.75	1.0	-	(TOKUHIRO; ISHIDA; KONDO, 2008)
Saccharomyces cerevisiae	LA	Expressão de *ldh* (*L. casei*)	Glicose	0.24	0.18*	-	(DEQUIN; BARRE, 1994)
Saccharomyces	LA	Expressão de *ldh* (*L. casei*)	Glicose	0.04		-	(DEQUIN; BAPTISTA;

cerevisiae							BARRE, 1999)
Saccharomyces cerevisiae	LA	Expressão de *ldh* (bovino)	Glicose	0.16	0.3*	-	(ADACHI et al., 1998)
Saccharomyces cerevisiae (pdc1)	LA	Expressão de *ldh* (bovino)	Glicose	0.20	0,23*	-	(ADACHI et al., 1998)
Saccharomyces cerevisiae (pdc1)	LA	Expressão de *ldh* (bovino) 2 cópias	Glicose	0.65	0.77*	-	(ISHIDA et al., 2005)
Saccharomyces cerevisiae (pdc1)	L-LA	Expressão de *ldh* (bovino) 6 cópias	Glicose	0.68*	2.5*	>99.9	(SAITOH et al., 2005)
Saccharomyces cerevisiae (pdc1)	D-LA	Expressão de *ldh* (*L. mesenteroides*) 2 cópias	Glicose	0.61	0.74 e 0.85*	99.9	(ISHIDA et al., 2006)
Saccharomyces cerevisiae (pdc1 pdc5)	LA	Expressão de *ldh* (bovino) 2 cópias e deleção piruvato descarboxilase 1 e 5	Glicose	0.82	0.38	-	(ISHIDA et al., 2006)
Saccharomyces cerevisiae (pdc1/adh1 duplo mutante*)*	LA	Expressão de *ldh* (bovino) 4 cópias e deleção piruvato descarboxilase e álcool desidrogenase	Glicose/ celobiose	0.75	1,14*	-	(TOKUHIRO; ISHIDA; KONDO, 2008)
Saccharomyces cerevisiae	LA	Expressão de *ldh* (bovino) - uma cópia integrada	Glicose	-	5*	-	(PORRO et al., 1995)
Saccharomyces cerevisiae	L-LA	Expressão L- *ldh L. plantarum* - uma cópia integrada	Glicose	Depende do parâmetro	Depende do parâmetro	-	(COLOMBIÉ; DEQUIN; SABLAYROLLES, 2003)
Saccharomyces cerevisiae	L-LA	Expressão L- *ldh R. oryzae* - plasmídeo multicópia	Glicose	0.44	1.25*	-	(SKORY, 2003)

Saccharomyces cerevisiae	L-LA	Expressão L- *ldh L. plantarum* - plasmídeo multicópia	-	-	-	-	(LIU; LIEVENSE, 2005)
***Saccharomyces cerevisiae* (*pdc1::loxP pdc5::loxP pdc6::loxP*)**	L-LA	Expressão L- *ldh L. plantarum* - plasmídeo multicópia	Glicose	0.93*	1*	-	(VALLI et al., 2006)

1.4 A levedura metilotrófica *Pichia pastoris* (*Komagataella phaffii*)

A levedura *Pichia pastoris*, recentemente reclassificada no novo gênero *Komagataella* e dividida em três espécies *K. pastoris*, *K. phaffii* e *K. pseudopastoris*, tem como característica fisiológica mais notável a capacidade de crescer em meio contendo metanol como única fonte de carbono, sendo assim classificada como metilotrófica. Este fato se dá pela presença da enzima peroxissomal álcool oxidase em seu metabolismo (Kurtzman, 2009). Sua taxomonia é descrita como sendo do reino *Fungi*; do filo *Ascomicota*; do sub-filo *Saccharomicotina*; da classe *Saccharomicetes*; da ordem *Saccharomicetales*; da família *Sacchamomicetaceae*; do gênero *Pichia* e da espécie *P. pastoris* (BARNETT; PAYNE; YARROW, 2000).

A utilização desta levedura no estabelecimento de bioprocessos tem várias vantagens tais como: produção intra e extracelular de proteínas heterólogas, fácil manipulação genética, capacidade de atingir altas densidades celulares em diferentes fontes de carbono, como glicose e glicerol, capacidade de fazer modificações pós-traducionais, como processamento proteolítico, viabilidade de produção de sistemas de expressão e possui genoma já sequenciado (CEREGHINO; CREGG, 2000). Além disso, *P. pastoris* possui status GRAS reconhecido pela agência americana regulatória de alimentos e medicamentos, FDA, desde 2006 (VOGL; HARTNER; GLIEDER, 2013).

A utilização de *P. pastoris* para produção heteróloga de proteínas foi relatada pela primeira vez há mais de 40 anos quando a companhia *Phillips Petroleum* juntamente com a *Salk Institute Biotechnology/Industrial Inc.* (SIBIA) a utilizaram em um processo de fermentação de alta densidade celular na produção de aditivo para alimentação animal (AHMAD; HIRZ; PICHLER, 2014). Em 1985, pela primeira vez uma cepa de *P. pastoris* foi desenvolvida como hospedeira para modificações genéticas. O sistema de expressão foi caracterizado por marcação auxotrófica pela deficiência no gene codificador da enzima histidinol desidrogenase presente na via de síntese do aminoácido histidina, permitindo, dessa forma, a utilização deste como marca auxotrófica (CREGG et al., 1985).

Atualmente, *P.pastoris* é a levedura mais utilizada na produção heteróloga de proteínas (GASSER et al., 2013). Este sistema é de particular interesse industrial devido ao seu poderoso e fortemente regulado promotor metanol induzível, álcool oxidase (AOX1) (POTVIN; AHMAD; ZHANG, 2012). Além disso, apresenta diversas vantagens quando comparada a *Saccharomyces cerevisiae*: maior produtividade, ausência de hiper-glicosilação,

crescimento em meio com metanol e integração estável de várias cópias do DNA transformante (CEREGHINO; CREGG, 2000; DEMAIN; VAISHNAV, 2009).

Diversos vetores foram desenvolvidos para produção heteróloga em *P. pastoris*, compartilhando algumas características comuns entre eles, sequência promotora, sítios de restrição único e sequência de terminação transcricional. Um sistema bastante utilizado para expressão em *P. pastoris* possui o promotor GAP (gliceraldeído – 3 – fosfato) que codifica a enzima glicolítica gliceraldeído – 3 – fosfato desidrogenase (GAPDH), promove alta expressão e tem sido testado como alternativa ao AOX1, por possuir expressão forte e constitutiva, ser mais adequado para produção em larga escala e ser mais eficiente para produção de proteínas heterólogas (ZHANG et al., 2009).

O sistema de expressão em *P. pastoris* é um dos mais produtivos sistemas de expressão eucariótico, existindo uma probabilidade de expressão da proteína de interesse em 50 a 70 %. Sendo o fator mais importante conseguir expressar sua proteína em qualquer nível, pois existem parâmetros bem definidos que possibilitam a otimização da expressão de proteínas heterólogas neste sistema (CEREGHINO et al., 2002).

1.5 Glicerol como fonte de carbono

O desenvolvimento de um processo eficiente para produção industrial de PLA depende diretamente da produção de ácido lático economicamente viável. Atualmente, um dos principais obstáculos para a produção em grande escala de ácido lático é o custo do substrato. O uso de resíduos agroindustriais demonstra-se vantajoso por utilizar matérias-primas que seriam descartadas e que não são utilizadas como alimentos (JOHN; NAMPOOTHIRI; PANDEY, 2006). Exemplos de resíduos já utilizados para produção de ácido lático estão listados na Tabela 2.

Tabela 2 - Resíduos utilizados como fonte de carbono e energia para produção de ácido lático

Substrato	Microrganismo	Rendimento (g/g)	Referência
Farelo de trigo	*Lactococcus lactis*	0.64	(AKERBERG; ZACCHI, 2000)
Papel de escritório	*Rhizopus oryzae*	0.59	(PARK; ANH; OKUDA, 2004)
Espiga de milho	*Rhizopus oryzae*	0.29	(RUENGRUGLIKIT; HANG, 2003)
Bagaço de cana-de-açucar	*Lactococcus lactis*	0.71	(LAOPAIBOON et al., 2010)
Glicerol	*E.coli* (modificada	0.80	(MAZUMDAR et al.,

geneticamente)	2013a)

O glicerol é um tri-álcool com 3 carbonos, tendo como nome sistemático da IUPAC 1,2,3-propanotriol. A presença de três grupos hidroxila na estrutura do glicerol é responsável pela solubilidade em água e sua natureza higroscópica. Além disso, é uma molécula altamente flexível formando ligações de hidrogênio tanto intra como intermoleculares (BEATRIZ et al., 2011).

O glicerol cru (ou glicerina) é o principal resíduo do processo de transesterificação, que consiste na conversão de óleos e/ou gorduras vegetais em biodiesel (LEONETI et al., 2012). Segundo a ANP (2016), em 2015 foram produzidos 3,9 milhões de m^3 de biodiesel no Brasil, o que corresponde a 53,3% da capacidade total de produção, o qual gerou um acúmulo de 346,8 mil m^3 de glicerol. Sendo assim uma fonte de carbono extremamente disponível e, consequentemente, de baixo custo. Além disso, a utilização do glicerol é considerada vantajosa por tornar a cadeia de produção de biodiesel mais rentável por maximizar a utilização da biomassa.

O uso do glicerol cru como fonte de carbono para produção de ácido lático já foi relatatado por Mazundar e colaboradores (2013) em fermentação utilizando cepa de *E. coli* modificada geneticamente. A composição química do glicerol cru contém 30% de impurezas, como sabão, catalisadores, sais, matéria orgânica, e impurezas da água trazidas do processo de conversão. Assim, para melhor rendimento do processo fermentativo, é imprescindível a escolha de microrganismos robustos (Yang *et al.*, 2012).

1.6 Metabolismo de glicerol em *Pichia pastoris*

A levedura metilotrófica *P. pastoris* naturalmente cresce em diversas fontes de carbono, como glicose, metanol e glicerol (LOOSER et al., 2015). Por exibir um metabolismo predominantemente oxidativo, com alto rendimento em biomassa mesmo em altas concentrações de açúcar (Crabtree negativo), *P. pastoris* tem se tornado cada vez mais promissora em usos biotecnológicos (PFEIFFER; MORLEY, 2014).

A regulação central do metabolismo do carbono em *P. pastoris* e *S. cerevisiae* é muito semelhante e o crescimento de ambas as leveduras seguem perfis muito similares em relação à taxa de biossíntese de aminoácidos (SOLÀ et al., 2007). Porém, quando comparadas em relação à produção de proteínas heterólogas, *P. pastoris* possui características importantes,

como não ser considerada forte fermentadora, render mais biomassa e não gerar etanol, que acumulado pode atingir níveis tóxicos rapidamente (efeito crabtree) (CEREGHINO; CREGG, 2000).

O glicerol bruto é originado durante o processo de transesterificação de óleos e gorduras, ou seja, processo de obtenção de um éster (biodiesel) a partir de outro éster com álcool (metanol ou etanol), sendo catalisada na presença de ácidos ou bases fortes. Assim, o glicerol proveniente da indústria de biodiesel possui vários contaminantes, sendo o metanol encontrado em maior quantidade. Por possuir a enzima álcool oxidase 1 (AOX1), *P. pastoris* é capaz de converter metanol em formaldeído, sendo capaz de crescer em altas concentrações deste álcool, podendo utilizar o glicerol bruto de forma eficaz (TANG et al., 2009).

Corroborando esta hipótese, Anastácio e colaboradores (2014) demonstraram maior produção de biomassa de *P. pastoris* utilizando glicerol bruto em comparação a glicerol puro, indicando que este microrganismo pode também utilizar os contaminantes do glicerol como fontes de carbono. Além disso, o processo de produção de proteínas heterólogas reguladas pelo promotor AOX1 em *P. pastoris* é, comumente, feito em três etapas de alimentação: (1) fase de crescimento em glicerol; (2) fase de adaptação ao metanol, e; (3) fase de indução em metanol (UNREAN, 2013).

Como outras moléculas neutras pequenas, o glicerol pode atravessar a membrana citoplasmática através da difusão facilitada. Esta é realizada por uma proteína integral de membrana, GlpF, que atua como um canal altamente seletivo, conduzindo poli álcoois e derivados de ureia. Em leveduras, o fluxo de glicerol através da membrana plasmática é controlado por difusão passiva, uma proteína de canal ou mecanismo de absorção ativo (DA SILVA; MACK; CONTIERO, 2009).

Em uma célula microbiana, durante o catabolismo aeróbico, o glicerol entra por difusão facilitada e/ou transporte ativo e é então fosforilado em glicerol-3-fosfato, seguido por oxidação de 3-dihidroxiacetona, que é convertida em gliceraldeído-3-fosfato. Este último entra na via glicolítica e transforma-se em ácido pirúvico, e em seguida em acetilCoA, um precursor de uma variedade de produtos metabólicos (CHATZIFRAGKOU et al., 2011).

Diversos trabalhos encontrados na literatura demonstram a eficiente utilização de glicerol bruto e/ou puro por *P. pastoris* na produção de proteínas heterólogas, enzimas industriais e químicos de alto valor agregado (INAN et al., 1999; ZHANG et al., 2003; JUNGO; MARISON; STOCKAR, 2007; SOLÀ et al., 2007; TANG et al., 2009; LIMA et al., 2016).

A partir da análise do genoma das cepas GS115 e DSMZ 70382 de *P. pastoris*, Damasceno e colaboradores (2012) afirmam que apesar desta levedura ter um sistema transportador de glicose menos eficiente do que *S. cerevisiae, P. pastoris* possui quatro supostos transportadores de H^+/glicerol, os quais permitem que esta tenha uma alta taxa de crescimento quando glicerol é usado como fonte única de carbono.

1.7 Transporte de ácidos orgânicos

O transporte rápido de monocarboxilatos (MCT) tais como piruvato, ácido lático e os corpos cetônicos através da membrana plasmática celular é essencial para o metabolismo de carboidratos, gorduras e aminoácidos, sendo facilitados por transportadores (HALESTRAP; PRICE, 1999).

Cada MCT catalisa o transporte de prótons ligados a rede ou troca de monocarboxilatos de cadeias curtas, geralmente substituído sobre os dois ou três átomos de carbono com um grupo ceto (por exemplo, piruvato e acetoacetato) ou grupo hidroxilo (por exemplo, L-lactato e Db-hidroxibutirato). Ambos, influxo e efluxo de monocarboxilatos são facilitados, com a direção de transporte de líquido a ser determinado unicamente pelos gradientes de concentração de prótons e monocarboxilato através da membrana plasmática (HALESTRAP; WILSON, 2012).

Essencialmente, os mecanismos de transporte de ácidos carboxílicos podem ser divididos em dois grupos principais: independente de energia (passivo) ou dependente de energia (ativo). No primeiro caso, difusão simples e/ou difusão facilitada (um canal ou uma permease) envolve o transporte sem carga, ou seja, forma não dissociada do ácido. Em pH baixo a forma não dissociada é favorecida e, sendo lipossolúvel, pode atravessar a membrana celular por simples difusão. Uma vez dentro da célula, o pH neutro conduz à dissociação do ácido, enquanto que o ânion ácido dificilmente difundem para fora da célula, ficando acumulados os prótons liberados acidificam o citoplasma e podem alterar várias vias metabólicas (SOARES-SILVA; CASAL; PAIVA, 2008).

O transportador JEN1P, encontrado no genoma da levedura *Saccharomyces cerevisae* transporta monocarboxilatos incluindo ácido lático, piruvato e outros ácidos carboxílicos de cadeia curta, e sua expressão é estreitamente regulada (CASAL et al., 1999). Além disso, já é bem caracterizado e tem sido tema de vários trabalhos publicados (MCDERMOTT; ROSEN; LIU, 2010; PAIVA et al., 2013; PAIVA; KRUCKEBERG; CASAL, 2002). Além destes,

Soares-Silva e colaboradores (2003) expressaram o gene que codifica o transportador JEN1P em *P. pastoris* comprovando sua atividade.

Recentemente, Lima e colaboradores (2016) demonstraram aumento na produção de ácido lático utilizando uma cepa de *P. pastoris* com superexpressão do gene codificador do transportador putativo PAS, homólogo ao JEN1p, confirmando sua eficiência no transporte de ácido lático.

Apesar de o ácido lático ser o monocarboxilato cujo transporte através da membrana plasmática seja quantitativamente maior, os MCTs são também essenciais para o transporte de muitos outros monocarboxilatos metabolicamente importantes, tais como o piruvato, o ácidos de cadeia ramificada derivados de leucina, valina e isoleucina, e corpos cetónicos acetoacetato, β-hidroxibutirato e acetato (HALESTRAP; PRICE, 1999).

2. Justificativa

O acúmulo de plásticos derivados do petróleo tem gerado um montante de 140 milhões de toneladas no ambiente com perspectiva de degradação em até 1000 anos, além de ser dependente de matéria prima não renovável. A substituição dos plásticos petroquímicos por bioplásticos é vantajosa, pois além de ser proveniente de fonte renovável, é biodegradável. O ácido lático é a unidade monomérica do poli (ácido lático), um tipo de bioplástico amplamente usado na fabricação de embalagens, desde fibras a espumas. Além disto, este químico tem sido cada vez mais utilizado nas indústrias de alimentos, farmacêuticas e cosméticas. O desenvolvimento de um processo nacional em escala industrial para produção eficiente e economicamente viável de ácido lático faz-se necessário para atender à atual demanda. Para isto, é interessante que o processo de produção tenha baixo custo, o que depende diretamente da fonte utilizada para este fim. O glicerol cru tem sido um excelente candidato como fonte de carbono em processos de produção de químicos de alto valor agregado. Por ser um co-produto gerado pela transesterificação, ou seja, conversão de óleos vegetais à biodiesel, o aumento da produção deste biocombustível implica em maior quantidade de glicerol gerado, tornando-o assim uma fonte renovável de baixo custo. A levedura metilotrófica *P. pastoris* é capaz de metabolizar rapidamente grandes quantidades desse substrato e atingir altos níveis de densidade celular em meio com pH ácido, porém não é capaz de produzir ácido lático. A utilização da engenharia genética permite o desenvolvimento de microrganismos modificados geneticamente para produção heteróloga de químicos de interesse, possibilitando o desenvolvimento de uma cepa de *P. pastoris* capaz de converter glicerol cru em ácido lático.

3. Objetivos

Construir cepas recombinantes de *P. pastoris* e otimizar o processo fermentativo visando a produção de ácido L-lático a partir de glicerol bruto, principal resíduo da indústria de biodiesel. Para atingir estes objetivos, foram propostas as seguintes metas:

1. Introdução do gene codificador da enzima LDH na cepa X-33

2. Seleção das cepas geneticamente modificadas por testes de atividade de LDH.

3. Avaliar produção de ácido lático a partir de diferentes fontes de carbono

4. Otimização dos parâmetros de fermentação utilizando a cepa de *P. pastoris* com atividade da enzima LDH

5. Inserção dos genes codificadores de transportadores de ácido lático na cepa GS115

6. Cálculo de Km e Vmáx das cepas com transportadores de ácido lático

7. Construção de cepas com atividade LDH e transportadores de lactato

8. Comparar produção de ácido lático pelas cepas com atividade LDH com e sem inserção de transportadores

4. Material e métodos

4.1 Plasmídeos e microrganismos

Os plasmídeos e microrganismos utilizados para clonagem e expressão estão listados na tabela 3.

Tabela 3 - Plasmídeos e microrganismos utilizados para clonagem e expressão de genes heterólogos

Plasmídeos/cepas	Genótipo	Referência
Plasmídeos		
pGAPZB	Marca de seleção: Zeocina®; Expressão controlada pelo promotor constitutivo GAPp	Invitrogen®
pPICPGKGFP	Gene codificador de fluorescência verde clonado sob controle do promotor PGK	Comunicação pessoal
pGAP-LDH	LDH^+, gene de *Bos taurus* codificador da enzima LDH	(LIMA et al., 2016)
pPGK-JEN1	$JEN1^+$, gene de *S. cerevisiae* codificador do transportador de lactato Jen1p	(LIMA et al., 2016)
pPGK-PAS	PAS^+, gene de *P. pastoris* codificador do transportador putativo de lactato PAS	(LIMA et al., 2016)
E. coli		
DH5α™	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Life technology
DH10B™	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *end*A1 *ara*D 139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–	Life technology

P. pastoris		
X-33	Cepa sem modificação genética	Life technology
XL	X-33 + pGAP-LDH	(LIMA et al., 2016)
GS115	*his4*$^{-}$	Life technology
GJ	*his4*$^{-}$ + pPGK-JEN	(LIMA et al., 2016)
GLJ	*his4*$^{-}$ + pGAP-LDH + pPGK-JEN	(LIMA et al., 2016)
GS	*his4*$^{-}$ + pPGK-PAS	(LIMA et al., 2016)
GLS	*his4*$^{-}$ + pGAP-LDH + pPGK-PAS	(LIMA et al., 2016)

4.2 Material

4.2.1 Oligonucleotídeos (*primers*)

Os oligonucleotídeos, mostrados na Tabela 4, foram sintetizados pela IDT (Integrated DNA Technologies) e foram utilizados na concentração de 10 pmoles/μL.

Tabela 4 - Oligonucleotídeos para amplificação de genes por PCR. As bases marcadas em vermelho representam os sítios de clivagem das endonucleases de restrição

Oligonucleotídeos	**Sequência 5'- 3'**	**Sítio de restrição**
LDH – F	CGAGAATTCATGGCAACTCTCAAGGATCAGCTGATTCAG	*EcoR*I
LDH – R	CGACTCGAGTCAAACAGGCCTCTTCTTCATGTCA	*Xho*I
JEN1P – F	ATTCGCGGCCGCATGTCGTCGTCAATTACA	*Not*I

JEN1P – R	TTAAACGGTCTCAATATGCTGAATTCATC	*EcoR*I
PAS – F	ATTCGCGGCCGCATGTCGCATTCAATCCATT	*Not*I
PAS – R	TTACTTATTTCCTTCAAAAGCCGAATTCATC	*EcoR*I
pGAP F	GTCCCTATTTCAATCAATTGAA	-------
3´ AOX1	GCAAATGGCATTCTGACATCC	-------

4.2.2 Endonucleases de restrição

As endonucleases de restrição foram obtidas de marcas comerciais, como descritos na tabela 5. Assim como as enzimas utilizadas na ligação entre vetor e inserto (Tabela 6).

Tabela 5 - Endonucleases de restrição usadas para clivagem dos genes obtidos por PCR

Enzima	Sítio de clivagem	Tampão	Temperatura de incubação	Origem
AvrII	C↓C T A G G	CutSmart™	37 °C	New England Biolabs
***EcoR*I**	G↓A A T T C	Tango	37 °C	Fermentas
***Not*I**	G C↓G G C C G C	0	37 °C	Fermentas
***Sac*I**	G A G C T↓C	Tango	37 °C	Fermentas

Tabela 6 - Outras enzimas utilizadas

Enzima	Concentração	Origem
T4 DNA ligase	5 (U/uL)	Invitrogen®
***Taq* DNA polimerase**	5 (U/uL)	Fermentas

4.2.3 Tampões e Soluções

Ampicilina

Ampicilina 100 mg/mL

Dissolvido em água ultra-pura

Brometo de etídeo

EtBr 10 mg/mL

Dissolvido em água

Clorofane [1:1 v/v]

Fenol	1 parte
Clorofórmio	1 parte

Liticase

Liticase	20 mg/ml

Dissolvida em água

RNAse

RNAse A (DNAse *free*)	10 mg/ml
Acetato de sódio (pH 4,8)	50 mM

Fervida em banho-maria por 10 minutos

Tampão de amostra para gel de agarose

TAE 20X	50% (v/v)
Glicerol	30% (v/v)
Azul de bromofenol	0,25% (p/v)

Tampão Fosfato

A) Fosfato de potássio monobásico	13,61g/L
B) Fosfato de sódio dibásico dodecahidratado	35,81g/L

Misturar 96,4 mL da solução A e 3,6 mL da solução B

Ajustar pH para 8,0

Tampão SE

Sorbitol	0,9 M
EDTA	100 mM

pH 7,5

Tampão TE

Tris – HCL	10 mM
EDTA	20 mM

Diluídos em água

pH 7,5

Tampão TAE 50X

Tris-acetato	2M
EDTA	0,05M

pH 8,0

Zeocina

100 mg/mL

Obtida comercialmente em solução (invitrogen)

4.2.3.1 Soluções para extração de DNA plasmidial

Solução I

Tris-HCl (pH 8,0)	10 mM
EDTA	1 mM

Diluídos em água destilada

Solução II

NaOH	0,2 mM
SDS	1 % (p/v)

Dissolvidos em água destilada

Preparada para uso imediato

Solução III

Acetato de sódio	3 M
Ácido acético	2 M

pH 4,8

Dissolvidos em água destilada

4.2.5 Meios de cultura

Os reagentes foram dissolvidos em água destilada e esterilizados por autoclavagem durante 20 minutos à 120^{o}C.

A) Cultura bacteriana

Meio LB

Extrato de levedura	0,5%
Peptona	1,0%
NaCl	1,0%

Ao meio sólido foram adicionados 1,5% de ágar bacteriológico (p/v)

Meio LBLS

Extrato de levedura	0,5%
Peptona	1,0%
NaCl	0,5%

Ao meio sólido foram adicionados 1,5% de ágar bacteriológico (p/v)

Meio SOB

Bacto-triptona	2,0%
Extrato de levedura	0,5%
NaCl	0,05%
KCl	0,01%

B) Cultura de leveduras

Meio YNB

Yeast Nitrogen Base	1,34% (p/v)
Biotina	4 x 10^{-5}% (v/v)
Fonte de carbono (glicose ou glicerol)	2,0% (p/v)

Meio YNB sem aminoácidos

Yeast Nitrogen Base (w/o aminoacids)	1,34% (p/v)
Biotina	4 x 10^{-5}% (v/v)

Fonte de carbono (glicose ou glicerol) 2,0% (p/v)

Meio YPD (Meio extrato de levedura peptona glicose)

Extrato de levedura 1 % (p/v)

Peptona de caseína 2 % (p/v)

Dextrose 2 % (p/v)

Para meio YPD sólido foi adicionado ágar bacteriológico a 2 % (p/v)

4.3 Métodos

4.3.1 Cultivo de microrganismos

As bactérias foram cultivadas em meio LB ou LBLS a 37^{o}C. As leveduras foram cultivadas em meio específico para cada experimento a 30 ^{o}C. Para seleção de bactérias resistentes à ampicilina, foram adicionados 20 μg/mL deste antibiótico ao meio de cultura. Enquanto que para a seleção de cepas resistentes a zeocina foram adicionados 50 μg/mL para *E. coli* e 100 μg/mL para *P.pastoris* ao meio utilizado.

4.3.2 Extração de DNA genômico de leveduras

Foi inuculado 1% do pré- inóculo em 40 mL de meio YPD em Erlemeyer de 250 mL, mantido sob agitação a 30 °C para crescimento celular até a saturação. As células foram coletadas por centrifugação a 6,100 x g por 5 min em temperatura ambiente e logo após, ressuspendidas em 3 mL de tampão. Foi adicionado 100 μL de liticase 20 mg/mL, misturou por pipetagem e incubado a 37 °C por 60 min. A suspensão foi centrifugada e as células ressuspendidas em 3 mL de TE20. Após adicionar 0,5 mL de SDS 10% a suspensão foi incubada a 65 °C por 30 min. Adicionou-se 1,5 mL de acetato de potássio 5M (pH 8,9) e a suspensão foi incubada no gelo por 30 minutos. Centrifugou-se a mistura a 16,000 x g por 5 minutos a 4 °C, transferiu-se o sobrenadante para um tubo *falcon* limpo e adicionou 1 volume de clorofane. Extraiu uma vez gentilmente por inversão dos tubos, centrifugou-se novamente a 6,100 x g por 10 min. A fase aquosa foi transferida para um tubo novo e adicionou-se 2,5 volumes de etanol 100%, sendo incubado a temperatura ambiente por 5 min e então centrifugado a 16,000 x g por 15 min a 4 °C. O *pellet* foi lavado uma vez com etanol

70% e após secagem e foi ressuspenso em 200-500 μL de TE + RNAse. O DNA genômico foi armazenado a 4 ^{o}C até utilização.

4.3.3 PCR

As reações de PCR foram constituídas de: 1x tampão de reação, 1dNTP, 0,5 μM de *primer forward* e *reverse* (tabela 3) e 10 ng de DNA *template* (DNA genômico). As condições foram 30 ciclos de 94 ^{o}C/30 segundos, 58 ^{o}C/ 15 segundos, 72 ^{o}C/ 60 segundos, seguindo uma extensão final de 72 ^{o}C/10 minutos.

4.3.4 Análise em gel de agarose

As análises dos fragmentos de DNA amplificados por PCR foram feitas em gel de agarose 0,8% (p/v). A agarose foi dissolvida em tampão de corrida TAE 1x e adicionado brometo de etídeo na concentração final de 0,5 μg/mL. O tampão de amostra foi diluído para concentração final de 1x na amostra analisada. As amostras e marcadores foram aplicados no gel e submetidos à eletroforese. Ao final da corrida, o gel foi exposto à luz ultravioleta para análise.

4.3.5 Purificação dos produtos de PCR

A purificação dos produtos de PCR foi realizada com o *kit* QIAquick PCR purification (QIAGEN). A quantificação do produto de PCR purificado foi realizada em gel de agarose 0,8% por comparação de intensidade de bandas de DNA (λ) com concentração conhecida.

4.3.6 Preparação de células bacterianas competentes para eletroporação ($MgCl_2$)

(Adaptado de SAMBROOK et al., 1989).

Após preparo de inóculo de uma colônia bacteriana (isolada em 5 mL de meio LB líquido, o qual foi mantido sob agitação em shaker e 37°C *overnight*. O $MgCl_2$ foi adicionado a 500 mL de meio SOB e logo após, uma alíquota de 1 mL do inóculo, sendo mantido sob

agitação e temperatura de 37°C até atingir OD_{600nm} de 0,5. Após atingir a OD ideal, a cultura foi mantida em gelo por 30 minutos, para cessar o crescimento celular, e logo após as células foram coletadas por centrifugação a 6,100 x g por 20 minutos a 4°C. O sobrenadante foi descartado e as células ressuspendidas em 500 mL de glicerol 10% gelado, para retirada de todo meio SOB. Os frascos foram centrifugados a 6,100 x g por 20 minutos a 4°C e, novamente as células foram ressuspendidas em 30 mL de glicerol gelado para cada 500 mL de inóculo inicial. Após centrifugação a 6,100 x g por 20 minutos a 4°C, as células foram ressuspendidas em 5 mL de glicerol 10 % gelado e alíquotadas em tubos de micro centrífuga de 1,5 mL no volume de 50 μL cada. Imediatamente após as células foram congeladas em nitrogênio líquido e armazenadas em *freezer* -80°C.

4.3.7 Ligação de fragmentos de DNA (vetor-inserto)

Os sistemas de ligação foram feitos com a razão molar entre vetor e inserto nas proporções 1:3 e 1:10. A enzima T4 DNA ligase (Tabela 6) foi utilizada com o tampão fornecido pelo fabricante, os sistemas foram mantidos em termociclador a 16 ^{o}C por 16 horas. Após este período, mantidas a 4 ^{o}C até a transformação em *E. coli*.

4.3.8 Obtenção dos vetores de expressão

4.3.8.1 Gene codificador da enzima Lactato desidrogenase

O esquema de construção do vetor contendo o gene codificador da enzima L-lactato desidrogenase de *Bos taurus*, o qual foi selecionado por ter sido funcionalmente expresso em *S. cerevisiae* (BRANDUARDI et al., 2006) e, ainda, por produzir a forma enantiomérica pura de L-ácido lático, está representada na Figura 4.

Foi utilizado o vetor de expressão constitutiva pGAPZB (Invitrogen), tendo o promotor do gene da gliceraldeído – 3 – fosfato desidrogenase (GAPp), sendo expresso de forma constitutiva na presença de glicerol como fonte de carbono. Os fragmentos de DNA amplificados por PCR foram tratados com as enzimas de restrição *Eco R*I e *Xho* I para posterior ligação no vetor utilizando a enzima T4 DNA ligase. O plasmídeo resultante (pGAP-LDH) foi sintetizado pela Genome Company (Madison, WI, USA).

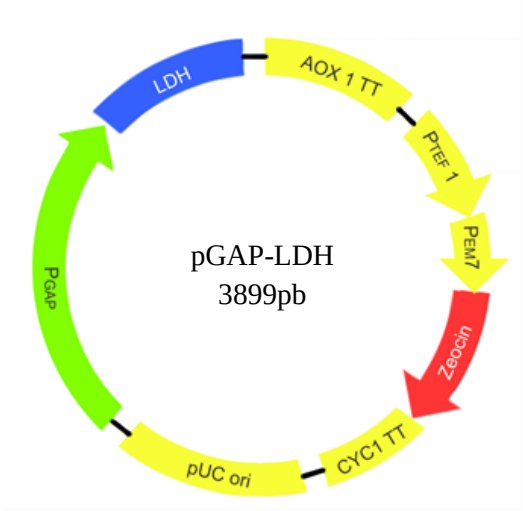


Figura 4 - Vetor pGAPZB com gene *ldh*, posteriormente linearizado com endonuclease *Avr*II (sítio de restrição contido no promotor GAP) para inserção no genoma de *P. pastoris*.

4.3.8.2 Gene codificador dos transportadores de lactato PAS e JEN1P

Os genes codificadores dos transportadores PAS e JEN1 foram clonados no vetor pPIC-PGK (UnB) como no esquema mostrado na Figura 5, o qual é expresso de forma constitutiva na presença de glicerol como fonte de carbono. Os fragmentos de DNA foram amplificados por PCR a partir de DNA genômico de *P. pastoris* e *S. cerevisiae*, respectivamente, utilizando os *primers* descritos na tabela 3. Para inserção em *P. pastoris*, os plasmídeos foram linearizados com a endonuclease de restrição BglII.

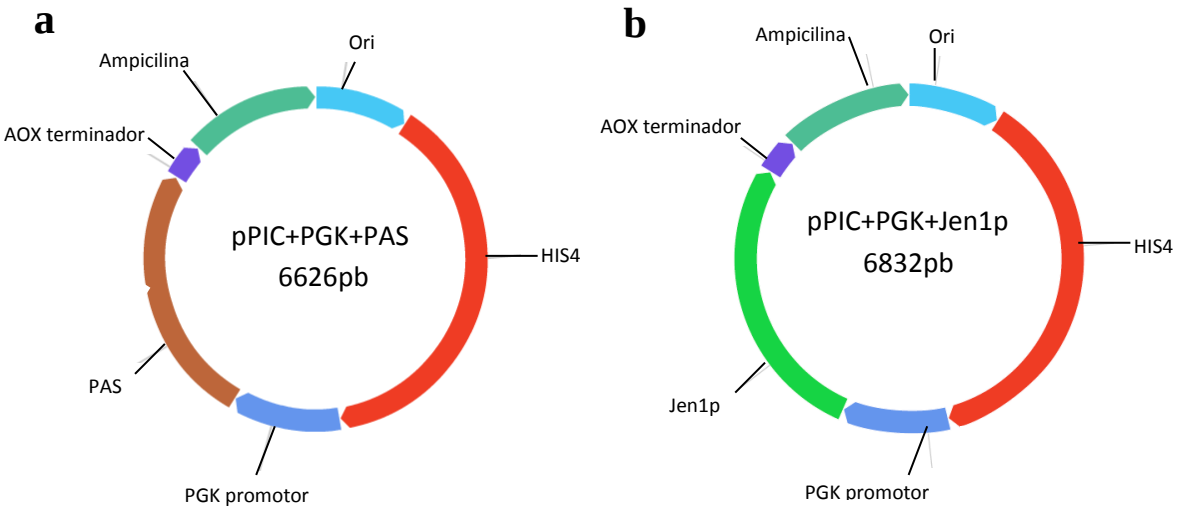


Figura 5 - Plasmídeo pPIC-PGK contendo gene codificador do (a) transportador de lactato PAS; (b) transportador de lactato JEN1P.

