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Rafael Barros de Souza

Analise fisiológica e transcriptômica de *Saccharomyces cerevisiae* em resposta celular à composição mineral da cana de açúcar

RECIFE, 2016

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Tese apresentada à coordenação do programa de Pós-graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos à obtenção do grau de doutorado em Ciências Biológicas, área de concentração: Biotecnologia.

Prof. Dr. Marcos Antonio de Morais Junior
Orientador
Prof. Dr. Will de Barros Pita
Co-orientador

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Catalogação na fonte
Elaine Barroso
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Souza, Rafael Barros de

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BANCA EXAMINADORA:

_____

Prof. Dr. Roberto Dias Lins Neto

Departamento de Virologia – Centro de Pesquisa Aggeu Magalhães – FIOCRUZ

_____

Profa. Dra. Fernanda Cristina Bezerra Leite

Departamento de Biologia - UFRPE

_____

Prof. Dr. Antônio Carlos de freitas

Departamento de Genética - UFPE

_____

Prof. Dr. Tercílio Calsa Junior

Departamento de Genética – UFPE

_____

Prof. Dr. Marcos Antonio de Morais Junior

Departamento de Genética - UFPE

RECIFE, 2016

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RESUMO

Neste trabalho analisamos o efeito da composição mineral do caldo de cana sobre a biologia da levedura *Saccharomyces cerevisiae* relacionado com os principais aspectos metabólicos da produção de etanol combustível. No primeiro capítulo avaliamos a composição mineral global de diferentes amostras de caldo de cana fornecidos por diferentes fornecedores de cana e destilarias da região Nordeste do Brasil. Os efeitos desses minerais em conjunto e individualmente foram analisados em ensaios fermentativos que revelaram a ação indutora do magnésio e a ação inibidora do cobre sobre a fisiologia da levedura. Os dados indicaram a reversão do efeito do cobre pela presença de magnésio nas amostras industriais. Como aplicação, propusemos a adição pelas destilarias de magnésio na concentração de 600 mg L^{-1} durante a fermentação industrial. No segundo capítulo determinamos o efeito positivo do magnésio sobre os aspectos fisiológicos, como o aumento na produção de etanol e na resistência a diferentes formas de estresse do processo industrial, e sobre a expressão gênica com elevação na expressão dos genes relacionados com o metabolismo energético e de carboidratos, e de resposta a estresse. Além disso, comprovamos a supressão sobre o efeito tóxico do cobre. No terceiro capítulo analisamos o efeito tóxico do cobre sobre a fisiologia da levedura, como a diminuição da taxa de crescimento celular e produção de etanol. O padrão de expressão gênica mostrou que o cobre causa danos na estrutura do DNA. Os mecanismos de proteção da célula envolvem basicamente a inibição da síntese proteica para restauração da integridade do material genético.

Palavras chaves: Composição mineral. Caldo de cana-de-açúcar. Rendimento. Magnésio. Cobre.

ABSTRACT

In this work, we analyze the effect of the mineral composition of the sugarcane juice on the biology of the yeast *Saccharomyces cerevisiae* related to the main metabolic aspects of the ethanol fuel production. In the first chapter we evaluated the global mineral composition of different sugarcane juice samples supplied by different sugarcane suppliers and distillery in the Northeast region of Brazil. The effects of these minerals together and individually were analyzed in fermentative trials that revealed the action of magnesium and the inhibitory action of copper on the physiology of yeast. The data indicated the reversion of the effect of copper by the presence of magnesium in industrial samples. As an application, we proposed the addition of 600 mg L^{-1} magnesium distillates during industrial fermentation. In the second chapter we determined the positive effect of magnesium on the physiological aspects, such as the increase in ethanol production and the resistance to different forms of industrial process stress, and on the gene expression with elevation in the expression of genes related to energy metabolism and carbohydrates, and stress response. In addition, we have proven suppression of the toxic effect of copper. In the third chapter we analyze the toxic effect of copper on the physiology of yeast, such as the decrease in cell growth rate and ethanol production. The gene expression pattern showed that copper causes damage to the DNA structure. Cell protection mechanisms basically involve the inhibition of protein synthesis to restore the integrity of the genetic material.

Keyword: Mineral composition. Sugarcane juice. Yield. Magnesium. Copper

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group A (normal mineral) or group B (high mineral), as defined in the legend of Fig. 4. Synthetic medium without mineral supplementation was used as reference.

Figure 5. Fermentation kinetics of synthetic reference medium (a) and supplemented media with magnesium (b) and copper (c). All these fermentations were carried out with the industrial yeast JP1 (*S. cerevisiae*). Ethanol (white square) and glycerol produced (white diamond), as well as consumption of sucrose (black diamond), glucose (black square) and fructose (black triangle). Results represent the average value of four biological replicates.

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Figure 1. Fig. 1 Effect of Mg^{II} supplementation on *S. cerevisiae* JP1 strain growth containing ethanol or glycerol (square symbol) were supplemented with symbol) was used as the reference using ethanol (panel A) or glycerol (panel B) as a carbon source. YP media Mg^{II} at 500 mg L^{-1} (triangle symbol). Medium containing glucose (diamond

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Figure 3. Effect of Mg^{II} (500 mg L^{-1}) and/or Cu^{II} (15 mg L^{-1}) supplementation on the efficiency of ethanol production by the industrial *S. cerevisiae* JP1 strain using sugarcane juice (SCJ) or sugarcane molasses (SCM) as industrial substrates. (*) values show statistical differences ($p < 0.05$).

Figure 4. Effect of Mg^{II} supplementation on the growth inhibitory effect of sulphuric acid in the *S. cerevisiae* JP1 strain. The YPD medium was adjusted to pH 2.5 with sulphuric acid in the absence (square symbol) or in the presence of Mg^{II} (500 mg L^{-1}) (triangle symbol). Unadjusted YPD medium (diamond symbol) was used as the reference.

Figure 5. Effect of Mg^{II} supplementation on the growth repressive effect of 2-deoxyglucose (2DG) in the *S. cerevisiae* JP1 strain. YNB medium with glucose (20 g L^{-1}) (diamond symbol) was supplemented with 2-DG (1 mmol L^{-1}) in the absence (square symbol) or in the presence of Mg^{II} (500 mg L^{-1}) (triangle symbol).

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Figure 1. Effect of copper concentration on yeast growth. The reference medium (diamond symbol) was supplemented with copper at 5 mg L^{-1} (triangle symbol), 10 mg L^{-1} (square symbol) and 15 mg L^{-1} (circle symbol). The assay was carried out in triplicate.

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LISTA DE ABREVIATURAS

Al^{3+}	Alumínio trivalente
ATP	Trifosfato de adenosina
Ca^{2+}	Cálcio bivalente
CETENE	Centro de tecnologias estratégicas do Nordeste
Cu^{2+}	Cobre bivalente
Cy3	Cianina 3
Cy5	Cianina 5
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-acético (Ethylenediamine tetraacetic acid)
Fe^{2+}	Ferro bivalente
HPLC	Cromatografia líquida de alta pressão (High performance liquid chromatography)
K^{+}	Potássio
Kcal	Quilocaloria
Mg^{2+}	Magnésio bivalente
Mn^{2+}	Manganês bivalente
mRNA	Ácido ribonucleico mensageiro
NADH	Nicotinamida adenina de nucleotídeo reduzida
NEM	Núcleo de Engenharia Metabólica
OD	Densidade óptica (Optic density)
P	Fósforo
pH	Potencial Hidrogeniônico
PROÁLCOOL	Plano Nacional do Álcool
RNA	Ácido ribonucleico
RT-qPCR	Transcriptase Reversa – Reação em cadeia da polimerase quantitativa
SAGE	Análise serial da expressão do gene
SGD	Saccharomyces Genome Database
GO	Ontologia gênica
YNB	Nitrogênio levedura base (Yeast nitrogen base)
YPD	Levedura Peptona e Dextrose

SUMÁRIO

1. INTRODUÇÃO GERAL

Na produção de etanol combustível em destilarias brasileiras, a etapa de fermentação é crucial para a eficiência industrial devido principalmente à susceptibilidade de contaminação por bactérias e leveduras selvagens, uma vez que não há esterilização do caldo de cana de açúcar. Esses microrganismos contaminantes podem competir pelos nutrientes do meio e consumir o açúcar do substrato produzindo etanol com uma baixa produtividade, bem como, podem produzir outros compostos como ácido lático e ácido acético, causando uma queda significativa no rendimento industrial (Silva-Filho et al 2005a; Basso et al 2011).

Na tentativa de se explicar diminuição no rendimento industrial de algumas destilarias do nordeste brasileiro, o grupo do núcleo de engenharia metabólica da UFPE (NEM) decidiu analisar duas variáveis importantes na produção de álcool combustível, a massa de célula de levedura presente nas dornas de fermentação e o mosto de alimentação (caldo de cana de açúcar diluído). Através dos ensaios de fermentação e de determinações dos minerais no caldo de cana de açúcar foi possível observar a existência de uma correlação entre a queda do rendimento em etanol e a "fermentabilidade" dos mostos de alimentação, no qual é avaliada pela produção de etanol a partir de uma mesma cepa de levedura cultivada em laboratório. que consiste na capacidade desse mosto em ser fermentado pelas células de levedura. Mais ainda, nas amostras do fermento industrial foram detectadas ausência de contaminação tanto por levedura selvagem como também por bactérias. Assim sendo, esses resultados sugeriram que a composição mineral do mosto de alimentação estaria interferindo no metabolismo celular.

Portanto, o presente trabalho tem como objetivo geral avaliar a influência da composição mineral do mosto sobre o estado metabólico das células de levedura durante a fermentação alcoólica, analisando a fisiologia e a expressão gênica global da linhagem industrial *Saccharomyces cerevisiae* JP1. A compilação desses dados possibilitará um conhecimento mais aprofundado do metabolismo da levedura industrial JP1 submetida a variações da composição mineral do substrato, o qual poderá ajudar a indústria a elaborar estratégias de eliminação ou minimização dos possíveis efeitos danosos da composição nutricional do caldo de cana de açúcar ou, possivelmente, a identificação de fatores que potencializem o aumento da produção de etanol durante a fermentação.

2. REFERENCIAL TEÓRICO

2.1 Produção de etanol combustível no Brasil

O etanol é o biocombustível mais consumido no mundo, sendo o Brasil pioneiro na introdução desse combustível renovável na sua matriz energética e atualmente é o segundo maior produtor do mundo. A produção brasileira se deve inicialmente ao Plano Nacional do Álcool (PROÁLCOOL) lançado na década de 70, o qual visava estimular a produção de etanol pelos empresários, proporcionando empréstimos a juros baixos e alto preço de venda do produto (Basso et al., 2011). No território brasileiro a produção de etanol é bastante divergente entre as regiões Centro/Sul e N/NE (Tabela 1) sendo quase 10 vezes maior a produção na região Centro/Sul, principalmente por causa dos estados de São Paulo, Minas Gerais e Goiás (SINDAÇÚCAR, 2015). Na região nordeste, o crescimento da produção de etanol dependente dos níveis de produtividade da cultura de cana de açúcar na zona da mata, no qual necessita da ampliação de área de irrigação e de mecanização do setor agrícola. Além disso, ainda se faz necessário muitos ajustes na etapa industrial da produção de etanol, principalmente com relação a manutenção e aumento do rendimento fermentativo (Vidal et al., 2006). Apesar de uma produção relativamente baixa em comparação ao centro/sul do país, o setor sucroalcooleiro nordestino abastece o mercado externo com custos competitivos.

Tabela 1. Números finais da produção de cana de açúcar e etanol do setor sucroalcooleiro brasileiro (SINDAÇÚCAR, 2016).

Safra/Região	**N/NE**		**C/Sul**	
	Cana (t)*	**Etanol (m^3)**	**Cana (t)**	**Etanol (m^3)**
2009/2010	59.917.90,00	2.005.10,00	541.961.7,00	23.685.70,00
2010/2011	63.139.00,00	1.987.30,00	560.544.3,00	25.612.50,00
2011/2012	65.415.270,00	2.103.459,00	494.937.656,00	20.597.334,00
2012/2013	55.719.561,00	1.854.994,00	533.517.580,00	21.608.912,00
2013/2014	56.457.965,00	1.967.531,00	602.802.820,00	26.045.950,00
2014/2015	61.257.477,00	2.282.091,00	576.926.338,00	26.677.683,00

*Tonelada (t) de cana de açúcar colhida.

Tecnicamente, o etanol pode ser produzido de uma ampla variedade de matérias primas

renováveis que podem ser classificadas grosseiramente em três principais grupos: (1) aquelas que contêm quantidades consideráveis de açúcares facilmente fermentáveis (cana de açúcar, beterraba sacarina, sorgo sacarino), (2) por fontes de amido e polímeros de frutose (milho, batata, arroz, trigo, agave) e (3) por fonte celulósica (palha, capim, sabugo de milho, madeira, bagaço de cana) (Basso et al., 2011). Na tabela 2 segue um resumo dos tipos de açúcares utilizados para a produção de etanol, suas fontes industriais e as principais leveduras envolvidas. Dentre essas variedades de fontes de açúcar, o sorgo sacarino vem se destacando como um potencial substrato para a indústria alcooleira. O sorgo apresenta uma grande produtividade agrícola, tendo inclusive uma maior eficiência na utilização dos recursos hídricos do que a cana de açúcar e, portanto, sendo uma alternativa de cultivo em regiões secas como o nordeste brasileiro. Além disso, o caldo extraído do sorgo sacarino apresenta teores de açúcares e outros nutrientes adequados para serem utilizados na fermentação alcoólica. Já existem algumas cultivares de sorgo sacarino selecionadas para o cultivo na região nordeste do Brasil (Dutra et al 2013).