4.3.9 Transformação de *E. coli* por eletroporação (SAMBROOK et al., 2001)

Uma alíquota de *E. coli* competente previamente preparada foi retirada do *freezer* -80 ^{o}C e mantida no gelo até total descongelamento. Em seguida, foram adicionados 2 µL do sistema de ligação, posteriormente aplicados em cuveta de eletroporação para bactéria (1mm). A mesma foi colocada eletroporador modelo *Electroporator Cell Porator Pulser (GIBCO – BRL™*), com os parâmetros ajustados para 1800 V; 200 e 25 F. Após choque, as células foram transferidas para tubo de micro centrífuga 1,5 mL e adicionado 1 mL de meio LB e incubadas a 37 ^{o}C por 1 hora, para recuperação. Após o período de incubação, as células foram plaqueadas em meio LB com antibiótico para seleção de transformantes.

4.3.10 Preparação de DNA plasmidial (mini-prep) (SAMBROOK et al., 2001)

Foi preparado inóculo com 5 mL de meio LB contendo antibiótico adequado, mantido a 37 ^{o}C sob agitação overnight. As células foram coletadas por centrifugação 6,100 x g por 2 minutos. Após descartar o sobrenadante, o sedimentado foi ressuspendido com 250 µL de solução I em vortex, posteriormente adicionou-se 350 µL da solução II, esta suspensão foi homogeneizada por inversão dos tubos e incubada à temperatura ambiente por 5 minutos. Foram adicionados 300 µL da solução III gelada e a mostra foi novamente homogeneizada. A mistura foi incubada por 5 minutos no gelo e centrifugada a 16,110 x g por 5 minutos. O sobrenadante foi descartado e o sedimentado ressuspendido em 200 µL de tampão TE. Foram adicionados 100 µL de acetato de amônio 7,5 M e após agitação em *vórtex* centrifugados nas mesmas condições por 10 minutos. O sobrenadante foi passado para um tubo limpo e adicionado 700 µL de etanol gelado. Após centrifugação, o precipitado foi lavado com etanol 70% gelado e exposto à temperatura ambiente para secagem. Quando completamente seco, o material foi ressuspendido em 50 µL de água ultrapura com RNAse. O DNA plasmidial foi estocado à -20 ^{o}C até o uso.

4.3.11 Extração de DNA plasmidial em larga escala (maxiprep)

Foi utilizado o kit *(PureLink™) Quick Plasmid Miniprep Kit* seguindo protocolo fornecido pelo fabricante *Invitrogen*®.

4.3.12 Precipitação de DNA

Ao DNA eluído foi adicionado 1 volume de isopropanol gelado e NaAc 3M ajustado para concentração final de 0,3 M. Após 30 minutos incubado no gelo, foi centrifugado a 14,600 x g por 15 minutos. Posteriormente, o sobrenadante foi descartado e o sedimento lavado duas vezes com álcool 70%. Após secagem em temperatura ambiente, o mesmo foi ressuspendido em 50 μL de TE.

4.3.13 Tratamento com endonucleases de restrição

As clivagens foram feitas seguindo orientações do fabricante. O tempo de incubação, tampão e concentração variou de acordo com a eficácia de cada enzima.

4.3.14 Transformação em *P. pastoris* (SCORER et al., 1994)

A levedura foi inoculada em 50 mL de YPD e mantida sob agitação a 30ºC até atingir OD_{600nm} entre 1,3-1,5. As células foram coletadas por centrifugação por 5 minutos a 16,110 x g a 4ºC, logo após lavadas duas vezes em 50 mL e 25 mL de água gelada estéril, respectivamente. Após etapa de lavagem, as células foram ressuspendidas em 20 mL de sorbitol 1M gelado estéril. A suspensão de células foi transferida para tubo de micro centrífuga e centrifugadas utilizando as mesmas configurações das etapas anteriores. Por fim, ressuspendidas em 0,5 ml de sorbitol 1M e ajustadas para volume final de 1,5 mL. Foram misturados 80 μL de células competentes com aproximadamente 10 μg de DNA linearizado ressuspendido em água, adicionou-se 320 μl de sorbitol 1M e a suspensão foi transferida para cubeta de eletroporação 0,2 cm sendo mantida em gelo por 5 minutos. A eletroporação foi realizada com os seguintes parâmetos: 1500 V; 400 Ω e 25 μF. Imediatamente após o choque, foi adicionado 1 ml de sorbitol 1M gelado à cubeta e a suspensão foi transferida para um tubo de micro centrífuga de 1,5 ml estéril. As células foram incubadas a 30° C por 2 horas para recuperação e plaqueadas em meio YPD ágar com zeocina 100 μg/mL e mantidas em estufa a 37 ^{o}C por 72 horas. As cepas transformadas de GS115 foram plaqueadas em YNB (sem aminoácidos) com glicose 2% e incubadas nas mesmas condições.

4.3.15 Avaliação da expressão heteróloga em *P. pastoris*

4.3.15.1 Seleção de clones em placas de petri

Os clones de X33/ pGAPZB ligado ao gene LDH crescidos em meio YPD ágar suplementado com zeocina (100 μg/mL) foram replaqueados para análise de crescimento comparados à cepa selvagem. Os clones de GS115 transformados com vetor pPICPGK ligado aos genes de transportadores de lactato, foram transferidos para placas com YNB (*w/o aminoacids*) com glicose 2% para análise de crescimento.

4.3.15.2 Determinação de atividade enzimática da LDH

Os ensaios para determinação de atividade enzimática foram realizados como descrito previamente, com modificações (TARMY; KAPLAN, 1968). Foi feito um pré inoculo da levedura em 5 mL de meio YPD (glicose 2%) com zeocina (100 μg/mL) mantido a 30 °C, sob agitação em shaker por 16 horas. Após este período, as células foram recolhidas por centrifugação e re-inoculadas em um novo frasco para crescimento sob as mesmas condições até a fase exponencial. A cultura foi submetida a centrifugação (3000 x g / 5 min./4 °C). O sedimento foi ressuspendido em 3 volumes de Y- Per (*Yeast Protein Extraction Reagent*), transferido para um tubo de 2 mL e agitado em vórtex por 10 minutos, sendo resfriado a cada intervalo de 1 minuto. Posteriormente o material foi sedimentado (10,000 x g /20 min./4 °C). O sobrenadante foi removido e mantido em gelo durante todo o ensaio. A reação foi montada com extrato celular, 10 uL ; NADH , 8 mL ; tampão fosfato 50 mM (pH 8,0) , 800μL , e água ultra-pura para 1 mL de volume final. Depois de 150 segundos, foram adicionados 40 mL de piruvato e a reação foi concluída em 300 segundos. Em seguida, a atividade de LDH foi determinada a 30 ° C através da redução de absorvância 340 nm causada pela oxidação do co-fator NADH após a adição de piruvato como substrato. A unidade de atividade enzimática é definida como a quantidade de enzima necessária para oxidar 1 umol de NADH por minuto.

4.3.16 Ensaio com lactato radioativo

A absorção de ácido láctico marcado foi determinada como descrito anteriormente (SOARES-SILVA et al., 2003) com modificações. Um inóculo primário das cepas GS115

(controle), GJ e GS foi preparado em meio YPD, mantidos sob agitação em shaker a 30^{o}C até a fase de crescimento exponencial. Após este período, as células foram recolhidas por centrifugação e re-inoculadas em meio YNB (13,4 g/L) com L-(+)-ácido lático (5 g/L) (Sigma- Aldrich, USA) mantidos nas mesmas condições por 4 horas. Os inóculos foram centrifugados a 12000 g durante 5 min e lavados duas vezes com água gelada. As células foram ressuspendidas em tampão de fosfato de potássio monobásico (KH_2PO_4) 1 M, pH 5,0 e mantida à temperatura ambiente durante o ensaio. Uma mistura contendo 1µl de ácido lático redioativo [U-14C]lactic acid (sodium salt; 106.9 mCi/mmol [3.955 GBq/mmol], Perkin Elmer Life Science) e 9 µl L-(+)-ácido lático foi adicionada a 40 µl de células em tampão KH_2PO_4. Após 10s de incubação a reação foi parada com 1 mL de água gelada. As células foram rapidamente filtradas em filtro de nitrocelulose 0,45µm (Sartorius, Goettingen, Alemanha) ligado a um sistema de filtro de vácuo. Após extensivas lavagens e remoção do ácido láctico radioativo não incorporado, as membranas contedo as células lavadas foram transferidas para um tubo de cintilação contendo 3 mL de fluido de cintilação (ScintiSafe; Thermo Fisher Scientific, Whatman, MA, EUA). A radioatividade intracelular foi medida no espectrofotômetro líquido de cintilação Packard Tri-Carb 2200 CA com correção de desintegrações por minuto. Para determinar a adsorção não específica 14C, foi adicionado ácido lático marcado no tempo zero e mantido em água gelada por 10s. Para determinar a cinética de transporte que melhor representa os valores experimentais das taxas de absorção iniciais de ácido lático e estimar os parâmetros cinéticos, foi utilizada uma análise de regressão não linear assistida (GraphPad software). Todas as amostras foram repetidas pelo menos três vezes e os dados apresentados são os valores médios das repetições. Para comparação entre as cepas foi utilizado o Turkey Test ANOVA.

4.3.17 Cálculo do peso seco celular

Para o cálculo do peso seco celular foram utilizados diferentes inóculos com OD específicas. Filtros millipore de 0,45 µm foram secos por 12 h a 30 ^{o}C em estufas e, posteriormente, pesados em balança analítica. Utilizando estes filtros e sistema de vácuo, 1mL de cada inóculo foi filtrado e o material foi seco nas mesmas condições anteriores. O cálculo foi efetuado subtraindo o valor do filtro depois do processo de filtração do inóculo pelo valor do filtro antes da filtração. Os demais valores foram calculados a partir da equação relacionando os valores de OD e peso de células em gramas.

4.3.18 Parâmetros de fermentação

As fermentações foram feitas em biorreatores Infors HT (Infors HT, Bottmingen, Switzerland) utilizando meio YNB suplementado com 40 g/L de fonte de carbono, com OD_{600nm} inicial 0,2 e pH 5,5 controlado por adição de KOH 3M. Os reagentes utilizados foram obtidos da *Sigma Aldrich Co*. Todas as fermentações foram feitas em triplicatas e cada resultado apresentado é de uma das triplicatas com erro menor que 10% entre eles.

O crescimento celular, consumo da fonte de carbono e a síntese de produtos foram utilizados para determinação das taxas volumétricas (g/L/h), específicas (g/ g de células/ h) e de rendimento (g/g de produto).

4.3.19 Otimização do processo fermentativo

Para a otimização do processo fermentativo, os parâmetros de fermentação foram testados, sendo um único parâmetro modificado por fermentação.

Os principais parâmetros testados foram base, sendo estas hidróxido de amônio e hidróxido de potássio (NH_4OH e KOH), pH (3,0 e 5,0) e oxigênio dissolvido (3% e 5%) sendo em todas estas utilizado meio de cultura YNB (13,4 g/L) e glicerol (4%) como fonte de carbono.

4.3.20 Detecção e quantificação dos produtos de fermentação

O substrato e produtos produzidos na fermentação foram quantificados por HPLC (do inglês: Cromatografia líquida de alta precisão) *Shimadzu*. O sobrenadante foi analisado em duas proporções (água: sobrenadante) 1:1 e 1:20 e quantificados de acordo com curva de calibração com padrões nas seguintes concentrações: 0,25 g/L, 0,5 g/L, 1,0 g/L, 2,0 g/L, 3,0 g/L, 4,0 g/L e 5,0 g/L de cada componente analisado. Foi utilizada pré-coluna para total retirada de resíduos celulares antes da injeção da amostra na coluna de exclusão iônica Shim-pack SCR-101H (Shimadzu), específica para ácidos orgânicos. Para cada corrida, 20 uL de amostra foram injetados no sistema junto a fase móvel de H_2SO_4 5mM diluído em água ultra-pura com vazão 0,6 mL/min à 50 ^oC. As análises foram feitas em detector RID (*Refractive Index Detector*) e UV com comprimento de onda de 210 nm.

5. Resultados e Discussão

Para melhor entendimento dos experimentos executados neste trabalho, os resultados foram divididos em dois capítulos.

Capítulo 1: Produção de ácido lático por *P. pastoris* modificada geneticamente

5.1. Construção de cepas

5.1.1 Integração do gene *ldh* no genoma de *P. pastoris*

O gene de *B. taurus* codificador da enzima LDH foi otimizado para expressão em *P. pastoris* (Genone, Rio de janeiro, Brasil) e posteriormente, introduzido no genoma da levedura por recombinação homóloga. Para comprovar a integração do plasmídeo contendo o gene codificador da enzima LDH no genoma da X-33, foi realizada extração do DNA genômico dos clones obtidos, sendo estes utilizados como molde na reação de PCR. Os *primers* utilizados foram pGAP F e 3'AOX1 (Tabela 4) sintetizados a partir da sequência do promotor e terminador contidos no vetor pGAPzB. Para análise do tamanho (pb) do fragmento amplificado na reação de PCR (1163pb), o marcador molecular utilizado foi *1 kb Plus Ladder* (Invitrogen). A figura 6 mostra a eletroforese em gel de agarose 0,8% de produtos de PCR dos clones de *P. pastoris*, sendo o controle positivo o vetor pGAP+LDH e, os controles negativos cepas não transformadas.

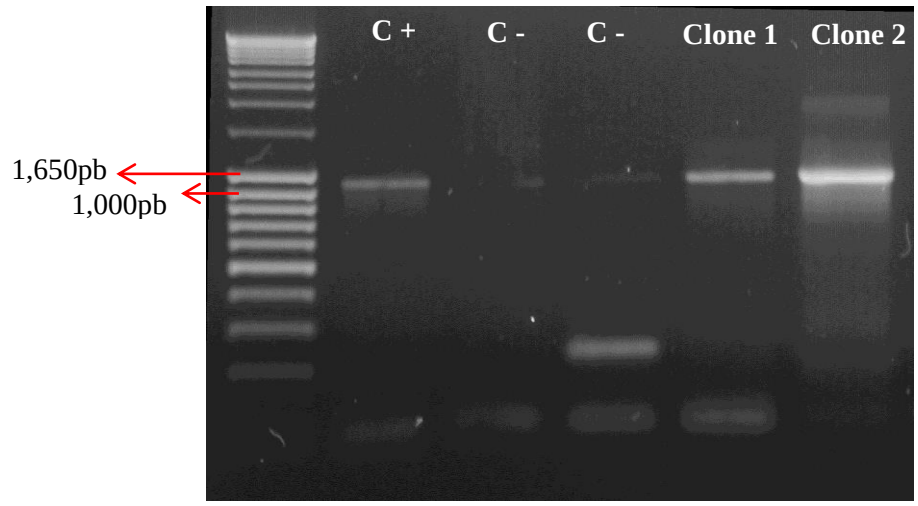


Figura 6 - Eletroforese em gel de agarose 0,8% de produtos de PCR de diferentes colônias de *P. pastoris*. (1) controle positivo usando o vetor pGAP-LDH; (2 e 3) controle negativo usando colônias de *P.pastoris* não transformadas e; (4 e 5) colônias de *P. pastoris* c com vetor pGAP-LDH integrado no genoma, resultando na cepa XL.

5.1.2 Perfil de crescimento em meio líquido

A fim de observar o perfil de crescimento dos clones de *P. pastoris* B1 a B6, assim denominados por conterem o gene bovino da LDH inseridos no genoma, as cepas foram crescidas em erlenmeyers aletados contendo meio YNB (10% do volume do frasco) adicionado de glicerol (20 g/L) e zeocina (100 μg/mL), tendo OD_{600nm} inicial 0.2. A figura 7 mostra a análise do crescimento celular, com pontos coletados para medição de OD a cada 3 horas, sendo o tempo total de crescimento de 48 horas.

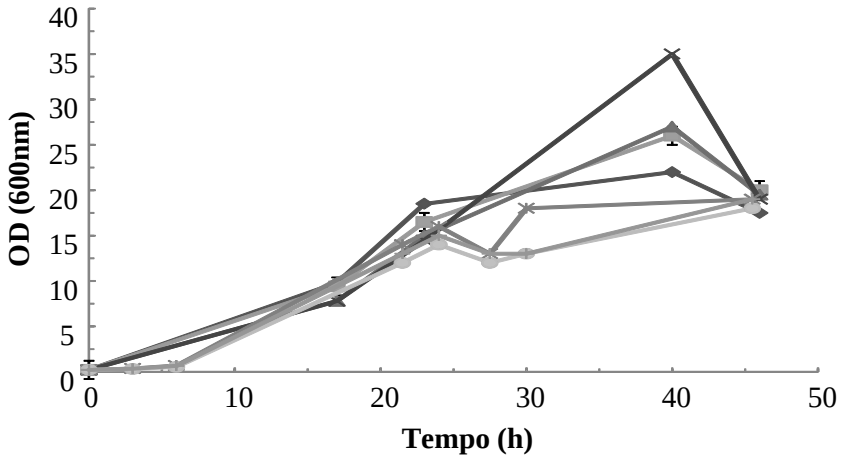


Figura 7 - Crescimento celular medido por densidade óptica (OD) dos clones de *P. pastoris* em meio YNB líquido utilizando glicerol como fonte de carbono. Sendo (losango) X33; (retângulo) B1; (triângulo) B2; (letra x) B3; (asterisco) B4; (quadrado) B5 e; (círculo) B6.

Apesar da inserção de uma via fermentativa, a figura 7 mostra que não há diferença significativa nos perfis de crescimentos entre os clones obtidos, assim como quando comparados à cepa selvagem, X33, nestas condições de crescimento.

5.1.3 Ensaio para análise de atividade da enzima LDH

Para avaliar a atividade enzimática específica dos clones B1 a B6, oxidação de NADH, diretamente ligada à redução de piruvato a ácido lático foi medida em espectrofotômetro. Além disso, a quantificação da atividade da enzima LDH possibilitou a comparação entre os clones obtidos após transformação e destes com a cepa selvagem. Sendo X-33 utilizada como controle negativo, por não apresentar nenhuma atividade relacionada a

esta enzima. Conforme a figura 8, todas as colônias apresentaram atividade de LDH com diferenças estatísticas comparadas à cepa X33, no entanto, o clone B5 demonstrou maior atividade. Sendo por isso, selecionada para os demais experimentos deste trabalho, posteriormente denominada, XL (Tabela 3).

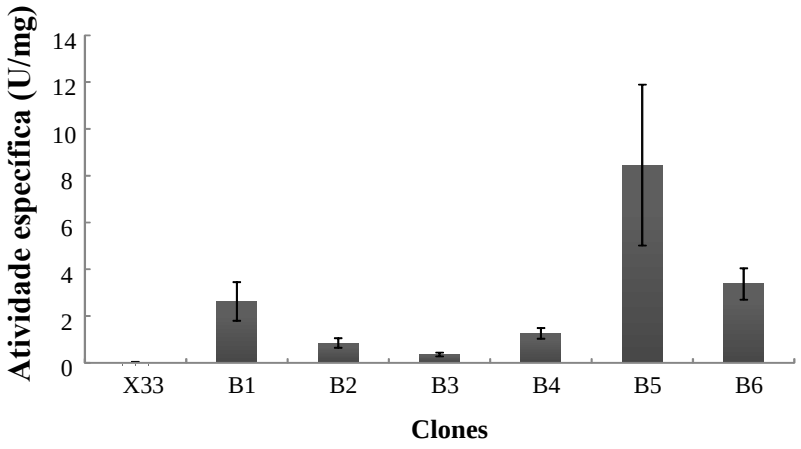


Figura 8 - Atividade enzimática específica da conversão de piruvato a ácido lático pela enzima LDH dos clones de *P. pastoris* e da cepa selvagem, X-33.

Assim como observado por Liaud e colaboradores (2015), a diferença na atividade intracelular de LDH entre os clones obtidos pode ser justificada pelo número de cópias do vetor integrados no genoma de *P. pastoris*, já que o vetor utilizado tem o promotor GAP, o qual permite expressão de múltiplas cópias em *P. pastoris.* No entanto vale ressaltar que nenhum experimento em nível de DNA para determinar o diferente número de cópias nos diferentes clones foi realizado.

5.1.4 Produção de ácido lático a partir de diferentes fontes de carbono

Atualmente, o processo principal usado para produzir ácido lático é a fermentação de glicose derivada de amido ou de celulose (WANG et al., 2014). Com o aumento da produção de biodiesel, a quantidade de glicerol gerado (346,8 mil m^3 em 2015) permitiu que este resíduo seja considerado uma fonte promissora para fins biotecnológicos. Assim, apesar do maior rendimento utilizando glicose como substrato, como mostra a figura 9, a utilização do glicerol pode ser viável já que este tem custo consideravelmente inferior. Além disso, estudos realizados na UnB demonstraram altas taxas de densidade celular de *P. pastoris* utilizando glicerol bruto como única fonte de carbono (ANASTÁCIO et al., 2014). Com o intuito de

quantificar a produção de ácido lático pela cepa XL, foi realizada fermentação em batelada utilizando exclusivamente glicose, glicerol ou sacarose como fonte de carbono.

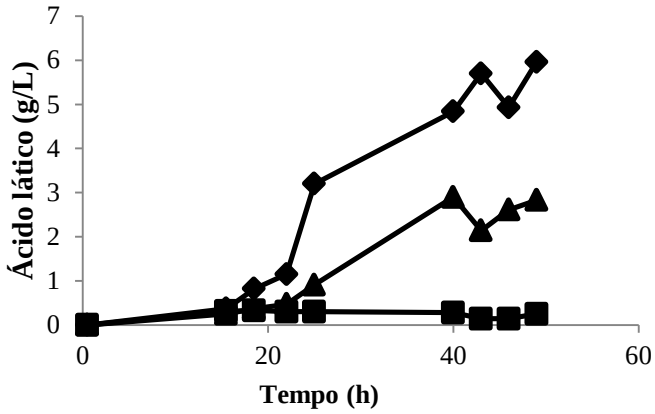


Figura 9 - Produção de ácido lático utilizando diferentes fontes de carbono, glicose (losango), glicerol (triângulo) e sacarose (quadrado).

Como apresentado na figura 9, utilizando glicose a cepa XL produziu pouco mais que o dobro de ácido lático quando comparada a produção a partir de glicerol, 5,96 g/L e 2,83 g/L, respectivamente. Sendo o rendimento em glicose, aproximadamente 3 vezes maior em comparação ao glicerol. Apesar disto, a introdução da atividade LDH de *B. taurus* em *P. pastoris* resultou em um rendimento de lactato ($Y_{lac/s}$) de 0,235 g/g, sendo este valor 52% superior à introdução do mesmo gene em *S. cerevisiae* ($Y_{lac/s}$ 0,155 g/g) usando glicose como substrato (BAUMANN et al., 2010).

5.1.5 Caracterização cinética de XL em batelada

A fim de testar o comportamento fisiológico de XL, foi realizada fermentação em batelada contendo 4% de glicerol. A Figura 10 mostra produção, aproximadamente, 4 vezes maior da cepa XL em comparação a cepa X33. Este aumento comprova a capacidade de conversão de glicerol em ácido lático, confirmando atividade heteróloga de LDH. A utilização de glicerol por *P. pastoris* na produção de outros produtos biotecnológicos já foi descrito anteriormente (ÇELIK et al., 2008; TANG et al., 2009), fazendo desse organismo uma via potencial na conversão de glicerol bruto em produtos de interesse biotecnológico.

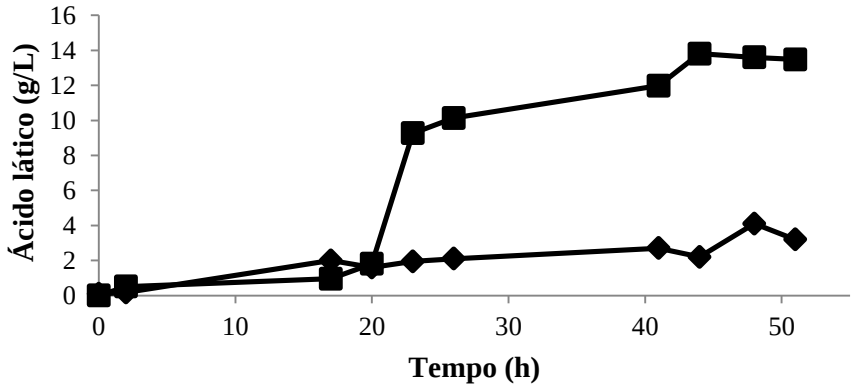


Figura 10 - Comparativo de produção de ácido lático em fermentação em bateada pela cepa XL (quadrado) e cepa X33 (losango) de *P. pastoris*.

5.1.6 Otimização do processo fermentativo

O principal objetivo do biorreator é promover um ambiente ideal para o microrganismo, já que o metabolismo celular depende diretamente das funções fornecidas pelo reator. Assim, vários parâmetros podem ser modificados a fim de fornecer um ambiente com condições ótimas para melhor crescimento celular e consequente aumento da produção do produto de interesse (HANSMANN et al., 2013).

O pH do ambiente é particularmente significativo na determinação do crescimento de leveduras na presença de ácidos orgânicos. Sendo o pH do meio abaixo do pKa do ácido produzido, a forma não dissociada do ácido predomina e este pode atravessar a membrana celular por difusão facilitada. Um vez no citosol, a dissociação química do ácido ocorre liberando prótons (H^+) e um contra-íon. Devido a sua carga, estes íons são capazes de atravessar a camada lipídica e acumulam-se no interior da célula. Assim, além de afetar a homeostase do pH interno, estes ácidos têm um impacto na organização lipídica e função das membranas celulares (MIRA; TEIXEIRA; SA-CORREIA, 2010).

Outro fator determinante no processo fermentativo é a quantidade de oxigênio disponível. A limitação de oxigênio afeta fortemente o metabolismo no núcleo celular diminuindo a oferta de energia, assim, há uma diminuição na produçao de biomassa, devido à reducao de ATP disponível. Além disso, há um reajuste no fluxo metabólico, alternando de respiratório para fermentativo. Uma vez que a produção de ácido lático é um processo metabólico que requer ATP, uma mudança para o metabolismo fermentativo poderia ter impacto também na síntese e /ou secreção deste metabólito (BAUMANN et al., 2010).

5.1.6.1 Efeitos de pH na produção de ácido lático

P. pastoris é capaz de crescer em uma ampla faixa de pH, sendo tipicamente considerada entre 2,8 e 6,5 (WEGNER, 1983). Embora este intervalo tenha pouco ou nenhum efeito sobre o crescimento da levedura, este pode ter efeitos importantes dependendo da produção de interesse no processo fermentativo (CREGG et al., 1993). O consumo de substrato e a produção de ácido lático torna o meio ácido durante a fermentação. Além disso, o glicerol bruto pode ter pH baixo já que contém resíduos do processo de transesterificação. Para análise da influência do pH na produção de ácido lático, foram realizadas fermentações em batelada alimentada em pH 3,0 e 5,0. A figura 11 mostra o perfil da fermentação da cepa XL em diferentes pH. A tabela 7 apresenta os parâmetros cinéticos obtidos durante fermentação em batelada.

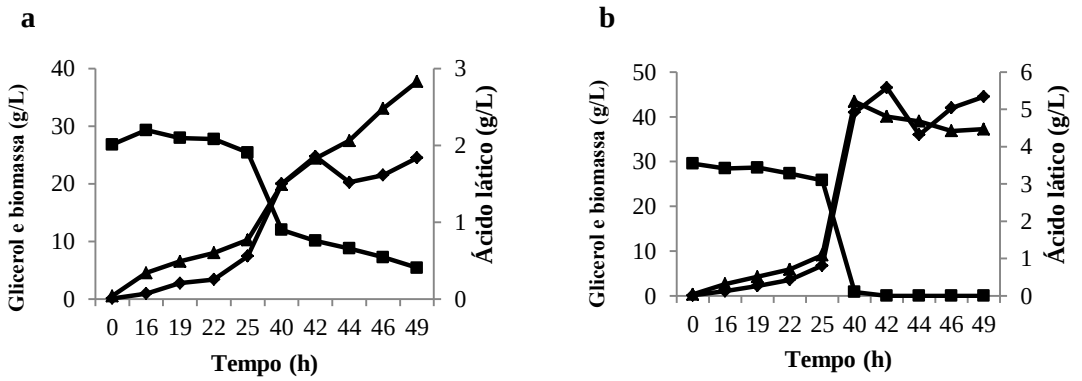


Figura 11 - Perfil da fermentação utilizando a cepa XL em (a) pH 3,0, e (b) pH 5,0, sendo considerado consumo de glicerol (quadrado), produção de ácido lático (losango) e biomassa (triângulo).

Tabela 7 - Parâmetros cinéticos durante fermentação em batelada em pH 3,0 e 5,0

	μ máx	**$Y_{x/s}$**	**$Y_{lac/s}$**	**Y_{total}**	**q_x**	**q_{lac}**	**r_x**	**r_{lac}**
pH 3,0	0,139	0,3472	0,0266	0,3738	0,1242	0,0033	0,2230	0,0059
pH 5,0	0,1303	0,2496	0,115	0,3672	0,0253	0,0029	0,1148	0,0132

Y rendimento; s substrato, x biomassa; lac lactato; Y g/g; q g/g/h; r g/L/h

Os parâmetros cinéticos apresentados na Tabela 7 mostram que em pH 3,0 houve maior direcionamento da fonte de carbono para produção de biomassa do que em pH 5,0, sendo o rendimento ($Y_{x/s}$) de 0,3472 e 0,2496, respectivamente. Enquanto que em pH 5,0 o perfil fermentativo da cepa XL foi melhor ativado, produzindo 4,16 vezes mais lactato do que quando o pH 3,0 foi mantido.

5.1.6.2 Efeitos de disponibilidade de nitrogênio na produção de ácido lático

Para manutenção do pH ótimo é necessária injeção de compostos básicos durante o processo fermentativo. A disponibilidade de nitrogênio pode afetar muitos aspectos no metabolismo de leveduras, além de regular o crescimento, pode causar menor produção de biomassa e diminuir o perfil fermentativo destas (VILANOVA et al., 2007). A utilização de KOH nas fermentações anteriores gerou a hipótese do aumento na produção de ácido lático com maior disponibilidade de nitrogênio no biorreator. Já que o ácido lático é produto prímario do metabolismo de *P. Pastoris* , o aumento da produção deste deveria ser paralelo a produção de biomassa (MATTANOVICH; SAUER; GASSER, 2014). Com o intuito de avaliar a produção de ácido lático em processo fermentativo com maior e menor níveis de nitrogênio disponível, as bases NH_4OH e KOH, ambas na concentração 3M, foram utilizadas para manutenção do pH ótimo na fermentação em batelada da cepa XL.

Apesar das implicações de suplementação com nitrogênio nos parâmetros cinéticos de fermentação serem evidentes na literatura, a figura 12 mostra que não há alteração destes em fermentações utilizando a cepa XL.

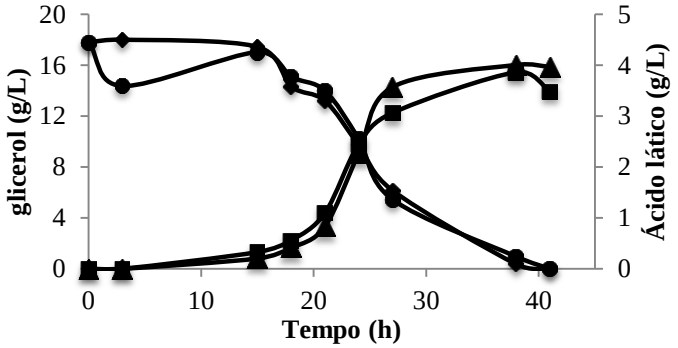


Figura 12 - Consumo de glicerol e produção de ácido lático pela cepa XL utilizando KOH (losango e quadrado, respectivamente) ou NH_4OH (círculo e triangulo, respectivamente) para manutenção do pH ótimo durante fermentação em batelada.

Por não apresentar nenhuma diferença estatística quanto à disponibilidade de nitrogênio fornecido por meio de composto básico durante o processo fermentativo e considerando a diminuição do custo de produção, para as demais fermentações deste trabalho o pH ótimo foi mantido utilizando-se KOH.

5.1.6.3 Efeitos da porcentagem de DO fornecida durante processo de produção de ácido lático

P. pastoris é uma levedura aeróbica obrigatória (BAUMANN et al., 2010). Ainda assim, após a introdução de uma via fermentativa, foram testados dois diferentes níveis do fornecimento de oxigênio dissolvido, 3% e 5%, para avaliar se a limitação de oxigênio poderia aumentar a produção de ácido lático por induzir o perfil fermentativo da levedura. A figura 12 mostra que quando fornecido 3% de oxigênio dissolvido, a cepa XL teve rendimento (lac/s) de 0,236 g/g, e quando fornecido 5%, esta cepa apresentou rendimento 20% inferior (Ylac/s de 0,196 g/g). Além disso, o μmax da cepa XL foi 10% menor em 3% do que em 5% de oxigênio dissolvido, sendo 0,174 e 0,189, respectivamente. Assim, indicando maior conversão de glicerol em biomassa quando fornecidas maiores quantidades de oxigênio dissolvido, diminuindo a produção de ácido lático.

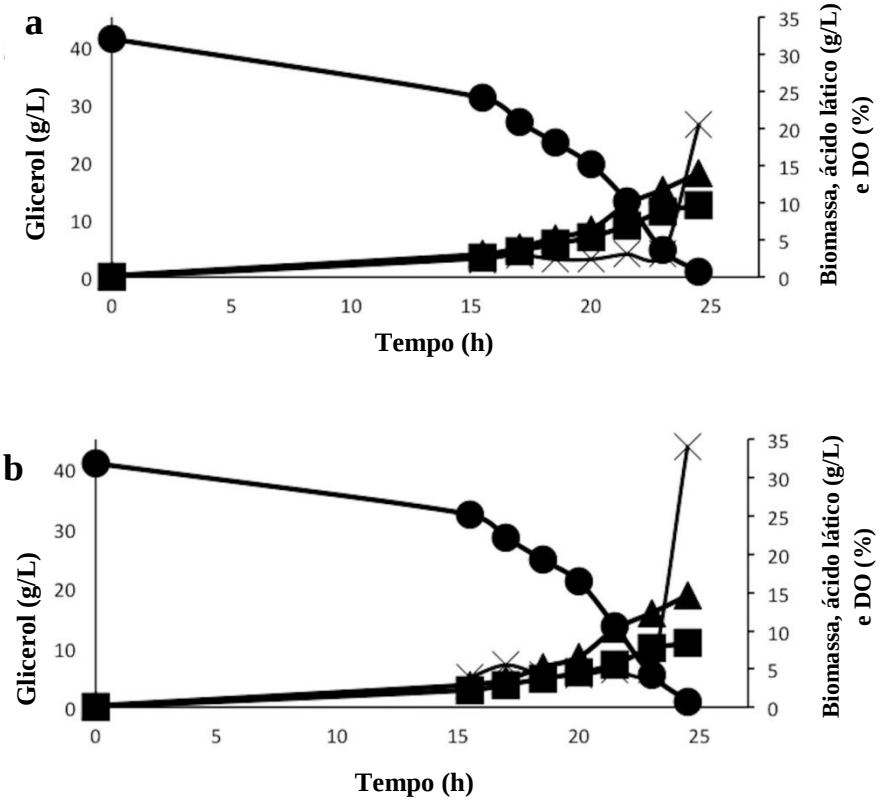


Figura 13 - Fermentação em batelada da cepa XL utilizando glicerol (círculo) como fonte de carbono e fornecimento limitado de oxigênio dissolvido, sendo 3% (a) e 5% (b), para produção de biomassa (triângulo) e ácido lático (quadrado).

É possível perceber que a maior produção de ácido lático ocorre quando a porcentagem de DO fornecido é menor, este efeito pode ser justificado pelo aumento da

capacidade de fermentação da cepa XL e pela regulação do promotor pGAP. Em estudos recentes, a produção heteróloga de fragmentos de ligação ao antígeno (Fab, do inglês: *fragment antigen-binding*) sob regulação do promotor pGAP e limitação de DO mostrou diminuição de 2 vezes na produção de biomassa, enquanto a produção da proteína heteróloga aumentou 2,5 vezes (BAUMANN et al., 2008). Gunes e Çalik (2016) avaliaram que a porcentagem de oxigênio fornecida também afeta a produção de proteínas heterólogas sob regulação do promotor pGAP em *P. pastoris.* Os resultados mostraram que com 20% de ar saturado houve maior atividade da enzima glicose isomerase do que quando 15% de DO foi fornecido. Apesar disto, neste trabalho não foi avaliada a atividade enzimática em diferentes condições de aeração, os resultados apresentados indicam que condições de limitação de oxigênio forçam o metabolismo fermentativo da levedura.

Capítulo 2: Transportadores de ácido lático

5.2 Identificação de um transportador de lactato putativo em *P. pastoris*

Em *S. cerevisiae*, a atividade do *lactate–proton symporter* foi descrita como dependente da expressão do gene *jen1* (PAIVA et al., 2013). Em membranas heterólogas de *P. pastoris* reconstituídas, o transportador de lactato de *S.cerevisiae*, Jen1p, mostrou-se funcional (SOARES-SILVA et al., 2003). Com o objetivo de identificar um gene ortólogo de *jen1* no genoma de *P. pastoris*, realizou-se um BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi). Este revelou uma ORF com similaridade significativa (50,19% de identidade), como mostrado na Tabela 8. Enquanto que

Tabela 8 - Alinhamento de sequências de nucleotídeos dos genes *jen1* e *pas*

```
Nome        Sequência
JEN1p       ATGTCGTCGTCAATTACAGATGAGAAAATATCTGGTGAACAGCAACAACCTGCTGGCAGA
PAS         ------------------------------------------------------------


JEN1p       AAACTATACTATAACACAAGTACATTTGCAGAGCCTCCTCTAGTGGACGGAGAAGGTAAC
PAS         ------------------------------------------------------------


JEN1p       CCTATAAATTATGAGCCGGAAGTTTACAACCCGGATCACGAAAAGCTATACCATAACCCA
PAS         -----------------------------------------------ATGTCGCATTCAA
                                                            **  *  *  * *

JEN1p       TCACTG--CCTGCACAATCAATTCAGGATACAAGAGATGATGAATTGCTGGAAAGAGTTT
PAS         TCCATTCTGCTGCTCACAAGAGTGTCATGACTGATATTGAAAAACAACACGACAGAATAC
            **  *    **** **    * *      **      ***  **   *  ** *** *

JEN1p       ATAGCCAGGATCAAGGTGTAGAGTATGAGGAAGATGAAGAGGATAAGCCAAACCTAAGCG
PAS         ATCCTCATGACCACCCAGTTA------------CTCCTTTGGAAAAGCCAGACTTGTCTG
            **   ** ** **    **              *     *** ****** ** *    *

JEN1p       CTGCGTCCATTAAAAGTTATGCTTTAACGAGATTTACGTCCTTACTGCACATCCAC----
PAS         CAAAATCGATAAAACATTACTTTGCTACTAGACTTTCCACGTTGGTGCCAAAAGCTGAAG
            *    ** ** ***  ***   *   ** *** ** *  * **  ***  *

JEN1p       -----GAGTTTTCTTGGGAGAATGTCAATCCCATACCCGAACTGCGCAAAATGACATGGC
PAS         ATTTCAAAGATAAGCGATACATTTTCAATCCTTTCATGGTGCTCCGTGAGTTGACACCCA
                  *   *    *  * * * *******  *    *  ** **  *  *****

JEN1p       AGAATTGGAACTATTTTTTTATGGGTTATTTTGCGTGGTTGTCTGCGGCTTGGGCCTTCT
PAS         AAAATTGGAACTTCTTTATGTGTGGTTTTGCTGCTTGGACTTGGGATGCTGTGGATTTTT
            * **********  *** *    **** *  *** ***   *  *  ***  **  ** *

JEN1p       TTTGCGTTTCAGTATCAGTCGCTCCATTGGCTGAACTATATGACAGACCAACCAAGGACA
PAS         TCTCTGTTTCTCTTAATGTAACAAATATTGCTAAAGACCTGAATGTCACCACCAAAGATG
            * *  *****  *    **  *     * *** **       *     * ***** **

JEN1p       TCACCTGGGGGTTGGGATTGGTGTTATTTGTTCGTTCAGCAGGTGCTGTCATATTTGGTT
PAS         TCACTTGGGGTTTAACTCTGGTGTTGATGCTAAGGTCAGTTGGTGCTATTTCGTTTGGTC
            **** ***** **     *******  *  *  * ****  ****** *    ******

JEN1p       TATGGACAGATAAGTCTTCCAGAAAGTGGCCGTACATTACATGTTTGTTCTTATTTGTCA
```

```
PAS         TGTGGGCAGATAGAAAAGGAAGAAAATGGCCTTTTATTTTCAACATGATTCTTCTCATTG
            * *** ******        ***** ***** *  ***       ** *  *  *  *

JEN1p       TTGCACAACTCTGTACTCCATGGTGTGACACATACGAGAAATTTCTGGGCGTAAGGTGGA
PAS         TTCTTCAAATTGGACTGGGATTTGTGAACACTTATAAACAATTCTTAGGAGTGAGAGCTG
            **   *** *  *      **      **** **  *  ****  * ** ** **

JEN1p       TAACCGGTATTGCTATGGGAGGAATTTACGGATGTGCTTCTGCAACAGCGATTGAAGATG
PAS         TATTTGGTATTGCTATGGGTGCTGTGTTTGGTAACGCATCCTCTCTAGCATTAGATGACT
            **   ************** *   * *  **    ** **  *   ***  * ** **

JEN1p       CACCTGTGAAAGCACGTTCGTTCCTATCAGGTCTATTTTTTTCTGCTTACGCTATGGGGT
PAS         GTCCTCCAAGAGCCAAGGGAATTGTTAGTGGTATATTCCAACAAGGATATGCCTTTGGTT
              ***   * ***        *  *    *** ****       *  ** **  * ** *

JEN1p       TCATATTTGCTATCATTTTTTACAGAGCCTTTGGCTACTTTAGGGATGATGGCTGGAAAA
PAS         ACCTTCTGGTTGTGGTGTTTCAACGTGCTATCACTGATAATGCTTCAAAAAGTTGGAGAG
             * *  * * * *  * *** *  * **  *     *   *       *  * **** *

JEN1p       TATTGTTTTGGTTTAGTATTTTTCTACCAATTCTACTAATTTTCTGGAGATTGTTATGGC
PAS         CGTTATTCTGGTTTAGTGCCGGTCCGCCAGTGCTTATCATATTATGGAGACTGGTTCTAC
              ** ** *********     **  *** * **  * ** ** ****** ** *    *

JEN1p       CTGAAACGAAATACTTCACCAAGGTTTTGAAAGCCCGTAAATTAATATTGAGTGACGCAG
PAS         CAGAAACAGATGCATTTATTG------------CCCAGCAAAAC---T------TGAGAA
            * *****  *    ** *               ***   **      *          *

JEN1p       TGAAAGCTAATGGTGGCGAGCCTCTACCAAAAGCCAACTTTAAACAAAAGATGGTATCCA
PAS         TGGAAACTAGCGATGGACAGACTGCCTCACAACAGTTCGTCAAACAGGGAAA--------
            ** ** ***  * ***  ** **    ** **     * * *****    *

JEN1p       TGAAGAGAACAGTTCAAAAGTACTGGTTGTTGTTCGCATATTTGGTTGTTTTATTGGTGG
PAS         ----AGACGCCTTGAGAGTCTACTGGTTGATGCTTATCTACATGATTATCATGATGGCCG
                     *  *   *   ********* ** *    **  ** ** *  *  ***  *

JEN1p       GTCCAAATTACTTGACTCATGCTTCTCAAGACTTGTTGCCAACCATGCTGCGTGCCCAAT
PAS         CATTCAACTTTAGTTCTCACTCCTCCCAAGATCTTTACCCTACTCTTCTAACAGTTCAAT
                 ** *      ****  * ** *****  * *  ** **  * **    *  ****

JEN1p       TAGGCCTATCCAAGGATGCTGTCACTGTCATTGTAGTGGTTACCAACATCGGTGCTATTT
PAS         ATGAATTTGGTAAAGACAGATCTACAGTTACTAACGTCGTGGCAAATCTTGGAGCCATTG
              *   *    ** **       ** ** * *   ** **  * **  * ** ** ***

JEN1p       GTGGGGGTATGATATTTGGACAGTTCATGGAAGTTACTGGAAGAAGATTAGGCCTATTGA
PAS         TCGGAGGTATTTTCTGGGGTCATATGTCCAACTTCATTGGTCGACGTCTTGCAGTTTTGC
              ** *****  * *  ** **  *     *  * * ***  ** *  * *   * ***

JEN1p       TTGCATGCACAATGGGTGGTTGCTTCACCTACCCTGCATTTATGTTGAGAAGCGAAAAGG
PAS         TCTGTTGTATAGTAGGTGGAGCTTTCATTTACCCTTGGGCAT---TTATAAATGGTTCAG
            *    ** * * * *****    ****  ******          * * **  *     *

JEN1p       CTATATTAGGTGCCGGTTTCATGTTATATTTTTGTGTCTTTGGTGTCTGGGGTATCCTGC
PAS         GGATCAATGCTGGTGCTTTCTTCTTACAATTTGCTGTCCAAGGTGGATGGGGTGTATGTC
              **    * **  * **** * *** * ***  ****   ****  ****** *    *

JEN1p       CCATTCACCTTGCAGAGTTGGCCCCTGCTGATGCAAGGGCTTTGGTTGCCGGTTTATCTT
PAS         CAGTTCATCTTGCTGAGATGTCTCCGCCCCATTTCCGAGCTTTTGTTACAGGAACCACCT
            *  **** ***** *** ** * **  *  **    * ***** *** * **     * *

JEN1p       ACCAGCTAGGTAATCTAGCTTCTGCAGCGGCTTCCACGATTGAGACACAGTTAGCTGATA
PAS         ACCAGCTAGGAAACCTGGCATCATCTGGATCATCTACTATCCTGGCCGATATTGGAGAAA
            ********** ** ** ** **  * *   * ** ** **   * *  *  * *  ** *

JEN1p       GATACCCATTAGAAAGAG---ATGCCTCTGGTGCTGTGATTAAAGAAGATTATGCCAAAG
PAS         GATTCCCCATTTACGACGAGAATGGAGTGCGGAAAGAGGGTGTTTATGACTATGCCAAAG
            *** ***  *  *    *   ***      *    * *  *    * ** **********

JEN1p       TTATGGCTATCTTGACTGGTTCTGTTTTCATCTTCACATTTGCTTGTGTTTTTGTTGGCC
```

```
PAS         TAATGGCTATATTTATGGGAGCTGTGTTTGCTTTCCTGTTTATTGTGATGTTGCTGGGTC
            * ******** ** *  **  **** **    ***   ***  *    * **  * ** *

JEN1p       ATGAGAAATTCCATCGTGATTTGTCCTCTCCTGTTATGAAGAAATATATAAACCAAGTGG
PAS         CAGAAAGAAGAG---GAGCCTCCAATCTTGATGATCTCCAGGATTACATTGACGATTGGG
              ** * *       * *  *       *  ** * *  ** * ** **  ** *   **

JEN1p       AAGAA---------------TACGAAGCCGATGGTCTTTCGATTAGTGACATTGTTGAAC
PAS         AGCAAAAAGATCTGGACAACAAAAAAGGAATTGAAACTGAGTTCATCGAAGATGTTCAGT
            *  **                *  ***    **    *  * * *  **   **** *

JEN1p       AAAAGACGGAATGTGCTTCAGTGAAGATGATTGATTCGAACGTCTCAAAGACATATGAGG
PAS         TAATGGAG---GCTGTCTC------------TAATGAGGACCTTTCTGAGAAAGGCAATG
             ** *  *     **  **            * **  * ** * **  *** *    * *

JEN1p       AGCATATTGAGACCGTTTAA------------
PAS         AGAAAGTAGAGGCTTTTGAAGGAAATAAGTAA
            ** * * *** * ** **
```

Prevê-se que a proteína do homologo, PAS, tenha 552 aminoácidos, pertença à família dos *proton-linked monocarboxylate transporter family* (número de acesso: XM_002492622.1) e contenha 10 domínios transmembrana, enquanto que Jen1p possui 12 domínios transmembranares.

5.2.1 Avaliação do transporte de ácido lático usando lactato 14C

Desde a identificação de *jen1* codificador de um transportador de lactato em *S. cerevisiae*, sabe-se que o transportador Jen1p é capaz de reconstituir a atividade da lactato permease em *P. pastoris* (SOARES-SILVA et al., 2003). Após as análises e alinhamento de sequências, o gene PAS foi inserido na cepa de *P. pastoris* GS115 sob o controle do promotor de fosfoglicerato quinase 1(PGK), resultando na cepa GS (Tabela 1). De forma semelhante, o gene *jen1* de *S. cerevisie* foi inserido na cepa GS115, resultando na cepa GJ (Tabela 1). Ambas as cepas, incluindo o controle (GS115), foram utilizadas para o ensaio de transporte de lactato ^{14}C (Fig. 13 e Tabela 9). No entanto, não foi encontrada diferença significativa na cepa que contém o gene *jen1* e, portanto, estes dados não são apresentados. Em relação às cepas com superexpressão do transportador putativo de lactato provenientes de *P. pastoris*, o transportador PAS (cepa GS) mostrou afinidade 3 vezes maior por lactato em comparação à cepa controle, GS115, como mostra a Figura 14. Sendo os valores de Km e Vmáx apresentados na tabela 9.

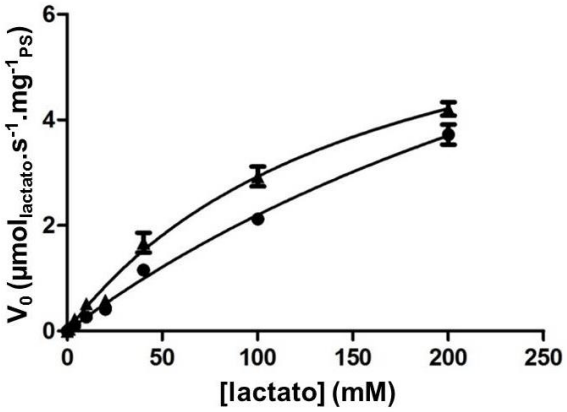


Figura 14 - Taxas de absorção de ácido láctico radioativo das cepas GS115 (círculo) e GS (triângulo).

A atividade da enzima foi medida através de Vmáx e também da constante de Michaelis-Menten, K_M, o qual demonstra a força de ligação de um substrato à enzima. A tabela 7 mostra os valores de Vmáx e Km do transportador de lactato de *P. pastoris* superexpresso na cepa recombinante.

Tabela 9 - Vmax e Km da cepa selvagem (GS115) e modificada (GS) com transportador PAS medidos com lactato radioativo.

	Vmax	**Km**
GS115	11,68 ± 2,09	430,70 ± 105,00
GS	7,54 ± 0,67	157,40 ± 25,46

Como pode ser visualizado, mesmo que a cepa modificada tenha reduzido sua velocidade máxima de transporte de 11,68 ± 2,09 µmol/seg.mg para 7,54 ± 0,67µmol/seg.mg, o valor está dentro da margem de erro e a diferença não é significativa. No entanto, transportador PAS demonstrou afinidade quase 3 vezes maior, o que pode ser observado pela redução do seu valor de Km de 430,70 ± 105,00 µmolar para 157,40 ± 25,46 µmolar.

5.2.2 Produção de ácido lático por cepas com transportadores superexpressos

Após confirmar que as cepas recombinantes de *P. pastoris* foram capazes de produzir ácido lático utilizando glicerol como fonte única de carbono e que as cepas GS e GJ apresentam maior afinidade por lactato quando comparadas a cepa controle, as cepas XL, GLS e GLJ (Tabela 1) foram avaliadas quanto à produção de ácido lático. Para isto, foram realizadas fermentações em batelada alimentada em duas etapas: (1) produção de biomassa em condições aeróbicas e; (2) iniciada pelo aumento do nível de pH (>5) com uma única adição de glicerol (4%) e baixa concentração de oxigênio dissolvido (2%).

Apesar da cepa GJ ter mostrado maior afinidade do que a cepa controle (GS115) no ensaio com lactato radioativo, quando avaliada em processo de fermentação por batelada alimentada, esta cepa apresentou rendimento pouco maior (2%). Enquanto que a cepa GLS apresentou produção de ácido lático 46% maior do que a cepa XL e 43% maior quando comparada a cepa GLJ, como mostra a Figura 15. Assim como obteve maiores taxas de produção específica (0,126 g/h) e volumétrica (0,673 g/L/h) (Tabela 10).

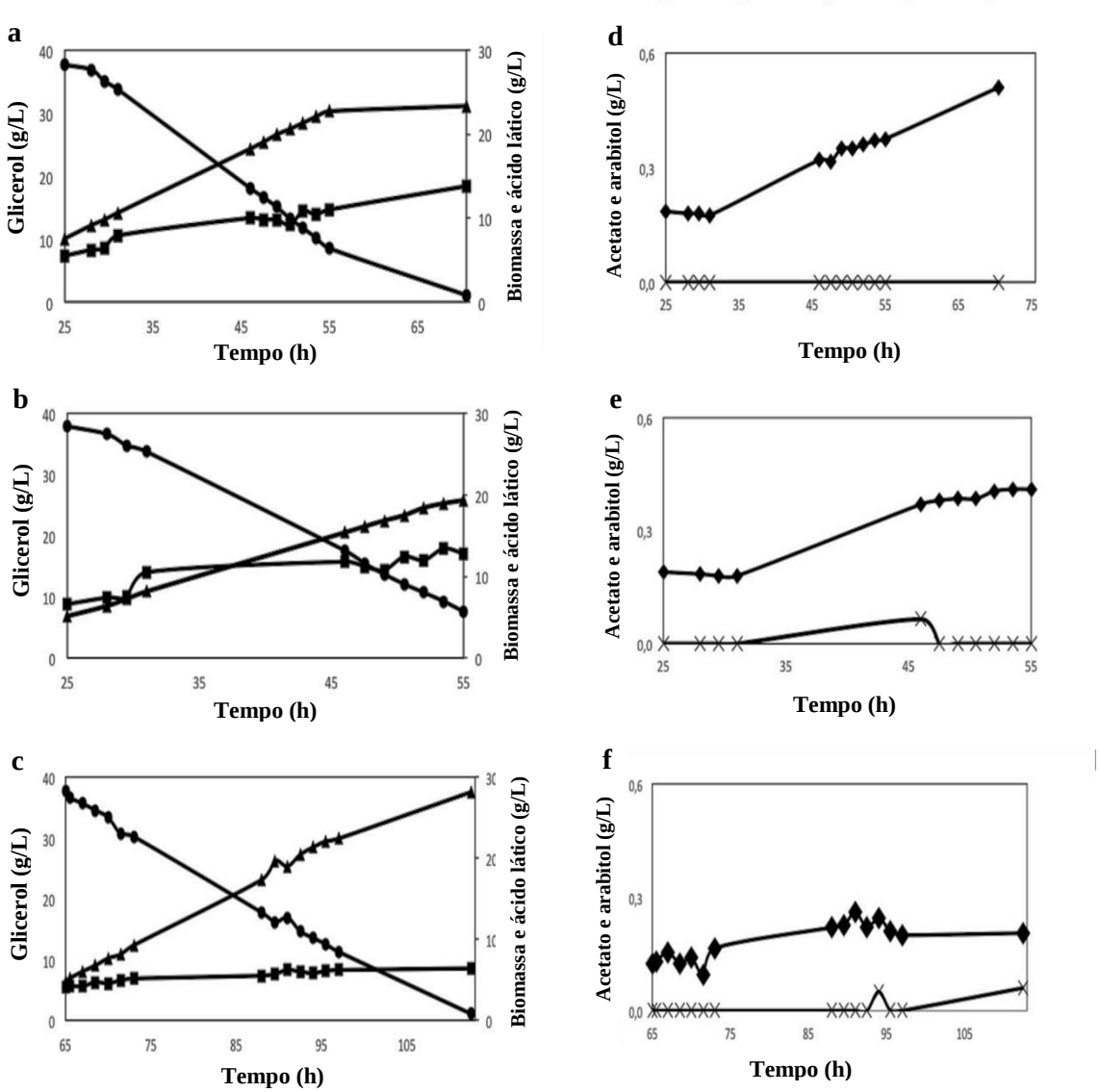


Figura 15 - Perfil de fermentação em batelada alimentada das cepas XL (a e d), GLJ (b e e) e GLS (c e f). As figuras a, b e c mostram o consumo de glicerol (círculo) e a produção de biomassa (quadrado) e lactato (triângulo). As figuras d, e e f, mostram a quantificação dos co-produtos acetato (losango) e arabitol (letra x).

Tabela 10 - Parâmetros cinéticos durante fermentação em batelada alimentada e fase aeróbica limitada.

Cepas	$Y_{x/s}$	$Y_{lac/s}$	$Y_{ac/s}$	$Y_{ara/s}$	q_x	q_{lac}	q_{ac}	q_{ara}	r_x	r_{lac}	r_{ac}	r_{ara}
XL	0,180± 0,004	0,460± 0,004	0,008± 0,001	0,000± 0,000	0,020± 0,004	0,053± 0,012	0,001± 0,000	0,000± 0,000	0,117± 0,025	0,348± 0,073	0,006± 0,001	0,000± 0,000
GLJ	0,177± 0,004	0,470± 0,035	0,010± 0,005	0,000± 0,000	0,024± 0,003	0,063± 0,003	0,001± 0,000	0,000± 0,000	0,160± 0,054	0,413± 0,101	0,001± 0,004	0,000± 0,004
GLS	0,066± 0,004	0,673± 0,033	0,004± 0,002	0,001± 0,001	0,014± 0,002	0,146± 0,016	0,001± 0,000	0,000± 0,000	0,065± 0,005	0,6743± 0,041	0,003± 0,001	0,001± 0,000

Y rendimento; s substrato, x biomassa; lac lactato; ac acetato; ara arabitol; Y g/g; q g/g/h; r g/L/h

Além disso, na fase alimentada, a cepa GLS apresentou a menor taxa de produção de biomassa (aproximadamente 3 vezes) e acetato (2,5 vezes), mostrando que esta cepa direciona a maior parte do carbono para produção de ácido lático ao invés de produção de biomassa. Os co-produtos acetato, arabitol e etanol também foram quantificados, e os parâmetros cinéticos da fase alimentada do processo fermentativo mostrou que as cepas XL e GLJ produziram, aproximadamente, 2 vezes mais acetato do que a cepa GLS, justificando a menor produção de ácido lático (Tabela 10).

Dentre as cepas e os modos de fermentação testados, a cepa GLS obteve os melhores resultados em fermentação com batelada alimentada com limitação de oxigênio. A produção de ácido lático utilizando glicerol como fonte de carbono já foi observada em outros organismos, como a bactéria Gram-positiva *Enterococcus faecalis* (MURAKAMI et al., 2015). Neste estudo foi utilizado o modo de batelada alimentada com 30 g/L de glicerol combinado com 22 g/L de ácido acético como substrato, neste experimento foi obtida taxa de produção volumétrica de ácido lático 7% maior do que nos experimentos com a cepa GLS. Outro estudo avaliou a produção de ácido lático pelo fungo *Rhizopus oryzae* em fermentação por batelada com 40 g/L de glicerol cru com adição de nutrientes inorgânicos de suco de *lucern* (VODNAR et al., 2013). Em ambos os estudos o total de ácido lático ao final do processo fermentativo foi de aproximadamente 12% menor do que a quantidade produzida neste trabalho. Embora estes estudos tenham apresentado dados de produção de ácido lático a

partir de glicerol cru ou puro, em nenhum deles foi feita modificação genética nas cepas utilizadas.

O custo da bio-produção de poli (ácido lático) depende diretamente do substrato utilizado (GHAFFAR et al., 2014). O glicerol cru é o resíduo obtido da conversão de óleos vegetais em biodiesel. Estima-se que a cada 9 kg de biodiesel produzido, 1kg de glicerol cru é gerado (BARBIER et al., 2011). Assim, tendo grande disponibilidade e baixo custo, o glicerol cru tornou-se um excelente candidato para produção de químicos de alto valor agregado, incluindo ácido lático (ANP,2016; DA SILVA et al., 2009; LEONETI et al., 2012; YANG et al., 2012). A cepa GLS é capaz de produzir ácido lático utilizando glicerol puro como única fonte de carbono, obtendo rendimento de 0,7 g/g, sendo este valor próximo ao rendimento teórico máximo de 1,0 g/g.

6. Conclusão

A cepa geneticamente modificada de *P. pastoris* desenvolvida neste trabalho é capaz de produzir ácido lático, fazendo deste organismo um potencial biocatalisador na conversão de glicerol em produtos biotecnológicos. Além disso, foi possível a identificação de um transportador putativo de lactato. Para isto, duas cepas geneticamente modificadas, GLS e GLJ, expressando genes codificadores de transportadores de lactato foram desenvolvidas no intuito de aumentar a produção de ácido lático. Ambas mostraram maior afinidade por lactato quando comparadas à cepa controle. As fermentações em batelada com 40 g/L de glicerol apresentaram aumento de 46 e 43% na produção de ácido lático pela cepa GLS quando comparada à cepa controle e GLJ, respectivamente. As taxas de produção volumétrica e específicas de ácido lático foram maiores quando utilizada a cepa GLS com diminuição simultânea de aproximadamente 60% na produção de biomassa.

7. Perspectivas

- Fermentar a partir de glicerol proveniente de diferentes indústrias de biodiesel;
- Modificação das vias metabólicas para redução de arabitaol e acetato como co-produtos;
- Verificar os paramentos fermentativos em escala de 100L.

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9. Produção

9.1 Curso

- Industrial Biotechnology for Lignocellulose Based Processes, Chalmers University of Technology, 2013.

9.2 Participação em eventos internacionais

- 36th Symposium on Biotechnology for Fuels and Chemicals (Flórida, EUA, 2014)
  Metabolic Engineering for L-lactic acid production in *Pichia pastoris*
- X Metabolic Engineering (Vancouver, Canadá, 2014)
  Characterization of LDH genes for L-lactic acid production in *Pichia pastoris*

9.3 Participação em eventos nacionais

- III Simpósio de Biologia Molecular (Universidade de Brasília, 2013)
  Engenharia metabólica em *Pichia pastoris* para produção de L-ácido lático
- IV Simpósio de Biologia Molecular (Universidade de Brasília, 2014)
  Estratégias de engenharia genética de leveduras para super produção de L-ácido lático
- V Simpósio de Biologia Molecular (Universidade de Brasília, 2015)
  Engenharia metabólica em *Pichia pastoris* para produção de L-ácido lático utilizando glicerol cru como fonte de carbono
- VI Simpósio de Biologia Molecular (Universidade de Brasília, 2016)
  Novo transportador de lactato aumenta a produção de L- ácido lático por cepas recombinantes de *Pichia pastoris* utilizando glicerol como fonte de carbono

9.4 Trabalhos apresentados em eventos nacionais

- XX Simpósio Nacional de Bioprocessos (Fortaleza, 2015)
  Metabolic engineering of *Pichia pastoris* for L-lactic acid production using glycerin as carbon source
- 5ª Jornada Sul Americana de Biologia e Biotecnologia de Leveduras (Olinda, 2015)
  Engenharia metabólica em *Pichia pastoris* para super produção de L-ácido lático

9.5 Patentes Depositadas

- BR1020130310522 - Levedura recombinante para produção de ácido lático utilizando glicerol bruto como fonte de carbono (2013)
- BR10201500745 - Levedura Recombinante Associada A Transportadores De Lactato Para Produção De Ácido Lático Utilizando Glicerol Bruto Como Fonte De Carbono (2015)

9.6 Artigos publicados

- Microbial Cell Factories (anexo A)

  Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*

- Biotechnology for Biofuels (anexo B)

  Identification and characterization of putative xylose and cellobiose transporters in *Aspergillus nidulans*

- Scientific Reports – Nature (anexo C)

  The low affinity glucose transporter HxtB is also involved in glucose signalling and metabolism in *Aspergillus nidulans*

10. Anexos

Anexo A - Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*

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Microbial Cell Factories

RESEARCH

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Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*

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Abstract

Background: Crude glycerol is the main byproduct of the biodiesel industry. Although it can have different applications, its purification is costly. Therefore, in this study a biotechnological route has been proposed for further utilization of crude glycerol in the fermentative production of lactic acid. This acid is largely utilized in food, pharmaceutical, textile, and chemical industries, making it the hydroxycarboxylic acid with the highest market potential worldwide. Currently, industrial production of lactic acid is done mainly using sugar as the substrate. Thus here, for the first time, *Pichia pastoris* has been engineered for heterologous L-lactic acid production using glycerol as a single carbon source. For that, the *Bos taurus* lactate dehydrogenase gene was introduced into *P. pastoris*. Moreover, a heterologous and a novel homologous lactate transporter have been evaluated for L-lactic acid production.

Results: Batch fermentation of the *P. pastoris* X-33 strain producing *LDHb* allowed for lactic acid production in this yeast. Although *P. pastoris* is known for its respiratory metabolism, batch fermentations were performed with different oxygenation levels, indicating that lower oxygen availability increased lactic acid production by 20 %, pushing the yeast towards a fermentative metabolism. Furthermore, a newly putative lactate transporter from *P. pastoris* named PAS has been identified by search similarity with the lactate transporter from *Saccharomyces cerevisiae* Jen1p. Both heterologous and homologous transporters, Jen1p and PAS, were evaluated in one strain already containing LDH activity. Fed-batch experiments of *P. pastoris* strains carrying the lactate transporter were performed with the batch phase at aerobic conditions followed by an aerobic oxygen-limited phase where production of lactic acid was favored. The results showed that the strain containing PAS presented the highest lactic acid titer, reaching a yield of approximately 0.7 g/g.

Conclusions: We showed that *P. pastoris* has a great potential as a fermentative organism for producing L-lactic acid using glycerol as the carbon source at limited oxygenation conditions (below 0.05 % DO in the bioreactor). The best strain had both the LDHb and the homologous lactate transporter encoding genes expressed, and reached a titer 1.5 times higher than the strain with the *S. cerevisiae* transporter. Finally, it was also shown that increased lactic acid production was concomitant to reduction of acetic acid formation by half.

Keywords: L-Lactic acid, *Pichia* (*Komagataella*) *pastoris*, Lactate transporter, Oxygen limited fermentation, Lactate dehydrogenase

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Background

Bio-based chemical production from renewable sources has received considerable attention in recent decades due to both economic and environmental concerns, such as the price of petroleum-derived compounds and the effect of residue accumulation on the Earth [1]. Therefore, biotechnological routes using raw materials and renewable sources for the production of bulk chemicals have been studied as an important alternative to conventional petroleum-based processes [2, 3].

Among available raw materials, crude glycerol has gained attention in recent years for being the main waste product in the conversion of vegetable oils into biodiesel. Brazil is the second largest biodiesel producer reaching a production capacity in May 2016 of 19,976.81 m^3/day of pure biodiesel, resulting in approximately 1997.7 m^3/day of crude glycerol [4]. Therefore, with the aim of developing strains that will further be able to use crude glycerol as a carbon source, glycerol was used in this study as the substrate for genetically engineered *Pichia pastoris* strains to produce lactic acid. Recent studies have reported metabolically-engineered microorganisms such as *Escherichia coli* [5, 6], *Rhyzopus oryzae* [7] and *Enterococcus faecalis* [8] for improving lactic acid (2-hydroxyproponoic acid) production using glycerol as a single carbon source. However, bacteria have to overcome low tolerances toward acidity when producing lactic acid, while yeasts are generally resistant to low pHs. In addition, yeasts are generally robust and resistant microorganisms which can survive in industrial conditions, and are thus easy to use in scale-up bioprocesses [9].

Lactic acid is an organic acid commonly produced by diverse organisms such as bacteria (e.g. *Corynebacterium glutamicum* and *Bacillus* strains), fungi (e.g. genus *Rhizopus*), yeasts (*Saccharomyces* and *Kluyveromyces* genera), and microalgae (e.g. *Scenedesmus obliquus*) [10]. It is currently utilized in food, pharmaceutical, textile, leather, and chemical industries, making it the hydroxycarboxylic acid with the highest market potential worldwide [11]. Moreover, lactic acid is the monomer used for the production of biodegradable poly-lactic acid (PLA) that can be used in automobile, packaging and cosmetic industries [10]. PLA is essentially produced by the direct polymerization of lactic acid, and has its physical and mechanical properties determined by the purity of the two lactic acid optical isomers, L- and D-lactic acid [12]. Therefore, depending on the characteristics of the desired PLA, both isoforms have to be produced independently so they can be used in correct proportions.

The methilotrophic yeast *Pichia pastoris* naturally grows in high densities on different carbon sources such as glucose, glycerol, methanol, and sugar alcohols [13]. On account of its ability to grow on defined medium achieving high cell densities and its preference for respiratory mode, decreasing the excretion of by-products like acetate and ethanol, this system is a powerful candidate for utilization at the industrial scale [14]. Moreover, methanol, the primary contaminant of crude glycerol, has no negative impact on *P. pastoris* growth [15]. In fact, this microorganism has higher biomass production growing in crude glycerol than in pure glycerol, indicating that *P. pastoris* can even utilize the contaminants from the biodiesel transesterification process for biomass formation [16].

In this study, for the first time, the *LDH* encoding gene of lactate dehydrogenase from *Bos taurus* was cloned under the control of the GAP constitutive promoter and introduced into *P. pastoris* strains. Nevertheless, the obtained yield was only 10 % of what is theoretically possible. In order to evaluate whether L-lactic acid production could be improved, different oxygenation conditions were tested. Moreover, two lactate transporter coding-genes were also evaluated: the lactate transporter Jen1p from *Saccharomyces cerevisiae* and the putative *P. pastoris* lactate transporter, identified for the first time in the present work. All constructed strains were evaluated in fed-batch experiments for glycerol consumption and lactic acid production. The best strain containing both LDH and Lactate transporter activity reached 70 % of yield.

Results

L-lactic acid production in *P. pastoris*

The codon-optimized *ldh* encoding-gene from *B. taurus* was introduced into the *P. pastoris* genome by homologous recombination. The selected colonies were grown in selective medium and were used to measure LDH-specific activity. All colonies showed LDH activity with statistical differences compared to the wild-type strain, however, one clone demonstrated higher activity among the selected clones (data not shown), here named XL (Table 1). In order to test the physiological behavior of XL, batch fermentation containing 4 % glycerol was performed and proved its ability to convert glycerol into lactic acid, confirming heterologous LDH production [17].

Pichia pastoris is an obligate aerobe yeast [18]. In order to evaluate whether oxygen limitation would improve lactic acid production, batch cultivations were performed at two different dissolved oxygen levels, 3 and 5 %. When supplied with 3 % dissolved oxygen, the XL strain had a lactate yield (lac/s) of 0.236 g/g, and when supplied with 5 % dissolved oxygen, it had 20 % lower yield ($Y_{lac/s}$ of 0.196 g/g). Moreover, XL μmax at 3 % was about 10 % lower (0.174) than at 5 % (0.189) indicating a higher conversion of glycerol into biomass when higher amounts of dissolved oxygen are supplied, lowering the production of lactic acid (Fig. 1).

Table 1 Plasmids and strains used in this work

Plasmids/strains	Genotype	Reference
Plasmids		
pGAPZB	*Pichia* integrative plasmid; Zeocin® selection; Expression controlled by constitutve GAP promoter	Invitrogen®
pPICPGKGFP	Green Fluorescente enconding gene cloned under the controlo f PGK promoter	Personal communication
pGAP-LDH	LDH$^+$, *Bos taurus* gene encoding for LDH enzyme	This work
pPGK-JEN	JEN1$^+$, *S. cerevisiae* gene-encoding for the lactate transporter Jen1p	This work
pPGK-PAS	PAS$^+$, *P. pastoris* gene-encoding for a putative lactate transporter PAS	This work
E. coli strains		
DH5α™	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK +) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Life technology
DH10B™	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–	Life technology
P. pastoris strains		
X-33	Wild type	Life technology
XL	X-33 + pGAP-LDH	
GS115	*his4*$^-$	Life technology
GJ	*his4*$^-$ + pPGK-JEN	This work
GLJ	*his4*$^-$ + pGAP-LDH + pPGK-JEN	This work
GS	*his4*$^-$ + pPGK-PAS	This work
GLS	*his4*$^-$ + pGAP-LDH + pPGK-PAS	This work

Identification of a putative lactate transporter in *P. pastoris* and ^{14}C lactate transport assay

In *S. cerevisiae,* the activity for the lactate–proton symporter has been described to be dependent on JEN1 gene expression [19]. Expression of *S. cerevisiae* Jen1p lactate transporter in reconstituted heterologous *P. pastoris* membrane vesicles demonstrated that Jen1p is a functional transporter [20]. A BLAST search (http://www.blast.ncbi.nlm.nih.gov/blast.cgi) aiming to identify the *jen1* orthologous gene in the *P. pastoris* genome was performed and revealed an open reading frame with significant similarity (50.19 % identity). The protein of the putative homologue, PAS, was predicted to have 552 amino acids in length and to belong to the proton-linked monocarboxylate transporter family (accession number: XM_002492622.1). The Jen1p proteins contained 12 of the predicted transmembrane segments, while PAS contained only 10 helices.