Tabela 2. Diversidade de fontes de carbono para produção de etanol e diferentes espécies de levedura empregadas (Walker, 2004).

Fonte de Carbono	Fontes Industriais	Leveduras
Xilose e Arabinose	Hidrolisado de madeira e bagaço/celulose e steep corn	*Pichia stipitis e Candida shehatae*
Sacarose, Maltose e Lactose	Cana de açúcar, beterraba, cereais, soro de queijo e sorgo sacarino	*S. cerevisisae e Kluyveromyces marxianus*
Amido e Inulina	Cereais e Tubérculos	*Schwanniomyces e Kluyveromyces*

Apesar das varias alternativas, a cana de açúcar ainda é largamente a matéria prima mais utilizada para a produção de açúcar e álcool. A produção de cana de açúcar é dominado pelo Brasil representando em 2015 cerca de 40% da produção mundial (Tabela 3). No Brasil, a colheita da cana tem a duração de cerca de 200 dias e na grande maioria é utilizado para fabricação de açúcar. Para a produção de etanol é utilizado tanto o caldo da cana como também o mel final ou melaço que é um subproduto da produção do açúcar cristal. A adição de mel na produção de etanol ocorre também com o intuito de diminuir os custos com a matéria prima, a qual pode incidir em 38 a 73% no custo total do etanol final (Schmidell,

2005).

Tabela 3. Dados de 2015 dos dez maiores produtores de cana de açúcar no mundo (Food and Agriculture Organization of the United Nations, 2015).

País	Produção (Mil toneladas)
Brasil	739.267,00
Índia	341.200,00
China	*125.536,00
Tailândia	100.096,00
Paquistão	63.750,00
México	61.182,00
Colômbia	34.876,00
Indonésia	*33.700,00
Filipinas	31.874
Estados Unidos	27.906
Mundo	1.877.105,00

*dado não oficial

O processamento da cana nas industriais brasileiras ocorre quase todas da mesma forma. A cana é plantada no sistema de talhões na qual são divididas por áreas de plantio de acordo com a variedade da cana e o planejamento de colheita e transporte para a fábrica. As variedades de cana podem variar de acordo com seu tempo de crescimento e maturação. A grande maioria das variedades de cana utilizadas nas destilarias do nordeste do Brasil foram desenvolvidas pelo programa de melhoramento genético de cana de açúcar gerenciado pela RIDESA (Rede Interinstitucional para o Desenvolvimento de Setor Sucro-Alcooleiro). Após o período de maturação, onde a planta atinge sua máxima produção de açúcar, ocorre a colheita do colmo da planta que pode ser feito de forma manual ou mecanizada. Em seguida, esses colmos são transportados para a fábrica onde serão moídos para a extração do caldo que servirá para o processo de extração do açúcar ou para a fermentação alcoólica. O bagaço da cana após a moagem é secado e levado para a caldeira da fábrica onde será queimado para a geração de energia, na qual será utilizada pela própria fábrica. No contexto da produção de etanol, algumas poucas destilarias possuem o processo de esterilização do caldo para evitar contaminação no processo de fermentação. Além disso, a única forma de avaliar previamente a qualidade da cana processada é a quantificação dos açúcares dissolvidos no caldo, essa metodologia pode causar erros de previsão na produção de etanol no qual será discutido melhor a seguir.

2.2 Fermentação Alcoólica Industrial

Em 75% das destilarias do país o processo é conduzido pelo método Melle-Boinot normalmente chamado de batelada alimentada ou no modelo contínuo, ambos utilizando o reciclo de células de levedura (Basso et al., 2011). O processo de batelada alimentada consiste basicamente na transferência do fermento tratado nos pré-fermentadores e do caldo, melaço e água, que formam o mosto de alimentação, para as dornas de fermentação através de bombeamento ou por gravidade. Com a transferência concluída, o substrato a ser fermentado começa a alimentar a dorna até preencher o volume adequado para o processo. No final da fermentação, caracterizada inicialmente pelo consumo total do açúcar, o mosto fermentado é enviado para centrífugas, onde ocorrerá a separação das células de levedura do mosto fermentado. A biomassa de células (denominado na indústria como Leite ou Creme) é enviada para os pré-fermentadores onde serão diluídas e tratadas, geralmente, com ácido sulfúrico para o controle de bactérias e depois iniciarem uma nova fermentação (ver esquema simplificado do processo na Figura 1). No pré-fermentador também pode ser adicionado nutrientes como forma de enriquecer as células com fontes de nitrogênio e minerais. O processo contínuo de fermentação é caracterizado pela utilização de várias dornas de fermentação conectadas em série no qual tanto o substrato de alimentação quanto as células de levedura tratadas são simultaneamente adicionados às dornas de fermentação de uma maneira contínua e controlada (ver esquema simplificado na Figura 2).

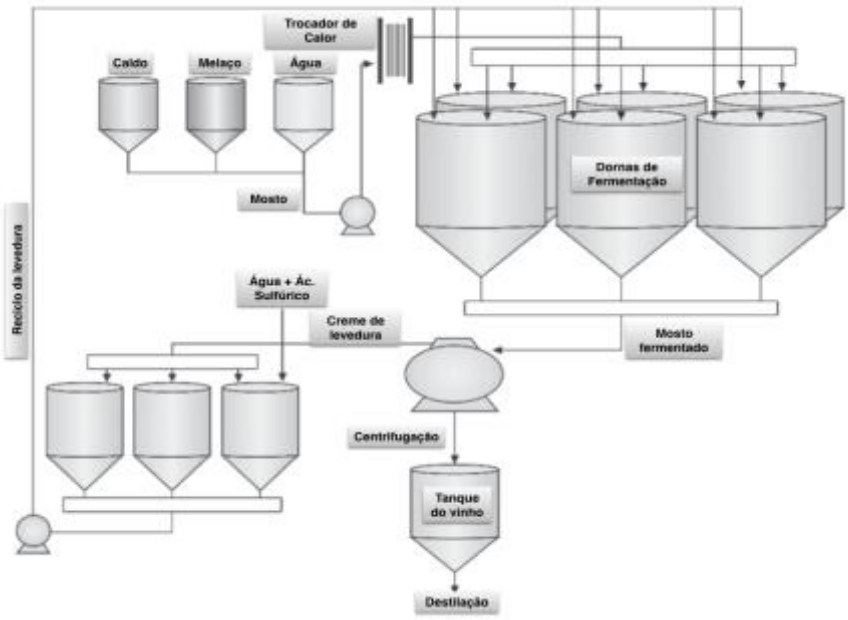

Figura 1. Esquema simplificado do processo de fermentação em batelada utilizado nas destilarias do Brasil (Adaptado de Amorim et al 2016).

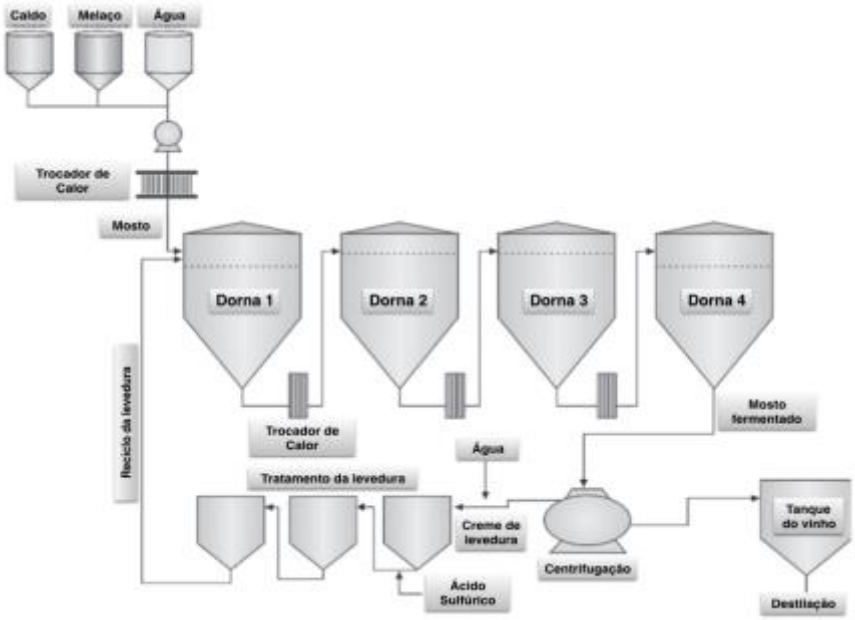

Figura 2. Esquema simplificado do processo continuo de fermentação utilizado nas destilarias do Brasil (Adaptado de Amorim et al 2016).

Em ambos os processos, cerca de 90 a 95% das células são recicladas resultando em uma concentração celular bastante elevada nas dornas. Essa reutilização reduz a necessidade de propagar nova biomassa celular diminuindo custo e tempo. Estima-se que durante o ciclo de fermentação ocorre um aumento da biomassa entre 5 a 10%, recuperando as células perdidas na centrifugação. A reutilização da biomassa de levedura ocorre até ser identificada alguma queda no rendimento fermentativo causada por contaminação microbiológica não controlável por biocidas ou por uma queda na viabilidade celular. Um ciclo de fermentação dura em torno de 6 a 10 horas e normalmente a temperatura é mantida entre 32 a 35ºC (Basso et al., 2011).

Do ponto de vista microbiológico, a produção de etanol em destilarias brasileiras é bastante dinâmica devido a ausência de esterilização do caldo de cana de açúcar no qual possibilita a entrada de bactérias e leveduras presentes na microbiota natural da planta bem como do solo onde foram cultivadas. Essa contaminação pode causar severos danos à fermentação diminuindo o rendimento em etanol (Silva-Filho et al., 2005a). Por outro lado, a competição durante a fermentação seleciona microrganismos mais adaptados e resistentes às condições adversas do processo. Nas destilarias do nordeste do Brasil o grupo de Engenharia Metabólica da UFPE vem isolando microrganismos presentes na fermentação e tem identificado espécies de leveduras e bactérias que são associadas tanto com alto rendimento

fermentativo, caso da linhagem industrial de *Saccharomyces cerevisiae* JP1, quanto associadas a episódios de queda na produção como a levedura *Dekkera bruxellensis* e várias espécies de bactérias, principalmente, pertencente ao gênero *Lactobacillus* (Silva-Filho et al., 2005a; Silva-Filho et al., 2005b; De Souza-Liberal et al., 2005; De Souza-Liberal et al., 2007; Basílio et al., 2008; Lucena et al., 2010).

2.2.1 Bioquímica e fisiologia da fermentação alcoólica

A fermentação alcoólica é um processo basicamente anaeróbico que consiste na metabolização de açúcares fermentáveis tendo como principal produto de interesse o etanol. As leveduras, especialmente a espécie *Saccharomyces cerevisiae*, são dotadas de grandes capacidades fermentativa e conseguem altos rendimentos na produção de etanol. Em condição estritamente anaeróbica, 1 mol de glicose (180 g) produz 2 moles de etanol (92 g), 2 moles de dióxido de carbono (88 g) e 57 Kcal de energia (Lehninger et al., 1995). Isto corresponde ao rendimento teórico máximo de 0,511 g de etanol produzido por grama de glicose consumida pelo microrganismo. Na prática industrial, valores de rendimento acima de 0,46 g etanol/g glicose são considerados ótimos, visto que parte do açúcar metabolizado pela levedura é desviada para gerar biomassa celular entre outros produtos, como gás carbônico, ácido acético, ácido pirúvico, ácidos orgânicos, fumárico, málico e succínico (Otero et al., 2007; Lima et al., 2001).

As células de *S. cerevisiae* são amplamente empregadas nesse tipo de processo devido à sua alta capacidade fermentativa. Essa levedura possui um metabolismo do tipo Crabtree positivo, no qual a presença de glicose ou de outros açúcares fermentáveis acima de certa concentração induz o metabolismo fermentativo mesmo na presença de oxigênio (Postma et al., 1989). Isso se dá pelo fato de que o excesso de glicose aumenta o fluxo pela via glicolítica e eleva a concentração do cofator NADH. As leveduras que possuem esse metabolismo parecem apresentar uma regulação que limita a re-oxidação desse NADH excedente pela cadeia respiratória, e leva ao redirecionamento da metabolização do piruvato pela via fermentativa (Pronk et al., 1996).

Apesar da alta capacidade fermentativa em condições industriais, *S. cerevisiae* pode ter seu rendimento em etanol diminuído em resposta a necessidade de adaptação a várias condições de estresse, como estresse osmótico, oxidativo, etanólico, ácido e térmico (Zhao and Bai, 2009), bem como, a condições de excesso ou escassez de nutrientes, como compostos nitrogenados e minerais (Walker, 2003). Para evitar alguns desses estresses são tomadas algumas medidas na indústria como a diminuição na adição de ácido sulfúrico no tratamento

do creme de levedura, bem como a utilização de trocadores de calor para manter a temperatura das dornas constante em 32ºC. Entretanto, influência nutricional, especialmente os minerais, é constantemente negligenciado pelos produtores de etanol combustível, apesar de terem um impacto tanto positivo quanto negativo no metabolismo das leveduras e diretamente na eficiência da fermentação (Walker, 2003).

2.3 Composição nutricional do caldo de cana de açúcar

Além dos açúcares, o caldo de cana de açúcar é formado por diversos grupos de compostos que podem ter significativa influência no rendimento da fermentação alcoólica. De acordo com Walford (1996) podem ser encontrados no caldo compostos pertencentes aos seguintes grupos: carboidratos (sacarose, glicose e frutose), aminoácidos (asparagina, glutamina, ácido aspártico, ácido glutâmico, alanina, valina, ácido aminobutírico, treonina, isoleucina e glicina), ácidos orgânicos não nitrogenados (oxálico, cítrico, tartárico, málico, aconítico, succínico, glicólico, acético e lático) e minerais (potássio, sódio, magnésio, cálcio, cobre, zinco, alumínio, cobalto, ferro, manganês, molibdênio, cloreto, fosfato e sulfato). Na tabela 3 segue a proporção desses nutrientes no caldo de cana de açúcar.

Tabela 4. Concentração dos nutrientes no caldo de cana de açúcar (Walfrod, 1996).

Grupos		**Concentração %**
Açúcares	Sacarose	81 - 87
	Glicose e Frutose	3 - 7
	Oligossacarídeos	0,06 - 0,6
	Polissacarídeos*	0,2 - 0,8
Sais	Sais inorgânicos (minerais)	1,5 - 3,7
Orgânicos	Ácidos Orgânicos	0,7 - 1,3
	Aminoácidos	0,5 - 2,5
	Ceras, ácidos graxos e fosfolipídios	0,05 - 0,15
Insolúveis	Areia, bagaço e outros	0,15 - 1

*incluindo gomas e dextrana.

Em destilarias do Brasil a composição mineral do mosto de alimentação pode variar de acordo com a proporção de melaço no mosto de alimentação, da qualidade nutricional do solo onde as plantas foram cultivadas e também pode variar de acordo com a variedade da cana de

açúcar. Apesar da sua grande relevância para o metabolismo da levedura, existem poucas descrições na literatura sobre a composição dos minerais no caldo de cana e no melaço. A tabela 4 mostra a faixa de concentração de alguns minerais no caldo de cana de açúcar, revelando que alguns nutrientes como o potássio, magnésio, enxofre, cálcio, zinco, cobre e alumínio podem apresentar uma faixa ampla de concentração. Essas variações podem ter consequências para o metabolismo das células de levedura e, consequentemente, na produção de etanol.

Tabela 5. Faixa de concentração de íons no caldo de cana de açúcar (Amorim e Leão, 2005).

Nutrientes	**Faixa de concentração (mg L^{-1})**
Nitrogênio total	70 - 350
Fósforo	20 - 200
Potássio	300 - 12.000
Magnésio	80 - 3.900
Enxofre	80 - 3.900
Cálcio	150 - 2.000
Zinco	0.45 - 9
Cobre	0.20 - 8
Manganês	2 - 8
Alumínio	2 - 500

2.4 Função dos minerais no metabolismo de levedura

As leveduras requerem uma vasta gama de metais para seu crescimento e suas funções metabólicas. A nutrição mineral da célula é muito importante para garantir o sucesso da fermentação, especialmente na produção de álcool (Walker, 2003). Nutrientes como nitrogênio, magnésio, manganês, zinco, cobre, ferro entre outros desempenham diversas funções, principalmente como cofatores de muitas enzimas e a homeostasia da concentração

intracelular desses compostos é estreitamente regulada pelos seus transportadores celulares (Stehlik-Thomas et al., 2004). A tabela 5 mostra a composição mineral de células de *Saccharomyces sp*. oriunda de um processo industrial de produção de cerveja (Caballero-Córdoba et al., 1997). É importante destacar que a composição mineral de qualquer biomassa celular é dependente do substrato utilizado para o cultivo das células. Portanto, essa composição pode variar bastante entre células cultivadas em malte de cevada (Tabela 5) e em células cultivadas em caldo de cana de açúcar (dados não encontrados na literatura).

Tabela 6. Composição mineral da biomassa de levedura cervejeira *Saccharomyces sp* (Caballero-Córdoba et al., 1997).

Nutrientes	**mg/100g**
Fósforo	16,9
Potássio	13,6
Magnésio	2,1
Ferro	0,10
Cálcio	0,73
Zinco	4,56
Cobre	4,54
Manganês	15,9
Alumínio	0,95

O nitrogênio pode ser assimilado por *Saccharomyces cerevisiae* nas formas amoniacal (NH^{4+}), amídica (ureia) ou amínica (na forma de aminoácidos), mas não em forma de nitrato (Roitman et al., 1988). A habilidade de assimilar nitrogênio por diferentes fontes pode conferir uma vantagem de sobrevivência no ambiente industrial como é o caso da levedura *Dekkera bruxellensis*, a qual é capaz de assimilar nitrogênio na forma de nitrato, conferindo-lhe uma vantagem competitiva em relação a *S. cerevisiae* no processo industrial de etanol combustível (De Barros Pita et al., 2011). A disponibilidade de nitrogênio regula a taxa de multiplicação celular, bem como desempenha um importante papel para a fermentação alcoólica através da

ativação de sistemas de transporte de açúcares, aumentando a influxo no interior da célula (Lagunas et al., 1982).

O magnésio atua como cofator enzimático para mais de 300 enzimas envolvidas em diferentes vias metabólicas e bioenergéticas. O magnésio desempenha papéis multifacetados na fisiologia das células de levedura nos níveis citológico, bioquímico e biofísico sendo muito importante no processo de fermentação na ativação de várias enzimas glicolíticas (Walker, 2003) e na proteção a estresses ambientais durante a fermentação industrial, tais como aqueles causados por etanol, temperaturas elevadas e alta pressão osmótico (Dombek, 1986; D'Amore et al., 1988). Portanto, a biodisponibilidade no meio e a utilização metabólica de íons de Mg^{2+} é fundamental para uma produtiva e eficiente fermentação por células de levedura (Walker e Maynard, 1997).

Diferente do magnésio, o cálcio é exigido em quantidades muito menores pela célula (Youatt, 1993), porém, é bastante requisitado na fermentação, estando envolvido na proteção celular ao estresse causado pela elevada concentração de etanol (Vasconcelos, 1987; Chourchesne et al., 2010). Quando se liga na parede celular de certas linhagens de levedura, o cálcio e outros íons divalentes causam a floculação, característica importante em fermentações de cerveja (Walker, 2003). Cálcio também está associado à proteção e tolerância a pH ácido, uma vez que este mineral atua como um sinalizador intracelular (De Lucena et al., 2012; Cyert, 2003).

O potássio é o cátion celular mais abundante em leveduras, constituindo 1-2% do peso seco da célula, sendo importante na osmorregulação, equilíbrio de carga de macromoléculas, regulação de fosfato, absorção de cátions divalente e ativador de uma série de reações na via glicolítica (Walker, 2003; Jones e Greenfield, 1994).

O fósforo é essencial para o metabolismo energético, sendo fundamental na produção de ATP e na síntese de ácidos nucleicos. Esse elemento é considerado indispensável para a absorção e metabolização dos açúcares tendo uma influência significativa no metabolismo fermentativo (Amorim, 1985, Vasconcelos, 1987).

O alumínio é um metal tóxico que inibe a produtividade de muitas culturas, e por isso uma quantidade considerável de informação está disponível na literatura sobre o seu efeito em plantas (Haug, 1984). Mais recente, o Al^{3+} foi identificado em certas concentrações como um elemento estressante da levedura, em condições de fermentação industrial, acarretando diminuição simultânea da viabilidade e dos teores de trealose da levedura (Oliveira et al., 2009).

O zinco é um elemento essencial na fisiologia da célula de levedura, assim como os

demais íons atuando como cofator para muitas enzimas (Zhao et al., 2011). Íons de zinco em quantidades adequadas no meio promovem aumento na taxa de crescimento celular de levedura, bem como na produção de etanol, sendo essencial para a atividade da álcool desidrogenase e crucial na estrutura de enzimas e proteínas não catalíticas (Jones e Gadd, 1990; Magonet et al., 1992; Berg et al., 2002). Deficiência de zinco diminui o crescimento celular e a atividade fermentativa e concentrações elevadas pode exercer um efeito tóxico na célula, afetando a permeabilidade da membrana ao potássio (Stehlik-Thomas et al., 2004).

Outros metais podem influenciar a fermentação, tais como: ferro, manganês e cobre. O ferro é essencial para as leveduras, mas em excesso também pode ser tóxico. Por este motivo, a incorporação e utilização do ferro pelas leveduras são bem reguladas através de mecanismos de transporte, armazenamento e mobilização (Pas et al., 2007; Philpott e Protchenko, 2008). O manganês é essencial para a célula de levedura como um elemento traço, desempenhando também funções importantes no metabolismo de *S. cerevisiae*, como parte de algumas enzimas, por exemplo, a piruvato carboxilase (Stehlik-Thomas et al., 2004), sendo necessário em concentrações bem menores do que o magnésio. Manganês e magnésio têm uma conhecida concorrência intracelular por sítios de ligação de enzimas devido principalmente às suas propriedades químicas semelhantes. A competição entre os dois íons pode ocorrer em resposta à disponibilidade desses minerais e ter uma grande importância regulatória (Blackwell et al., 1997). Por sua vez, o cobre é um cátion divalente vital para células de leveduras na qualidade de um cofator de algumas enzimas, tais como: citocromo C oxidase, lactase e Cu-Zn-superóxido dismutase (Stehlik-Thomas et al., 2004).

Entre todos esses minerais, o magnésio apresenta uma significativa relevância na produção de etanol não somente pelas suas inúmeras contribuições para o metabolismo celular, como veremos mais detalhado no tópico seguinte, mas também por ser um dos metais mais abundante tanto no caldo de cana de açúcar quanto no melaço.

Os cálculos de produtividade e rendimento da fermentação alcoólica na indústria sucroalcooleira são exclusivamente determinados pela quantidade de açúcar que entra na fermentação e pela quantidade de etanol produzida no final. Porém, a concentração desses minerais pode ser fundamental para garantir a eficiência do processo devido à sua influência no metabolismo das células de levedura. Nesse contexto, técnicas moleculares modernas podem ajudar a elucidar o efeito desses íons no metabolismo fermentativo de leveduras industriais utilizadas na produção de etanol, avaliando a sua influência sobre os níveis de transcrição dos genes, através da técnica de microarranjo de DNA.

2.5 Análise Transcriptômica

O estudo da genômica funcional, a qual visa o entendimento da interação entre o genótipo e o fenótipo, se tornou possível através do desenvolvimento de metodologias como SAGE (*serial analysis of gene expression*) e de microarranjo de DNA as quais possibilitam a identificação e mensuração de diferentes níveis de expressão de mRNAs (RNA mensageiros), em diferentes condições e organismos.

Ao contrário de técnicas como Northern Blot e RT-qPCR que analisam a expressão de um único gene, a técnica de microarranjo de DNA, desenvolvida por Schena et al. (1995), permite a análise da expressão de milhares de genes simultaneamente em um único ensaio. Essa técnica consiste na hibridização específica do DNA (fragmentos de até 60 bases) com uma fita de DNA de acordo com sua sequência de nucleotídeos, ou seja, o princípio da técnica é baseado na complementariedade específica de uma molécula de ácido nucleico marcada com fluoróforos, por exemplo, cianina 3 (Cy3) ou cianina 5 (Cy5) com pequenas moléculas de DNA (fragmentos de até 60 bases) pré-definidas correspondentes a cada um dos genes em estudo. A hibridização das moléculas ocorre numa lâmina, em geral de vidro, construídas por um robô altamente preciso que distribui as bases nucleotídicas (adenina, citosina, guanina e timina) formando pontos (spots) que correspondem a um gene específico e o conjunto desses pontos forma um arranjo genômico (arrays).

Para a análise de expressão gênica global com marcação dupla as amostras testes são marcadas com Cy3 (verde) e a amostra de referencia com Cy5 (vermelho). Após hibridização dessas amostras com os arrays, a lâmina será lida por um escâner que varia o espectro do vermelho intenso ao verde intenso. A partir dessa variação de tonalidade de cada spot é possível determinar, comparativamente, o nível de expressão dos genes das amostras teste com a de referência.

Essa ferramenta possibilita analisar a expressão simultânea de mais de 6 mil genes, no caso da levedura *Saccharomyces cerevisisae*. E para melhor entender e interpretar os genes considerados induzidos e reprimidos, existem banco de dados para *S. cerevisisae* disponíveis online e gratuitos com informações especificas sobre os genes baseadas em artigos científicos. Por exemplo, o banco de dados genômico de *Saccharomyces cerevisiae* (*Saccharomyces* Genome Database - SGD) fornece detalhadas informações biológicas dos genes dessa levedura e também fornece relações funcionais entre a sequência e os produtos dos genes nos fungos e nos organismos superiores. Nessa plataforma é possível também ter acesso aos fenótipos dos mutantes para cada gene e também as condições em que os genes são induzidos

ou reprimidos.