Subsequently, the *PAS* gene was inserted into the *P. pastoris* GS115 strain under the control of the phosphoglycerate kinase 1 promoter, resulting in the GS strain (Table 1). Similarly, the *jen1* gene of *S. cerevisie* was inserted into GS115 strain, resulting in the GJ strain (Table 1). Both strains, including the control (GS115), were used for the ^{14}C lactate transport assay (Fig. 2 and Table 2). Nevertheless, no significant difference was found in the strain containing the *Jen1* gene and therefore this data is not shown.

The lactate transport by the GS115 and GS strains was assayed using radiolabeled lactate. Both GS and the wild-type strain (GS115) were incubated in the presence of ^{14}C lactate, and the uptake capacity was further measured (Fig. 2). As can be seen in Fig. 2, the *P. pastoris* GS115 strain showed a higher velocity of transport, indicating that the GS strain rather increased the affinity for lactate. When Km and Vmax were calculated for all strains, these results were confirmed, where GS showed an increase of about threefold in affinity for lactate when compared with GS115 (Table 3).

Insertion of lactate-transporters results in increased lactate production in fed-batch fermentation

Once confirmed that *P. pastoris* could produce lactic acid using glycerol as a single carbon source and that the strains GS and GJ presented a higher affinity towards lactate when compared to the control strain, all constructed strains where evaluated for lactic acid production. To that end, fed-batch fermentation of the XL, GLJ and GLS strains was performed. Fed-batch was composed of two phases: the first one favoring biomass formation in aerobic conditions and the second initiated by the pH level change (>5) with a single pulse addition of 4 % glycerol and hypoxia conditions.

It can be observed that the GJ strain, although having shown higher affinity than the control strain towards lactate in the radiolabeled assay, presented a slightly higher

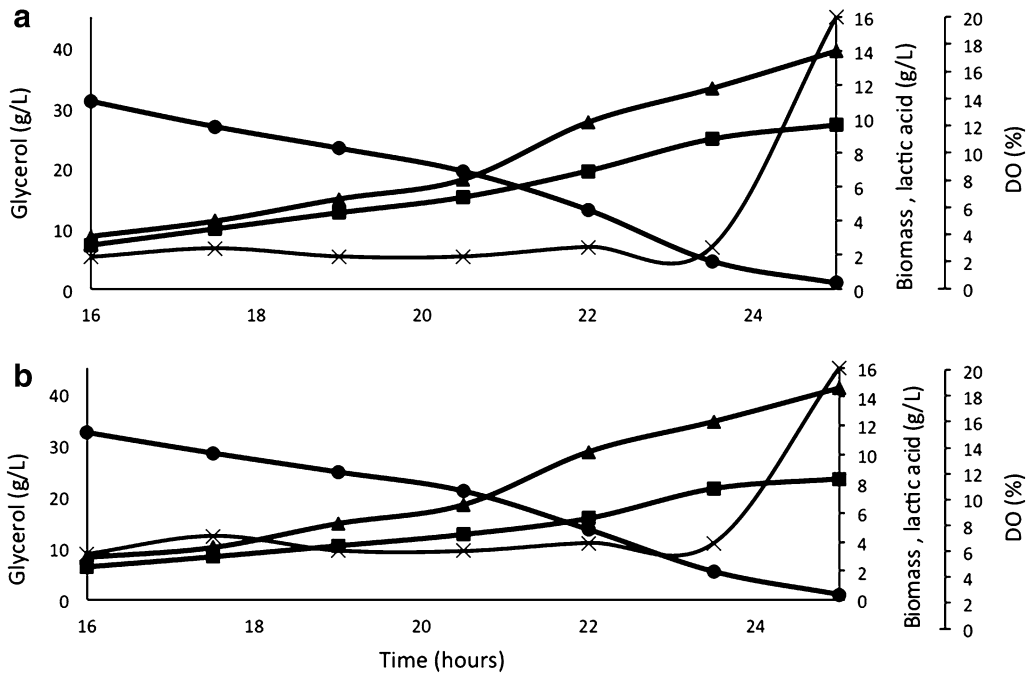

Fig. 1 XL batch fermentation using glycerol (*filled circle*) as carbon source and limited dissolved oxygen supply by 3 % (**a**) and 5 % (**b**) for the production of biomass (*filled triangle*) and lactate (*filled square*). Experiments were performed in triplicate. The figures show the profile of one of the fermentation within 10 % error

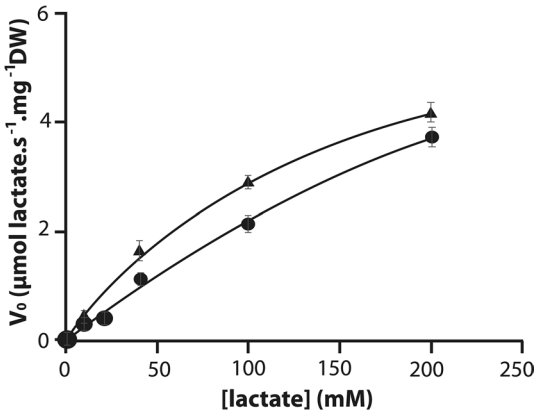

Fig. 2 Uptake rates of labeled lactic acid measured in GS115- (*filled circle*) and GS (*filled triangle*) strains. Experiments performed in triplicate

Table 2 Vmax and Km of GS115 and GLS strains determined by using radiolabeled lactate

Strain	Vmax (µmol/s mg)	Km (µmolar)
GS115	11.68 ± 2.09	430.70 ± 105.00
GLS	7.54 ± 0.67	157.40 ± 25.46

lactate yield (2 %) when evaluated in a fed-batch experiment. On the other hand, GLS presented the highest lactate yield compared to XL and GLJ (46 and 43 % higher, respectively) (Fig. 3), as well as the highest lactate specific (0.126/h) and volumetric (0.673 g/L/h) productivity rates (Table 3). Moreover, at the fed-phase, GLS presented simultaneously the lowest biomass (approximately threefold) and acetate (2.5-fold) yields, showing that this strain is directing carbon towards lactate production instead of biomass formation. By-products such as acetate, arabitol and ethanol were also evaluated, and the kinetic parameters of the fed-phase showed that both XL and GLJ strains presented acetate approximately twofold higher yields, which is an indication of their lower lactate production (Table 3). For GLS, the increase in lactate yield was simultaneous with the reduction in arabitol, was close to zero in all strains and ethanol was never detected (data not shown).

Discussion

Limiting oxygen increases the production of L-lactic acid by *P. pastoris*

Pichia pastoris is a highly successful candidate for the production of heterologous protein (for review, see [21, 22]), however, few studies have been performed using the metabolic engineering of this microorganism [23–25].

Table 3 Kinetic parameters during fed-batch experiments at limited aerobic phase

Strain	$Y_{x/s}$	$Y_{lac/s}$	$Y_{ac/s}$	$Y_{ara/s}$	q_x	q_{lac}	q_{ac}	q_{ara}	r_x	r_{lac}	r_{ac}	r_{ara}
XL	0.180 ± 0.004	0.460 ± 0.004	0.008 ± 0.001	0.000 ± 0.000	0.020 ± 0.004	0.053 ± 0.012	0.001 ± 0.000	0.000 ± 0.000	0.117 ± 0.025	0.348 ± 0.073	0.006 ± 0.001	0.000 ± 0.000
GLJ	0.177 ± 0.004	0.470 ± 0.035	0.010 ± 0.005	0.000 ± 0.000	0.024 ± 0.003	0.063 ± 0.003	0.001 ± 0.000	0.000 ± 0.000	0.160 ± 0.054	0.413 ± 0.101	0.001 ± 0.004	0.000 ± 0.004
GLS	0.066 ± 0.004	0.673 ± 0.033	0.004 ± 0.002	0.001 ± 0.001	0.014 ± 0.002	0.146 ± 0.016	0.001 ± 0.000	0.000 ± 0.000	0.065 ± 0.005	0.673 ± 0.041	0.003 ± 0.001	0.001 ± 0.000

Y yield, *s* substrate, *x* biomass, *lac* lactate, *ac* acetate, *ara* arabitol, *Y* g/g, *q* g/g/h, *r* g/L/h

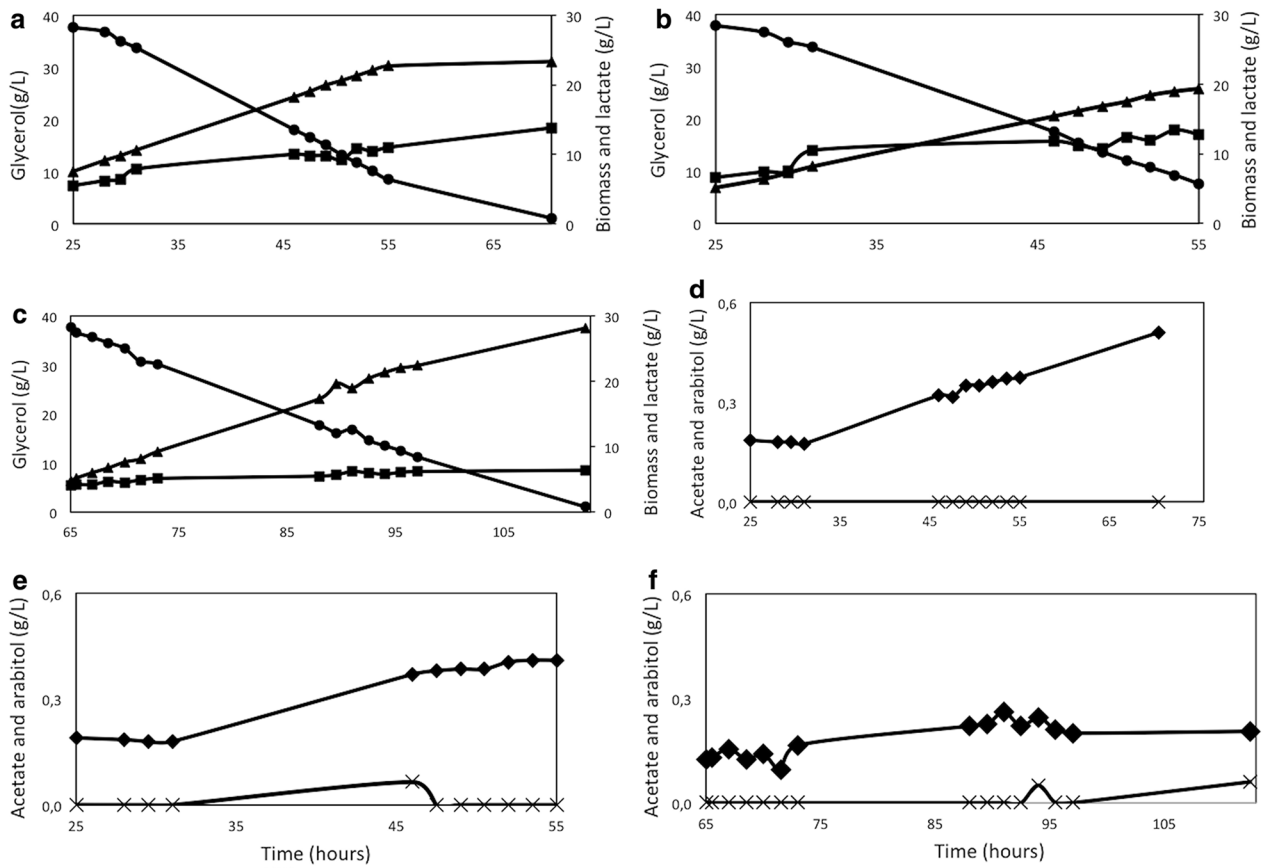

Fig. 3 Fed-batch fermentation profile of the strains XL (**a** and **d**), GLJ (**b** and **e**) and GLS (**c** and **f**). **a**–**c** show the consumption of glycerol (*filled circle*) and the production of biomass (*filled square*) and lactate (*filled triangle*). The **c**–**f** show the formation of the products acetate (*filled diamond*) and arabitol (*cross*). Experiments were performed in triplicate. The figures show the profile of one of the fermentation within 10 % error

Our study shows for the first time that genetically engineered *P. pastoris* strains containing LDH activity are able to produce lactic acid from glycerol. The use of glycerol by *P. pastoris* for the production of other biotechnological products such as phytase and recombinant human erythropoietin production has been previously reported [15, 26], making this organism a potential route for the conversion of crude glycerol into products of biotechnological interest. Introduction of LDH activity from *B. taurus* resulted in a lactate yield per consumed substrate 52 % higher than what has been previously found for the introduction of the same gene in *S. cerevisiae* ($Y_{lac/s}$ 0.155 g/g) using glucose as substrate [18]. Furthermore, lactate production could be further improved by restricting oxygen limitation in the bioreactor [27, 28]. Our results suggest that a lower dissolved oxygen

supply can provide higher production of heterologous lactic acid, which can be an effect of both increased yeast fermentation capacity and up regulation of the pGAP promoter. In a previous study, evaluation of heterologous production of a Fab fragment under the control of a pGAP promoter and limited oxygen conditions (8.39 and 5.87 %) showed that biomass yield decreased twofold while heterologous protein production increased by 2.5-fold [29]. It has been recently evaluated whether oxygen transfer affects protein production under the control of the pGAP by *P. pastoris*. It was observed that 20 % air saturation showed the highest volumetric activity of glucose isomerase when compared to 15 % DO [30]. Although we have not evaluated the enzyme activity in different aeration conditions, our result indicates that limiting oxygen availability pushes the yeast metabolism towards the fermentative pathway.

New putative lactate transporter from *P. pastoris*

Since the identification of *JEN1* coding for the lactate transporter in *S. cerevisiae* [19], it was reported that the Jen1p transporter was able to reconstitute the lactate permease activity in *P. pastoris* [20]. Moreover, in the previous study the background activity of the control strain (KM71H) could not be measured and the kinetic parameters Vmax (2.15 ± 0.14 nmol of lactic acid/s mg of dry weight) and Km (0.54 ± 0.08 mM) could only be determined for the recombinant strain producing the Jen1p transporter [20]. In the present study we did not see a significant difference between the control strain and the one over producing Jen1P, despite a tendency of the GJ strain has a greater affinity to lactate. Regarding the strains overproducing the putative lactate transporter from *P. pastoris*, GS strain, it showed a threefold higher affinity to lactate compared with control strain.

GLS strain provides the highest lactate secretion in fed-batch

Among tested strains and fermentation modes, the best performance was achieved by GLS in oxygen limited fed-batch fermentation. Production of lactic acid using glycerol has been reported using other organisms such as the Gram-positive lactic acid bacteria *E. faecalis* [3]. This later study used fed batch with 30 g/L of glycerol coupled with 22 g/L of acetic acid as substrate and they achieved a lactic acid volumetric productivity rate 7 % higher than what was achieved in our study using the GLS strain. Another study evaluated the production of lactic acid using the fungi *Rhizopus oryzae* in a batch fermentation with 40 g/L of crude glycerol plus either inorganic nutrients of lucern green juice [7]. In both cases the total amount of lactic acid at the end of the fermentation was approximately 12 % lower than what has been found in the present study. Although these studies have shown the production of lactic acid using either crude or pure glycerol, they did not use engineered strains like the one presented here.

It has been previously reported that the cost of bio-production of polylactic acid depends on the substrate used in its process [31]. Crude glycerol is obtained as a residue from the conversion of vegetable oils into biodiesel. It is estimated that for every 9 kg of biodiesel produced, 1 kg of crude glycerol is generated [32]. Thus, for its high availability and low cost, crude glycerol is an excellent candidate for high-added-value chemical production including lactic acid [4, 33, 34, 35]. We have developed a novel *P. pastoris* engineered strain that produces lactic acid using pure glycerol as unique carbon source. Our strain GLS, which has achieved the highest L-lactic acid yield of 0.7 g/g, shows a result close to the maximum theoretical yield of 1.0 g/g.

Conclusions

This study has shown for the first time that genetically engineered *P. pastoris* strains are able to produce L-lactic acid, making this organism a potential biocatalyst for the conversion of crude glycerol into products of biotechnological interest. Another novelty of this study is the identification of a putative lactate transporter in *P. pastoris.* Two genetically modified strains carrying lactate transporters were developed in order to improve the secretion of L-lactic acid. Both strains named GLJ and GLS showed higher affinity towards lactate when compared to the control strain. Fed-batch fermentation processes fed with 40 g/L glycerol showed that GLS presented an increase of 46 % in lactate yield compared to the control strain and 43 % higher than GLJ. The lactate volumetric and specific productivity rates were higher in GLS with a concomitant decrease in biomass and lactate yields of approximately 60 % each were also observed.

Methods

Plasmids and strains

The plasmids and strains used in this study are listed in Table 1. The bacterial strains were grown at 37 °C in Luria broth medium (0.5 % yeast extract, 1 % peptone and 1 % sodium chloride), and the yeast strains were grown at 30 °C in YPD (0.5 % yeast extract, 1 % peptone and 2 % dextrose). When required, the medium were supplemented with the appropriate antibiotics: ampicillin for *E. coli* cultivation (100 μg/mL) and zeocin for *P. pastoris* cultivation (100 μg/mL).

Strain construction

In order to produce lactic acid, the plasmid pGAPZB (Life Technologies, Carlsbad, CA, USA) containing the *Bos*

Taurus ldh codon-optimized gene was linearized with *Avr*II restriction enzyme (Life technology, San Diego, CA, USA) and integrated by homologous recombination into the *P. pastoris* X-33 chromosomal DNA. The resulting plasmid named pGAP-LDH was synthesized by Genome Company (Madison, WI, USA). The integration was confirmed by resistant clones selected in zeocin (100 μg/mL). The resulting strain was named XL (Table 1). In order to evaluate the influence of the lactate transporter in *P. pastoris* strains producing LDH, the pPICPGKGFP plasmid (derived from pPIC9 k-Life technology) was used. pPICPGKGFP containing either the codon-optimized gene encoding for the lactic acid transporter *Jen1p* from *S. cerevisiae* or the codon-optimized gene encoding for the putative transporter of *P. pastoris Pas* were developed, resulting in the plasmids pPGK-JEN and pPGK-PAS, respectively (Table 1). The primers used for the construction of pPGK-JEN were JEN-F 5′ATTCGCGGCCGCATGTCGTCGTCAATTACA3' and JEN-R5' TTAAACGGTCTCAATATGCTGAATTCATC3' (*Not*RI and *Eco*RI restriction sites underlined, respectively), and the primers used for the construction of pPGK-PAS were PAS-F5' ATTCGCGGCCGCATGTCGCATTCAATCCATT3' and PAS-R5' TTACTTATTTCCTTCAAAAGCCGAATTCATC3′ (*Not*RI and *Eco*RI restriction sites underlined, respectively). The plasmids pPGK-JEN and pPGK-PAS were integrated into the GS115 (*his*4) strains using the restriction enzyme *Bgl*II (Life technology, San Diego, CA, USA). Positive clones were selected in YNB solid medium without amino acids, and the resulting strains were respectively named GJ and GS. Next, GJ and GS strains were transformed with pGAP-LDH, resulting in the strains GLJ and GLS, respectively (Table 1).

Enzyme activities

Enzyme assays were carried out as described previously with modifications [36]. Briefly, a primary inoculum culture was prepared in YPD medium, with zeocin (100 μg/mL), and maintained at 30 °C and 180 rpm overnight. Cells were harvested, re-inoculated in a new flask, and grown in a shaker at 30 °C until the exponential phase. After centrifugation, cells were ressuspended in Yeast Protein Extraction Reagent (Y-Per, Life Technologies) for 10 min. The reaction was assembled with cellular extract, 10 μL; NADH, 8 μL; 50 mM phosphate buffer (pH 8.0), 800 μL, and ultra-pure water for a 1 mL final volume. After 150 s, pyruvate 40 μL was added and the reaction was completed in 300 s. Then, LDH activity was determined at 30 °C through the absorbance reduction at 340 nm caused by oxidation of NADH cofactor after pyruvate addition as substrate. The unit of enzyme activity was defined as the amount of enzyme necessary to oxidize 1 μmol NADH per minute.

^{14}C-lactate transport in the strains containing Jen1p and PAS

Radiolabelled lactic acid uptake was determined as described previously [20] with modifications. Briefly, a primary inoculum culture for GS115 (control), GJ and GS strains were prepared in YPD medium and grown until the exponential phase of growth at 30 °C and 180 rpm. Cells were harvested, re-inoculated in YNB medium with 0.5 % L-(+)-lactic acid (Sigma Aldrich Co., USA) for glucose starvation during 4 h in shake at 30 °C. The inoculums were centrifuged at 12,000*g* for 5 min and washed twice with ice-cold water. Cells were ressuspended in KH_2PO_4 1 M buffer, pH 5.0 and kept at room temperature during experiment. A mix containing 1 uL of [U-14C] lactic acid (sodium salt; 106.9 mCi/mmol [3.955 GBq/mmol], Perkin Elmer Life Science) and 9 uL of different concentrations of non-radiolabeled lactic acid (Sigma Aldrich Co., USA) was added to 40 μL of washed cells. After 10 s of incubation, the uptake reaction was quenched by the addition of 1 mL of ice-cold water. Cells were quickly filtered through a nitrocellulose filter 0.45 μm (Sartorius, Gottingen, Germany) linked to a vacuum filter system. After extensive washes and removal of the non-incorporated radiolabelled lactic acid, the membranes containing the washed cells were transferred to a scintillation tube containing 3 mL of scintillation fluid (ScientiSafe; Thermo Fisher Scientific, Whatman, MA, USA). The intracellular radioactivity was measured in the Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer with disintegrations per minute correction. To determine the non-specific 14C adsorption, the labeled lactic acid was added at zero time after the addition of ice-cold water. To determine the transport kinetics that best fit with the experimental values of initial lactate uptake rates and to estimate the kinetic parameters, a computer-assisted nonlinear regression analysis was used (GraphPAD software). All experiments were repeated at least three times and the data reported are the average values. For comparison between strains, an ANOVA Tukey Test was used.

Medium for batch and fed-batch

In batch and fed-batch experiments, a defined medium was utilized as previously described with modifications [37]. The composition of the medium (per liter) was: 20 or 40 g glycerol·$1H_2O$, 1.8 g $C_6H_8O_7$, 0.02 g $CaCl_2{\cdot}2H_2O$, 12.6 g $(NH_4)2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.9 g KCl, and 4.35 mL PTM1 trace salts stock solution, and pH was set to 5.0 with 25 % HCl. PTM1 trace salts stock solution (per liter) was composed by: 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 14.3 g $FeSO_4$ and 5.0 mL H_2SO_4 (95–98 %), 0.4 g biotin. 0.04 g histidin was supplemented for the strain GS115.

Fermentation parameters for batch and fed-batch

For batch experiments, the pre-inoculum culture was prepared with 20 g/L glycerol in 100 mL defined medium in a 1 L flask, and it grew for approximately 48 h at 30 °C and 200 rpm. This pre-culture was then used to inoculate 500 ml defined medium at an initial OD_{600nm} of 2.0 into 1 L Infors HT fermentor (Infors HT, Bottmingen, Switzerland). The glycerol concentration in the defined medium for batch experiments was 40 g/L. To evaluate the production of lactic acid in XL, a batch experiment was performed at the following conditions: 30 °C, 500 rpm, air flow 0.05 vvm, dissolved oxygen at either 5 or 3 % and pH 5 controlled with 5 M NH_4OH. To evaluate the correlation between amount of dissolved oxygen and lactic acid production, a batch fermentation in cascade mode was performed at the following conditions: pH 5 controlled with 5 M NH_4OH, 30 °C, and dissolved oxygen at 3 or 5 % measured by a sterilized electrode (Mettler-Toledo, Moburn, MA, USA), maintained by computational adjustment of the rotation speed (minimum 350/set 500/maximum 900 rpm) and air flow (minimum 0.05/set 0.05/maximum 0.5 vvm). The batch fermentations were run for 30 h. Samples were collected every 90 min and centrifuged at 12,000*g* for 2 min, then the supernatant was stored at −20 °C for HPLC analysis. For fed-batch, the pre-inoculum was prepared as described above. The fed-batch fermentations were performed with an initial glycerol concentration of 20 g/L at the following conditions: 30 °C, 500 rpm, air flow 0.05 vvm, dissolved oxygen at 30 % and pH 5 controlled with 5 M NH_4OH. When pH went above 5, the feeding step was initiated with the addition of 40 g/L glycerol in a single pulse. Samples were collected every 90 min and centrifuged at 12,000*g* for 2 min. The supernatant was stored at −20 °C for HPLC analysis.

Substrate consumption, lactic acid, biomass and by-product formation

Acetic acid, ethanol, arabitol, lactic acid and glycerol were quantified using a Hewlett-Packard High-performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with UV (210-nm) and refractive index detectors. A pre-column Guard Column SCR (H) (50 mm × 4 mm id) with stationary phase sulphonated styrene–divinylbenzene copolymer resin was used. The chromatography was performed using a Shim-pack SCR-101H (Shimadzu) (300 mm x 7.9 mm id) column equilibrated at 60 °C with 5 mM H_2SO_4 as the mobile phase at flow rate of 0.6 mL/min, and an injection volume of 20 μL. The run was 26 min long. For the analysis of biomass, dry cell weight (DCW) samples were collected for OD_{600nm} measurement and the same sample was dried then weighed. OD_{600nm} was converted to DCW (g/L) using the appropriate calibration curve; 1 unit of OD_{600nm} corresponded to 0.390 g DCW/L.

Authors' contributions

PBAL and KCLM contributed equally to this work and were mainly responsible for writing the manuscript. NTMM constructed the XL strain. LSC, TFR and GHG performed the ^{14}C lactate transport assay. BSM performed HPLC analysis. VHC, GSA, EM and KCLM performed the fermentations and their data interpretation. NSP designed and coordinated the study, supervised data interpretation and writing the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The patent on which the conclusion of the manuscript relied is deposited publicly under the number BR1020130310522. Information about accession number of the sequence acquired by the BLAST toll (NCBI) is given in the manuscript.

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Anexo B – Identification and characterization of putative xylose and cellobiose transporters in *Aspergillus nidulans*

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RESEARCH

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Identification and characterization of putative xylose and cellobiose transporters in *Aspergillus nidulans*

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Abstract

Background: The conversion of lignocellulosic biomass to biofuels (second-generation biofuel production) is an environmentally friendlier alternative to petroleum-based energy sources. Enzymatic deconstruction of lignocellulose, catalyzed by filamentous fungi such as *Aspergillus nidulans*, releases a mixture of mono- and polysaccharides, including hexose (glucose) and pentose (xylose) sugars, cellodextrins (cellobiose), and xylooligosaccharides (xylobiose). These sugars can subsequently be fermented by yeast cells to ethanol. One of the major drawbacks in this process lies in the inability of yeast, such as *Saccharomyces cerevisiae*, to successfully internalize sugars other than glucose. The aim of this study was, therefore, to screen the genome of *A. nidulans*, which encodes a multitude of sugar transporters, for transporters able to internalize non-glucose sugars and characterize them when introduced into *S. cerevisiae*.

Results: This work identified two proteins in *A. nidulans*, CltA and CltB, with roles in cellobiose transport and cellulose signaling, respectively. CltA, when introduced into *S. cerevisiae*, conferred growth on low and high concentrations of cellobiose. Deletion of *cltB* resulted in reduced growth and extracellular cellulase activity in *A. nidulans* in the presence of cellobiose. CltB, when introduced into *S. cerevisiae*, was not able to confer growth on cellobiose, suggesting that this protein is a sensor rather than a transporter. However, we have shown that the introduction of additional functional copies of CltB increases the growth in the presence of low concentrations of cellobiose, strongly indicating CltB is able to transport cellobiose. Furthermore, a previously identified glucose transporter, HxtB, was also found to be a major xylose transporter in *A. nidulans*. In *S. cerevisiae*, HxtB conferred growth on xylose which was accompanied by ethanol production.

Conclusions: This work identified a cellobiose transporter, a xylose transporter, and a putative cellulose transceptor in *A. nidulans*. This is the first time that a sensor role for a protein in *A. nidulans* has been proposed. Both transporters are also able to transport glucose, highlighting the preference of *A. nidulans* for this carbon source. This work provides a basis for future studies which aim at characterizing and/or genetically engineering *Aspergillus* spp. transporters, which, in addition to glucose, can also internalize other carbon sources, to improve transport and fermentation of non-glucose sugars in *S. cerevisiae*.

Keywords: *Aspergillus nidulans*, *Saccharomyces cerevisiae*, Xylose, Cellobiose, Sugar transport

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Background

An increase in energy demands, a depletion in fossil fuels, and high emissions of greenhouse gases have led to the search for alternative and environmentally friendlier energy sources. One alternative energy source is lignocellulose which is found in the cell walls of all plants, such as hardwoods, softwoods, crops, and grasses, thus making it the most abundant organic material on the planet [1–4]. Lignocellulosic wastes are produced by the forestry, pulp and paper, and agriculture industries in addition to municipal and animal wastes [5]. The main components of lignocellulosic biomass are cellulose (40–50 %), hemicelluloses (25–35 %) and lignin (15–20 %) [6]. Cellulose consists of long chains of the hexose sugar glucose, which represents the most abundant simple sugar in the plant cell wall, whereas the main sugar of hemicelluloses is xylose, although other sugars, such as arabinose and galactose, also make up considerable fractions of this polysaccharide. The production of biofuels from lignocellulose, in a process called 2nd generation (2G) biofuel production, aims at converting these sugars into ethanol [7]. Lignocellulosic biomass is deconstructed by enzymatic degradation into a mixture of hexose (e.g., glucose) sugars, pentose (e.g., xylose) sugars, cellodextrins (e.g., cellobiose), and xylooligosaccharides. Cellodextrins are glucose polymers of varying lengths (e.g., cellobiose is a glucose dimer), released during cellulose degradation by cellobiohydrolases and which are subsequently cleaved into glucose monomers by β-glucosidases [5, 8, 9]. Once these simpler sugars have been released from the complex lignocellulosic polymers by enzymatic deconstruction, they can be converted into ethanol by fermenting organisms.

The preferred organism for fermentation of lignocellulosic sugars to ethanol is the budding yeast *Saccharomyces cerevisiae*, which is substantially used in several industrial processes, such as baking, brewing, and wine making [10]. *S. cerevisiae* primarily uses glucose monomers for fermentation and is unable to ferment cellobiose. Furthermore, *S. cerevisiae* is also unable to grow efficiently on xylose as the sole carbon source, although its genome appears to encode all components necessary for metabolizing xylose [11]. Genetic engineering of *S. cerevisiae* has introduced components into the yeast cells that allowed fermentation of cellobiose and xylose [12, 13], but transport of these sugars into the cell is still a limiting factor for successful conversion to ethanol. Complete fermentation of all the sugars found in lignocellulose is desired to reduce the costs of 2G biofuel production and make it an economically feasible process [14]. Therefore, one of the bottlenecks of the conversion of lignocellulose to ethanol lies in the engineering of yeast strains, which can efficiently transport xylose, cellobiose and other lignocellulosic sugars into the cell [15].

Transport of carbon sources is mainly carried out by single polypeptide secondary carriers belonging to the major facilitator superfamily (MFS) of transporters and which transport small soluble molecules in response to ion gradients [16, 17]. The MFS of transporters is divided into 17 families of which families 1, 5, and 7 are involved in sugar transport [16, 17]. Domestic and wild-type *S. cerevisiae* species transport xylose into the cell with low affinity (K_M = 100–190 mM) via the expression of native high-affinity hexose transporter-encoding genes, such as *GAL2* and *HXT7* [18, 19], highlighting the preference of *S. cerevisiae* for glucose. Although specific pentose transporters have not been described in yeast, engineering of hexose transporters has been shown to significantly improve xylose transport [20–22]. Furthermore, heterologous introduction of specific D-xylose transporters, derived from other organisms, can improve the growth rate of *S. cerevisiae* on xylose, increasing V_{max} (maximum reaction velocity rate) values and displaying an increase in high cell density sugar consumption [23]. However, this heterologous system only supports low rates of D-xylose transport [24, 25] and may not be perfectly integrated in the endogenous carbon metabolism regulatory network of *S. cerevisiae*. Similar to xylose transporters, cellodextrin transporters from *Neurospora crassa* have also been introduced into *S. cerevisiae* together with a β-glucosidase-encoding gene; they conferred the ability of *S. cerevisiae* to grow on cellobiose [26]. The advantages of *S. cerevisiae* being able to directly use cellobiose for growth are that this does not require adding large quantities of β-glucosidases into the cultures and it also prevents the build-up of glucose in the culture medium that is repressive for cellulase and hemicellulase-encoding genes [27]. However, further engineering is required to optimize cellobiose transport and metabolism in *S. cerevisiae*. The search to find xylose and cellobiose-specific transporters is, therefore, of importance for bioethanol production from lignocellulose.

Filamentous fungi degrade lignocellulosic biomass through secreting a large repertoire of hydrolytic enzymes that break down lignocellulosic sugar polymers into simple sugars which subsequently can be taken up by the cell [28, 29]. Accordingly, the genomes of filamentous fungi also encode large numbers of MFS transporters. Currently, the genomes of *Trichoderma reesei* and *A. nidulans* are predicted to encode 164 and 357 proteins, respectively, belonging to the MFS, although it is not known how many of these are exactly involved in sugar transport [30, 31]. In addition, filamentous fungi, such as *N. crassa* and *T. reesei*, are able to transport disaccharides such as cellobiose into the cell through cellobiose-specific transporters; once internalized, cellobiose has been shown to play an important role in signaling the

presence of cellulose [12, 19, 32]. Furthermore, transporters expressed by filamentous fungi often can transport more than one type of sugar; for example, the *A. nidulans* transporter XtrD was shown to be able to transport, in addition to xylose and glucose, several other monosaccharides, whereas the *T. reesei* STP1 transporter is involved in glucose and cellobiose uptake [32, 33]. However, a very few sugar transporters have been functionally characterized in filamentous fungi [34–40].

The aim of this work was, therefore, to identify and characterize *A. nidulans* transporters involved in cellobiose and xylose uptake and heterologously introduce them into *S. cerevisiae*. This study identified several transporters with roles in pentose or cellodextrin transport. Characterization of the cellobiose transporter CltA showed increased efficiency in cellobiose transport than when compared to a previously identified *N. crassa* cellobiose transporter. Furthermore, this work identified CltB as a putative cellobiose transceptor. In addition, a previously described glucose transporter was identified as playing a major role in xylose transport.

Results

Identification of CltA and CltB with roles in cellobiose transport or signaling

We have previously used genome-wide transcriptional profiling to identify 12 transporters, belonging to the major facilitator superfamily (MFS) that have increased mRNA accumulation in xylose-rich conditions [33]. One of these transporters, named XtrD, was identified as a xylose-specific transporter [33]. We, therefore, started by characterizing three other randomly chosen transporters (*xtrF-H* that correspond to AN0332, AN8347, and AN9173, respectively) that belong to this series of putative xylose transporters. Although these three genes were upregulated in the presence of xylose, deletion of these genes in *A. nidulans* did not have a significant effect on growth in the presence of xylose and glucose nor could they confer growth, when heterologously introduced, of *S. cerevisiae* in the presence of xylose, glucose, and other monosaccharides (data not shown). Since these transporters were not able to transport either hexoses or pentoses, we hypothesized if they could be involved in the transport of more complex sugars, such as cellodextrins (e.g., cellobiose) or xylooligosaccharides. The genome of *N. crassa* encodes two cellobiose transporters termed *CDT-1* and *CDT-2* which transport and internalize cellodextrin molecules [26, 41] and which also appear to have transceptor activity and, therefore, play a role in cellulose signaling [42]. The here identified, supposed xylose transporter-encoding gene *xtrG* (AN8347) has identity with the *N. crassa* cellobiose transporter *CDT-2* (44 % identity, 61 % similarity, e-value of 4e−137). BLASTp search of the *Aspergillus* genome database (www.aspgd.org) using *N. crassa CDT-1* as a query allowed us to identify a second gene, AN2814, with high identity to the *N. crassa* cellodextrin transporter (61 % identity, 75 % similarity, e-value of 0.0).