Outra análise dos genes pode ser feita através da plataforma *Gene Ontology (GO) consortium*. Essa ferramenta de bioinformática analisa a ontologia gênica de vários organismos diferentes, possibilitando o estudo dos genes e de seus produtos em três domínios: componente celular, função molecular e processos biológicos. O primeiro agrupa os genes de acordo com as partes das células, o segundo de acordo com as atividades elementares que os produtos gênicos estão envolvidos, tal como ligações moleculares e catalises. O terceiro agrupa os genes de acordo com eventos moleculares com início e fim bem definidos necessários para a integridade de unidades vivas como células, tecidos, órgãos e organismos.

Além dessas duas ferramentas já apresentadas, há disponível o banco de dados YEASTRACT que permite a analise de mais de 48 mil associações regulatórias entre fatores de transcrição e genes alvos em *S. cerevisiae*. Essa ferramenta está associada com os bancos de dados SGD e GO consortium. Portanto com essa ferramenta é possível detectar redes regulatórias baseadas nos genes induzidos e reprimidos identificados na técnica de microarranjo de DNA.

Enfim, a utilização da técnica de microarranjo de DNA e das ferramentas de bioinformática associada com experimentos fisiológicos em *S. cerevisiae* poderia aumentar o entendimento sobre a resposta celular à composição mineral do substrato, em especial sobre o metabolismo fermentativo.

3 CONSIDERAÇÕES

O grupo de pesquisa em Biologia Molecular e Engenharia Metabólica da UFPE vem executando atividades de pesquisa na investigação dos fatores microbiológicos associados com a produção de etanol combustível em destilarias da região nordeste do Brasil há mais de uma década, gerando resultados sobre a composição e dinâmica da população de leveduras (Silva-Filho et al., 2005a,b; Liberal et al., 2007; Basílio et al., 2008) e de bactérias (Lucena et al 2010). O possível efeito dos contaminantes biológicos sobre o rendimento industrial já vem sendo bem discutido na literatura. Entretanto, ao longo desses anos foram observados momentos de queda nos rendimentos industriais em várias destilarias sem que houvesse relação direta com a presença dos agentes biológicos. Assim, iniciou-se uma linha de pesquisa para investigação dos parâmetros físico-químicos sobre a produção de etanol. Pouco se sabe sobre o efeito da composição mineral do caldo de cana sobre o rendimento fermentativo industrial, apesar da literatura mostrar que esses minerais podem agir como ativadores e/ou

inibidores da fermentação. Portanto, o presente trabalho busca aprofundar os conhecimentos sobre o efeito da composição mineral do caldo de cana sobre o metabolismo fermentativo da levedura industrial *S. cerevisiae* JP1 no intuito de contribuir com o aprimoramento deste setor industrial de grande relevância socioeconômica para o Brasil. A presente tese de doutorado gerou três artigos científicos nos quais foram organizados cronologicamente e estão nos anexos da presente tese. O primeiro artigo intitulado "**Mineral Composition of the Sugarcane Juice and Its Influence on the ethanol fermentation**" foi publicado em 2015 na revista *Applied of Biochemistry and Biotechnology*. O segundo artigo intitulado "**Magnesium ions in yeast: setting free the metabolism from glucose catabolite repression**" foi publicado em 2016 na revista *Metallomics*. O terceiro artigo intitulado "**Yeast genes regulated by copper under fermentation condition**" foi submetido para publicação em 2017 para a revista *Metallomics*.

4 OBJETIVO GERAL

Avaliar a influência da composição mineral do caldo de cana de açúcar no metabolismo fermentativo da levedura industrial *Saccharomyces cerevisisae* JP1.

4.1 Objetivos específicos

- Determinar a composição mineral do caldo de cana de açúcar e sua influência na produção dos principais metabólitos da fermentação e
- Identificar os genes diferencialmente expressos na exposição aos minerais e associá-los aos parâmetros fermentativos.

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6 ANEXO

6.1 Anexo I

Mineral Composition of the Sugarcane Juice and Its Influence on the ethanol fermentation

Rafael Barros de Souza, João Assis S. de Menezes, Raquel de Fátima Rodrigues de Souza, Emmanuel D. Dutra e Marcos Antonio de Morais Jr.

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Mineral Composition of the Sugarcane Juice and Its Influence on the Ethanol Fermentation

Rafael Barros de Souza · João Assis S. de Menezes · Raquel de Fátima Rodrigues de Souza · Emmanuel D. Dutra · Marcos Antonio de Morais Jr

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Abstract In the present work, we evaluated the mineral composition of three sugarcane varieties from different areas in northeast Brazil and their influence on the fermentation performance of *Saccharomyces cerevisiae*. The mineral composition was homogeneous in the different areas investigated. However, large variation coefficients were observed for concentrations of copper, magnesium, zinc and phosphorus. Regarding the fermentation performances, the sugarcane juices with the highest magnesium concentration showed the highest ethanol yield. Synthetic media supplemented with magnesium also showed the highest yield (0.45 g g^{-1}) while the excess of copper led to the lowest yield (0.35 g g^{-1}). According to our results, the magnesium is the principal responsible for the increase on the ethanol yield, and it also seems to be able to disguise the inhibitory effects of the toxic minerals present in the sugarcane juice.

Keywords Mineral composition · Magnesium · Copper · Fermentation · Ethanol yield · Sugarcane juice

Introduction

The composition of sugarcane juice may vary, affecting several industrial operational parameters, such as the fermentation yield. Several parameters are implicated in determining the final composition of the sugarcane juice, such as the variety of sugarcane, soil type, fertilization

R. B. de Souza · R. d. F. R. de Souza · M. A. de Morais Jr (✉)
Interdepartmental Research Group in Metabolic Engineering, Department of Genetics, Federal University of Pernambuco, Av. Prof. Moraes Rego, 1235, 50670-901 Recife, PE, Brazil
e-mail: marcos.morais@pesquisador.cnpq.br

E. D. Dutra
Biomass Energy Research Group, Department of Nuclear Energy, Federal University of Pernambuco, Av. Prof. Moraes Rego, 1235, 50670-901 Recife, PE, Brazil

J. A. S. de Menezes
Fermenta Industrial Biotechnology and Environment ME, Incubatep/ITEP, Av Luiz Freire, 700, room 10, 50740-540 Recife, PE, Brazil

strategies, climatic conditions, maturity level of the sugarcane, type of harvest, period of time between burning, cutting and processing, content tips and straw and also by the use or not of vinasse irrigation [1].

Although the ash content in sugarcane juice is somewhat constant, its mineral composition can vary widely, depending on the source of raw material. In order to minimize these differences, many distilleries use the strategy of adding molasses to the broth [1]. This mixture is the most recommended since some stocks have deficient nutritional level and molasses usually contains high concentrations of minerals in its composition [2]. In spite of the importance, the mineral composition in broths of sugarcane and molasses used in distilleries in Brazil are often overlooked by the producers. Several minerals are important in the nutrition of yeast by allowing proper cell metabolism and growth. The major components are ammonium, phosphorus, sulphur, potassium, magnesium, calcium, zinc, manganese, copper and iron. Additionally, in the case of fermentative metabolism, minerals such as potassium, magnesium, calcium, manganese, iron, zinc and copper are somehow essential [3].

Some of these minerals, such as magnesium, manganese, zinc, copper and iron, play important roles as enzyme cofactors and in maintaining cell homeostasis [4]. However, excessive amounts of these components can be toxic and cause damage to the functions they are associated. For example, high levels of potassium and calcium present in sugarcane molasses can cause osmotic stress that impairs yeast fermentation performance [2]. Nitrogen can be assimilated by *Saccharomyces cerevisiae* as ammonium ion (NH_4^+), amide (urea) or aminic (amino acids) forms, but not in the form of nitrate and little or none in the form of proteins. These components have a direct influence on the budding and specific growth rates and transport of sugars [5].

Potassium (K^+) is the most abundant monovalent cation in yeast, consisting of 1–2 % of the yeast cell dry weight [3]. This is important in osmoregulation, charge balancing of macromolecules, regulation of phosphate and absorption of divalent cations [6]. It also acts as an activator of a series of reactions in glycolysis and other steps of metabolism, and it is also involved in ionic balance. The monovalent cation phosphorous (P^+) is essential for the energy metabolism and the synthesis of nucleic acids. This element is considered essential for carbohydrate absorption and transformation of sugar into alcohol and for the production of ATP from the glycolysis and the respiratory chain, and of course for the regulation of enzymes and metabolic mechanisms by kinases [3]. Magnesium (Mg^{2+}) is the most abundant intracellular divalent cation and represents around 0.3 % of the yeast cell dry weight. This mineral acts as a cofactor for over 300 enzymes involved in different metabolic reactions, such as DNA and ATP synthesis. Magnesium plays multifaceted roles in the physiology of yeast cells at cytological, biochemical and biophysical levels and is very important in industrial fermentation processes, in which it is necessary for activation of several glycolytic enzymes and in protecting from environmental stresses during fermentation, such as those caused by ethanol, high temperatures or high osmotic pressure [3]. Therefore, its availability in the medium, cellular absorption and subsequent metabolic utilization seems to be a prerequisite for achieving the maximum fermentation activity of the yeast cell [3].

On the other hand, the divalent cation calcium (Ca^{2+}) presents relatively few and more specific biochemical functions, being required in much smaller quantities by the cell when compared with Mg^{2+} [7]. It can induce connections between surface proteins of different cells, causing flocculation that is important in beer fermentation [3]. Although Ca^{2+} is apparently not necessary for the growth of yeast cells [8], it is important in fermentation for their involvement in cellular protection to ethanol stress [9]. It was recently suggested that the assimilation of Ca^{2+} could help in the protection and tolerance to medium acidification [10], since this mineral acts as an intracellular marker of homeostatic regulation [11]. As another relevant divalent

cation, zinc (Zn^{2+}) is an essential element for normal growth, metabolism and physiology of the yeast cell, besides acting as a cofactor for many proteins [12]. It also plays an important role in the metabolism of the yeast fermentation, because it is essential for the activity of alcohol dehydrogenase [13], also presenting a crucial role in the structure of enzymes and non-catalytic proteins [14]. Zinc in appropriate amounts in the medium promotes the growth of yeast cell and ethanol production [15]. In contrast, deficiency of Zn^{2+} blocks cell growth and fermentative activity, while high concentrations of Zn^{2+} can be toxic to the cell, affecting the membrane permeability to K^{+} causing decrease in cell growth and fermentative activity [4].

Other metals can influence the fermentation, such as iron (Fe^{3+}), manganese (Mn^{2+}) and copper (Cu^{2+}). These are required as cofactors of enzymes (especially Mn^{+2}) and yeast respiration as components of redoxisomes (Cu^{+2} and Fe^{+3}) [16]. Copper acts as cofactor of some enzymes, such as cytochrome C oxidase and Cu/Zn-superoxide dismutase [4]. Manganese is essential for yeast cell as a trace element, playing important roles in the metabolism as part of some enzymes, such as pyruvate carboxylase [4], but also acting as competitor of Mg^{2+} for some enzymes of ATP and DNA synthesis [17]. Moreover, iron is essential for the yeast but may also be toxic, and its utilization by the yeast cells is quite well regulated [18]. *S. cerevisiae* can easily grow in culture media in which Fe^{3+} is very scarce or very abundant. Yeast cells are also able to grow in Fe^{3+}-free medium for several generations, indicating that they express efficient mechanisms for storage and mobilization of iron [19].

At last, it should be pointed out the toxic effects of aluminium (Al^{3+}) that inhibits productivity of many crops, and thus, for economic reasons, considerable amount of information is available in the literature about its effects on plants [20]. It was recently identified as stressful element of the yeast in industrial fermentation conditions, causing a simultaneous reduction of the viability and concentration of the yeast trehalose [21].

In the past few years, we have dedicated efforts to study the composition of microbial fermentation processes for ethanol fuel production, especially in the northeast region of Brazil, describing the main species of yeasts and lactic acid bacteria whose presence could risk the efficiency of the industrial process [22–25]. However, in some of these studies, we could observe that the microbiota of these processes was not associated with problems in fermentation. For this reason, we turned our attention to the analysis of the mineral composition of the substrate and also how changes in this composition may influence the fermentative capacity of yeast.

Materials and Methods

Sugarcane Varieties and Juices

Three different varieties of sugarcane that represented the most commonly found in farms providing for the distilleries in the northeast Brazil were analysed. These varieties were bred by the RIDESA consortium (Brazilian Interuniversity Network for the Development of Sugarcane). The sugarcane varieties RB867515, RB92579 and RB863129 had been collected from agricultural fields of three distilleries. The first distillery (site IT) is located in the municipality of Aldeias Altas (04° 37′ 40″ S 43° 28′ 15″ O), state of Maranhão, which presents a yellow latosol-type soil, a typical coastal plain soil with poor nutrient clay material and high cohesion of structural aggregates [26]. The second distillery (site TB) is localized in the municipality of Caaporã (07° 30′ 57″ S 34° 54′ 28″ O), state of Paraiba, and the third distillery (site ST) is in the municipality of Goiana (07° 33′ 39″ S 35° 00′ 10″ O), state of Pernambuco. In both sites TB and ST, the soil is classified as spodosols, a strongly leached

ashy grey-type acidic soil with limited suitability for cultivation that normally depends of correct fertilization [26]. Sites TB and ST are close to each other approximately by 17 km, and both are 1,300 km far away from IT site. These sugarcane varieties are commonly cultivated in all the sites, and it has to be stated that those sugarcanes were cultivated under strict controlled conditions of irrigation and fertilization by the distilleries. Samples were collected in the beginning of the harvest season 2011/2012, in august, in a randomized design. It only collected the maturated samples; in other words, the plants had an equally distributed sugar content (BRIX°) in the whole stem. In these analyses, the handheld refractometer was used (IPS 10 T-Impac), and the samples were collected from the apex, middle, and bottom of the stem and then measured the maturation level. Twenty-seven samples were collected for each variety. Then, the broth were extracted from the sugarcane and stored in a freezer (−20) for the subsequent analysis. Part of the sugarcane juice was subjected to digestion with sulphuric acid and hydrogen peroxide to measure the following nutrients [27]: total nitrogen (TN) by Kjeldahl method; total phosphorus by colorimetric method; potassium contents by flame photometer [28]; and calcium, magnesium, iron, zinc, aluminium, copper and manganese by atomic absorption spectrometry.

Samples from 30 independent farms that supplied sugarcane from different varieties to distillery Miriri Agroindustrial, Santa Rita, Paraíba state, were collected in the harvest season 2009/2010 and divided into two groups according to the average of industrial fermentation yield in the moment in which their canes were used by the distilleries: group A corresponded to the group of farms that supplied cane when the industrial yield in two distilleries was coincidently above 85 % and group B corresponded to the farms that supplied sugarcane when the industrial yield was coincidently below 80 %. The blends of group A and group B were used in the fermentation assays.

Yeast Strain and Media of Growth and Fermentation

Industrial strain *S. cerevisiae* JP1 was used in the present study, since this yeast is the most used in the distilleries of northeastern Brazil [29]. In some experiments, we used the industrial strain *S. cerevisiae* PE-2 that is commonly used by distilleries in southern Brazil [30]. The cells were maintained and pre-grown in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 20 g L^{-1} bacteriological peptone, 20 g L^{-1} agar) at 32 °C. For the fermentations with sugarcane juice, the broths from the RB varieties were unfrozen and sterilized by autoclavation (121 °C, 15 min). Afterwards, the juices were centrifuged to remove suspended solids, and the supernatant was used for the fermentation. The synthetic media for the fermentations were prepared with YNB (Yeast Nitrogen Base, Difco) without ammonium sulphate and amino acids to final concentration of 1.6 g L^{-1} and sterilized by filtration (sterile Millipore membrane 0.22 μm). Sucrose was used as carbon source to final concentration of 155 g L^{-1}. Mineral supplementation was elaborated with minerals in the form of salts as NH_4Cl, KH_2PO_4, KCl, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}2H_2O$, $AlCl_3$, $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$ and $FeSO_4{\cdot}H_2O$. The highest concentration of each mineral was based on the average of their concentrations in sugarcane juice in the group A and group B of distilleries described above. The amount of minerals present in the YNB medium formulation was incorporated in this calculation.

Fermentation Assays

The yeast cells were pre-grown in YPD at 32 °C for successive cycles of 24 h in a rotator shaker at 140 rpm. After each cycle, cells were recovered by centrifugation (1,200×*g* for 5 min

at room temperature) and suspended in fresh medium for a new cycle. It was repeated until accumulation of enough cells to perform the fermentation assays. All the fermentations were carried out on a single-batch in a 250-mL flasks containing 200 mL of sugarcane substrate separated in two sets of tubes. In the first set of tubes, a blend of juices from different cane varieties were added cultivated in the same geographical site. In the second set of tubes, a blend of juices from the same cane varieties were added cultivated in different geographical sites. Cells from pre-cultures were suspended to 10^8 cells mL^{-1} in each blend and left to ferment for 6 h at 32 °C without agitation. At defined times, samples were withdrawn, centrifuged and the supernatant taken for metabolite analysis. The measurement of carbohydrates, glycerol, acetate and ethanol were carried out by HPLC (Waters Co., USA) using an Aminex HPX-87H column (BioRad, USA) heated at 60 °C. Sulphuric acid at 5 mM was used as mobile phase at a flux of 0.6 L min^{-1} [31]. The yeast cells were evaluated to viability by direct microscopic count on a Neubauer chamber after dying with methylene blue. The production of CO_2 was measured by the weight loss approach [25] before each sampling, taking into account the loss of volume in the previous sampling.

Statistical Analysis

The mineral composition determination in each distillery was performed in nine samples from each sugarcane variety. Fermentations with sugarcane juice substrates were carried out in duplicate while fermentations with the synthetic media were performed with four biological replicates. The results were subjected to analysis of variance (ANOVA) and were compared by the Tukey test (α=0.05) using the software ASSISTAT [32].

Results and Discussion

Variation in Mineral Composition of Sugarcane Juice Changes the Yeast Fermentation Performance

During the monitoring of eight distilleries from 2004 to 2012, samples of the feeding substrate (sugarcane juice diluted to around 12 °Brix) were collected throughout the harvesting periods, and fermentation tests were performed in laboratory using fresh cell biomass of the industrial strain *S. cerevisiae* JP1. This strain was isolated as dominant yeast in industrial processes in northeast of Brazil [29], and it has been used as fermenting strain in several distilleries ever since. Typical results from two distilleries from the state of Paraiba showed the significant variation in the efficiency of fermentation by JP1 strain when using the industrial substrate (Fig. 1). Given that in such experiments, the only yeast was the cells of JP1 strain and that the bacterial population was at low count ($<10^4$ cells/mL), thus, the straight explanation for the fall in the ethanol yield was the chemical composition of the industrial substrate.

Our research group has surveyed for a long time the industrial fermentation processes for fuel ethanol production in the northeast Brazil, as well as in others regions, in the attempt to identify the most important events of microbial contamination that can affect their yields [24, 25, 29, 31]. However, we have observed along these years several episodes of drop in the industrial production of ethanol that could not be simply explained by microbial infection episodes. Despite the sense settled in the literature and among the distilleries worldwide that some contaminating micro-organisms are responsible for these problems, this relationship does not seem to be absolute. For example, declines in industrial yields were identified in distilleries in the northeast of Brazil that are commonly susceptible to episodes of contamination by the

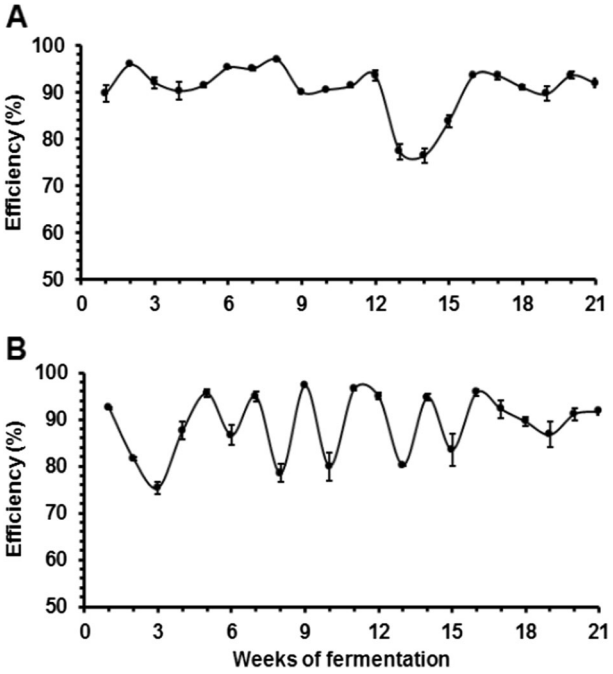

Fig. 1 Fermentation efficiencies of *S. cerevisiae* JP1 strain when using sugarcane juice supplied by distilleries Japungu Agroindustrial (**a**) and Miriri Agroenergy (**b**) along 21 weeks of harvesting season 2009–2010

yeast *Dekkera bruxellensis* in periods where the population of this yeast was below the level considered critical or even when the population of *S. cerevisiae* was dominating the process [33]. From the constant monitoring of several distilleries in the past harvest seasons of sugarcane, it was possible to identify that the quality of the fermentation substrate also has an important role in industrial yield and can interfere with the fermentation capacity of the yeast cells.

From this finding, we conducted a randomized analysis of feeding sugarcane juice belonging to distilleries from the state of Paraiba (recorded as TB samples) and extended the analysis to sugarcane from distilleries in the neighbour states of Pernambuco (ST samples) and Maranhão (IT samples). The analysis of the mineral composition of sugarcane juice in these three sites showed that there is certain homogeneity among substrates. However, large coefficients of variation were observed for concentrations of Cu^{2+}, Mg^{2+}, Zn^{2+} and P^{+} (Table 1). In a previous work, we showed that sugarcane juice can vary in the content of nitrate assimilated by the cane depending on the physic-chemical composition of the soil, such as total acidity and pH, and such variation could be one of the causes of the settlement of *D. bruxellensis* contamination in the process [34]. It can be observed the lower concentration of Mg^{2+} and P^{+} in samples from the site IT than in the two other sites (Table 1). It may be a cause of lower production of ethanol observed in fermentation assays of this substrate using industrial strain JP1 (Fig. 2a). It is known that these minerals are essential for the functioning of the enzymes in the central metabolism and fermentation, cell energy metabolism and maintenance of intracellular pH [3]. The higher Cu^{2+} content in the samples of the ST site than in two other sites is also worth noting (Table 1). The optimal concentration of Cu^{2+} in the medium for yeast growth is in the range of 0.09 mg L^{-1}, and higher concentrations of this

Table 1 Mineral composition of sugarcane juices in three different locations in the northeast of Brazil and its influence in the fermentation efficiency of *S. cerevisiae* JP1 industrial strain

Parameters	Concentrations (mg L^{-1})			VC%[b]
	ST[a]	IT[a]	TB[a]	
Calcium	108±10 a	90±6.0 a	106±29 a	17
Copper	5.8±6 a	2.1±1.3 a	0.7±0.1 b	126
Iron	43±21 a	37±8.0 a	24±2.8 a	38
Magnesium	1397±34 a	117±6 b	1356±119 a	75
Manganese	4.7±1.5 a	6.4±2 a	4.0±0.9 a	29
Zinc	15±12 a	15±5.1 a	9.9±3.7 a	58
Phosphorus	421±26.7 a	153±42 a	390±39.8 a	68
Potassium	650±21.8 a	954±32 a	778±18.4 a	21
Total nitrogen	420±92 a	313±99 a	266±48 a	25

[a] Average values for sugarcane juices supplied by distilleries in the states of Maranhão (IT), Paraiba (TB) and Pernambuco (ST). Standard deviations (±) were calculated from nine samples for each distillery and small letters refer to the statistical significance of the differences as calculated by ANOVA ($p<0.05$)

[b] Variation coefficient among the sites, considering the average of nine samples each

mineral can lead to metabolic changes with negative effect on yeast growth [16]. The protective effect of Mg^{2+} over toxic effect of Cu^{2+} has already been reported [35], but not using industrial substrates or simulating industrial conditions. Thus, this result corroborates the finding that such inhibitory effect of Cu^{2+} could be suppressed by the presence in high concentrations of some divalent cations in the industrial medium, such as Mg^{2+} (Table 1), leading to increased fermentation yield (Fig. 2a).

Additionally, samples from these three sites were also grouped according to the three cane variety commonly cultivated: RB92579, RB863129 and RB867515. The blend of the sugarcane juices for variety RB867515 from three different sites showed higher content of Cu^{2+} (4.9 mg L^{-1}) than the varieties RB92579 (2.23 mg L^{-1}) and RB863129 (1.43 mg L^{-1}). This could be the cause of the lower fermentation efficiency of JP1 strain when the juice of

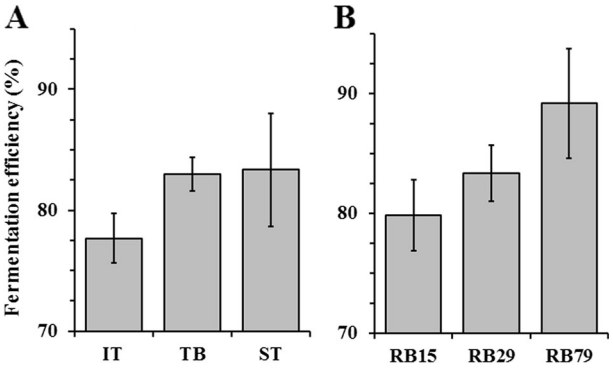

Fig. 2 Fermentation efficiencies of the *S. cerevisiae* industrial strain JP1 using sugarcane juice from different origins. **a** Juice from three different sites of study in the states of Maranhão (IT), Paraiba (TB) and Pernambuco (ST) were composed by the pool of samples from three different sugarcane varieties. **b** Juice from the three different sugarcane varieties RB867515 (RB15), RB863129 (RB29) and RB92579 (RB79) were composed by the pool of samples from three different sites of study

RB867515 was used as substrate (Fig. 2b). Moreover, the juice from RB863129 variety was converted to ethanol at lower efficiency than the one from RB92579 (Fig. 2b), probably as the result of its lower content of Mg^{2+} (528 and 957 mg L^{-1}, respectively). Thus, it seems clear that there is a balance between the concentrations of these two minerals in sugarcane juice that affects fermentative ability of yeast cells. Once again, the results indicate that Mg^{2+} supplementation of the industrial wort that presents Cu^{2+} higher than 3 mg L^{-1} would help to mitigate the inhibitory effect of the metal.