To further characterize these potential cellobiose transporter-encoding genes (here now named *cltA* and *cltB*, respectively), we evaluated their expression patterns in the presence of 1 % cellobiose (Fig. 1a, b). The expression of *cltA* increased gradually (about 4.8-fold) over a time period of 4 h, whereas expression of *cltB* varied during the same time period (Fig. 1a, b). Next, both genes were deleted in *A. nidulans* and a Δ*cltA* Δ*cltB* double deletion strain was constructed. The wild-type, *ΔcltA*, *ΔcltB,* and the double *ΔcltA* Δ*cltB* strains were grown in 1 % glucose and 1 % cellobiose for 48 and 72 h, and biomass was determined (Fig. 1c, d). All the mutant strains had a similar biomass than the wild-type strain when grown in 1 % glucose (Fig. 1c). However, in the presence of cellobiose, the *ΔcltB* strain showed a ~50 % reduction in biomass after 48 h growth when compared to the wild-type strain, whereas there was no significant difference between the Δ*cltA* and wild-type strains (Fig. 1d). The double mutant showed a ~75 % reduction in biomass when compared to the wild-type strain after 48-h growth in 1 % cellobiose (Fig. 1d). These results suggest that CltA and CltB could collaborate towards cellobiose transport. Interestingly, there is also a reduction in cellulase activity in the *ΔcltB* and *ΔcltA* Δ*cltB* mutants of 50 and 70 %, respectively, than when compared to the wild-type strain (Fig. 1e), suggesting that these transporters play a role in the regulation/signaling of cellulase production.

We decided to investigate in more detail the phenotype provided by Δ*cltB* by complementing and overexpressing the *cltB*. First, we complemented the Δ*cltB* with a wild-type copy of *cltB* integrated ectopically, creating a strain Δ*cltB::cltB*$^{+}$. Subsequently, we transformed the wild-type GR5 strain with CltB::GFP and selected for transformants with a single homologous integration and multiple ectopic integrations (Additional file 1). We selected single candidates for homologous (named CltB::GFP) and multiple ectopic integrations (named oCltB3::GFP). Growth phenotypes of Δ*cltB::cltB*$^{+}$, CltB::GFP, and oCltB3::GFP were identical to the wild-type strain on MM with glucose as single carbon source (data not shown). Expression measured by qRT-PCR experiments showed that oCltB3::GFP has about eightfold more *cltB* expression than the wild-type strain in the presence of cellobiose (Fig. 2a). To verify the cellular localization and expression of CltB:GFP, the GFP strain was grown for 16 h in fructose and transferred to either 0.1 or 1 % cellobiose for 4 or 8 h (Fig. 2b). We have not observed any fluorescence in fructose (data not shown), but in contrast in 1 % cellobiose, we were able to

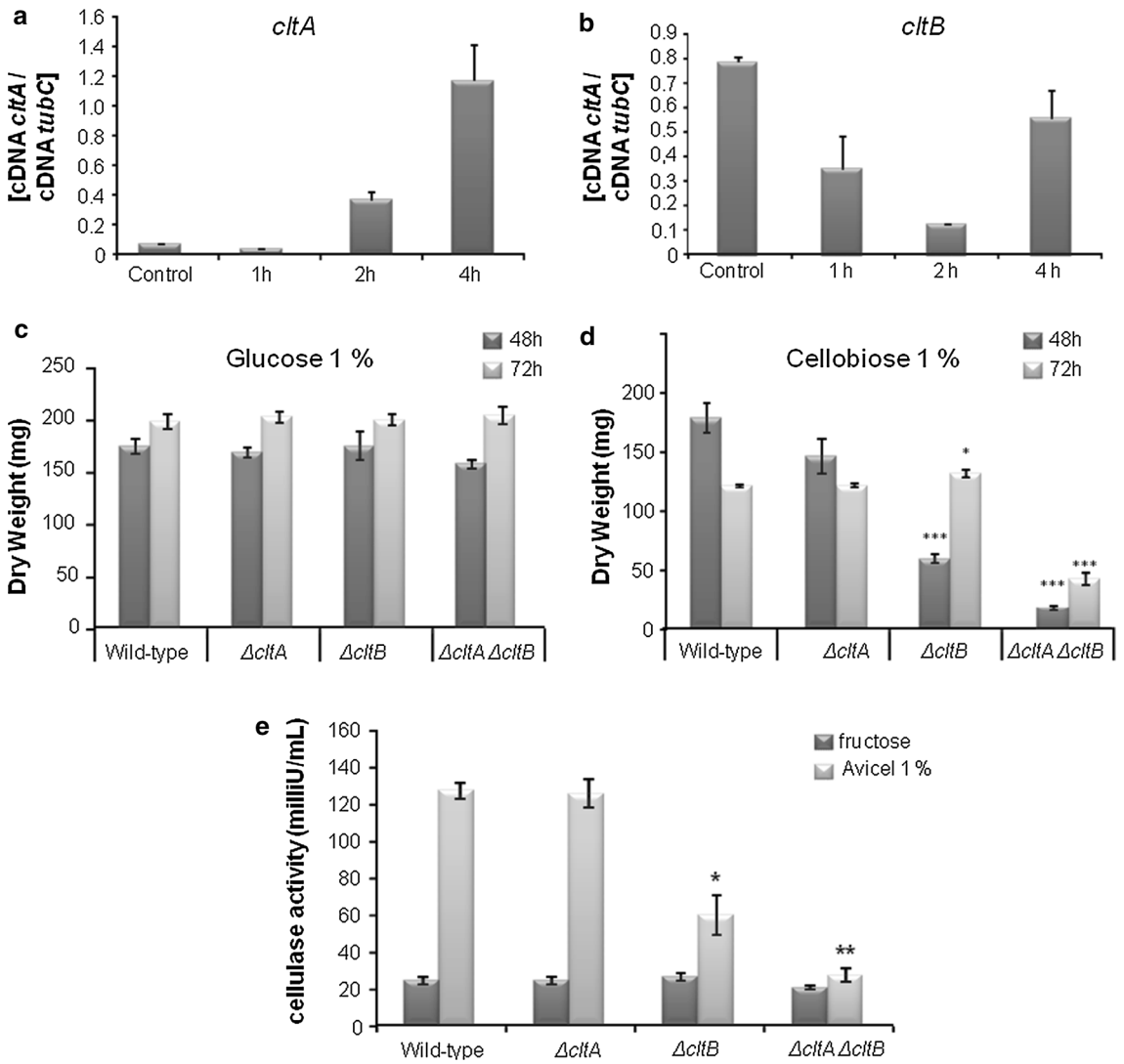


Fig. 1 CltA and CltB are cellobiose transporters which are involved in the signaling response to cellulose. The expression of *cltA* (**a**) and *cltB* (**b**) was assessed by RT-qPCR in the presence of 1 % cellobiose. The effect of the single deletions of *cltA* and *cltB* and the double deletion *cltA cltB* was assessed on fungal biomass accumulation (*DW* dry weight) when grown for 48 or 72 h in the presence of 1 % glucose (**c**) or 1 % cellobiose (**d**). Cellulase activity (**e**) was measure in the supernatants of the same strains when grown in minimal medium supplemented with 1 % fructose for 16 h and then transferred to minimal medium containing 1 % avicel for 5 days. *Error bars* indicate standard deviation for three biological replicates

see a weak fluorescence in oCltB3::GFP, mostly localized in the cytoplasm and in the cell membrane (Fig. 2b). To evaluate the impact of overexpressing $cltB^{+}$ on growth in the presence of 0.5 and 1 % cellobiose as a single carbon source, the wild-type, Δ*cltB*, Δ*cltB::cltB*$^{+}$, and oCltB3::GFP were grown for 24 h in MM + 0.5 or 1 % cellobiose (Fig. 2c). There is no significant difference in the growth (as evalutated by dry weight) of the wild type and Δ*cltB::cltB*$^{+}$ in both 0.5 and 1 % cellobiose (Fig. 2c); in contrast, as it is also shown in Fig. 1d, we have observed a significant differential reduced growth in Δ*cltB* in both cellobiose concentrations (Fig. 2c). The overexpression strain oCltB3::GFP has shown more growth than the wild type only in 0.5 % but not in 1 % cellobiose (Fig. 2c). Taken together, these results suggest that CltB is able to transport cellobiose.

CltA and CltB confer the ability of *S. cerevisiae* to grow in the presence of cellobiose as sole carbon source

To evaluate the ability of CltA and CltB to transport cellobiose, both genes were cloned into *S. cerevisiae* SC9721_pGH1-1, a SC9721 strain previously transformed with the *N. crassa* β-glucosidase-encoding gene *gh1-1* (NCU00130) [26]. Both *cltA* and *cltB* were fused to *gfp,* and plasma

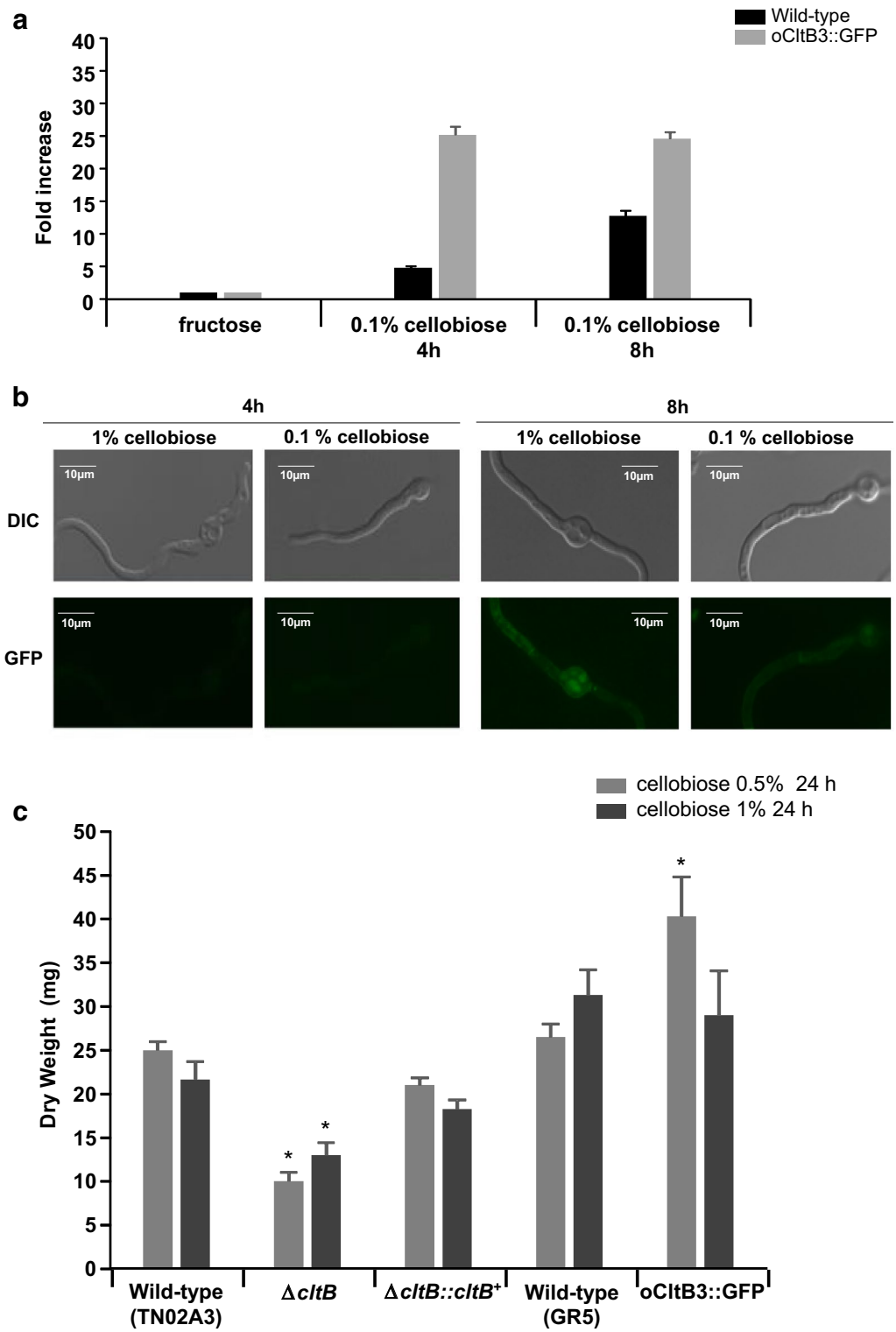


Fig. 2 CltB overexpression increases the growth on cellobiose. **a** The expression of *cltB* was assessed by qRT-PCR in the presence of 0.1 % cellobiose. **b** Germlings of oCltB3::GFP were grown for 16 h in fructose 1 % and transferred to 0.1 or 1 % cellobiose. **c** The fungal biomass accumulation (dry weight) in the wild-type (TN02A3 and GR5), Δ*cltB*, Δ*cltB::cltB*$^{+}$, and oCltB3::GFP strains was assessed for 24 h in the presence of 0.5 or 1 % cellobiose. *$p < 0.005$

membrane localization of CltA and CltB was confirmed by fluorescence microscopy when grown for 24 h in YNB supplemented with 1 % glucose medium (Fig. 3a). The *S. cerevisiae* CltA::GFP and CltB::GFP strains were then grown in liquid YNB medium supplemented with 1 % glucose for 24 h at 30 °C, before cells were washed and spotted in a serial dilution onto YNB solid medium containing either 1 % glucose or varying concentrations of cellobiose. Yeast strains containing only CltA or CltB (no β-glucosidase) were used as negative controls as they were unable to grow on cellobiose as sole carbon source. *S. cerevisiae* transformed with *N. crassa cdt-1* and the β-glucosidase-encoding gene (*gh1-1*) was used as a positive control (Fig. 3b). The drop-out assay clearly shows that CltA, and to a lesser extent CltB, is able to transport cellobiose and, thus, enable *S. cerevisiae* to grow on cellobiose as sole carbon source.

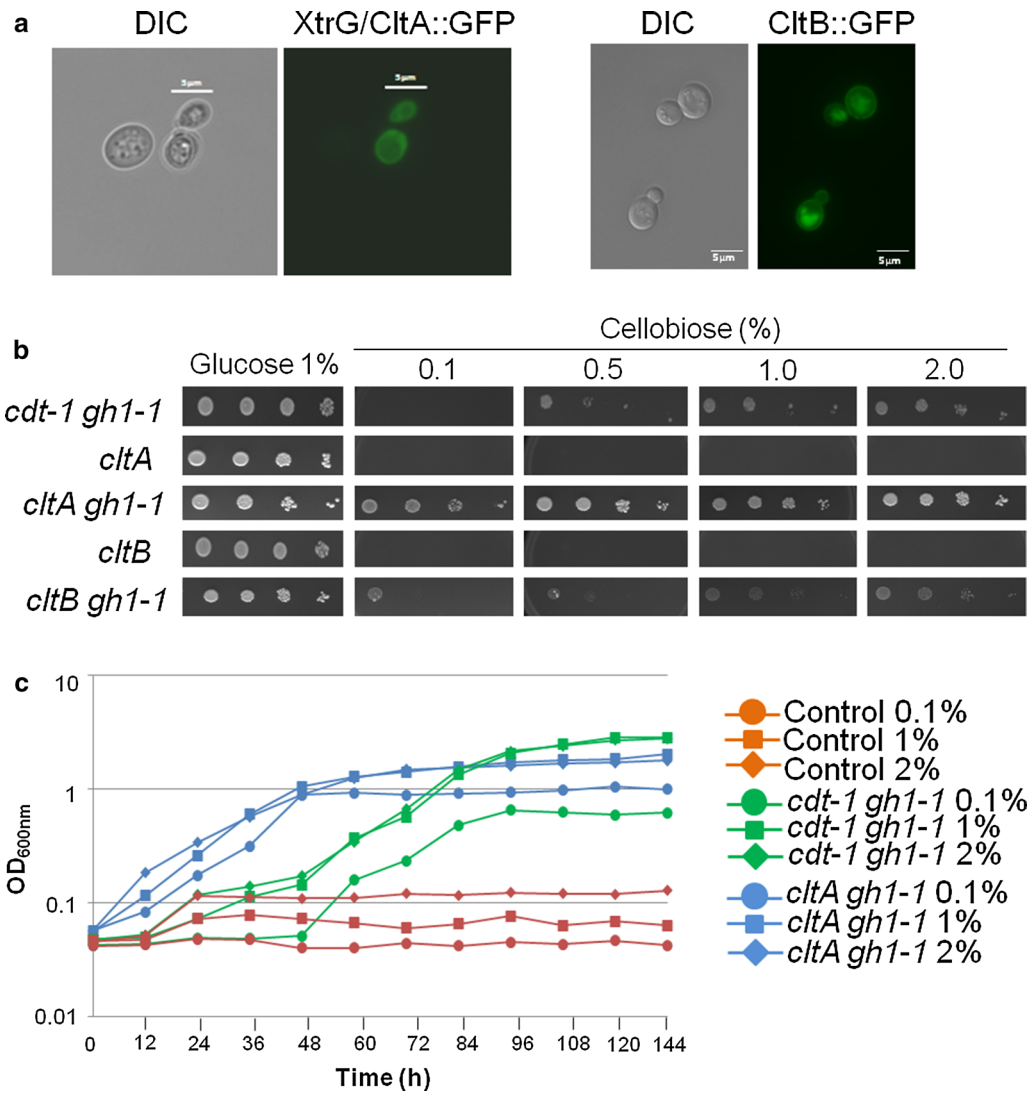


Fig. 3 CltA and CltB confer growth of *S. cerevisiae* in the presence of low and high concentrations of cellobiose. **a** CltA and CltB localize to the plasma membrane in *S. cerevisiae*. Strains were grown for 16 h at 30 °C in YNB supplemented with 2 % glucose before pictures were taken without (DIC) and with (GFP) fluorescence. **b** *S. cerevisiae* strains containing *A. nidulans cltA*, *cltB,* and *N. crassa cdt-1* with and without the β-glucosidase-encoding (*gh1-1*) gene were pre-grown for 24 h in YNB media containing 1 % glucose before a serial dilution was made (1:10 dilution starting at optical density OD_{600nm} 1.0) and diluted cells were grown on YNB plates containing different concentrations of cellobiose (0.1–2 %). **c** Growth curves of *S. cerevisiae* strains containing the *N. crassa cdt-1* and the *A. nidulans cltA* transporter-encoding genes. Both strains also contained the β-glucosidase-encoding gene *gh1-1*. Strains were grown for 144 h in the presence of different concentrations of cellobiose (0.1, 1, and 2 %) at 30 °C. Growth was assessed by measuring the OD at 600 nm. The yeast strain expressing CltB has not grown in liquid medium, and it was not used in this experiment

To compare the efficiency of these cellobiose transporters, the growth rates of *S. cerevisiae* strains harboring either the *A. nidulans* CltA or the *N. crassa* CDT-1 transporters were compared in the presence of different concentrations of cellobiose. Although *cltA* was expressed from a weaker yeast promoter than *cdt-1* [43–45], the *S. cerevisiae* CltA strain grew much faster during the first 36 h incubation in different concentrations of cellobiose, suggesting that CltA appears to transport different concentrations of cellobiose faster into the yeast cell than when compared to the yeast strain containing CDT-1 (Fig. 3c).

Deletion of the hexose transporter-encoding gene hxtB results in reduced xylose uptake

As mentioned in the introduction, many sugar transporters expressed by filamentous fungi are capable of transporting more than one type of monosaccharide across the fungal membrane. As a next step, we decided to investigate the possibility that *A. nidulans* hexose transporters could be involved in xylose uptake. In a previous study, four hexose transporters, termed HxtB-E were shown to confer growth of *S. cerevisiae* strain EBY.VW4000 in the presence of glucose, fructose, mannose, and galactose [46]. These transporters, therefore, seem to accept multiple sugars as substrates, although xylose as a potential substrate for these transporters was not characterized at that time. In *A. niger,* on the other hand, MstA, the orthologue of HxtB in *A. nidulans* was shown to have high affinity for xylose when introduced into *S. cerevisiae* [47]. We, therefore, decided to investigate whether these four transporters were able to transport xylose into the cell.

The *A. nidulans* wild-type strain was first grown from spores in fructose-rich media before being transferred to media containing either 0.2 or 2 % xylose for 6, 12, 18 and 24 h (Fig. 4). Gene expression of *hxtB-E* was assessed by RT-qPCR in these conditions. All four genes were induced to a different extent in the presence of low concentrations of xylose (0.2 %) but not in the presence of 2 % xylose (Fig. 4a–d). Next, the four transporter-encoding genes were knocked out in *A. nidulans* and growth of these strains in the presence of glucose and xylose was assessed. The wild-type and the four deletion strains were grown in liquid minimal medium supplemented with 1 % glucose, 0.2 % xylose or 2 % xylose for 24 and 48 h before fungal dry weight was measured (Fig. 5). All strains showed a similar biomass when grown in 1 % glucose for 24 and 48 h (Fig. 5a). The Δ*hxtB* and Δ*hxtE* strains showed significantly reduced biomass when grown in 2 % xylose for 48 h (Fig. 5b). However, after 72 h of growth, all the mutant strains had a similar dry weight to the wild-type strain (data not shown). To further characterize xylose uptake, the concentration of xylose was measured in the supernatants of the wild-type, Δ*hxtB* and Δ*hxtE* strains when grown for 72 h in medium supplemented with either 1 or 2 % xylose. After 72 h, the wild-type strain and Δ*hxtE* strains had consumed all the xylose in the extracellular medium, whereas xylose consumption was much slower in the Δ*hxtB* strain and residual xylose could still be detected after 72 h in the supernatant of this strain (Table 1).

To confirm the above described results, the capacity of xylose uptake was studied in both the wild-type and Δ*hxtB* strains using ^{14}C-xylose. In the wild-type strain, ^{14}C-xylose uptake obeyed single saturation kinetics with a K_m value of 56.17 ± 11.9 mM and a V_{max} of 0.27 µmol of xylose h^{-1} per 2.5 × 10^7 conidia (Fig. 5c; Additional file 2 for Eadie-Hofstee and Lineweaver–Burk plots). The *ΔhxtB* mutant strain showed both a decreased affinity for xylose (K_m = 100.4 ± 17.92 mM) and a slight reduction in transport capacity (V_{max} = 0.20 µmol of xylose per hour per 2.5 × 10^7 conidia; Fig. 5c; Additional file 1 for Eadie-Hofstee and Lineweaver–Burk plots).

HxtB confers growth of *S. cerevisiae* in the presence of xylose

To confirm the presence and the cellular localization of HxtB, the HxtB::GFP and HxtE::GFP strains were constructed. Growth phenotypes of HxtB::GFP and HxtE::GFP were identical to the wild-type strain (data not shown). Both strains were grown for 10, 15, 20, and 24 h in minimal medium containing 0.1 or 1 % xylose. HxtB::GFP and HxtE::GFP were expressed in the presence of low and high concentrations of xylose upon which it localized to the fungal plasma membrane and small vacuoles (Fig. 6; Additional file 3). To confirm the xylose-transporting capacity of HxtB::GFP, it was introduced into *S. cerevisiae* EBY.VW4000 strain which was previously transformed with all the components necessary for the xylose metabolic pathway (see "Methods" section). The *S. cerevisiae* EBY.VW4000 strain lacks around 20 glucose transporters and is unable to grow on various hexose and pentose monosaccharides, including glucose, fructose, mannose, galactose, and xylose [48]. This strain is, therefore, a good tool for evaluating the ability of heterologous introduced transporter to take up various sugars thus conferring growth to *S. cerevisiae* in the presence of various pentose and hexose sugars. HxtB::GFP localized to the plasma membrane in *S. cerevisiae* when grown in maltose-rich conditions (Fig. 7a). Furthermore, when transferred from maltose-rich media to media containing low and high concentrations of xylose, *S. cerevisiae* strain HxtB::GFP was able to grow in both 0.1 and 1 % xylose, whereas the strain which lacked HxtB::GFP (control) was not able to do it (Fig. 7b). Furthermore, ^{14}C-xylose

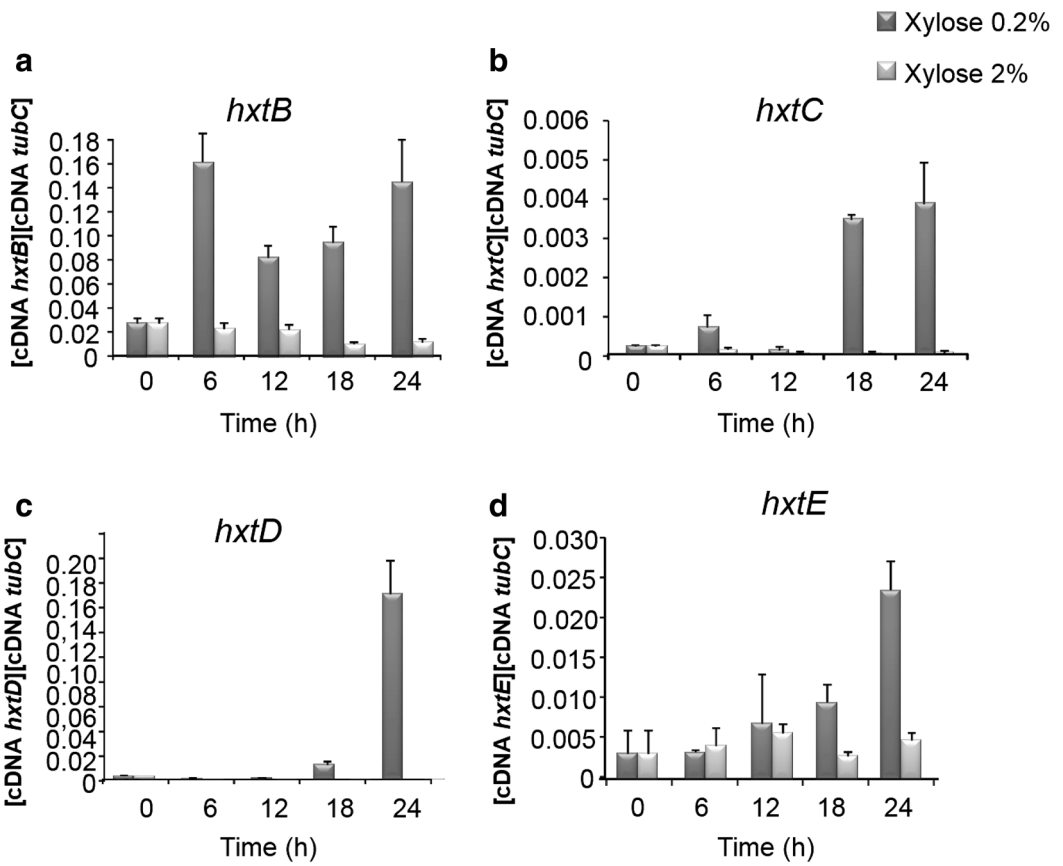
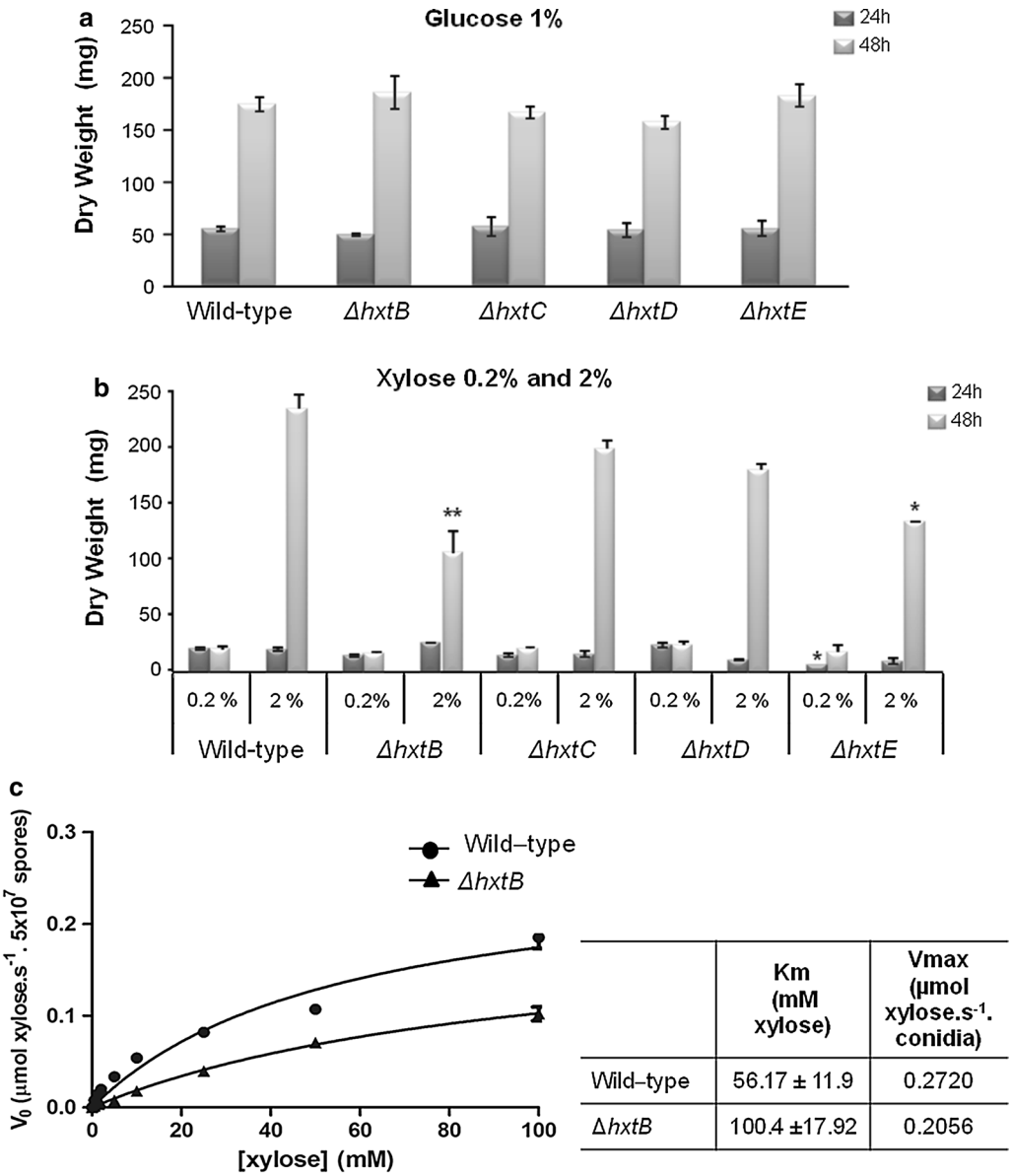


Fig. 4 The expression of the *A. nidulans* hexose transporter-encoding genes *hxtB-E* is upregulated in the presence of low concentrations of xylose. Transcript levels of *hxtB-E* (**a**–**d**) were determined by RT-qPCR in the wild-type strain when grown for 0, 6, 12, 18, and 24 h in the presence of 0.2 and 2 % xylose. *Error bars* indicate the standard deviation for three replicates

uptake in *S. cerevisiae* HxtB::GFP followed single saturation kinetics with a K_m value of 0.54 ± 0.08 mM and a V_{max} of 1.14 ± 0.08 μm of xylose h^{-1} per mg cell dry weight (Fig. 7c). In contrast, the *S. cerevisiae* strain which does not contain *hxtB* was unable to transport xylose (Fig. 7c). In agreement, *S. cerevisiae* HxtB::GFP was able to grow and consume around 90 % of extracellular xylose after 192 h of growth in xylose-rich medium and at the same time produce ethanol (Fig. 7d). The control strain, which did not contain the HxtB transporter, did not grow in the presence of xylose and, hence, did not consume xylose and did not produce ethanol (Fig. 7d). In addition, as previously reported, *S. cerevisiae* strains containing the transporters HxtB, HxtC, and HxtE are able to grow in the presence of glucose, galactose, fructose, and mannose, while HxtD was not able to use any of these monosaccharides for growth [46]. In contrast, we were not able to see any xylose transport by *S. cerevisiae* strains harboring HxtC, -D, or -E (data not shown). Taken together, these results suggest that HxtB plays, in addition to being a glucose transporter, a major role in xylose uptake.

Discussion

One of the major drawbacks in biofuel production from lignocellulosic plant material is the inability of fermenting organisms to produce ethanol when growing on sugars other than glucose. Lignocellulose is composed of hexose (glucose) and pentose sugars (mainly xylose) and enzymatic deconstruction of it by, for example, filamentous fungi results in the release of these monosaccharides as well as in the release of oligosaccharides (e.g., the glucose dimer cellobiose) [6]. More specifically, most fermenting organisms are not very efficient at transporting pentose sugars and oligosaccharides into the cell. Complete conversion of all the sugars found in lignocellulose is desired to make 2G biofuel production an economically feasible process [14]. *S. cerevisiae* is one of the preferred organisms for fermentation as it is already applied



	Km (mM xylose)	Vmax (µmol xylose.s^{-1}. conidia)
Wild–type	56.17 ± 11.9	0.2720
Δ*hxtB*	100.4 ±17.92	0.2056

Fig. 5 HxtB is a xylose transporter. Fungal dry weight of the wild-type, Δ*hxtB*, Δ*hxtC*, Δ*hxtD,* and Δ*hxtE* strains was measured after strains were grown for 24 or 48 h in the presence of 1 % glucose (**a**) or in the presence of 0.2 and 2 % xylose (**b**). Xylose transport, as assessed by ^{14}C-xylose uptake, was measured in the wild-type and Δ*hxtB* strains in the presence of different concentrations of xylose (**c**)

in various industrial processes and is generally regarded as safe [10].

One successful strategy to improve non-glucose uptake in *S. cerevisiae* is to introduce transporters, from other organisms into its genome. The genomes of filamentous fungi, which are able to internalize a wide variety of mono- and oligosaccharides, are, therefore, screened to find transporters which are able to transport non-glucose sugars. Although this has greatly improved the ability of *S. cerevisiae* to take up pentose sugars such as xylose or cellodextrins such as cellobiose [13, 20, 21, 26], further genetic engineering is required to optimize non-glucose sugar transport. In addition, most transporters encoded by filamentous fungi have not been characterized yet, although these organisms also play a major role in 2G biofuel production. This work, therefore, aimed at identifying xylose- and cellobiose-specific transporters through screening the genome of the filamentous fungus *A. nidulans* and characterizing them when introduced into *S. cerevisiae*.