We did not detect any differences in the production of glycerol or acetate, two metabolites related to redox balance of the yeast cells, when the juices of different sites or different varieties of sugarcane were tested (data not shown). No statistical differences to the results for all these fermentation parameters showed in Fig. 2 were observed for the industrial strain PE-2 (data not shown), widely used in industrial processes in southern Brazil [30].

Excess of Minerals in the Sugarcane Negatively Affects Yeast Fermentation

After analysing samples of sugarcane juice with no apparent history of negative influence on industrial fermentation (Table 1), we evaluated samples of sugarcane juice that are constantly related to fermentation problems without any clear relationship with the settlement of contamination episodes either by bacteria or yeast (see Material and Methods). The blends of juices from groups A and B of farms (see Material and Methods for definitions) were analysed for mineral composition. A large variation among the mineral concentrations was observed when comparing samples from groups A and B (Fig. 3). In this case, the concentration of Cu^{2+} did not differ between these two groups, and so, this mineral could not be responsible alone for the fall in the industrial yield used as the initial parameter for definition of sugarcane group. However, it can be verified that in the group B, there was an excess of nitrogen (total and ammonium), P^{+}, Ca^{2+}, Mn^{2+} and Fe^{3+} (Fig. 3). Total nitrogen content of the juice may consider the presence of ammonium, free amino acids, nitrate and other forms of nitrogenous compounds in minor concentrations. Ammonium and nitrate can be found up to 250 mg L^{-1} each in sugarcane juices, depending on the type of soil in which sugarcane is cultivated [34]. However, *S. cerevisiae* is incapable to assimilate nitrate and has very little capacity to assimilate traces of proteins, peptides and peptide-like compound that can be present in the medium [36]. Therefore, only ammonium may account as nitrogen source for the observed effect on fermentation.

Afterwards, two synthetic fermentation media that were formulated by mimicking the mineral composition of juices from groups A and B were used for fermentation assays. Their fermentation efficiency yields were compared to those using complete synthetic laboratory medium (YNB medium). The results showed fermentation efficiencies around 90 % when the cells of JP1 and PE-2 industrial strains fermented group A-like media, similar to that observed for complete synthetic medium (Fig. 4). On the other hand, the combination of various minerals in excess recorded in the pool of juices from group B promoted a reduction of the yeast fermentative capacity for the two strains for less than 50 % of the theoretical maximum (Fig. 4). The average of cell viability at the end of the fermentation assays was 85 % for the complete synthetic and group A-like media, while it was around 65 % in the group B-like media. This last group also showed higher buffering effect that avoided the drop of external pH below 4.0, which may be also detrimental for fermentation. Residual sucrose of 2.3 to 2.5 % was detected at the end of fermentation, which is more than ten times higher than the acceptable level of residual assimilable sugar in the distilleries. Even though the Mg^{2+} concentration was higher in the group B-like than that in group A-like media (Fig. 4), this was probably not enough to suppress the inhibitory effects of other minerals in excess. It is

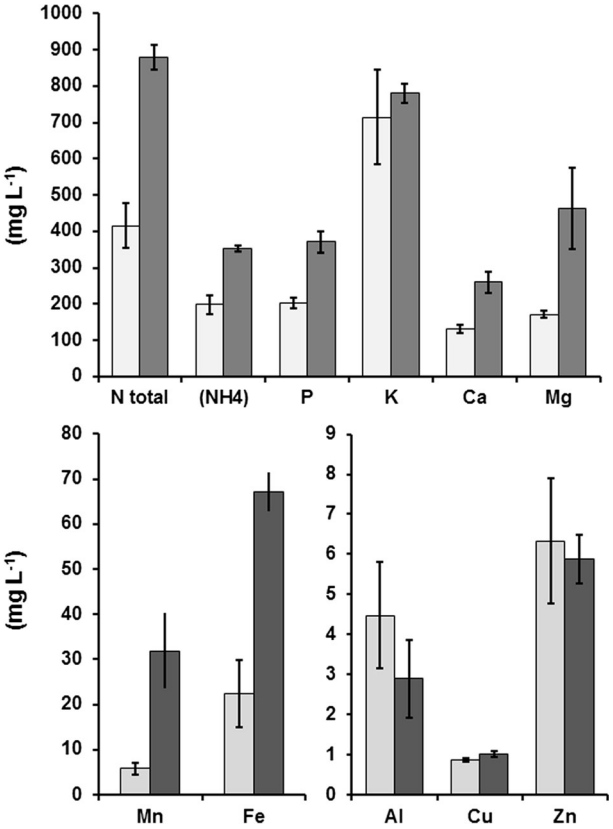

Fig. 3 Mineral composition of the pool of juices from two groups of sugarcane supplier farms divided according to their influence in the industrial fermentation yield. Group A corresponded to the group of farms supplying cane when the industrial yield was above 85 % (*light grey columns*) and group B corresponded to the farms supplying sugarcane when the industrial output was below 80 % (*dark grey columns*)

noteworthy that the Mg^{2+} concentration in group B-like medium was lower than observed in juice from ST or TB sites (Table 1).

Effects of Individual Minerals in the Fermentation

With the aim to study the effects of each mineral on the fermentation capacity of the yeast cells, we formulated ten synthetic fermentation media containing only one element in high concentration and one synthetic medium containing very low concentration of all the minerals (Table 2).

From this point, we defined high concentration of a mineral based on its average concentration in juices from over 30 sugarcane farms studied (Fig. 3) and its effect on the fermentation yield (Fig. 4). In the period of 6 h of fermentation, the yeast biomass did not vary significantly among the media tested (data not shown). The media could be sorted in four groups regarding the ethanol yields (Table 3). The highest ethanol yield as well as ethanol-specific productivity was observed in high-Mg^{2+} and in high-Mn^{2+}medium, respectively (Table 3). The kinetics of fermentation in this medium showed no statistical difference in the consumption of sucrose (Fig. 5b) and the specific consumption rates of glucose

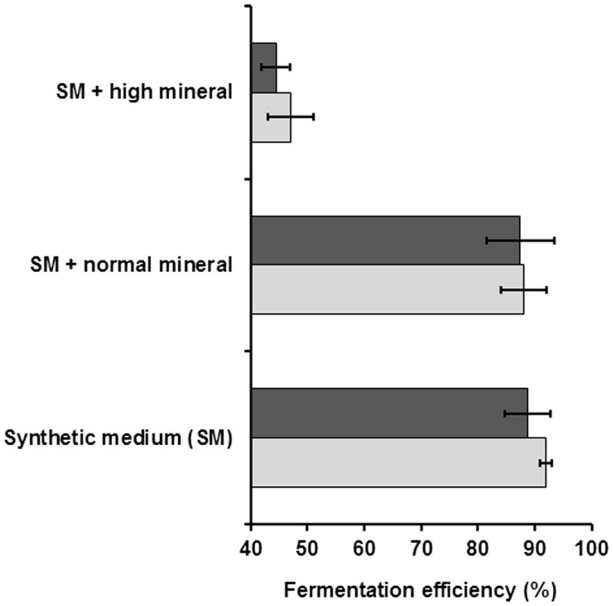

Fig. 4 Fermentation efficiencies of the *S. cerevisiae* industrial strains JP1 (*light grey columns*) and PE-2 (*dark grey columns*) when using synthetic media supplemented with minerals according to the composition of sugarcane juice from group A (*normal mineral*) or group B (*high mineral*), as defined in the legend of Fig. 4. Synthetic medium without mineral supplementation was used as reference

(98 mg h^{-1} g DW^{-1}) and fructose (115 mg h^{-1} g DW^{-1}) released by the extracellular invertase activity compared to the fermentation kinetics in the reference medium (Fig. 5a) with specific consumption rates of glucose (105 mg h^{-1} g DW^{-1}) and fructose (120 mg h^{-1} g DW^{-1}). Magnesium absorption and subsequent metabolic utilization seem to be a prerequisite for achieving the maximum fermentation activity of the yeast cell [37]. Three key enzymes that

Table 2 Formulation of synthetic fermentation media containing the excess of one of the minerals measured in sugarcane juice samples provided by different farms in the city of Santa Rita, Paraiba state, Brazil

Minerals	Fermentation media (mg L^{-1})										
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
NH_4	40.0	**360**	40	40	40	40	40	40	40	40	40
P	17.9	17.9	**270**	17.9	17.9	17.9	17.9	17.9	17.9	17.9	17.9
K	150.0	150	150	**750**	150	150	150	150	150	150	150
Ca	24.4	24.4	24.4	24.4	**262**	24.4	24.4	24.4	24.4	24.4	24.4
Mg	26	26	26	26	26	**400**	26	26	26	26	26
Al	0.7	0.7	0.7	0.7	0.7	0.7	**3.7**	0.7	0.7	0.7	0.7
Cu	0.2	0.2	0.2	0.2	0.2	0.2	0.2	**1.0**	0.2	0.2	0.2
Zn	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	**5.9**	1.2	1.2
Mn	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	**30**	1.2
Fe	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	**68**

Bold entries highlight the mineral that is in excess in each medium

Table 3 Physiological parameters of batch fermentation of *S. cerevisiae* JP1 strain in synthetic medium with excess of one compound

Media	Compound in excess	Ethanol ($g\ L^{-1}$)	CO_2 ($g\ L^{-1}$)	Glycerol ($g\ L^{-1}$)	$Y_{E/S}$ ($g\ g^{-1}$)	Qp ($g\ EtOH\ h^{-1}\ gDW^{-1}$)	Cell viability (%)
M1	–	53.0 bc	51.1 a	5.1 ab	0.38 cde	2.08	93
M2	NH_4	55.0 abc	62.2 a	4.6 ab	0.37 de	2.15	91
M3	P	52.0 bc	51.0 a	4.9 ab	0.39 cd	2.02	91
M4	K	51.7 bc	46.0 a	3.5 d	0.38 cde	1.93	93
M5	Ca	53.0 abc	52.0 a	3.7 cd	0.41 abcd	1.99	94
M6	Mg	57.9 ab	56.7 a	5.3 a	0.45 a	2.22	97
M7	Al	54.0 abc	55.0 a	4.3 bc	0.40 bcd	2.04	92
M8	Cu	48.0 c	48.7 a	3.1 d	0.35 e	1.81	94
M9	Zn	52.0 bc	48.5 a	5.0 ab	0.41 abc	2.05	90
M10	Mn	59.0 ab	62.2 a	4.7 ab	0.42 abc	2.31	90
M11	Fe	52.0 bc	53.7 a	3.7 cd	0.39 cd	1.98	93

*Average of four biological replicates, with small numbers representing the statistical significance of the differences calculated by ANOVA ($p<0.05$)

$Y_{E/S}$ ethanol yield from consumed sucrose, *Qp* specific ethanol production rate calculated per gram of cell dry weight (DW) at the end of fermentation

subsequently work for ethanol biosynthesis are dependent on the Mg^{2+}: enolase that converts 2-phosphoglycerate into 2-phosphoenolpyruvate [38], pyruvate kinase that converts 2-phophoenolpyruvate into pyruvate [39] and pyruvate decarboxylase that further converts pyruvate into acetaldehyde [40]. Therefore, we concluded that the high concentration of Mg^{2+} in the fermentation medium did not increase the transport of sugars, but rather incremented the metabolic conversion of sugar into ethanol. This might explain the highest ethanol yield achieved when fermenting sugarcane juices with higher contents of Mg^{2+}, as we observed for the juice from RB92579 sugarcane variety (Table 1).

The second group corresponded to synthetic media containing high concentrations of Zn^{2+}, Mn^{2+}, Ca^{2+} and Al^{3+} that showed ethanol yield in the range of 0.40 to 0.42 g g^{-1} (Table 3). This value corresponds to the average range of industrial fermentation processes amongst the production plants. Zinc is an important cofactor for the activity of alcohol dehydrogenase [13], which converts acetaldehyde into ethanol, and thus acts synergistically with Mg^{2+}. Manganese

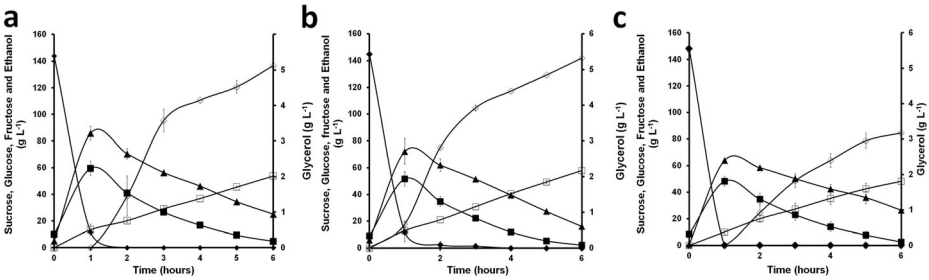

Fig. 5 Fermentation kinetics of synthetic reference medium (**a**) and supplemented media with magnesium (**b**) and copper (**c**). All these fermentations were carried out with the industrial yeast JP1 (*S. cerevisiae*). Ethanol (*white square*) and glycerol produced (white diamond), as well as consumption of sucrose (*black diamond*), glucose (*black square*) and fructose (*black triangle*). Results represent the average value of four biological replicates

is a potential competitor of Mg^{2+} in binding to enzymes, and its excess in the medium can lead to reduced ethanol production [3]. We observed that the highest Mn^{2+}/Mg^{2+} ratio in the juice, the lower the ethanol yield, as in the case of the juice of IT site (Table 1; Fig. 2a). Calcium is present at higher concentrations in sugarcane molasses than in sugarcane juice [2]. It counteracts with Mg^{2+} by competitively inhibiting the activity of some key enzymes, and its toxic effect is suppressed by increasing the availability of Mg^{2+} in the fermentation medium [37]. In the present work, Ca^{2+} concentration is low and adequate for the maintenance of cellular homeostasis [11] and might not affect yeast fermentation performance. Therefore, this cation may be only relevant in molasses-fermenting process. Al^{3+} was reported as only mildly toxic for yeast cells at 21.5 mg L^{-1} [41] and inhibited yeast by 72 % at concentration of 54 mg L^{-1} [21], which are four to 12 times higher than the highest concentration we found in sugarcane juices (Fig. 3). Thus, like Ca^{2+}, this cation seems not to be a concern for sugarcane juice fermentation.

The third group was composed by media containing high concentrations of P^{+}, Fe^{3+}, K^{+} and $NH4^{+}$, showing lower ethanol yields in the range of 0.37 to 0.39 g g^{-1} (Table 3). However, the consumption of sugars in these media was slightly higher (88.5 %±2.5) than that in Mg^{2+}-enriched medium (83 %). It seems that sugar was diverted to a metabolite other than glycerol (Table 3). Moreover, acetate and lactate were not produced, and yeast biomass remained unaltered in these media (data not shown). Thus, CO_2 production may be increased in this medium. In the case of Fe^{3+}, the reduction in ethanol yield could be explained by the induction of oxidative stress in cell yeast [16]. The ideal concentration of K^{+} for yeast growth is around 10 g L^{-1}, while no toxic effect was observed even at 100 g L^{-1} in the medium due to the strict control of the membrane permeability to this ion by the action of Na^{+}/K^{+} pump [42]. These concentrations are far higher than we observed in sugarcane juices, and so, its effect on the reduction of ethanol yield could not be determined. Tthe negative effect of phosphorus in the fermentation yield remains unclear, since no toxic effect was reported for the concentration range used in the present work. Instead, taking the results from P^{+} concentrations in juices from different locations (Table 1) and the yields of the correspondent juices (Fig. 2a), we can assume that such concentrations are still below the optimal for ethanol production, especially when in combination with low concentration of Mg^{2+}.

At last, the fourth group refers to fermentation media containing high concentrations of Cu^{2+} with ethanol yield as low as 0.35 g g^{-1} (Table 3). In an industrial point of view, the results represent an industrial efficiency of only 68 % of sugar conversion, imposing severe economic loss to the industry. Therefore, the use of sugarcane varieties that accumulate high Cu^{+2} content in their juices independently of the site of cultivation, such as the RB867515 variety (Fig. 2b), can be problematic for the industrial process. The kinetic analysis of the fermentation showed that sucrose consumption was similar to that of Mg^{2+}-enriched medium (Fig. 5c). However, a low specific consumption rate of glucose was calculated (89 mg h^{-1} g DW^{-1}) and even lower specific consumption rate of fructose (75 mg h^{-1} g DW^{-1}), when compared to the reference and Mg^{2+}-enriched media. Thus, it seems that Cu^{2+} significantly reduced fructose uptake by the yeast cells, among other metabolic effects. Early report showed that supplementation of the medium with Cu^{2+} to 43 mg L^{-1} stimulated ethanol production in YNB medium with glucose, although it extended the time of the experiments to days of fermentations [43]. This result is incompatible with the industrial approach of 8 to 12 h of fermentation. Moreover, the production of glycerol was lower than that in other media (Table 3), which indicates that Cu^{+2} affected the redox metabolism. It is known that Cu^{2+} acts protecting the yeast cells against oxidative damages by the activation of Cu-Zn superoxide dismutase. Thus, the elevated external Cu^{2+} concentration can deregulate the delicate balance that separates its metabolic beneficial from deleterious effects for the yeast cells [44]. As stated above, such

toxic effect could be suppressed by the adequate supplementation of Mg^{2+} in the fermentation medium.

Conclusion

In view of the results, we propose that the monitoring of Mg^{2+} concentration in the wort is the most important issue for industrial control of fuel ethanol fermentation process when using sugarcane juice. Keeping its concentration in the level above 600 mg L^{-1} may prevent falls in ethanol production by the presence of toxic cations in the fermentation substrate.

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6.2 Anexo II

Magnesium ions in yeast: setting free the metabolismo from glucose catabolite repression

Rafael Barros de Souza, Rayssa Karla Silva, Dayvison Soares Ferreira, Sérgio de Sá Leitão Paiva Junior, Will de Barros Pita e Marcos Antonio de Morais Junior

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Magnesium ions in yeast: setting free the metabolism from glucose catabolite repression†

Rafael Barros de Souza,‡*[a] Rayssa Karla Silva,[a] Dayvison Soares Ferreira,[a] Sérgio de Sá Leitão Paiva Junior,[b] Will de Barros Pita[ac] and Marcos Antonio de Morais Junior[ad]

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In a recent work we showed that magnesium (Mg^{II}) plays an important role in industrial ethanol production, overcoming the negative effect of the excess of minerals, particularly copper, present in sugarcane juice, with a consequent increase in ethanol yield. This cation has been reported to be involved in several steps of yeast metabolism, acting mainly as a co-factor of several enzymes of fermentation metabolism and protecting yeast cells from stressful conditions. However, despite many physiological investigations, its effect in the molecular mechanisms that control such metabolic activities remains unclear and to date no information concerning its influence on gene expression has been provided. The present work took advantage of the DNA microarray technology to analyse the global gene expression in yeast cells upon fermentation in Mg^{II}-supplemented medium. The results of the fermentation parameters confirmed the previous report on the increase in ethanol yield by Mg^{II}. Moreover, the gene expression data revealed an unexpected set of up-regulated genes currently assigned as being negatively-regulated by glucose, which belong to respiratory and energy metabolism, the stress response and the glyoxalate cycle. On the other hand, genes involved in ribosome biogenesis were down-regulated. Computational analysis provided evidence for a regulatory network commanded by key transcriptional factors that may be responsible for the biological action of Mg^{II} in yeast cells. In this scenario, Mg^{II} seems to act by reprogramming the yeast metabolism by releasing many genes from glucose catabolite repression with positive consequences for ethanol production and maintenance of cell viability.

Significance to metallomics

Magnesium is important due to its participation in various physiological processes in yeast cells, which are related to fermentative metabolism and protection against several forms of environmental stress. In the present paper we extend the biological action of Mg^{II} by showing its influence on yeast gene expression and presenting a model of metabolic regulation associated with this cation in fermentation and stress tolerance based on the relief of the glucose catabolite repression mechanism. The relevance to industrial processes is discussed.

Introduction

The mineral composition of sugarcane juice in the fuel ethanol industry has been suggested as an important factor influencing the fermentation process.[1–3] In a recent study, we reported a wide variation in the mineral concentration of broths from different sugarcane varieties cultivated in different regions in northeast Brazil.[4] This variation negatively influenced ethanol production, but could be overcome by the presence of a considerable concentration of magnesium in the fermentation substrate, which restored high ethanol yields.[4]

Magnesium (Mg^{II}) is referred to as an important component for the regulation of cell cycle, the control of many things from

[a] Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil
[b] Laboratory of Bioinformatics and Evolutionary Biology, Federal Rural University Pernambuco. Rua Dom Manoel de Medeiros, s/n, Recife, PE 52171-900, Brazil
[c] Department of Antibiotics, Federal University of Pernambuco. Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil
[d] Department of Genetic and Federal University of Pernambuco. Avenida Moraes Rego, No. 1235, Recife, PE 50760-901, Brazil

† Electronic supplementary information (ESI) available: Supplementary material and gene ontology category of genes up-regulated with magnesium supplementation, displaying only results with $p < 0.05$. See DOI: 10.1039/c6mt00157b

‡ Rafael Barros de Souza, Departamento de Genética – Universidade Federal de Pernambuco, A.v. Moraes rego, No. 1235, Cidade Universitária, 50670-901 Recife, PE, Brazil. E-mail: barrosrs87@gmail.com. Fax: +55-81-21268622, Tel: +55-81-21267817.

cell size and central metabolism functions to chromosome condensation and mitosis.[5] It is the fourth most abundant cation in nature, including biotic and abiotic systems, and its physicochemical characteristics, such as a relatively low ionic radius, makes it more stable in biological systems than other divalent cations, such as Ca^{II}.[6] Therefore, it can be used in a wide range of biological functions as a cofactor for over 300 enzymes in different metabolic reactions, such as DNA and ATP synthesis, in the activation of several glycolytic enzymes, as well as for the assembly and stabilization of cell membranes.[2,7] It also participates in the TCA cycle, β-oxidation, and in ion transport across cell membranes; furthermore, it plays an important role in mitochondrial functions.[8] Magnesium also binds to ATP molecules at physiological pH to stabilise phosphate groups used for phosphorylation and other ATP-dependent reactions like DNA polymerisation.

In yeast, the transport of Mg^{II} from the medium is dependent on two carrier proteins Alr1p and Alr2p, and the action of the mitochondrial inner membrane protein Mrs2p is associated with Mg^{II} intramitochondrial homeostasis.[2,9,10] Once inside, it participates in several biological processes, protecting cells from several stressful conditions, such as dehydration–rehydration treatments, high ethanol exposure levels and thermal shock, as well as the metal toxicity effect mainly caused by aluminium and copper.[11–14] It also acts on the central metabolism controlling the carbon flux either for respiration or fermentation, possibly through the modulation of the activities of pyruvate decarboxylase and pyruvate dehydrogenase.[15,16] The influx of Mg^{II} to yeast cells occurs during the period of maximal sugar consumption and growth rate, and the cation is almost completely expelled from the cells upon entrance to the stationary phase or when sugar is exhausted from the medium.[17] However, the residual ions into the cells are important for the maintenance of this induced physiological state, as we observed for the recycling of yeast biomass pre-cultivated in Mg^{II}-enriched medium.[4] The increment in glucose uptake and ethanol production, without affecting the yield, when the fermentation medium contains Mg^{II} in concentrations as low as 347 μmol L^{-1} (8.3 mg L^{-1}), was reported.[17] Besides, there are reports on stimulation of ethanol production when industrial substrates are supplemented with this cation.[15]

Despite the wide range of biological activities, to date, there has been no indication that Mg^{II} can control gene expression or the molecular mechanisms behind these phenomena, although it has been suggested that it changes the pattern of histone phosphorylation.[6] As a consequence, one could foresee its involvement in chromatin reorganization and re-modelling with an influence on gene activity. In the present work we performed a study connecting physiological data with the global transcriptional response of *Saccharomyces cerevisiae* JP1 industrial strain under fermentation conditions with supplementation of magnesium. The results provide the first evidence that in yeast cells Mg^{II} controls the expression of a subset of genes involved in chromatin organisation, carbohydrate metabolism, respiration and energetic metabolism, stress response and protein synthesis, possibly by modulating the activity of regulatory proteins, and thereby their biological mechanisms. As a consequence, yeast cells become more efficient in ethanol production under anaerobiosis and in oxidative metabolism, and stress resistant under aerobiosis as observed from physiological data. The (partial) relief of glucose catabolite repression that was observed herein may represent a milestone in the biological actions of Mg^{II} that is bioavailable in the environment for the yeast cells. The significance of these findings for the industrial production of ethanol is further discussed.

Methods

Yeast strain and media for growth and fermentation assays

Industrial strain *Saccharomyces cerevisiae* JP1, which is employed as a fermentation strain in many distilleries of Northeast Brazil,[4] was used in the present study. Cells were maintained and cultivated in YPD medium (yeast extract: 10 g L^{-1}; glucose: 20 g L^{-1}; bacteriological peptone: 20 g L^{-1}) at 32 °C. The solid medium contained an agar concentration of 20 g L^{-1}. The reference synthetic media were prepared with YNB (Yeast Nitrogen Base, Difco) without ammonium sulphate and amino acids at 1.6 g L^{-1}, glucose at 20 g L^{-1} for growth experiments or 140 g L^{-1} for fermentation assays and minerals: urea (80 mg L^{-1}), $(NH_4)_2SO_4$ (40 mgN L^{-1}), P (17.9 mg L^{-1}), K (150 mg L^{-1}), Ca^{II} (24.4 mg L^{-1}), Mg^{II} (26 mg L^{-1}), Al^{III} (0.7 mg L^{-1}), Cu^{II} (0.2 mg L^{-1}), Zn^{II} (1.2 mg L^{-1}), Mn^{II} (1.2 mg L^{-1}) and Fe^{III} (4.4 mg L^{-1}).[4] The amount of minerals initially present in the YNB medium formulation was taken into consideration. The effect of supplementation was evaluated by increasing the Mg^{II} and Cu^{II} concentrations to 500 mg L^{-1} and 15 mg L^{-1}, respectively, which was provided to the medium as a sulphate salt. Sugarcane juice and molasses were provided by Distillery Japungu Agroindustrial (Santa Rita, State of Paraiba, Brazil), diluted to an equivalent of 120 grams of sugar per litre and prepared as previously reported.[4] All the media were filtered and sterilized prior to use (Millipore membrane 0.22 μm).