Table 1 Residual xylose in the supernatant during *A. nidulans* growth

Time (h)	Wild-type	*ΔhxtB*	*ΔhxtE*
1 % xylose			
0	100	100	100
24	58.7 ± 6.6	60.7 ± 2.1	56.5 ± 2.0
48	23.8 ± 1.6	49.9 ± 7.5*	27.8 ± 0.2
72	0	17.4 ± 2.9*	0
2 % xylose			
0	100	100	100
24	75.9 ± 3.7	85.6 ± 4.2	73.3 ± 1.6
48	15.4 ± 4.6	37.3 ± 0.6*	19.3 ± 1.2
72	0	6.2 ± 3.4*	0

* $p < 0.01$

A BLAST analysis of XtrG, encoded by *xtrG* and identified as being upregulated in the presence of xylose [33], showed similarity to the N. crassa cellobiose transporter CDT-2. The name of XtrG was subsequently changed to CltA (cellobiose transporter A). Furthermore, another *A. nidulans* protein, encoded by AN2814, showed high identity to the *N. crassa* cellodextrin transporter CDT-1 and was, therefore, termed CltB. Cellooligosaccharides, such as cellobiose, released during enzymatic degradation of cellulose, have been shown to be important molecules for cellulase gene induction in filamentous fungi, such as *T. reesei, N. crassa,* and *P. oxalicum* [49–53]. The expression of *cltA* increased in the presence of cellobiose. Deletion of *cltB* and of *cltA* and *cltB* simultaneously, but not of *cltA*, resulted in reduced growth and cellulase secretion in the presence of cellobiose during the first 48 h, although this growth was restored after 72 h in the Δ*cltB* strain. In contrast, when introduced into *S. cerevisiae* strain SC9721 together with a β-glucosidase-encoding gene, CltB conferred only slow growth in the presence of different concentrations of cellobiose. These results suggest that the main function of CltB may not be cellobiose transport; it may also function as a transceptor involved in signaling the presence of lignocellulosic biomass. However, we have shown that the introduction of additional functional copies of CltB increases the growth in the presence of low concentrations of cellobiose, strongly indicating CltB is able to transport cellobiose. In *N. crassa*, CDT-1 and CDT-2, in addition to

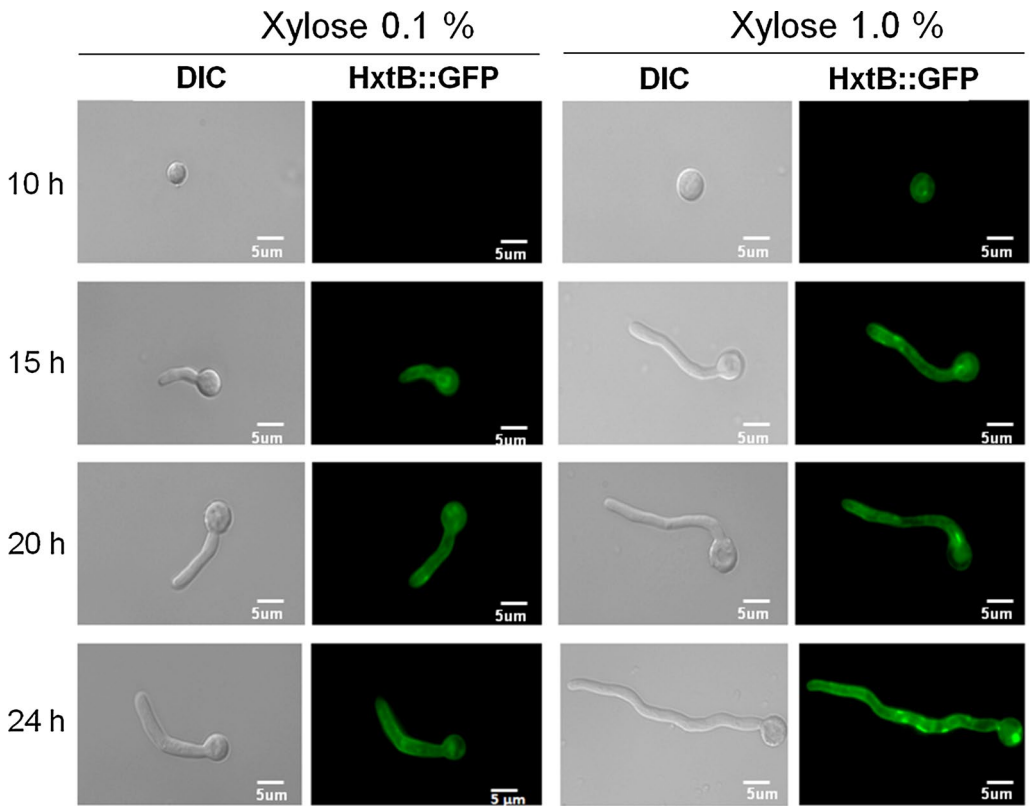


Fig. 6 HxtB localizes to the plasma membrane in *A. nidulans* in the presence of xylose. The *A. nidulans* HxtB::GFP strain was grown from conidia in minimal media supplemented with 0.1 or 1 % of xylose for 10, 15, 20, and 24 h. DIC (differential interference contrast) was applied to view unstained hyphae

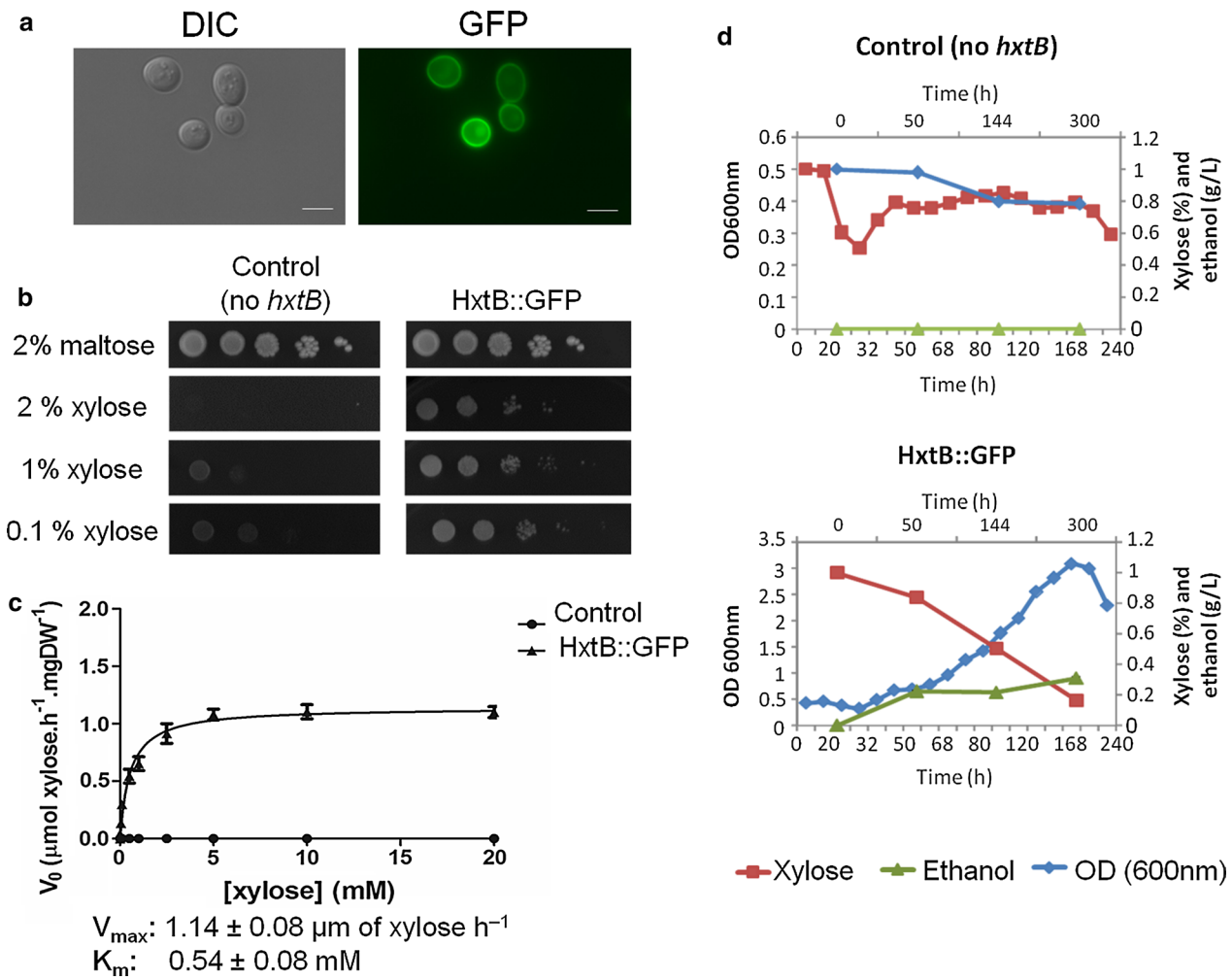


Fig. 7 HxtB confers growth of *S. cerevisiae* in the presence of xylose. **a** HxtB::GFP localizes to the plasma membrane in *S. cerevisiae* when grown in maltose-rich media. Microscopy pictures were taken in the absence (DIC) and presence of fluorescence (GFP). **b** Growth of *S. cerevisiae* strain EBY.VW4000 transformed with (*hxtB*) and without (control) the hexose transporter *hxtB* gene in the presence of low (0.1 %) and high (1 %) concentrations of xylose. Both the control and *hxtB* strains also contained the genes encoding enzymes of the xylose metabolic pathway. Yeast strains were first pre-grown in maltose-rich media before a serial dilution was made and cells were incubated on plates containing the different carbon sources at 30 °C for 144 h. **c** Xylose transport as determined by [^{14}C] xylose uptake in the *S. cerevisiae hxtB* strain in the presence of different concentrations of xylose. A *S. cerevisiae* strain which did not contain the HxtB hexose transporter was used as the control strain. Standard deviations were obtained from three biological replicates. **d** Growth, as determined by optical density at 600 nm (OD_{600nm}), ethanol production and xylose consumption of the *S. cerevisiae* control (does not contain *hxtB*) and HxtB strains when incubated in YNB supplemented with 1 % xylose

being cellobiose transporters, have been hypothetized to having a role in downstream signaling upon the detection of cellulose by the fungus [42]. Furthermore, the *T. reesei* transporters STP1 and CRT1 were also proposed to play an important role in the induction of cellulase-encoding genes [32]. This is the first time that a potential transceptor role has been identified for a protein in *A. nidulans* which is involved in the signaling process of cellulose.

In contrast, deletion of *cltA* did not result in reduced biomass accumulation and cellulase activity in the presence of cellobiose, but introduction of CltA into *S. cerevisiae* conferred growth in the presence of different concentrations of cellobiose. These results indicate that CltA (formerly XtrG) is a cellobiose transporter and this is the first time that a cellobiose-specific transporter has been identified in *A. nidulans*. The genome of *A. nidulans* encodes 357 MFS transporters and redundancy is very likely to exist between these transporters. This redundancy could compensate for the individual loss of CltA (growth not affected) and CltB (growth restored after 72 h), suggesting that other transporters exist with transceptor activities. Deletion of both CltA and CltB had a

more severe impact on fungal growth in the presence of cellobiose, suggesting that these two proteins do play major roles in cellobiose signaling and uptake and may work together to ensure growth on cellobiose. An intriguing aspect of the biology of *A. nidulans* CltA is why it is also induced in the presence of xylose when it actually is a cellobiose transporter. It is possible that cellobiose transporters could also transport xylooligosaccharides or that the main transcriptional activator of genes encoding proteins required for xylose and xylan metabolism, XlnR (on which CltA was shown to be dependent) can also induce genes encoding cellobiose transporters. Actually, it has already been demonstrated that *N. crassa* CDT-2 is able to transport both cellodextrins and xylodextrins [54]. Indeed, in *A. niger*, *cbhA,* and *cbhB*, encoding cellobiohydrolases which catalyze the depolymerization of cellulose were shown to be expressed at much higher levels in the presence of xylose and xylan than when compared to sophorose and cellulose [55]. Furthermore, when grown in the presence of xylan or cellulose, *A. nidulans* always secretes both cellulases and xylanases (data not shown). This is probably due to the fact that cellulose and hemicelluloses are always found together as they make up the plant cell wall.

Another characteristic of sugar transporters of the MFS is that they very often accept multiple monosaccharides and are, therefore, capable of transporting different sugars into the fungal cells; for example, XtrD transports various monosaccharides [33]. Previously, four glucose transporters were identified in *A. nidulans* (HxtB-HxtE) and we decided to verify if they could be involved in xylose transport. Deletion of *hxtB*, and to some extent *hxtE*, resulted in significantly reduced growth in the presence of high (2 % w/v) concentrations of xylose after 48 h. HxtB (also named MstC) has previously been shown to be a high-affinity glucose transporter which also appears to be able to translocate mannose, galactose, fructose, and xylose [56]. The expression of *hxtB* is not induced in the presence of glucose but rather in the absence of it, and this gene is also subject to CreA-mediated carbon catabolite repression [56]. This difference in growth and gene expression between high and low concentrations of xylose may therefore be explained by the high-affinity uptake system in which HxtB plays a role or due to the different time points at which both assays were carried out. Furthermore, gene transcription does not necessarily reflect protein secretion and growth. Growth on glucose was not affected, probably, because HxtB is a high affinity glucose transporter which is expressed when glucose is present in low concentrations. Glucose uptake in the presence of high concentrations of this sugar occurs via low affinity transporters such as MstE, which is induced by glucose in *A. nidulans* [57]. Furthermore, deletion of *hxtB* resulted in decreased affinity for and decreased transport capacity of xylose, indicating that HxtB is also a xylose transporter in addition to being a glucose transporter. In agreement, when HxtB::GFP was introduced into *S. cerevisiae*, where it located to the plasma membrane, it conferred growth of *S. cerevisiae* in the presence of different concentrations of xylose and was capable of successfully transporting xylose into the yeast cell. Furthermore, the *S. cerevisiae* HxtB::GFP strain produced ethanol when growing in xylose-rich media. This work, therefore, identified an *A. nidulans* transporter which in addition to taking up glucose was also efficient at transporting xylose.

Glucose is the preferred carbon source for most microorganisms as it provides rapid energy for survival and niche colonization. Hence, most fungi, including *A. nidulans* are specialized in taking up glucose as soon as it is detected in the environment through high and low affinity uptake systems. As shown in this work, *A. nidulans* transporters have preferentiality for glucose but when this sugar is not available, switch to transporting other sugars, such as xylose, arabinose, or galactose. In addition to the search for transporters which can also translocate pentose or cellodextrins, the focus of research should be directed to the molecular engineering of individual transporters to render them "blind" to glucose and increase affinity and specificity for alternative, non-glucose sugars as was done by [23, 58]. In addition, yeast strains, which already harbor heterologous introduced transporters, can be genetically modified through directed evolution to improve growth in the presence of pentose sugars or cellodextrins [33]. At the same time, introducing components required for the efficient transport and metabolism of various different sugars into *S. cerevisiae*, thus allowing the co-fermentation of multiple carbohydrates, has also proved to be a successful strategy [59, 60]. Furthermore, although the genome of *A. nidulans* (and other filamentous fungi) encodes a multitude of MFS sugar transporters, they have scarcely been characterized and further studies, including those on carbon source sensing and signaling, are required to confirm or reject the above proposed hypothesis. This work identified a cellobiose transporter and a potential cellobiose transceptor in *A. nidulans*, a role which has also been associated with cellobiose transporters in *N. crassa* and *T. reesei*. Furthermore, this study provided further characterization of a glucose/xylose transporter. Taken together, this work provides a preliminary screening and characterization of MFS transporters in *A. nidulans* and lays a basis for further exploration of sugar sensing and transport in industrially relevant fungi.

Conclusions

The knowledge on sugar transport in fungi is very limited, although it presents a key step in the conversion of lignocellulosic biomass to biofuels. In this work, a cellobiose transporter, a xylose transporter, and a putative cellobiose transceptor were identified and characterized in *A. nidulans*. This is the first time that a sensory role for a sugar has been associated to a protein in this fungus. This study, therefore, highlights the importance of continuously screening fungal genomes for transporter-encoding genes and in addition, functionally characterizing these proteins. Furthermore, another drawback in the second-generation bioethanol production is the presence of glucose which represses proteins required for the utilization of alternative carbon sources. The identified xylose transporter is also a major glucose transporter, highlighting the preference of *A. nidulans* for this sugar. Furthermore, targeted molecular protein engineering could render these transporters more specific for non-glucose carbon sources. This work, therefore, presents a preliminary basis for further studies which would characterize and engineer known and novel transporters with the aim to introduce them into fermenting yeast strains to successfully convert a large amount of plant cell wall sugars into ethanol.

Methods

Strains, media, and culture methods

A list of all the strains used in this work is given in Table 2. All *A. nidulans* strains were grown at 37 °C in either liquid (without agar) or solid (with 20 g/l agar) minimal medium [MM: 1 % (w/v) carbon source, 50 ml of a 20× salt solution (120 g/l $NaNO_3$, 10.4 g/l KCl, 30 g/l KH_2PO_4, 10.4 g/l $MgSO_4$), 1 ml of 5× trace elements (22.0 g/l $ZnSO_4$, 11 g/l boric acid, 5 g/l $MnCl_2$, 5 g/l $FeSO_4$, 1.6 g/l $CoCl_2$, 1.6 g/l $CuSO_4$, 1.1 g/l $(NH_4)_2MoO_4$, 50 g/l ethylenediaminetetraacetic acid (EDTA)] and adjusted to pH 6.5 with NaOH. Depending on the auxotrophy of the strain, uridine (1.2 g/l), uracil (1.2 g/l) or pyridoxine (0.005 mg/µl) were added. All *S. cerevisiae* strains were grown at 30 °C in liquid (no agar) or solid (20 g/l agar) YNB medium (7 g/l yeast nitrogen base without amino acids, 0.05 g/l histidine, 0.1 g/l lysine, 0.1 g/l leucine, 0.1 g/l tryptophan, 0.1 g/l uridine, and 0.1 g/l uracil). All reagents were obtained from Sigma Aldrich (St. Louis, MO, USA), except where stated.

Construction of *Aspergillus nidulans* null mutants

Standard genetic techniques for *A. nidulans* strain constructions, transformations, and DNA manipulations were done according to [61]. PCR reactions were performed using Phusion High-Fidelity DNA polymerase (New England Biolabs) or *TaKaRa Ex Taq DNA Polymerase* (Clontech USA). A list of all primer pairs can be found in Additional file 4. The gene knock-out strains *ΔxtrF*, *ΔxtrG*, *ΔxtrH*, and *ΔcltB* (AN0332, AN8347, AN9173 and AN2814, respectively) were obtained through replacing each gene with a prototrophic marker gene. Gene replacement cassettes were generated by in vivo recombination in *S. cerevisiae* as previously described by [62]. Briefly, the 5′ UTR of each target gene was PCR amplified using specific primers: *xtrF* (primers P1 and P2), *xtrG* (primers P7 and P8), *xtrH* (primers P13 and P14), and *cltB* (primers P27 and P28). Similarly, the 3′UTR regions of each gene were amplified by PCR: *xtrF* (primers P3 and P4), *xtrG* (primers P9 and P10), *xtrH* (primers P15 and P16), and *cltB* (primers P29 and P30). Pyridoxine or uridine/uracil were used as prototrophic markers, and their respective genes (*pyroA* and *pyrG*) were amplified by PCR from plasmids pAFpyro (primers P37 and P38) and pCDA21 (primers P35 and P36), respectively (Table 2). The individual gene fragments (5′ and 3′ UTRs and prototrophic marker gene) were transformed, together with plasmid pRS426, which was linearized with *EcoR*I and *BamH*I, into *S. cerevisiae* SC9721 using the lithium acetate method [63]. Positive *S. cerevisiae* transformation candidates were grown in YNB-URA medium, before gDNA was extracted and PCRs were run to confirm the correct construction. The cassettes were then PCR-amplified from *S. cerevisiae* genomic DNA, purified and used to transform *A. nidulans* TN02A3 strain, according to [64]. Positive *A. nidulans* transformation candidates were selected and purified through three rounds of growth on plates and gDNA was extracted. Gene deletions were confirmed by Southern blots (Additional file 5).

To construct the complemented strain Δ*cltB::cltB*$^+$, the complementing cassette containing the 5′ UTR region plus the cltB gene and the 3′ UTR region was amplified by PCR from *A. nidulans* genomic DNA using specific primers (P27 and P30). The *A. nidulans ΔcltB Ku80*$^+$ mutant was co-transformed with pCDA21 plasmid and the *cltB*$^+$ complementing cassette. Positive *A. nidulans* complemented candidates were selected and purified through three rounds of growth on plates, gDNA was extracted, and the candidates were confirmed by PCR (Additional file 6).

Construction of Aspergillus nidulans GFP- tagged strains

All *A. nidulans* GFP-tagged strains were constructed as described in the previous section ("Construction of *A. nidulans* null mutants") with the exception that genes were not replaced by prototrophic markers but were instead C-terminally tagged with GFP. The selective marker gene *pyrG* was also introduced. A list of all primers used for strain constructions can be found in

Table 2 Strains and plasmids used in this work

Strains/plasmids	Genotype	Reference
S. cerevisiae		
EBY.VW4000	CEN.PK2-1C *hxt13Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10Δ::loxP hxt8Δ::loxP hxt514Δ::loxP hxt2Δ::loxP hxt367Δ::loxP gal2 Δ stl1Δ::loxP agt1Δ::loxP ydl247wΔ::loxP yjr160cΔ::loxP*	[48]
SC9721	MATa *his 3-D200 URA 3-52 leu2D1 lys 2D202 trp 1D63*	FGSC
EBY.VW4000 +pRH195m +pRH274	EBYVW4000 pRH195 pRH274	[33]
hxtBGFP EBY.VW4000	EBYVW4000 pRH195 *hxtB* pRH274	This work
xtrF::GFP EBY.VW4000	EBYVW4000 pRH195 *xtrF* pRH274	This work
xtrG::GFP EBY.VW4000	EBYVW4000 pRH195 *xtrG* pRH274	This work
xtrH::GFP EBY.VW4000	EBYVW4000 pRH195 *xtrH* pRH274	This work
cltB::GFP EBY.VW4000	EBYVW4000 pRH195 *cltB* pRH274	This work
SC9721 cltA::GFP gh1-1	SC9721 pRH195 *cltA* pGH1	This work
SC9721 cltB::GFP gh1-1	SC9721 pRH195 *cltB* pGH1	This work
SC9721_pGH1	SC9721 pGH1	This work
SC9721_pCDT-1 gh1-1	SC9721 pCDT-1 pGH1	This work
A. nidulans		
TN02A3	*pyroA4 pyrG89; chaA1; ΔnKuA::argB*	[70]
HxtB::GFP TN02A3	pyrG89; pyroA4; Δnku70::argB; hxtB::GFP::pyrG	This work
HxtE::GFP TN02A3	pyrG89; pyroA4; Δnku70::argB; hxtE::GFP::pyrG	This work
ΔxtrF	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔxtrF::pyrG*	This work
ΔxtrG/ΔcltA	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔxtrG::pyrG*	This work
ΔxtrH	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔxtrH::pyrG*	This work
ΔcltB	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔcltB::pyroA4*	This work
ΔhxtB	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔhxtB::pyroA4*	[46]
ΔhxtE	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔhxtE::pyroA4*	[46]
ΔcltA ΔcltB	*pyroA4 pyrG89; chaA1; ΔnKuA::argB; ΔcltA::pyrG89; ΔcltB::pyroA4*	This work
GR5	*wA1 pyroA1pyrG89*	FGSC
ΔcltB::cltB^{+}	*ΔcltB::cltB*$^{+}$*::pyrG*$^{+}$ *pyrG89*	This work
oCltB3	*wA1 pyroA1 pyrG89 cltB3::gfp::pyrG*$^{+}$	This work
Plasmids		
pRH195[a]	pBluescript II SK+, *TRP1, CEN6, ARSH4+ PHXT7-XKS1-THXT7*	[24]
pRH274	pBluescript II SK+, *URA3, CEN6, ARSH4* + P*PGK1-XYL1-TPGK1*; P*ADH1-XYL2-TADH1*; P*HXT7-XKS1-THXT7*	[66]
pRS426	*ampR lacZ* URA3	[65]
pCDA21	*Zeo::pyr ampR*	[71]
pGH1-1	pRS425 *PGK1p-gh1-1-CYC1t*	[26]
pCDT-1	pRS426 *PGK1p-cdt-1-CYC1t*	[26]

[a] The original vector pRH195 carries the XKS1 gene which was released after digestion with Spe*I* and Sal*I*. The resultant vector without the XKS1 gene was used in this work for compl ementation assays

Additional file 4. The xtrF-H, hxtB and hxtE genes were amplified by PCR using primers P5/P6, P11/P12, P17/P18, P42/P43, and P48/P49, respectively. The 3′ UTRs of genes *xtrF-H*, *hxtB,* and *hxtE* constructions were amplified by PCR using primers P3/P4 (*xtrF*), P9/P10 (*xtrG*), P15/P16 (*xtrH*), P30/P32 (*cltB*), P44/P45 (*hxtB*), and P50/P51 (*hxtE*). The *gfp* gene was separated from the target gene by four additional codon triplets that after translation produce a four amino acid residue linker (glycine–threonine–arginine–glycine) region termed Spacer-GFP [65]. To allow fusion of GFP to our protein of interest, the stop codon of the gene ORF was removed when designing the primers. The GFP was amplified from the pMCB17apx plasmid (kindly provided by Vladimir P. Efimov) with primers P39/P40. The *pyrG* gene was amplified as described above. GFP-tagged gene constructions were confirmed by PCR in *A. nidulans*.

In addition, the *cltB* overexpression strain (GR5 CltB::GFP strain) was constructed in the *A. nidulans* GR5 background, because this strain allows multiple non-homologous ectopic integrations. Again, the *A. nidulans* GFP-tagged strains were constructed as described

in the previous section ("Construction of *A. nidulans* null mutants") with the exception that genes were not replaced by prototrophic markers but were instead C-terminally tagged with GFP and the selective marker gene *pyrG* was also introduced. The cltB gene (primers P27/P31) and the 3′ UTR (primers P30/P32) were amplified by PCR. The multiple integrations of CltB::GFP cassette were confirmed by Southern blot (Additional file 1).

For all constructions above described, the *gfp* gene was separated from the target gene by four additional codon triplets that after translation produce a four amino acid residue linker (glycine–threonine–arginine–glycine) region termed Spacer-GFP [65]. To allow fusion of GFP to our protein of interest, the stop codon of the gene ORF was removed when designing the primers. The GFP was amplified from the pMCB17apx plasmid (kindly provided by Vladimir P. Efimov) with primers P39/P40. The *pyrG* gene was amplified as described above. GFP-tagged gene constructions were confirmed by PCR in *A. nidulans* or southern blot. The GFP was amplified from the pMCB17apx plasmid (kindly provided by Vladimir P. Efimov) with primers P39/P40. The *pyrG* gene was amplified as described above. For *xtrF-H*, *hxtB* and *hxtE* mutants, GFP-tagged gene constructions were confirmed by PCR in *A. nidulans*.

Construction of *Saccharomyces cerevisiae* strains

Strain EBY.VW4000 (Table 2) was used for the *Saccharomyces cerevisiae* complementation assays [48]. A list of all primers can be found in Additional file 4. The *xtrF-H*, *cltB,* and *hxtB* ORFs were amplified by PCR from cDNA obtained from *A. nidulans* strains using primers P19/P20 (*xtrF*), P21/P22 (*xtrG*), P23/P24 (*xtrH*), P33/P34 (*cltB*), and P46/P47 (*hxtB*), respectively. Plasmid pRH195 was double digested with *Spe*I and *Sal*I for linearization and release of the *XKS1* gene (generating the pRH195 m). For in vivo recombination, plasmid pRH195 m was transformed into *S. cerevisiae E*BY.VW4000, which already contained plasmid pRH274 [33], together with all the PCR-amplified sugar transporters and GFP fragments using the lithium acetate method [63]. The *gfp* gene was amplified from plasmid pMCB17apx using primers P25/P26. *S. cerevisiae* EBY.VW4000 is unable to metabolize xylose and in addition to being transformed with the *A. nidulans* transporter-encoding genes, it was also transformed with genes encoding enzymes of the xylose metabolic pathway. *Saccharomyces stipitis* xylose reductase (XR) and xylose dehydrogenase (XDH) as well as *S. cerevisiae* xylulose kinase (XK) were introduced in EBY.VW4000 via plasmid pRH274 (Table 2), where the three enzyme-encoding genes were placed under the control of the *PGK1*, *ADH1,* and *HXT7* constitutive promoters, respectively [66]. Transformants were selected for tryptophan and uridine prototrophy on solid YNB lacking both tryptophan and uridine and supplemented with 2 % maltose.

Saccharomyces cerevisiae SC9721 strain was used to construct the yeast strains expressing the cellobiose transporters *cltA* and *cltB*. The SC9721 strain was first transformed with the pGH1 plasmid which contains the β-glucosidase-encoding gene *gh1-1* from *N. crassa* [26]. The *cltA* and *cltB* cellobiose transporter genes were amplified from cDNA of *A. nidulans* using primers P52/P22 (*cltA*) and P53/P34 (*cltB*). Plasmid pRH195 m was used to transform *S. cerevisiae* with the respective transporter genes. Furthermore, *S. cerevisiae* strain SC9721 was also transformed with plasmid pCDT-1, containing the already characterized *N. crassa cdt-1* cellobiose transporter-encoding gene, which was used as a positive control in ours assays [12]. All *S. cerevisiae* transformations were carried out using the lithium acetate method [63] and strain constructions were confirmed by PCR.

gDNA extraction from *A. nidulans and S. cerevisiae*

Genomic DNA extractions of *A. nidulans* and *S. cerevisiae* were performed according to [67] and [63].

Microscopy

A. nidulans strains HxtB::GFP, HxtE::GFP, XtrF::GFP, XtrG::GFP, XtrH::GFP, and CltB::GFP were grown from spores in 3 ml of MM containing 0.1 and 1 % xylose for 10, 15, 20, and 24 at 30 °C in a small Petri dish containing a microscopy cover slip. The oCltB3::GFP strain was previously inoculated 16 h at 30 °C in a small Petri dish containing a microscopy coverslip and 3 mL of MM supplemented with 1 % fructose as a carbon source. After 16 h, the germlings were washed with 1X PBS and transferred to 0.5 and 1 % cellobiose for 4 or 8 h. Coverslips were washed with 1× PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate) and viewed under the microscope. *S. cerevisiae* EBY.VW400 strain harboring the *hxtB*, *xtrF-H,* and *cltB* tagged to GFP constructions was grown 48 h in 0.5 ml of liquid YNB-trp-ura medium supplemented with 2 % maltose for 24 h at 30 °C in a 24-wells plate. Cells were washed with PBS and viewed under the microscope. All slides were viewed with a Carl Zeiss (Jena, Germany) microscope using the 100× magnification oil immersion objective lens (EC Plan-Neofluar, NA 1.3) equipped with a 100-W HBO mercury lamp epifluorescence module. Phase contrast brightfield and fluorescent images were taken with an AxioCam camera (Carl Zeiss), and images were processed using the AxioVision software version 3.1 and saved as TIFF files. Further processing was performed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, CA).

Dry weight measurement

A total of 5×10^7 spores of *A. nidulans* wild-type (TN02A3) and mutant strains (*ΔxtrF-H* and *ΔhxtB*) were inoculated in 50 ml of liquid minimal medium supplemented with 1 % glucose, 2 % xylose or 0.2 % xylose. 2.5×10^7 spores of *A. nidulans* wild-type (TN02A3) and mutant strains (Δ*cltA*, *ΔcltB and ΔcltA ΔcltB)* were inoculated in 50 mL of liquid minimal medium supplemented with 1 % glucose or 1 % cellobiose. Strains were grown for 24 and 48 h in xylose-rich media and for 48 and 72 h in cellobiose-rich media at 37 °C, 180 rpm. Mycelia were harvested by vacuum filtration, snap-frozen in liquid N_2, freeze-dried and subsequently weighed.

RNA extraction and real-time PCR reactions

To measure the expression of *xtrF-H*, a total of 10^7 spores from the *A. nidulans* wild-type, *creAd30* or Δ*xlnR* strains were inoculated in 50 ml of liquid MM containing 1 % fructose for 16 h at 37 °C, 180 rpm. Mycelia were washed with sterile water and transferred to MM supplemented with 1 % xylose or 1 % xylose and 1 % glucose for 6, 12 and 24 h at 37 °C, 180 rpm. Alternatively, 10^7 spores from the wild-type *A. nidulans* strain was inoculated in 50 ml of liquid MM supplemented with 1 % glucose, 1 % sorbitol, 1 % xylose, 1 % fructose, 1 % maltose, 1 % galactose, and 1 % mannose at 37 °C, 180 rpm for 8 or 16 h. All mycelia were harvested by vacuum filtration, snap-frozen in liquid N_2, and stored at −80 °C.

To measure the expression of *cltA* and *cltB*, a total of 10^7 spores from the *A. nidulans* wild-type strain were inoculated in 50 ml of liquid MM containing 1 % fructose for 16 h at 37 °C, 180 rpm. Mycelia were washed with sterile water and transferred to MM supplemented with 1 % cellobiose for additional 1, 2, and 4 h at 37 °C, 180 rpm. Mycelia were harvested by vacuum filtration, snap frozen in liquid N_2, and stored at −80 °C. cltA and cltB

Mycelia were ground to a fine powder under liquid N_2, and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. The quality of the RNA (10 µg) was checked by running them through the Bioanalyzer. RNA samples were DNAse-treated as previously described by [67], purified with the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) and quantified on the NanoDrop® 2000 (Thermo Scientific) machine. RNA integrity was confirmed using the Bioanalyser Nano Kit (Agilent Technologies) and the Agilent Bioanalyser 2100, using an RIM value of 6.0 as a threshold.

RNA was then reverse transcribed to cDNA using the Superscript III Reverse transcriptase kit (Invitrogen), according to manufacturer's instructions. All RT-qPCR reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Green PCR Master Mix kit (Applied Biosystems), according to manufacturer's instructions. Reactions and calculations were performed as previously described [68]. All primers are listed in Additional file 4.

Xylose uptake assay

Xylose uptake rates were measured by monitoring the incorporation of D-[U-^{14}C] xylose [289.0 mCi/mmol (10.693 GBq)/mmol] (Perkin Elmer Life Sciences) into germinating conidia in the presence of various D-xylose concentrations according to [33] with modifications. A total of 1.2×10^9 Δ*hxtB* conidia were inoculated in 600 ml MM containing 1 % glycerol (w/v) for 5 h at 37 °C, 180 rpm. Swollen conidia were harvested by vacuum filtration through nitrocellulose filters (Fisherbrand) and washed twice with ice-cold water. Conidia were re-supended in water to get a concentration of 2.5×10^7 conidia/250 µl. A total of 2.5×10^7 spores were inoculated with different concentrations of D-xylose (0.1–100 mM) in 1.5 ml tubes together with 1 µl of radiolabelled ^{14}C-xylose (0.2 µCi) and incubated at room temperature for 30–60 s. Xylose uptake was stopped by adding 1.5 ml ice-cold water and conidia were immediately harvested by vacuum filtration through nitrocellulose filters. Conidia were washed again two times with 1.5 ml ice-cold water.

Saccharomyces cerevisiae HxtB::GFP EBY.VW400 strain was inoculated in 300 ml of YNB medium supplemented with 2 % maltose until they reached the exponential growth phase (OD_{600nm} of 0.6). Yeast cells were pelleted by centrifugation at 4000 rpm for 5 min, washed twice with 50 ml ice-cold water, and then re-suspended in 4.5 ml of ice-cold water. 40 µl of this cell suspension were transferred to 1.5 ml Eppendorf tubes which were then incubated at 30 °C for 5 min for temperature equilibration. 10 µl of different concentrations of xylose (0.1 to 100 mM xylose) and 0.2 µCi of ^{14}C-xylose were added to the yeast cells. Xylose uptake was allowed to proceed for 10 s through vigorous vortexing before the reaction was stopped through the addition of 1.5 ml ice-cold water. Cells were harvested by vacuum filtration through nitrocellulose filters and washed two times with ice-cold water.

All nitrocellulose filters containing the fungal cells were transferred to 3 ml of ScintiSafeTM Econo1 scintillation liquid (Fisher Scientific), and the D-[U-^{14}C] xylose taken up by the cells was measured using the Tri-Carb® 2100TR Liquid Scintillation Counter.

Assaying extracellular xylose concentrations

The EBY.VW4000 + pRH195 m + pRH274 (control) and the HxtB::GFP yeast strains were inoculated (initial OD_{600} 0.5) in 50 ml YNB-trp-ura medium supplemented with 1 % (w/v) xylose at 30 °C, 150 rpm for 300 h. At each

time point, 2.0 ml of the culture was collected, centrifuged, and the supernatants were stored at −80 °C. The xylose concentration in the supernatants was measured using the D-xylose assay kit (Megazyme) following manufacturer's instructions. Absorbance was measured at 340 nm in a 96-well polystyrene plate (Corning) using the SpectraMax I3 spectrometer (molecular devices).

Assaying extracellular ethanol concentrations

The EBY.VW4000 + pRH195 m + pRH274 (control) and HxtB::GFP yeast strains were inoculated (initial OD_{600} 0.5) in 50 ml YNB-trp-ura medium supplemented with 1 % (w/v) xylose at 30 °C, 150 rpm for 300 h. At each time point, 2.0 ml of the culture was collected, centrifuged, and the supernatants were stored at −80 °C. Ethanol production was determined by measuring the absorption of NADH at 340 nm as previously described [69] with modifications. Reactions were started through mixing 100 µl assay buffer (50 mM pyrophosphate, 50 mM semicarbazide, and 20 mM glycine, pH 8.8) with 0.643 mM NAD^+, 5 U alcohol dehydrogenase and 10 µl sample supernatant in a 96-well polystyrene plate (Corning). Samples were incubated at 30 °C for 5 min, and then, the ethanol concentration was measured at 340 nm using the SpectraMax I3 spectrometer (Molecular devices).

Growth of *Saccharomyces cerevisiae* strains on solid medium

S. cerevisiae strains were inoculated in 50 ml YNB medium supplemented with 2 % maltose or 1 % glucose for 24 h at 30 °C, 150 rpm until an OD_{600nm} of 0.1. Yeast cells were centrifuged at 4.000 rpm for 5 min, washed two times with water, and re-suspended in water to a final concentration of 1.0 at OD_{600nm}. A serial dilution of 1:10 of the yeast cells was made, and 5 µl of the cell suspensions were spotted on plates containing 1 % glucose, 0.1, 1 or 2 % cellobiose. Plates were incubated at 30 °C for 168 h.

Yeast growth rates

The SC9721 cltA::GFP gh1-1, SC9721 cltB::GFP gh1-1 and SC9721 pCDT-1 gh1-1 strains were grown in YNB medium supplemented with 1 % glucose for 24 h at 30 °C, 150 rpm. The OD at 600 nm was measured; the cell cultures were diluted to OD_{600nm} of 0.1 and transferred to 50 ml YNB medium supplemented with the respective carbon source (glucose 1 % or cellobiose 0.1–2 %) at 30 °C, 150 rpm for 144 h. The OD_{600nm} was measured periodically at the indicated time points.

Cellulase assays

A total of 10^7 spores from wild-type, *ΔcltA*, *ΔcltB* and *ΔcltA ΔcltB* mutant strains were inoculated in liquid MM supplemented with 1 % fructose at 37 °C, 180 rpm for 16 h. Mycelia were washed with sterile water and transferred to MM supplemented with 1 % Avicel™ for 5 days at 37 °C, 180 rpm. The supernatant was separated from the mycelia using miracloth. Cellulase (endo-1,4-β-glucanase) activity in the supernatants was measured using Azo-CM-Cellulose (Megazyme International, Bray, Ireland) as a substrate, according to manufacturer's instructions.

Additional files

Additional file 1. Construction of an overexpressing CltB::GFP mutant strain. (A) Genomic DNA from *A. nidulans* wild-type GR5 and CltB::GFP transformant strains were isolated and cleaved with the enzyme *Pst*I; a 2.7-kb DNA fragment from the 5′-noncoding region plus the *cltB* gene was used as a hybridization probe. This fragment recognizes a single DNA band (about 5.0 kb) in the wild-type strain and a single DNA band (about 3.0 kb) in the CltB::GFP homologously integrated cassette. Different size of bands indicates the multiple integration of the GFP cassette. (B) Southern blot.

Additional file 2. Enzymatic kinetics. (A) Eadie–Hofstee and (B) Lineweaver–Burk plots for the data of the Fig. 6c.

Additional file 3. HxtE is target to the plasma membrane in *A. nidulans* in the presence of xylose. The *A. nidulans* HxtE::GFP strain was grown from conidia in minimal media supplemented with 0.1 % or 1 % of xylose for 10 h, 15 h, 20 h, and 24 h. DIC (differential interference contrast) was applied to view unstained hyphae.

Additional file 4. Primers used in this work.

Additional file 5. Genomic DNA from the *A. nidulans* wild-type, Δ*xtrG* (AN8347), Δ*xtrH* (AN9173), Δ*cltB* (AN2814) and the double Δ*cltA* Δ*cltB* strains was extracted and digested with different restriction enzymes to confirm the deletion strains. Diagram (A.) and Southern blot (B.) of the wild-type and Δ*xtrG* strains when digested with *Sac*I. A 1-kb DNA fragment from the *xtrG* 3′UTR (untranslated) region was used as a hybridization probe. The probe recognizes a single 10.0-kb band in the wild-type strain and a single 6.4-kb band in the Δ*xtrG* strain. Diagram (C.) and Southern blot (D.) of the wild-type and Δ*xtrH* strains when digested with *EcoR*I. A 1-kb DNA fragment from the *xtrH* 5′UTR (untranslated) region was used as a hybridization probe. The probe recognizes a single 3.4-kb band in the wild-type strain and a single 3.0-kb band in the Δ*xtrH* strain. Diagram (E.) and Southern blot (F.) of the wild-type and Δ*cltB* strains when digested with *Xba*I. A 1-kb DNA fragment from the *cltB* 5′UTR (untranslated) region was used as a hybridization probe. The probe recognizes a single 2.0-kb band in the wild-type strain and a single 3.3-kb band in the Δ*cltB* strain. Diagram (G.) and Southern blot (H.) of the wild-type and Δ*cltA* Δ*cltB* strains when digested with *Kpn*I. A 1-kb DNA fragment from the *cltB* 3′UTR (untranslated) region was used as a hybridization probe. The probe recognizes a single 2.0-kb band in the wild-type strain and a single 2.5-kb band in the Δ*xtrG* strain.

Additional file 6. PCR confirmation of the Δ*cltB::cltB*$^+$ strain. Genomic DNA from *A. nidulans* wild-type, complementing strains Δ*cltB::cltB*$^+$ (candidates 1, 2, and 3) and deletion strain Δ*cltB* were isolated and used as template for PCR reactions. (A) Specific primers P70 and P31 were used to amplify a DNA fragment of about 3.1 kb. (B) Specific primers P70 and P37 were used to amplify a DNA fragment of about 3.0 kb. Lanes 1 and 7: C-negative control with no DNA as a template; lanes 2 and 8: genomic DNA from the Δ*cltB::cltB* candidate 1; lanes 3 and 9: genomic DNA from the Δ*cltB::cltB* candidate 2; lanes 4 and 10: genomic DNA from the Δ*cltB::cltB* candidate 3; lanes 5 and 11: genomic DNA from the Δ*cltB* deletion strain; lanes 6 and 12: genomic DNA from the wild-type strain. The * indicates the candidate containing the homologous integration of the complementing cassette and (C^-) the negative control.

Abbreviations
2G: second-generation bioethanol; BP: base pairs; cDNA: complementary DNA; CDT: cellodextrin transporter; CLT: cellobiose transporter; CMC: carboxymethylcellulose; EDTA: ethylenediaminetetraacetic acid; GFP: green fluorescent protein; HXT: hexose transporter; Kb: kilo bases; K_m: Michaelis constant; M: molar; MFS: major facilitator superfamily; MM: minimal medium; Nm: nanometre; OD: optical densisty; ORF: open reading frame; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; pyrG: orotidine-5′-phosphate decarboxylase gene; Pyro: pyridoxine gene; RT-qPCR: quantitative reverse transcription PCR; TRP: tryptophan; V_{max}: maximum reaction velocity rate; XDH: xylitol dehydrogenase; XKS1: xylulose kinase; XR: xylose reductase; XTR: xylose transporter; YNB: yeast nitrogen base.

Authors' contributions
GHG, TFR, NSP, PBAL, FBM, and JVCO contributed to design, acquisition and analysis of data. LNAR, TFR, and GHG contributed to the concept and design of the investigation in addition to the preparation of the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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Anexo C - The low affinity glucose transporter HxtB is also involved in glucose signalling and metabolism in *Aspergillus nidulans*

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The low affinity glucose transporter HxtB is also involved in glucose signalling and metabolism in *Aspergillus nidulans*

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One of the drawbacks during second-generation biofuel production from plant lignocellulosic biomass is the accumulation of glucose, the preferred carbon source of microorganisms, which causes the repression of hydrolytic enzyme secretion by industrially relevant filamentous fungi. Glucose sensing, subsequent transport and cellular signalling pathways have been barely elucidated in these organisms. This study therefore characterized the transcriptional response of the filamentous fungus *Aspergillus nidulans* to the presence of high and low glucose concentrations under continuous chemostat cultivation with the aim to identify novel factors involved in glucose sensing and signalling. Several transcription factor- and transporter-encoding genes were identified as being differentially regulated, including the previously characterized glucose and xylose transporter HxtB. HxtB was confirmed to be a low affinity glucose transporter, localizing to the plasma membrane under low- and high-glucose conditions. Furthermore, HxtB was shown to be involved in conidiation-related processes and may play a role in downstream glucose signalling. A gene predicted to encode the protein kinase PskA was also identified as being important for glucose metabolism. This study identified several proteins with predicted roles in glucose metabolic processes and provides a foundation for further investigation into the response of biotechnologically important filamentous fungi to glucose.

Second generation (2G) biofuel production from lignocellulose-rich plant biomass has gained considerable interest as a renewable, environmentally friendly and food crop non-competitive energy source[1]. Biofuel production from lignocellulose encompasses 4 steps: (i) pre-treatment of the raw lignocellulosic material, (ii) enzymatic hydrolysis and release of simple sugars, (iii) fermentation of these sugars and (iv) purification and distillation of the biofuels[2]. Enzymatic hydrolysis of lignocellulosic polysaccharides and subsequent fermentation are of great research interest in order to make 2G biofuel production a cost-effective process.

Filamentous saprotrophic fungi, such as *Aspergillus spp*. and *Trichoderma reesei*, which are used in various industrial applications, are currently being investigated for the improved production of enzymes that hydrolyse plant sugar polymers[3]. Lignocellulose is composed of a wide range of different polysaccharides and saprotrophic fungi secrete a diverse array of enzymes that can degrade complex lignocellulose saccharides into simpler, fermentable sugars[4,5]. One of the drawbacks of this procedure is the inhibition of enzyme secretion in the presence of glucose, a hexose sugar which constitutes the basic unit of cellulose; a polymer which makes up 40–60% of the plant cell wall[4,5].

Glucose is the preferred carbon source for most microorganisms as it is easily metabolized and provides quick energy for growth and niche colonization[6]. In *Aspergillus spp*. and *T. reesei*, the detection of glucose

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triggers the repression of genes encoding enzymes required for lignocellulose degradation, a mechanism known as carbon catabolite repression (CCR) and mediated by the transcription factor CreA/CRE1. Although CreA/CRE1-mediated transcriptional repression has been extensively studied[6–10], sensing of extracellular glucose concentrations and subsequent transport have been barely characterized in filamentous fungi.

In *S. cerevisiae*, Rgt2p and Snf3p are two sensors that upon glucose detection increase the expression of transporters, therefore enhancing glucose uptake[11]. In the absence of glucose, Rgt1p forms a repressor complex with Mth1p, Std1p and Ssn6p-Tup1p, inhibiting the expression of *HXT* transporter-encoding genes[11]. Upon glucose detection, Rgt2p and Snf3p mediate phosphorylation via casein kinase I (Yck1p/Yck2p) of Mth1p and Std1p, leading to their proteasomal degradation, therefore preventing binding of Rgt1p to the *HXT* promoter regions[11]. Yeast cells can sense a wide range of glucose concentrations and subsequently express transporters required for the amount of glucose present; e.g. low affinity glucose transporters (Hxt1p) when extracellular glucose is high, high affinity glucose transporters (Hxt2p and Hxt4p) when extracellular glucose is low and intermediate glucose transporters (Hxt3p) when extracellular glucose concentrations are neither high nor low[11]. In addition, extracellular glucose concentrations are also detected by the GPCR (G-protein coupled receptor) Gpr1p[12]. In *S. cerevisiae*, the cellular response upon glucose detection is mediated by the cAMP/PKA (protein kinase A) pathway (for an extensive review see ref. 13). The adenylate cyclase Cyr1p is activated by the heterotrimeric Gα protein Gpa2p, which is released from the Gpr1p upon glucose sensing. Cyr1p then increases intracellular cAMP concentrations[14]. Rgt1p is a target of PKA phosphorylation, which causes de-repression of the *HXT* genes. An alternative pathway for the induction of adenylate cyclase activity is via the small GTPases Ras1p/Ras2p, which are activated by phosphorylated glucose[15]. In filamentous fungi, the cAMP/PKA pathway has also been shown to mediate the response to glucose sensing[13,16,17]. In the presence of glucose, PKA is important for spore germination, hyphal growth, cell wall homeostasis and secondary metabolite production whilst inhibiting alternative carbon source usage[17–20].

In contrast to *S. cerevisiae*, glucose sensing and the pathways leading to the transcriptional up-regulation of glucose transporter-encoding genes have not been elucidated in *Aspergillus spp*. and *T. reesei*. To date, several proteins have been described as having a sensory function for non-glucose sugars such as cellobiose and xylose[21–23]. Orthologues of the *S. cerevisiae* glucose sensors Rgt2p and Snf3p have not been described in these fungi. In *A. nidulans*, the G-alpha subunit GanB and the G-protein coupled receptor (GPCR) GprH have been shown to play a role in glucose sensing[24,25]. GanB is involved in mediating activation of cAMP synthesis and subsequent PKA activation in the presence of glucose during early conidial germination events[24]. In *Neurospora crassa*, *rco-3* was shown to be involved in glucose sensing[26].

The genomes of saprotrophic filamentous fungi encode a large number of transporters responsible for the uptake of different sugars found in plant biomass. Similar to *S. cerevisiae*, a high affinity and a low affinity glucose uptake system have been described for filamentous fungi. In *A. niger*, MstA and MstF were determined to be high affinity glucose transporters whereas *mstC* encodes a low affinity glucose transporter[27,28]. In *T. reesei*, STP1 and HXT1 were identified as glucose transporters[29,30]. In *A. nidulans*, MstE (AN5860) is a low affinity glucose transporter and HxtA, MstA/HxtD (AN8737) and MstC/HxtB (AN6669) were described as high affinity glucose transporters[31–33]. Additionally, two transporters, HxtC (AN10891) and HxtE (AN1797) have been identified as glucose transporters although it is currently not known with what affinity they transport glucose[32]. In addition to transporting glucose into the cell, these transporters (except for HxtA) are also able to carry other pentose and hexose sugars across the membrane[32,33]. Furthermore, HxtA was proposed to be important for sugar metabolism during *A. nidulans* sexual development[31], whereas deletion of *hxtB* caused a reduction in glucose consumption in the presence of low concentrations of glucose as well as increased resistance to the non-metabolisable glucose analogue 2-deoxyglucose (2DG)[32]. The aforementioned studies[32,33] therefore suggest that HxtB, HxtC, HxtD and HxtE are capable of transporting sugars other than glucose, into the cell whereas HxtA and HxtB may also be involved in fungal developmental processes and/or glucose metabolism as suggested by increased expression of HxtA::GFP during cleistothecia formation[32] and increased resistance of strain Δ*hxtB* to 2DG[33].

The aim of this work was therefore to study the transcriptional response of the filamentous fungus *A. nidulans* to high and low concentrations of glucose with the purpose of identifying novel factors involved in glucose sensing and metabolism. This study employed bioreactor-controlled chemostat cultivation, allowing for controlled culture conditions and reduced fluctuation in growth rate and extracellular glucose concentrations[34]. RNA-sequencing was performed on *A. nidulans* grown in low- and high-glucose chemostat conditions. The low-affinity glucose transporter MstC/HxtB was highly induced under glucose-limiting conditions and was shown to be involved in fungal developmental processes, cAMP production and PKA activity. Furthermore, the protein kinase PskA was found to be crucial for glucose uptake and metabolism under high and low glucose conditions.

Results

Transcriptional response of *A. nidulans* grown in glucose-limiting and glucose-abundant conditions. In order to investigate the transcriptional response of *A. nidulans* in low- and high-glucose conditions, RNAseq of the wild-type strain under continuous batch cultivation was carried out. The dilution rates (D) were set at 0.05 h^{-1} and 0.15 h^{-1} respectively, which corresponded to 25% and 75% of the maximum specific growth rate (μmax, see Materials and Methods). For the lower dilution rate (D = 0.05), the glucose-containing medium was slowly changed (5 l medium/20 h), allowing fungal biomass accumulation and establishing glucose-limiting conditions (~2 μM extracellular glucose), whereas at the higher dilution rate (D = 0.15), the medium was quickly changed (5 l medium/6.5 h) preventing biomass accumulation and allowing high glucose concentrations in the medium (~33 μM extracellular glucose) (Fig. 1a and b). The wild-type strain reached the steady state phase of growth after ~42 h and ~21 h at the lower and higher dilution rates respectively, as determined by measuring the dry weight of samples drawn at different time points from biological triplicates (Fig. 1a). RNAseq was performed

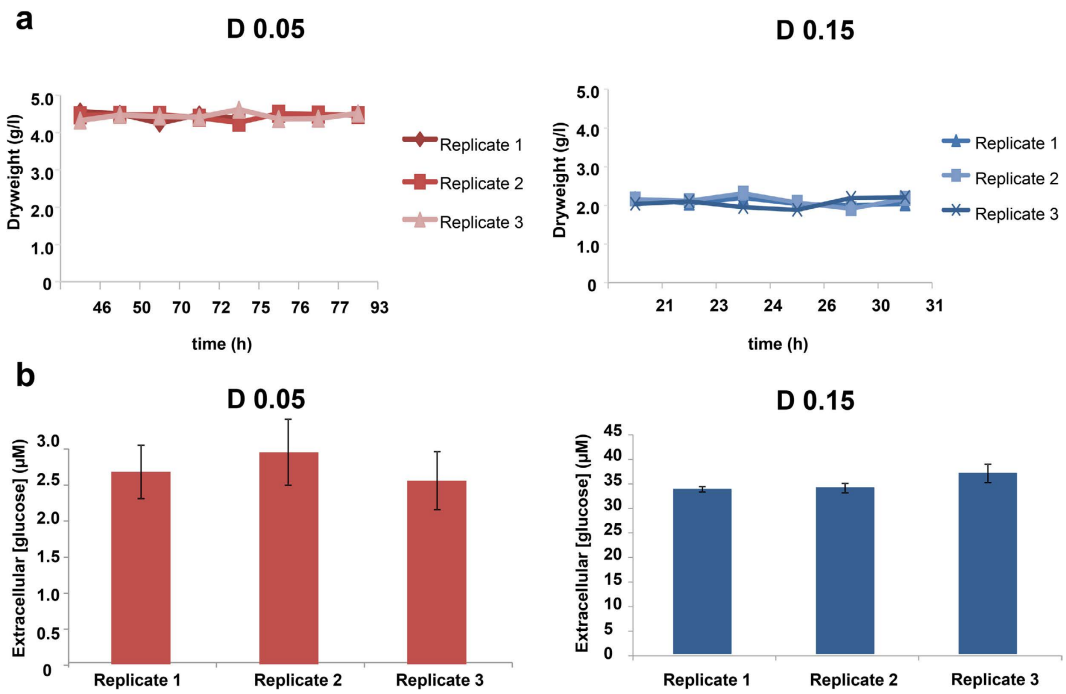

Figure 1. Steady-state growth of *A. nidulans* under low (D = 0.05) and high (0.15) glucose concentrations in chemostat cultures. (**a**) Fungal dryweight and (**b**) residual extracellular glucose concentrations were measured to confirm steady state, which was achieved after ~42 h under low glucose levels and after ~21 h in high concentrations of glucose. All experiments were performed in triplicate. Standard deviations present technical triplicates for each biological replicate.

on biological triplicates of the wild-type strain in the steady-state phase of growth in low- and high-glucose conditions (from 90–100 h for low and from 30–40 h for high-glucose).

A total of 3086 genes were identified as responding to differences in glucose availability by RNA-sequencing [Supplementary Table S1; False Discovery Rate (FDR) of <0.05]. In this set, 1265 genes had a strong effect, with a fold change of at least 2 times in either direction [log2 fold change <-1 and >1]. Of these, 554 genes were down-regulated (log2 fold change ≤ -1) and 711 genes were up-regulated (log2 fold change ≥ 1), taking as reference the low glucose condition (Fig. 2a and b). The RNAseq data was validated by performing qRT-PCR on 5 randomly chosen transcription factor-encoding genes (Supplementary Figure S1). All genes showed a similar expression pattern as was observed for the RNAseq data.

Gene ontology (GO) enrichment analysis (FetGOat) was performed on the set of 1265 significantly differently regulated genes with log2FC ≤ -1 or ≥ 1. Enrichment of terms involved in catabolic processes of various energy sources such as carboxylic and organic acids, amines, polysaccharides and amino acids was observed under low-glucose conditions. The term "sugar transmembrane transporter activity" was also significantly over-represented (Table 1). A more in-depth analysis of the significantly differently regulated genes showed that genes encoding proteins involved in the glyoxylate cycle (succinate semi-aldehyde dehydrogenase), genes involved in fatty acid synthesis and degradation, ammonium and amino acid uptake, glutamate and glutamine metabolism, aromatic amino acid metabolism, protease production and in the utilization of various intracellular storage compounds (trehalose, starch) as well as acetate metabolism and gluconeogenesis displayed increased expression under low-glucose conditions (Supplementary Table S1).

On the other hand, a transcriptional down-regulation of genes encoding proteins involved in monosaccharide catabolic processes, pyruvate and acetyl-coA metabolic and biosynthetic processes, gene expression, RNA processing and translation was observed in low-glucose conditions (Table 1). A more in depth analysis of these categories showed repression of the gene cluster required for the biosynthesis of the secondary metabolite asperidine A, for quinic acid utilization, fatty acid degradation, genes encoding kinases with roles in pyruvate metabolism, glycolysis and gluconeogenesis as well as amino acid and TCA cycle metabolism (Supplementary Table S1). Altogether, this data suggests that *A. nidulans* responds to low amounts of available glucose with the transcriptional down-regulation of glycolytic and protein biosynthetic genes paralleled by increased transcription of genes predicted to function in the degradation of alternative carbon sources and biosynthesis of secondary metabolites.

Interestingly, 40 genes encoding proteins required for the production of secondary metabolites showed altered expression (26 were up-regulated and 14 were down-regulated) (Supplementary Table S1). Five genes of the monodictyphenone (mdp) biosynthetic gene cluster were induced under low-glucose conditions. In addition, genes involved in the biosynthesis of asparthecin, aspyridone, derivative of benzaldehyde (dba), and sterigmatocystin, were also up-regulated (Supplementary Table S1). Asparthecin, aspyridone (cytotoxic), sterigmatocystin (carcinogenic) and mdp are polyketides synthesized from acyl-CoA units and are thought to provide *A. nidulans* with a competitive advantage in the soil; some have gained medical interest due to their cancer

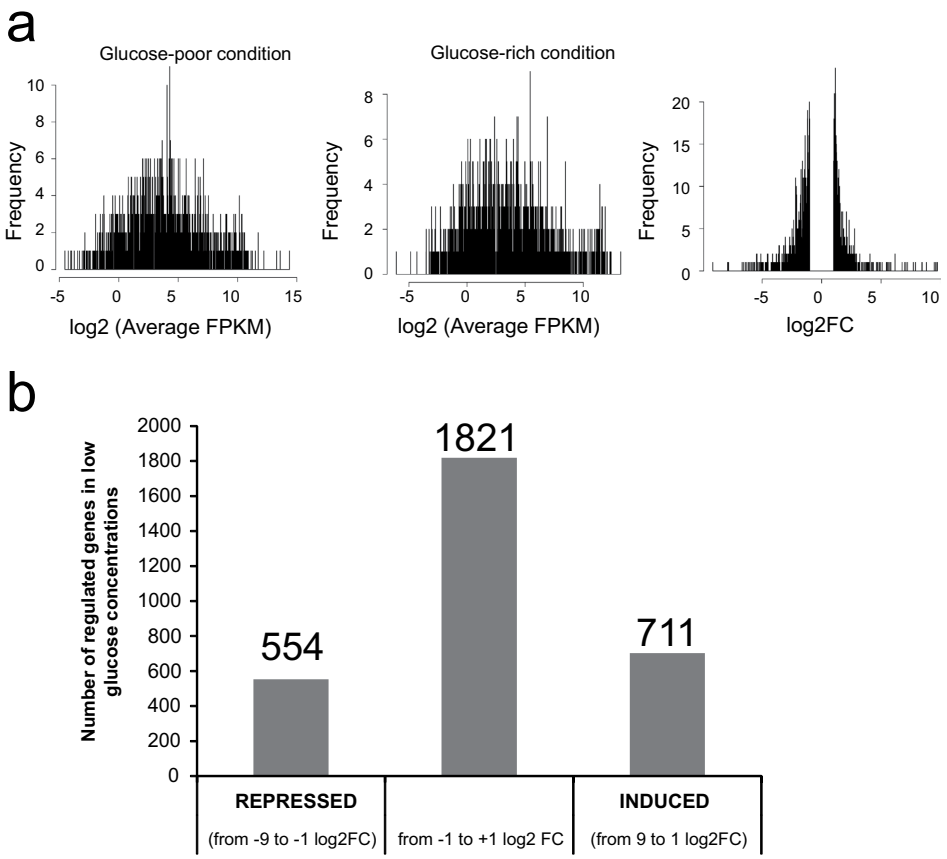

Figure 2. RNA-sequencing of *A. nidulans* when grown in chemostat cultures in the presence of low and high concentrations of glucose. (**a**) FPKM (fragments per kilobase of exon per million mappes reads) and subsequently calculated Log2-fold change (FC) of all the genes under low and high glucose conditions. (**b**) Number of genes which were significantly differently regulated ($-1 > \log2\text{-FC} > 1$) in glucose-limiting conditions.

preventive and anti-bacterial properties[35]. Furthermore, the gene encoding a polyketide synthase involved in the formation of the conidial green pigment was also significantly up-regulated under glucose-limiting conditions (Supplementary Table S1).

To further analyse the transcriptional response of *A. nidulans* to glucose, the RNAseq dataset comprising genes exclusively expressed (or exclusively induced) in either low-glucose or high-glucose conditions, e.g. which were condition-specific, were analysed. Considering the FPKM value higher than 1 in low or high glucose conditions ($p < 0.00005$), a total of 4 genes were specifically modulated in high glucose concentrations and 10 genes were specifically up-regulated in low glucose conditions (Supplementary Table S1). Interesting, genes encoding transporters, monooxygenases, proteins involved in secondary metabolism, methyltransferases as well as uncharacterized proteins were specifically up regulated in low-glucose conditions. Additionally, in high-glucose conditions, genes encoding a protein with a role in conidium formation, another encoding a putative alpha-1,3-glucanase (agnE), and two genes not yet characterized were also up-regulated.

The hexose transporter-encoding gene *hxtB* is highly up-regulated under glucose-limiting conditions. With our particular interest in glucose signaling and transport, the RNAseq data was mined for differential expression of genes encoding known or predicted transcription factors and transporters between low- and high-glucose conditions. A total of 14 putative transcription factor-encoding genes were differentially expressed under both conditions (Fig. 3a and b). Of these, 10 displayed increased and 4 displayed decreased expression under low-glucose conditions (Fig. 3a, Supplementary Table S1). Among the 10 up-regulated genes, three encode the previously, partially, characterized transcription factors MsnA (AN1652), AmyR (AN2016) and DbaG (AN7901) whereas the remaining six encode putative, uncharacterized transcription factors. MsnA was shown to be induced under high salt conditions and is thought to be targeted by the HOG (high osmolarity glycerol) pathway[36]. AmyR is involved in the induction of amylolytic genes required for starch utilization[37], whereas DbaG is a transcription factor that belongs to the dba secondary metabolite gene cluster (see previous results section). Of the four down-regulated genes in low-glucose conditions, two encode the transcription factors QutH (AN1139) and PbcR (AN1599) whereas the remaining two encode putative, uncharacterized transcription factors (Fig. 3a). QutH is a zinc finger-containing protein of the quinic acid utilization gene cluster, whereas PbcR is a transcription factor which activates the diterpene compound gene cluster responsible for the production of the secondary

GO term	Description	P-value	FDR	Ontology
Up-regulated under low glucose concentrations				
GO:0016054	organic acid catabolic process	0.000154	0.007646	BP
GO:0006725	cellular aromatic compound metabolic process	0.001114	0.036879	BP
GO:0046417	chorismate metabolic process	0.000576	0.023261	BP
GO:0019748	secondary metabolic process	4.68E-09	7.72E-07	BP
GO:0005975	carbohydrate metabolic process	4.49E-05	0.002871	BP
GO:0009063	cellular amino acid catabolic process	8.64E-06	0.000777	BP
GO:0043648	dicarboxylic acid metabolic process	7.73E-06	0.000765	BP
GO:0046395	carboxylic acid catabolic process	0.000154	0.007646	BP
GO:0016491	oxidoreductase activity	1.68E-07	6.14E-05	MF
GO:0051119	sugar transmembrane transporter activity	0.000312	0.045562	MF
Down-regulated under low glucose concentrations				
GO:0008152	metabolic process	0.001096	0.028103	BP
GO:0046365	monosaccharide catabolic process	0.001836	0.039959	BP
GO:0010467	gene expression	2.55E-08	2.97E-06	BP
GO:0071841	cellular component organization or biogenesis at cellular level	0.00096	0.026033	BP
GO:0034641	cellular nitrogen compound metabolic process	0.000842	0.024157	BP
GO:0006090	pyruvate metabolic process	0.001836	0.039959	BP
GO:0051169	nuclear transport	0.000587	0.019051	BP
GO:0006396	RNA processing	4.87E-12	8.04E-10	BP
GO:0070925	organelle assembly	0.000648	0.019982	BP
GO:0034660	ncRNA metabolic process	3.69E-16	7.32E-14	BP
GO:0006412	translation	1.61E-06	0.000114	BP
GO:0006084	acetyl-CoA metabolic process	0.002115	0.045024	BP

Table 1. FetGOat analysis of genes which were significantly differently regulated in low- and high-glucose conditions (FDR = false discovery rate, BP = biological process, MF = molecular function).

metabolite ent-pimara-8(14), 15-diene. Like the polyketides (see above), the terpenes are another group of secondary metabolites that are of particular interest due to their pharmaceutical properties[38]. The modulation of the same transcription factor-encoding genes is also shown in absolute FPKM (fragments per kilobase of exon per million mapped reads values) (Fig. 3b) and results are the same as presented in Fig. 3a.

A total of 89 transporter-encoding genes were significantly modulated in low-glucose conditions (Fig. 3c and d). Of these, 33 genes were down-regulated and 56 were up-regulated. Genes encoding the two transporters AtrA and MstE, presented the highest down-regulation (log2 fold-change of −9.47 and −7.89 respectively). The former is involved in multidrug resistance[39], whereas the latter has previously been characterized as a low affinity glucose transporter[40]. The transporter-encoding genes *hxtA*, *hxtD*/*mstA* and AN3915 (not yet characterized) presented the highest up-regulation among the 56 genes (Fig. 3c). HxtA and HxtD/MstA have previously been described as high affinity glucose transporters[31–33]. The expression of *hxtB* was the highest among all transporter-encoding genes in glucose-limiting conditions, when looking at the data in absolute FPKM values (Fig. 3d). Although *hxtB* was previously shown to be highly up-regulated in low glucose (0.1%) concentrations during batch cultivation[32], this work shows that it is also greatly induced during chemostat cultivation. In summary, the analysis presented here identified several putative, uncharacterised transcription factor and transporter-encoding genes.

HxtB is a low affinity glucose transporter and localizes to the plasma membrane in glucose starvation conditions. The RNAseq data presented here identified the transporter HxtB as up-regulated under low-glucose conditions, with the largest expression among all transporter-encoding genes. HxtB was previously identified as a xylose and glucose transporter with a possible role in glucose metabolism[31,38]. Deletion of *hxtB* in *A. nidulans* caused a reduced affinity for glucose and was therefore classified as a high affinity glucose transporter[32,33]. To further characterize the glucose affinity of HxtB, it was heterologously expressed in *S. cerevisiae* strain EBY.VW4000 as described previously[32]. Glucose uptake of the HxtB-expressing *S. cerevisiae* strain was measured and Km and Vmax values were derived (Supplementary Figure S2). HxtB was found to be a low affinity glucose transporter with a Km of ~15 mM which is similar to the Km (~10 mM) of the *S. cerevisiae* intermediate glucose transporter Hxt2[41]. To confirm HxtB expression, the protein was tagged with GFP. The *hxtB* gene was replaced with the *hxtB*::*gfp* fragment under the control of the native promoter and strain construction was confirmed by PCR before cellular localization of HxtB::GFP was microscopically assessed. In the presence of low concentrations of glucose (0.1% w/v) and when inoculated in media without any carbon source (starvation), a strong fluorescent signal of HxtB::GFP was observed in the hyphal membranes and septa (Fig. 4a). On the other hand, when the HxtB::GFP strain was inoculated in complete medium (2% w/v glucose), 1% w/v glucose and in the

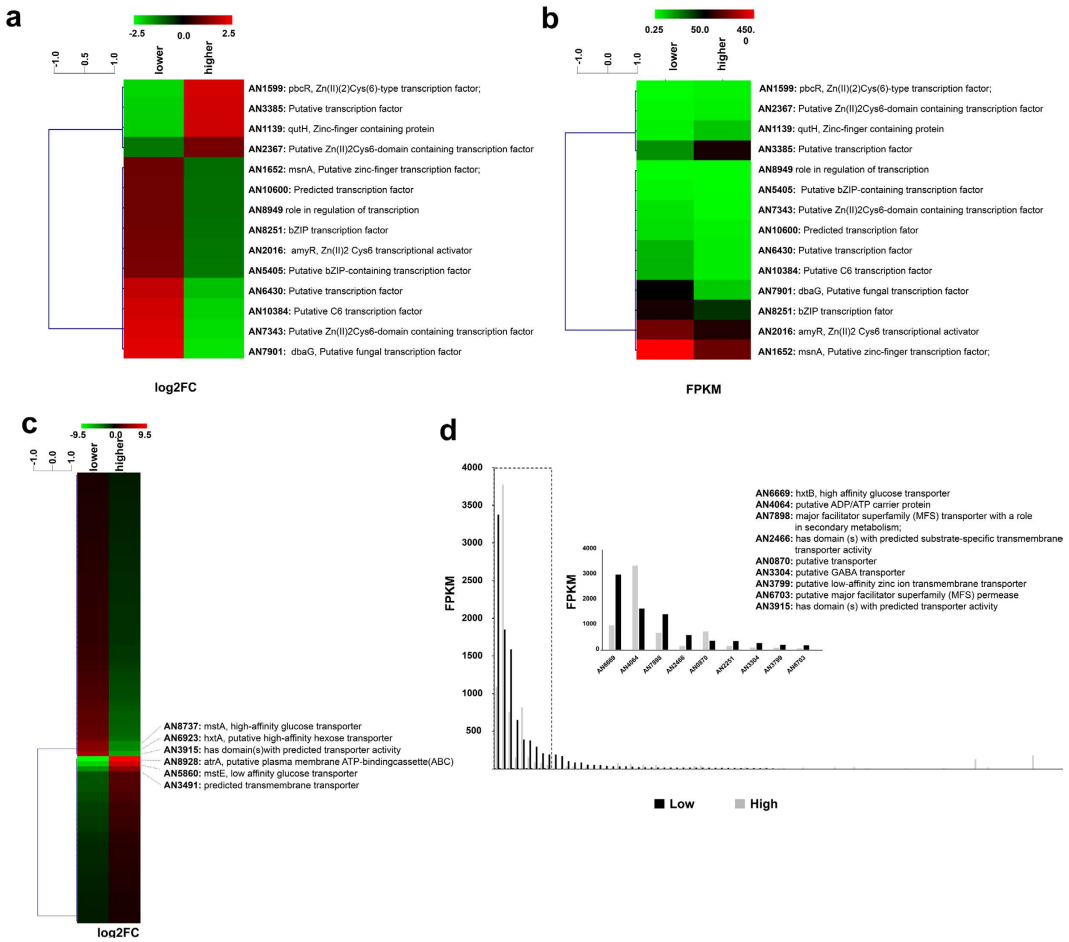

Figure 3. Heat maps of significantly differently regulated transcription factor and transporters. (**a**,**b**) transcription factor, (**c**,**d**) transporter encoding genes according to the Log2 fold-change (**a,c**) or FPKM (fragments per kilobase of exon per million mappes reads) (**b**,**d**) values.

presence of 1% w/v of the pentose sugar xylose, fluorescence was present but weak (Fig. 4b), indicating a reduced accumulation of HxtB::GFP in the fungal membranes in these conditions. A somewhat stronger fluorescence was also observed when hyphae were inoculated in the presence of the complex carbon source carboxymethylcellulose (CMC) (Fig. 4b). These results indicate HxtB is a low affinity glucose transporter which is strongly expressed under glucose-limiting conditions in agreement with previous studies[32,33].

Deletion of HxtB causes a hyperconidiation phenotype under continuous supply of low and high amounts of glucose. In order to further evaluate its glucose-transporting capabilities and any involvement in glucose metabolism, the Δ*hxtB* strain[32] was grown in low- and high-glucose conditions in chemostat cultures as described above for the wild-type strain. In contrast to the wild-type strain, the *hxtB* deletion mutant started to conidiate heavily, turning the bioreactor dark green after 24 h of continuous cultivation under low- (D0.05, ~2 μM glucose) and high glucose (D0.15, ~33 μM) concentrations conditions (data not shown). To confirm the hyperconidiating phenotype, samples were taken from both the wild-type and Δ*hxtB* chemostat cultures after 4 h at the high dilution rate (*i.e.*, high-glucose conditions) and analysed by microscopy (Fig. 4c). In addition, qRT-PCR of *brlA*, encoding a transcription factor involved in conidiophore development[14], was performed under the same conditions (Fig. 4d). Indeed, the Δ*hxtB* strain formed conidiophores after 4 h chemostat cultivation and the expression of *brlA* was almost 10 times higher in this strain than when compared to the wild-type strain (Fig. 4c and d).

Unfortunately, this hyperconidiating phenotype prevented reliable RNAseq analysis on the Δ*hxtB* strain when cultivated under chemostat conditions. Interestingly, we have not observed this conidiation phenotype during batch cultivation, where the Δ*hxtB* strain was grown for 32 h in 0.1% w/v glucose or for 72 h in 1% w/v glucose[32]. Taken together, these results indicate that HxtB potentially regulates the induction of asexual development in high and low-glucose conditions under chemostat cultivation in *A. nidulans*.

HxtB is involved in cAMP accumulation, protein kinase A activity and the Ras signaling pathway. The results described above suggest that HxtB may be involved in cellular signalling events that control fungal developmental processes. C^{14}-glucose transport experiments revealed a significant delay in glucose

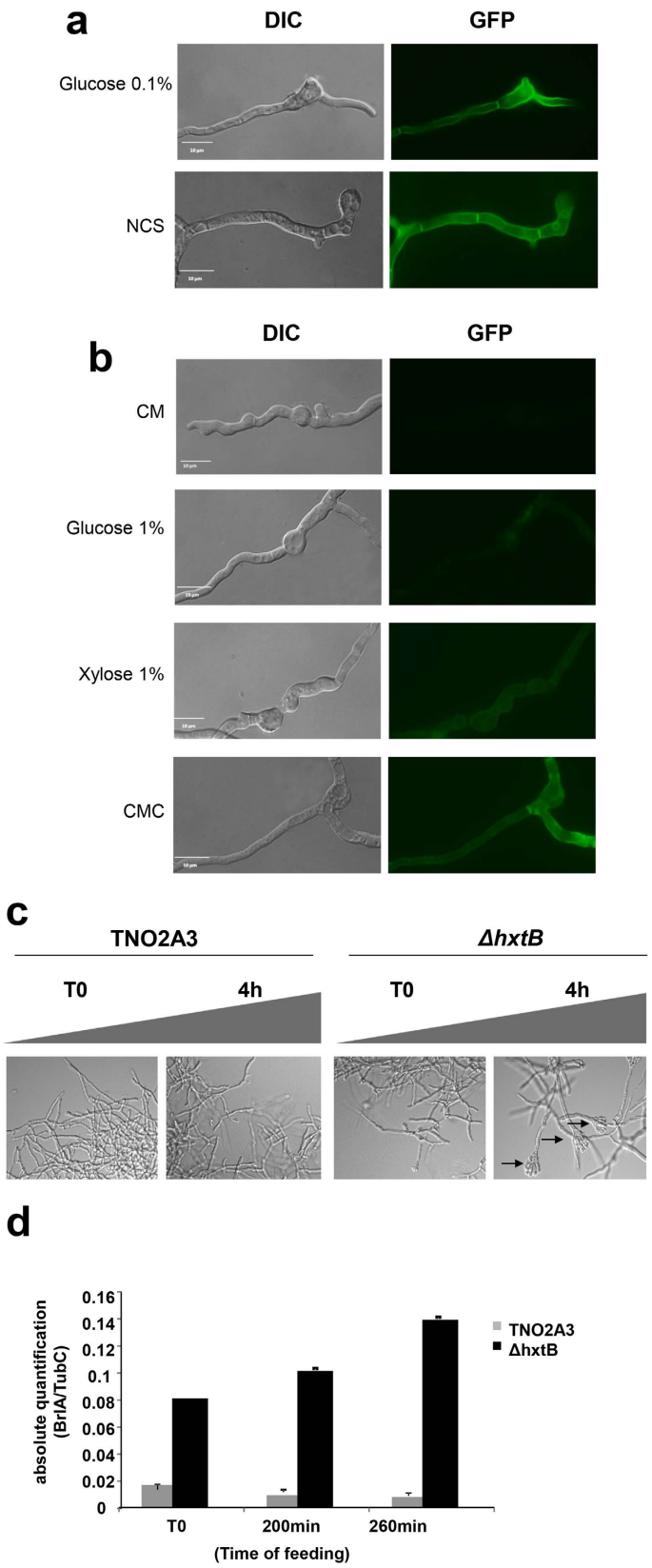

Figure 4. HxtB is involved in fungal developmental processes. (**a**,**b**) Microscopy of the HxtB::GFP strain in the presence of different glucose concentrations and carbon sources. (**c**) Microscopy of the wild-type and Δ*hxtB* strains after 4 h chemostat cultivation. Arrows indicate conidiophore structures. (**d**) Expression of the developmental regulator *brlA*, as determined by qRT-PCR, in the wild-type and Δ*hxtB* strains after 4 h chemostat cultivation.

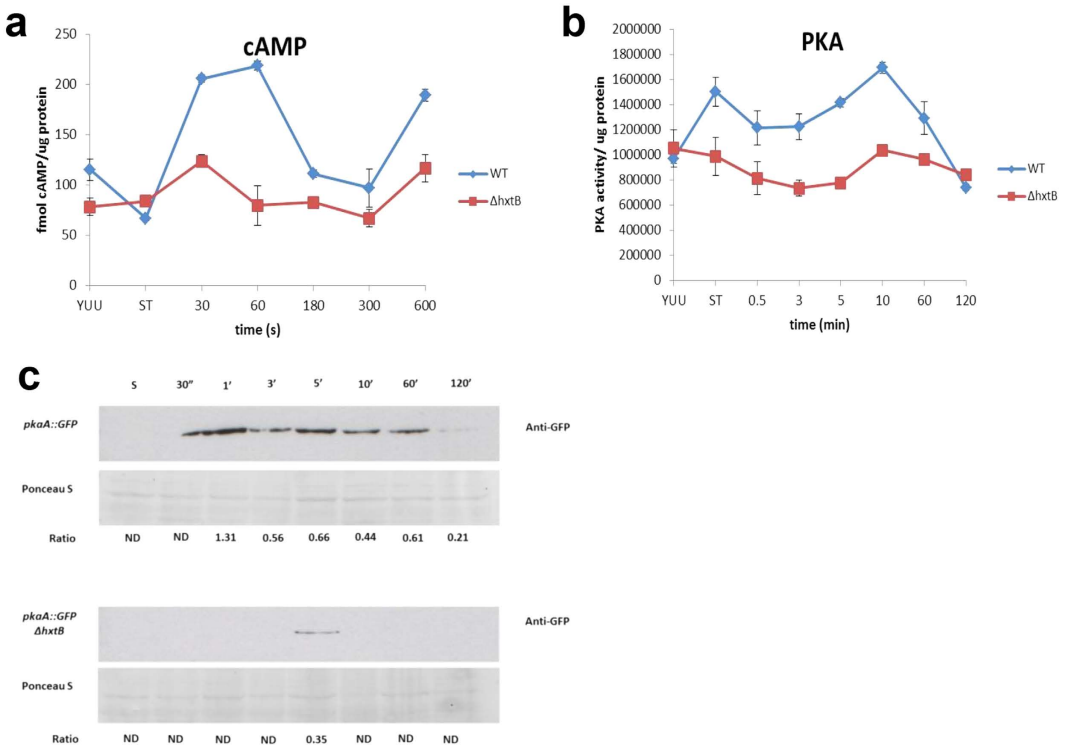

Figure 5. HxtB is involved in glucose signalling events. cAMP levels (**a**) and PKA (**b**) activities were measured in the wild-type and Δ*hxtB* strains when grown for 24 h in complete medium and after the addition of glucose for the indicated amounts of time. (**c**) Western blot of PkaA::GFP in the wild-type and Δ*hxtB* strains when grown in the same conditions as described above.

transport in the *hxtB* null mutant under high- and low-glucose concentrations, although eventually this strain consumed all extracellular glucose[32]. The increased conidiation phenotype of this mutant in high and low-glucose conditions suggests that HxtB could play a role in glucose signalling events. The cellular response to glucose sensing is mediated by the cAMP/PKA pathway[13,15]. Therefore, cAMP accumulation and PKA activity were determined in both the wild-type and the Δ*hxtB* strains. Both strains were grown overnight in complete medium before being transferred to minimal medium without any carbon source for 4 h. Glucose was then added to a final concentration of 2% w/v for different amounts of time before cAMP concentrations and PKA activities were measured (Fig. 5a and b). In the wild-type strain, addition of glucose caused an immediate increase of cAMP which thereafter oscillated between high and low amounts and which was absent in the Δ*hxtB* strain (Fig. 5a). Similarly, addition of glucose increased PKA activity in the wild-type strain but not in the Δ*hxtB* strain (Fig. 5b). In agreement, Western blot analysis of the PkaA::GFP (Supplementary Table S2) and the PkaA::GFP Δ*hxtB* (Supplementary Table S2) strains when grown in the same conditions as described for Fig. 5a and b, showed accumulation of PkaA after the addition of glucose in the wild-type strain but not in the Δ*hxtB* strain (Fig. 5c). These results indicate that HxtB influences glucose-related, downstream signalling events, although we cannot completely discard the possibility that a reduction of intracellular glucose concentrations due to a delay in glucose transport may also contribute to the observed phenotype.

An alternative pathway for increasing cellular cAMP concentrations is via Ras signalling[15]. RasA is a small GTPase involved in the control of fungal developmental processes such as spore swelling and germination, formation of vegetative and aerial hyphae and conidiation[42]. The RasA^{G17V} strain contains a mutation in the *rasA* gene (glycine to valine, position 17), which causes constitutive activation of RasA by locking it in its GTP-bound form[42]. Because *rasA*^{G17V} is under the control of the *alcA* promoter, cyclobutanone was used as a non-metabolisable, inducing compound[43]. In order to gather additional evidence for the involvement of HxtB in fungal developmental processes, the wild-type, RasA^{G17V}, Δ*hxtB* and RasA^{G17V} Δ*hxtB* strains were inoculated for 16 h in minimal medium without any carbon source and microscopically examined before the percentage of germinated spores was calculated for each strain. As expected, conidia of the wild-type strain had a very low germination rate (~15% germination) in no carbon source conditions. On the other hand, conidia of the RasA^{G17V} and Δ*hxtB* strains presented increased germination (~40% and ~55% respectively) when compared to the wild-type strain (Supplementary Figure S3B). The RasA^{G17V}Δ*hxtB* strain presented the highest percentage of germination (~70%), which suggests that HxtB and RasA function in parallel interacting pathways to regulate spore germination (Supplementary Figure S3B).

The protein kinase PskA is important for glucose uptake and metabolism. Protein kinases catalyse the phosphorylation of cellular proteins, therefore regulating the function, structure and/or localisation

of their target proteins[44]. They play an important role in the regulation of most cellular processes including signalling pathways. To further understand the response of *A. nidulans* to the presence of high- or low-glucose concentrations, the RNA dataset was screened (including not significantly differently regulated genes) for protein kinase-encoding genes. The PAS (Per-Arnt-Sim) domain-containing serine/threonine protein kinase PskA (AN4536) had a higher expression in low-glucose conditions (FPKM 13.8 low-glucose vs FPKM 9.0 high-glucose), although the difference was not statistically significant. In *S. cerevisiae*, there are two paralogues, termed *PSK1* and *PSK2* that arose from whole genome duplication. These two kinases are important for sugar metabolism, protein translation and glycogen biosynthesis, and thus play an important role in sugar consumption and storage and protein translation[45]. Likewise, PskA is predicted to be involved in the repression of glycogen synthesis and in (1–6)-beta-D-glucan biosynthesis in *A. nidulans*. Based on the aforementioned studies, we decided to characterize the effect of *pskA* deletion on growth, glucose uptake and intracellular glycogen and trehalose concentrations in *A. nidulans*.

Growth of the wild-type and the Δ*pskA* strains (Supplementary Table S2) were characterized when grown for 24 h and 48 h in minimal medium supplemented with 1% w/v glucose. Biomass accumulation was severely reduced in the Δ*pskA* strain when compared to the wild-type strain (Fig. 6a). Glucose uptake, as determined by measuring the residual glucose in the culture supernatants, was reduced in the Δ*pskA* mutant, suggesting difficulties with glucose transport (Fig. 6b). Furthermore, accumulation of intracellular glucose and glycogen (Fig. 6c and e) coupled with reduction in intracellular trehalose levels (Fig. 6d) in the Δ*pskA* strain imply that a general deregulation of glucose metabolism has occurred in the Δ*pskA* mutant. These results suggest an important role for PskA in glucose metabolism, including uptake and the generation of intracellular storage compounds.

Discussion

One of the drawbacks for the process of 2G biofuel production from plant lignocellulosic biomass is the inability of filamentous fungi to secrete key enzymes required for lignocellulose hydrolysis in the presence of glucose. Filamentous fungi such as *T. reesei* and *Aspergillus spp*., which are currently being investigated for hydrolytic enzyme production, prefer consuming simple, readily metabolized sugars such as glucose rather than alternative, more complex carbon sources such as those contained within lignocellulose; this preference is known as carbon catabolite repression (CCR) and is mediated by the transcription factor CRE1/CreA[6]. Although CRE1/CreA-mediated repression of genes encoding enzymes for lignocellulose degradation has been extensively studied[6–10], far less is known about how glucose is sensed and the corresponding downstream signalling events. The aim of this work was therefore to analyse the response of *A. nidulans* to low- and high-glucose conditions with the purpose to identify novel factors involved in glucose sensing and metabolism.

The genome-wide transcriptional response of *A. nidulans* when grown in the presence of low and high concentrations of glucose was assessed by RNA-sequencing (RNAseq). Continuous (chemostat) cultivation was performed which allowed for controlled culture conditions with reduced fluctuation in growth rate and extracellular glucose concentrations[33,46]. Furthermore, *A. nidulans* pellet formation was prevented allowing for homogenous, macroscopic morphology and glucose uptake throughout the culture. Continuous chemostat cultivation has been carried out for several filamentous fungi such as *A. niger*[34], *T. reesei*[47], *Penicillium chrysogenum*[48] and *N. crassa*[49] but until now has not been published for *A. nidulans*. This study shows that continuous batch and chemostat cultivation can also be applied to *A. nidulans*, which is considered an important 'reference' organism for studying various cellular processes in filamentous fungi.

In low-glucose conditions, there was an over-representation of Gene Ontology terms involved in catabolic processes of different, non-monosaccharide energy sources whereas pyruvate and acetyl-coA-related processes as well as protein synthesis were under-represented. These results indicate that the fungus is adapting to severe glucose limitation. In high-glucose conditions, genes encoding proteins involved in the same biological processes were modulated. Several transcription factor- and transporter-encoding genes that have not been characterized were identified as being significantly up-or down-regulated in low- and high-glucose conditions. These genes are potential targets for future detailed investigation of the transcription factors involved in cellular responses to starvation and/or glucose-abundant conditions as well as identifying additional sugar transporters that could be useful for biotechnological applications.

Furthermore, there was also a substantial regulation of genes encoding transcription factors and enzymes required for the biosynthesis of various secondary metabolites (SM). In particular, genes involved in polyketide synthesis, such as asparthecin, aspyridone, derivative of benzaldehyde (dba) and sterigmatocystin were induced whereas genes encoding proteins involved in aspernidine A, emericellamide, terpene biosynthesis and quinic acid utilization were down-regulated. An up-regulation of secondary metabolite production under carbon starvation conditions has been previously described in *A. nidulans*[50] and *A. niger*[51]. Nutrient limitation is accompanied by fungal developmental changes such as asexual structure formation and sporulation[25,51]. Polyketide synthases are essential for the production of secondary metabolites but have also been shown to be important for melanin production, a pigment pre-dominantly found in the conidia of *Aspergillus spp* and which protects against environmental stresses such as UV light and radiation[52–54]. Indeed, several polyketide synthase-encoding genes of different secondary metabolite clusters were up-regulated in low-glucose conditions in *A. nidulans*, including one polyketide synthase predicted to be involved in the formation of the conidial green pigment. The up-regulation of polyketide-type secondary metabolites under chemostat conditions could be due to the development of asexual structures. However, as we have not observed the formation of asexual structures during chemostat cultivation of wildtype *A. nidulans*, it is likely that the function of the produced secondary metabolites is linked to the survival of the fungus in a competitive, nutrient-limited environment as has been proposed previously[35].

One of the transporter-encoding genes which was up-regulated under chemostat carbon starvation conditions and whose expression level (FPKM) was highest among all transporter-encoding genes, was HxtB. HxtB

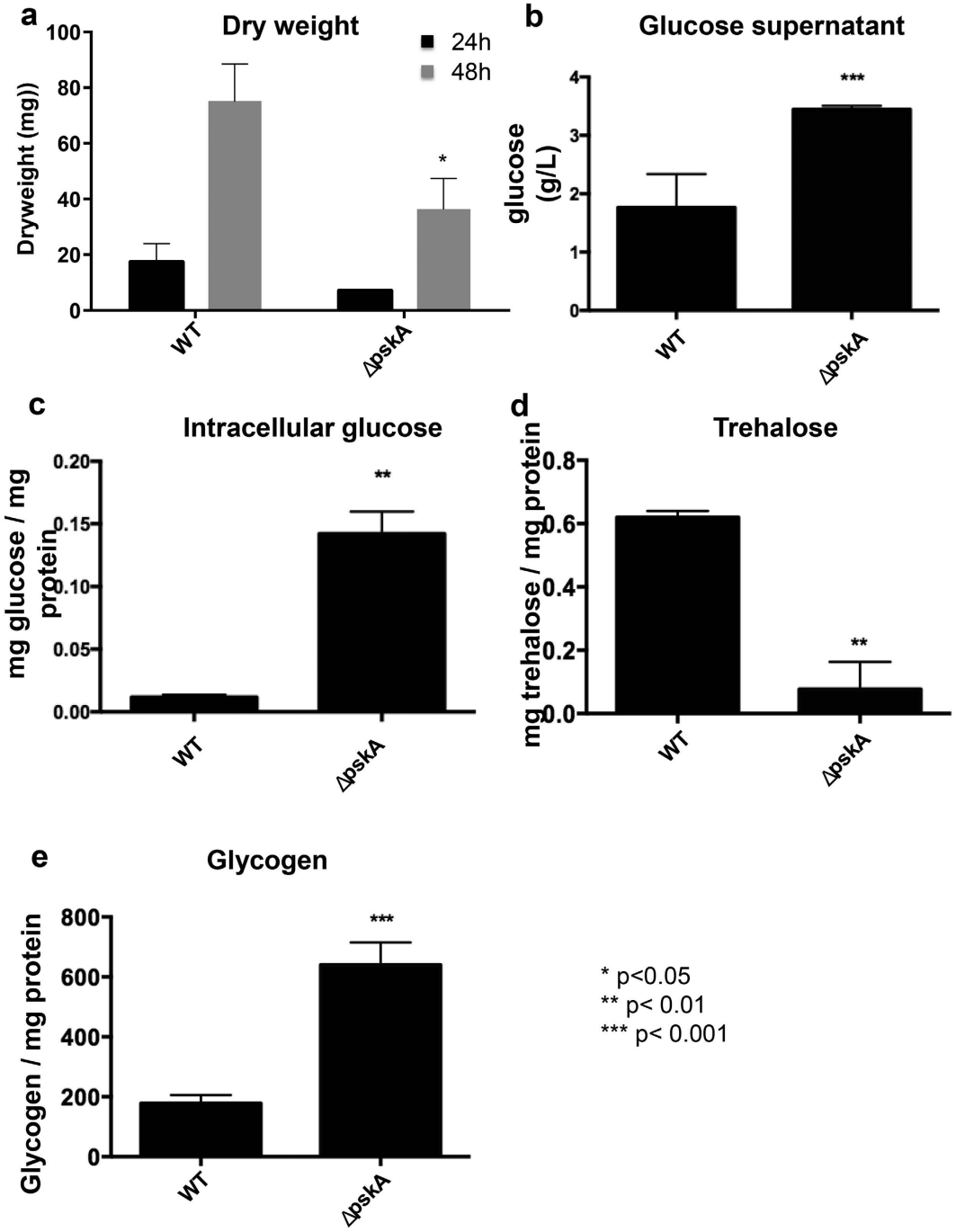

Figure 6. PskA is important for intracellular and extracellular glucose utilisation. Fungal dry weight (**a**), extracellular (**b**) and intracellular (**c**) glucose concentrations as well as intracellular trehalose (**d**) and glycogen levels (**e**) were measured in the wild-type and Δ*pskA* strains when grown for 24 h in complete medium and then transferred to minimal medium supplemented with glucose for 16 h.

was previously described as a high affinity glucose transporter[32,33] although heterologous expression in *S. cerevisiae* determined it to be a low affinity transporter. This discrepancy is likely due to the different systems used to characterize this transporter (e.g. deletion in a filamentous fungus vs. heterologous expression in a yeast). The expression of *hxtB* also increased during batch cultivation at low-glucose concentrations[32], suggesting that HxtB is important for growth in glucose-limiting concentrations during different cultivation conditions. HxtB was previously confirmed to be a glucose transporter that also accepts other monosaccharides, and deletion of *hxtB* resulted in delayed glucose uptake as well as increased resistance to the non-metabolizable glucose analogue 2-deoxy glucose (2DG)[32]. These results indicate that HxtB may be involved in cellular processes other than sugar transport. In agreement, this study found that deletion of *hxtB* caused hyperconidiation in the chemostat cultures under high and low glucose concentrations, a phenotype that was not observed during batch cultivation. One

important difference between both cultivation modes is that batch cultivation (irrespectively whether this is performed in shake flasks, bioreactors or during cultivation on agar plates) results in complete consumption of the initially added glucose and therefore adjusts carbon starvation after prolonged cultivation, whereas chemostat cultivations only limit the available glucose to a certain amount but provide the fungus with a continuous supply. It is therefore likely that the low amounts of glucose provided here during chemostat cultivation provide the necessary energy for increasing conidiation of the Δ*hxtB* strain. The deletion of *hxtB* was paralleled by an increased mRNA accumulation of the transcription factor *brlA*, which has been shown to play a central role in conidiophore development[14,55]. This is in contrast to a previous study where the expression of *hxtB* was found to decrease during sexual and asexual development[32]. This discrepancy may be due to the differences in the experimental setup (chemostat cultivation vs. solid media incubation). In summary, these results suggest a role for HxtB in asexual developmental processes during continuous chemostat cultivation in high- and low-glucose conditions.

In addition, the deletion of *hxtB* caused reduced cAMP levels and PKA activity suggesting a role for this transporter in downstream glucose signalling events, although it cannot be excluded that this may be due to delayed glucose uptake under high- and low-glucose conditions, as was previously shown for this strain[32]. The Δ*hxtB* mutant is able to transport glucose[32] and it is currently not known whether a certain threshold of intracellular glucose concentration triggers a spike in cAMP levels and PKA activity. Therefore, further studies are required to confirm/reject a role of HxtB in glucose sensing and signaling. In *T. reesei* and *N. crassa*, several transporters have been identified to play a role in cellulose sensing and to be important for regulating downstream signaling events that lead to proper utilization of this carbon source[21,22]. Studies on glucose sensing and subsequent activation of downstream targets are very limited in *Aspergillus spp*. but it is possible that, like in *T. reesei* and *N. crassa*, carbon source transporters can exert a sensory and regulatory function for the utilization of glucose. Furthermore, HxtB was also shown to be involved in RasA-mediated glucose signaling. Still, the molecular mechanism by which HxtB regulates cAMP levels and RasA activity remains subject to future studies.

This study also identified the protein kinase encoded by AN4536 as being important for glucose metabolism. We chose to name this protein kinase PskA due to its homologue Psk1p of *S. cerevisiae*. A pseudo-kinase has also been previously named PskA[56]. Although the two homologues of PskA have been characterized in *S. cerevisiae*[45], direct evidence for a role of PskA in glucose metabolism in *A. nidulans* is absent. This study found that *pskA* was expressed in both high- and low-glucose chemostat conditions. Furthermore, *pskA* was shown to be important for glucose uptake as well as the correct accumulation and utilization of intracellular storage compounds such as glycogen and trehalose. These results indicate that, like in *S. cerevisiae*, PskA may have a similar function in the control of sugar flux and metabolism in *A. nidulans*. PskA is therefore an interesting target for future carbon metabolism-related studies focused on elucidating cellular signalling pathways that could potentially be manipulated for biotechnological applications.

In conclusion, this study presents a detailed analysis of the genome-wide transcriptional response of *A. nidulans* to low- and high-glucose conditions using continuous chemostat cultivation. Several transcription factor- and sugar transporter-encoding genes were identified as being important for this response, representing novel, potential targets for future studies. The glucose and xylose transporter HxtB was shown to be important for downstream glucose signalling events and fungal developmental processes that suggest a transceptor-like role for this protein. Furthermore, the protein kinase PskA was also identified as being important for glucose-metabolism related processes. This study therefore lays a basis for further investigation of how biotechnologically important filamentous fungi respond to glucose, a carbon source which represses the production of industrially relevant enzymes from these organisms.

Methods

Strains and growth conditions. All strains (Supplementary Table S2) were grown at 37 °C (except where stated) in minimal medium or complete medium with (solid) or without (liquid) 2% w/v agar as previously described[32]. When necessary, uridine/uracil (1.2 g/L each) and/or pyridoxine (0.5 μg/mL) were added. For the batch and chemostat cultures, *A. nidulans* was grown for 3 days at 30 °C on plates containing minimal medium supplemented with glucose. Conidia were harvested with a 0.9% (w/v) NaCl solution and washed twice in the same solution. Batch and chemostat cultivations were performed using Ammonium Minimal Medium (4.5 g/L NH_4Cl, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4 \times 7H_2O$, 1 ml trace metal solution, 160 mg uridine, pH 3.0).

All fungal biomass was separated from the culture medium by vacuum filtration and snap-frozen in liquid N_2. The biomass was subsequently used for RNA/DNA extractions, biochemical assays or freeze-dried and weighed to determine the dry weight.

Batch and chemostat cultures. Chemostat cultures were performed in the BioFlo310 Fermentor/Bioreactor (6.6 l, New Brunswick Scientific, NJ, USA) as described previously with modifications[51]. First, batch cultivation of the *A. nidulans* FGSC A4 strain was carried out in 5 kg ammonium-supplemented minimal media which contained 1% w/v glucose and 0.003% w/v yeast extract. A total of 10^9 spores/L was inoculated and left to germinate for 8 h at 30 °C, 250 rpm whilst keeping the pH at 3.0. Polypropyleneglycol (PPG) was then added as an antifoam agent. The agitation was increased to 600 rpm before 20 g of NaOH were added, increasing the pH gradually from 3.0 to 5.5 over a time period of 4 h. The chemostat cultivation, using the same growth medium, was then started in the late-exponential growth phase of the fungus. The dilution rates (D) were set at 0.05 h^{-1} (lower) and 0.15 h^{-1} (higher). NaOH addition (to prevent acidification of the medium by the fungus) and CO_2 production data were used to define the maximum specific growth rate (μmax) which was 0.20 h^{-1} for the wild-type strain (data not shown). Steady state of growth was reached after ~45 h for the lower dilution rate and ~20 h for the higher dilution rate. Steady state [specific growth rate (μ) is equal to the dilution rate] was determined by monitoring CO_2 production, O_2 consumption, glucose consumption and by the addition of NaOH. All experiments were carried out in triplicate.

Determination of extracellular and intracellular glucose concentrations. Residual glucose in the supernatants of the chemostat cultures was determined enzymatically using the hexokinase and glucokinase method as previously described[57].

Intracellular and extracellular glucose concentrations in non-chemostat cultures were measured using the Glucose GOD-PAP Liquid Stable Mono-reagent kit (LaborLab Laboratories Ltd. Guarulhos, São Paulo, Brazil), according to the manufacturer's instructions. For the determination of intracellular glucose concentrations, whole cell extracts were generated by re-suspending mycelial powder in 1 mL of extraction buffer [50 mM Tris base pH 7.0, 50 mM NaF, 1 mM $NaVO_3$, 1 mM DTT, phosphatase inhibitor cocktail P0044 (Sigma) and an EDTA-free protease inhibitor cocktail (Roche)], followed by centrifugation and removal of the glucose-containing supernatants.

RNA extraction and qRT-PCR. Total cellular RNA was extracted using TRIZOL (Invitrogen) according to manufacturer's instructions. DNA was degraded with DNAse (Promega) according to manufacturer's instructions and RNA was subsequently purified using the RNeasy® Mini Kit (Qiagen), according to manufacturer's instructions. The quality of the RNA was verified using the Agilent Bioanalyser 2100 (Agilent technologies) and a RIM value of 8.0 as the RNA quality threshold. cDNA was synthesized from RNA using the ImProm-II™ Reverse Transcription System (Promega) according to manufacturer's instructions. qRT-PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Green PCR Master Mix kit (Applied Biosystems), according to manufacturer's instructions. Reactions and calculations were performed as previously described[58] and gene expression were normalised by the expression of *tubC*. Primer sequences used in this study are listed in Supplementary Table S3.

Library preparation and RNA sequencing. RNA was extracted from 100 mg of ground mycelial powder of the wild-type strain, when grown in biological triplicates in chemostat cultures during the steady state phase of growth, using the Qiagen RNeasy minikit according to the manufacturer's instructions. RNA quality was checked as described above.

RNA-seq libraries were prepared using Illumina TruSeq RNA library Prep kit v2 kit, which uses polyA-based mRNA enrichment. Sequencing was carried out in a HiSeq2000 using paired-end (2×50 bp) chemistry. Each condition was evaluated with three biological replicates. Initial quality check was carried out with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by cleaning step in Trimmomatic v.0.32[59]. Reads were mapped using Bowtie 2[60] and Tophat2[61] against the *Aspergillus nidulans* genome assembly version s10-m03-r07 (release on 02-Mar-2014, available in the AspGD). Saturation of known splice junctions was verified using the junction_saturation function from the RseQC package[62], in order to check for proper sequencing depth. Transcriptome analysis was carried out using Cufflinks v.2.1 as follows: transcript assembly and quantification for each library was performed with Cufflinks[63], followed by generation of a master transcriptome assembly by merging all libraries with cuffmerge. Transcript differential expression (DE) tests were carried using cuffdiff. Differentially expressed transcripts with q-values < 0.05 (adjusted p-values) were considered significant. The R package CummeRbund (http://compbio.mit.edu/cummeRbund/) was used for DE quality assessment (e.g. identification of outlier replicates). The RNAseq data was submitted as raw fastq files to the NCBI Short Read Archive (SRA) SRP090936, BioSamples SAMN05876028 (low glucose) and SAMN05876029 (high glucose) associated with BioProject PRJNA345604 (http://www.ncbi.nlm.nih.gov/bioproject/345604).

Trehalose assay. Trehalose concentrations were determined in 10 μg of total extracted cellular protein as described previously[64]. Mycelia were grown for 24 h in 50 ml complete medium or after transfer to minimal media supplemented with 1% glucose for an additional 16 h.

Glycogen assay. Mycelial glycogen concentrations were measured as previously described by ref. 65 with modifications. Mycelial powder was re-suspended in 1 mL of 80% ethanol and incubated for 1 min at 37 °C. Samples were centrifuged for 5 min at 14.000 g, before the pellets were re-suspended in 1 mL of 0.25 M Na_2CO_3. Samples were incubated for 90 min at 100 °C and then cooled down to room temperature. 200 μL of each sample was mixed with 50 μL of 3 M acetic acid and then with 700 μL of 0.2 M acetic acid pH 4.8. The glycogen within the samples was de-branched by incubating it with 10 μL of amyloglucosidase 75U solution (Sigma A7420) at 37 °C for 20 h. Reactions were stopped at 95 °C for 5 min and samples were precipitated by centrifugation at 14.000 g for 5 min at 4 °C. 20 μL were then used to measure the amount of free glucose using the Glucose GOD-PAP Liquid Stable Mono-reagent kit, according to manufacturer's instructions.

PKA activity and cAMP concentration. A total of 1×10^7 conidia were inoculated in complete media for 16 h, 37 °C, 180 rpm before being transferred to minimal media containing no carbon source for 4 h in the same conditions. Glucose was then added to a final concentration of 2% (w/v) for the indicated amounts of time. Cellular protein extracts were prepared according to ref. 20. A Bradford Assay was carried out to quantify the protein concentration in the samples (Bio-Rad, according to manufacturer's instructions). cAMP concentrations and PKA activities were measured in the cell extracts using the Amersham cAMP Biotrak EIA system assay (GE Healthcare) and the Peptag cAMP dependent PKA activity assay (Promega) according to manufacturer's instructions. The phosphorylated substrate was then run on a 1% w/v agarose gel and the intensity of the band was quantified using ImageJ. All samples were normalized by intracellular protein concentrations.

Western blotting. Mycelia were grown in the specified conditions and ground to a fine powder under liquid N_2 before being mixed with extraction buffer [50 mM Tris–HCl pH 7.0, 50 mM NaF, 1 mM $NaVO_3$, 1 mM DTT, phosphatase inhibitor cocktail P0044 (Sigma) and the complete mini EDTA-free protease inhibitor cocktail (Roche)]. Samples were centrifuged for 5 min at 14000 × *g* and the protein concentration in the supernatant

was determined as described above. 50 μg of total protein were run on pre-made gels, before being transferred to a membrane as described previously[10]. Membrane blocking and washes, primary and secondary antibody incubation as well as membrane signal detection were carried out as described previously[10]. The primary rabbit polyclonal IgG antibody anti-GFP (Abcam #ab290) was used in a 1:10000 dilution whereas a 1:5000 dilution was used for the secondary anti-rabbit IgG HRP linked antibody (Cell Signaling Technology, Beverly).

Microscopy studies. For fluorescent microscopy, 1×10^5 conidia were inoculated in 3 ml minimal medium supplemented with the respective carbon source, in small petri dishes containing a glass cover slip. Conidia were left to germinate overnight at 25 °C before coverslips were washed with 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and either viewed under the microscope or transferred to minimal medium without any carbon source. All slides were viewed with a Carl Zeiss (Jena, Germany) microscope, equipped with a 100 W HBO mercury lamp epifluorescence module, using the 100x magnification oil immersion objective lens (EC Plan-Neofluar, NA 1.3). Phase contrast brightfield (DIC) and fluorescent images (GFP, DAPI) were taken with an AxioCam camera (Carl Zeiss), and images were processed using the AxioVision software version 3.1 and saved as TIFF files. Further processing was performed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, CA).

To determine the germination capacity of the wild-type, *rasA*^{G17V}, *ΔhxtB* and *rasA*^{G17V} *ΔhxtB* strains, 1×10^5 conidia were inoculated into small petri dishes containing sterile coverslips and 3 ml no carbon source minimal medium supplemented with 10 μM cyclobutanone. Cells were incubated at 37 °C for 14 h before being viewed under the microscope. A minimum of 100 germinated and non-germinated conidia was counted. The experiment was carried out in triplicate.

***S. cerevisiae* glucose uptake assay.** The glucose uptake of the HxtB-expressing *S. cerevisiae* strain[32] was assessed by the incorporation of D-[U-^{14}C]glucose [289.0 mCi/mmol (10.693 GBq)/mmol]; Perkin Elmer Life Sciences] in different D-glucose concentration. Briefly, 500 ml of SC-Trp medium supplemented with glucose was inoculated at 30 °C with the EBY.WV4000 strain harbouring the *hxtB* gene[32]. Culture started from an initial OD_{600} 0.1 and were grown until reach $OD_{600} \sim 0.6$. Cells were harvested by centrifugation (4000 rpm), washed twice with 50 mL ice-cold water and resuspended in 1250 μL of cold water. A total of 400 μL of cells was then diluted in 800 μL of water and aliquots of 40 μL of this cellular suspension were transferred to 1.5 ml tubes and incubated at 30 °C for 5 min for temperature equilibration. After this period, 10 μl of water containing D-glucose [0.1–40 mM] plus 0.2 μCi of ^{14}C-glucose were added and the uptake was allowed for 10 seconds. After the incubation period, reaction was stopped by vigorous quenching with 1.5 ml ice-cold water. Cells were immediately harvested by vacuum filtration through nitrocellulose filters and washed two times with 1.5 ml ice-cold water. The nitrocellulose filters containing the yeast cells were transferred to 3 ml of ScintiSafeTM Econo1 scintillation liquid (Fisher Scientific), and the D-[U-^{14}C] glucose taken up by cells was measured using Tri-Carb® 2100TR Liquid Scintillation Counter.

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Author Contributions

T.F.R., B.M.N., P.B.A.L., L.J.A. and L.M. carried out the experiments. R.A.C.S. and D.M.P.R. carried out the RNASeq data analysis. T.F.R., V.M. and G.H.G. helped in designing the experiments. T.F.R., S.D.H., L.N.A.R. and G.H.G. wrote the manuscript.

Additional Information

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