Fermentation experiments

All the fermentations were carried out in a single-batch in 250 mL flasks containing a total of 200 mL of medium and yeast cells as previously described.[4] Cells were pre-grown in YNB medium to the beginning of the stationary growth phase, collected by centrifugation and suspended to 10^8 cells mL^{-1} in the fermentation media supplemented with Mg^{II} and/or Cu^{II}. The fermentations were carried out for 7 h at 32 °C without agitation.[4] At the end of the fermentations, samples were withdrawn, centrifuged and the supernatant taken for metabolite analysis *via* HLPC. Samples were taken to determine biomass production by cell dry weight and to evaluate cell viability by direct microscopic count in a Neubauer chamber after dying with methylene blue. In addition, cells collected from the Mg^{II}-supplemented synthetic media were immediately used for RNA extraction as described below. The measurement of carbohydrates, glycerol, acetate and ethanol was carried out using HPLC (Waters Co., USA) using an Aminex HPX-87H column (BioRad, USA) heated at 60 °C. Sulphuric acid at 5 mM was used as the mobile phase at a flux of 0.06 L min^{-1}. Fermentation experiments

were carried out in triplicate. Statistical analysis was performed as previously reported.[4]

Growth experiments

For the growth experiments, yeast cells were pre-grown overnight in synthetic reference medium at 32 °C with agitation (180 r.p.m.). Afterwards, cells were collected *via* centrifugation and re-suspended in fresh medium to an initial cell density (OD600) of 0.2 in sterile microtiter plates (96-well) for a total volume of 200 μL with specific media. For the growth assays with a respirable carbon source, ethanol (15.34 g L^{-1}) and glycerol (20.47 g L^{-1}) were added to reach the same initial carbon concentration of 666 mmol L^{-1} as in the glucose medium. These synthetic media were supplemented with yeast extract (10 g L^{-1}) and peptone (20 g L^{-1}) to fulfil the nutritional requirements of those respirable carbon sources. YPD and YPD adjusted to pH 2.5 with sulphuric acid were used when testing the inhibitory effect of medium acidification on yeast growth. For assessing the effect of glucose catabolite repression, YNB medium containing glucose or ethanol was supplemented with 2-deoxiglucose to 1 mmol L^{-1}. Cultivations were performed in a Synergy HT device (BioTek, Switzerland) at 32 °C and maximal speed with absorbance measurement every 10 minutes for 20 h. Growth curves were prepared from the average of three biological experiments and the growth rate was calculated from the slope of the exponential growth phase.

Transcriptome-wide expression microarray analyses

Collected cells were centrifuged and re-suspended in 400 μL AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3) and 80 μL of 10% SDS solution, mixed using a vortex and incubated at 65 °C for 10 min. The total RNA in the lysates was purified by using a RNAspin Mini RNA Isolation Kit (GE HealthCare, USA) and quantified on a NanoDrop ND-2000 UV-Vis spectrophotometer (ThermoFisher Scientifics, USA). Synthesis of cDNA and cRNA and labelling were performed using a Two-Color Low Input Quick Amp Labelling Kit (Agilent, USA) according to the manufacturer's instructions, with a Two-Color RNA spike-in kit as the internal control (Agilent, USA). cRNA from cells taken from the fermentation experiment supplemented with Mg^{II} was labelled in red with Cy-5 dye and assigned as the target samples, while cRNA from cells taken from the fermentation experiment in reference medium was labelled in green with Cy-3 and assigned as the reference sample. Target and reference cRNA samples were pooled and used to hybridise the yeast gene expression 8 × 15k spot slides (Agilent, USA) at 65 °C for 17 hours at 10 r.p.m. in a microarray hybridization oven (Agilent, USA). Fluorescent data from a 3 μm-resolution microarray scanner (Agilent, USA) were extracted as .txt files using the software Feature Extraction. Groups of genes with stringent statistical significance (concomitant Adj. $p < 0.05$ and $B \geq 3$) were utilized and their log FC values used to classify up- (≥ 0.5 log FC) and down-regulated (≤ -0.5 log FC) genes, a term meaning the abundance of transcripts under test conditions relative to the reference conditions.[18] Microarray data were submitted to Gene Expression Omnibus (GSE75803). The *Saccharomyces* Genome Database (SGD), Gene ontology Slim Mapper and YeastMine databases were used for the identification of encoded proteins and GO clustering of the genes. The analyses of transcription factor binding sites were carried out with the Yeastract database.

Results and discussion

Effect of magnesium on fermentation parameters

Recent analyses of the mineral composition in sugarcane juice used by ethanol distilleries in the northeast of Brazil revealed the negative effect of mineral excess on the yeast fermentation performance. This negative effect was overcome by the presence of Mg^{II} at concentrations above 500 mg L^{-1}.[4] In the present study we combined genetic and physiological data to determine the metabolic changes in the yeast cells triggered by this ion. A fermentation assay in the presence of Mg^{II} was performed. The first aspect to take into consideration is the absence of acetate in the medium supernatant (Table 1), which is indicative of the anaerobic condition of the experiment.[4] Similar to our previous report, Mg^{II} slightly increased glucose consumption as well as ethanol and glycerol production, while it decreased biomass generation (Table 1). No difference in cell viability was observed between the reference and the Mg^{II}-containing media (data not shown). Moreover, at the end of fermentation, glucose was still present at a concentration around 50 g L^{-1} in both media. Therefore, any effect that Mg^{II} might promote in the yeast cells was produced in the presence of a large amount of glucose in the medium.

Overview of gene expression in response to Mg^{II} supplementation

At the end of fermentation, RNA was purified from cells exposed to Mg^{II} supplementation and compared to samples withdrawn from the reference medium. Relative gene expression analyses were performed following strict parameters, regarding primarily genes associated with well-defined metabolic pathways or biological processes, as we recently reported.[18] A total of 222 genes showed significant changes in their expression, 130 genes up-regulated and 92 down-regulated (consult GEO GSE75803 for a complete list), which were categorised by Gene ontology (GO) analysis. It is worth noting that we did not observe the differential expression of genes involved in sulphur metabolism.

Table 1 Physiological parameters of the batch fermentation of *S. cerevisiae* JP1 strain in synthetic medium with magnesium supplementation

	Carbon (mol L^{-1})		Yield	
	Reference	Magnesium	Reference	Magnesium
Glucose consumption	3.00	2.96		
Ethanol produced[a]	1.69	1.73	0.43	0.45
Glycerol produced	0.18	0.21	0.06	0.07
CO_2 estimated[b]	0.85	0.86	0.42	0.43
Biomass produced[a]	0.13	0.07	0.04	0.02
Balance	95%	97%	95%	97%

[a] Yield values show statistical differences ($p < 0.05$). [b] Theoretical CO_2 calculated on the basis of ethanol produced.

Table 2 Ribosomal protein genes down-regulated in the fermentation supplemented with magnesium ions

Genes	Fold change	SGD description
Rpl33A	1.8	Ribosomal 60S subunit protein L33A
Rps15	1.6	Protein component of the small 40S ribosomal subunit
Rpl16B	1.6	Ribosomal 60S subunit protein L16B
Rps14a	1.6	Protein component of the small 40S ribosomal subunit
Rpb3	1.5	RNA polymerase II third largest subunit B44
Rpl14B	1.5	Ribosomal 60S subunit protein L14B
Rpl1A	1.4	Ribosomal 60S subunit protein L1A
Rpl9B	1.4	Ribosomal 60S subunit protein L9B

Besides, as a relative analysis of a gene-wide expression approach, any putative effect of sulphate in the Mg^{II}-rich medium shall be abolished by its effect in the reference medium that also contains ammonium sulphate.

According to the stringency applied, genes exhibiting down-regulation could not be placed in the category of the biological processes. However, two types of genes were worth highlighting: the CDC28 gene (with a −1.9 fold change relative to the reference condition) and eight genes that encode for ribosomal proteins (RP) (with a −1.55 fold change on average relative to the reference conditions) (Table 2). The CDC28 gene encodes for the Cdc28p cyclin-dependent kinase that acts as the major regulator for the progression of the mitotic and meiotic cell cycle, regulating the subset of genes involved in DNA replication and segregation, chromosome dynamics and cell morphogenesis, among others.[19,20] Alterations in either its transcriptional activity or function of its enzyme are associated with the arrest at G1/S or G2/M checkpoints, with a consequent decrease in cell cycle progression. Moreover, eight out of the 138 RP genes were also down-regulated upon exposure to Mg^{II}. These RP genes were reported to be co-regulated in response to growth conditions since the protein synthesis rate might be accelerated during the full exponential growth phase.[21] In addition to a lower biomass production in Mg^{II}-supplemented medium (Table 1), these results indicate a reprogramming response for a decrease in cell growth under anaerobic fermentative conditions. On the other hand, as will be presented later, the aerobic growth was not affected by the presence of this ion. This represents an apparent contradiction to the current view on the importance of Mg^{II} to cell cycle progression.[5] However, it is paramount to stress the differences among the culture conditions in the literature (mostly aerobic and growth-stimulating) and the present condition (anaerobic and growth-limited). In the latter, the excess of Mg^{II} might favour the synchronization of cell culture, uncoupling the cell cycle from cell division as previously proposed,[5] and decelerating biomass formation as reported in Table 1.

Regarding the up-regulated genes, GO analysis revealed consistent biological processes affected by the supplementation of Mg^{II}. Thirty-two out of the 130 up-regulated genes were grouped into energy generation processes such as oxidative phosphorylation, ATP metabolism, respiration, energy derivation by oxidation of organic compounds and generation of precursor metabolites for energy production. These genes are organized in the following subsections according to their metabolic pathway and more details about their roles are provided (see the ESI†).

Mitochondrial functions are affected by Mg^{II} even in anaerobiosis

The majority of the up-regulated genes encode mitochondrial membrane transporters and proteins of the TCA cycle, respiratory chain and ATP synthase complex. We attempted to place these genes based on the order of metabolic events they are involved in. First, we observed the co-regulation of genes encoding carriers responsible for the uptake of acetate by the mitochondrion. The genes YAT1 (3.8×) and YAT2 (1.6×) encode for the outer-membrane carrier of acetyl-carnitine, while the gene CRC1 (1.4×) encodes for the inner-membrane carrier of acetyl-carnitine.[22,23] The coordinated action of this shuttle may provide acetate as a substrate for the beginning of the TCA cycle, bypassing the pyruvate dehydrogenase complex, since cells were under anaerobic conditions. Once inside, acetate must be activated prior to its use in the TCA cycle, which could be done by the action of a CoA transferase. In the present work, we observed a consistent up-regulation of its gene ACH1 (3.0×), which might indicate the tendency for acetate assimilation metabolism. Ach1p transfers the CoA group from succinyl-CoA to acetate resulting in the production of acetyl-CoA which feeds the TCA cycle.[24] Further on, two genes involved in the TCA cycle were significantly up-regulated by Mg^{II}. First, the CIT3 gene (2.2×) that encodes for an alternative citrate synthase,[25] the enzyme responsible for the condensation of acetyl-CoA with oxaloacetate to produce citrate and free CoA. Second, the SDH1 gene (1.9×), coding for the flavoprotein of the succinate dehydrogenase complex, responsible for the FAD^{+}-dependent oxidation of succinate to fumarate with a consequent transfer of electrons to ubiquinone *via* $FADH_2$.[26] This reaction corresponds to the complex II of the respiratory chain. In addition, Mg^{II} led to the up-regulation of the SFC1 gene (2.2×), which encodes for a succinate–fumarate transporter and is required for ethanol and acetate utilization by the yeast cells.[27] Thus, that succinate–fumarate shuttle should provide cytosolic fumarate for gluconeogenesis when cells are growing on ethanol, acetate or glycerol (non-fermentable carbon sources).

Regarding the respiratory chain, the following genes showed coordinated up-regulation by Mg^{II}: NDE1 (1.4×) from complex I; COB (4.3×) and CYT1 (1.4×) from complex III; COX1 (4.0×), COX2 (3.0×), COX3 (5.8×), and COX7 (2.2×) from complex IV; and OLI1 (2.9×) that encodes the F0 subunit of the ATP synthase complex, also known as complex V. Thus, the likely increase of the metabolic flux through the respiratory chain could result in the increment of ATP production in yeast cells if they were growing in aerobiosis or in the presence of an alternative electron acceptor during anaerobiosis. Moreover, the up-regulation of the PET9 (2.0×) gene, coding for the major ATP/ADP carrier that exchanges the mitochondrial ATP by the cytosolic ADP, was also observed.[28] There is an indication in the literature that the PET9 gene is essential for growth on non-fermentable carbon sources, such as ethanol and glycerol.[29] In addition, the up-regulation of the RGI1 (2.0×) and RGI2

(4.0×) genes, coding for proteins of unknown functions associated with respiratory growth, was also observed.[30] This is a clear indication that Mg^{II} drives the respiratory metabolism in yeast cells even under anaerobic fermentation conditions. In order to evaluate this hypothesis, we carried out aerobic growth experiments with the industrial yeast *S. cerevisiae* JP1 in media containing respirable carbon sources. The results clearly showed that Mg^{II} increased the growth rate from 0.06 h^{-1} to 0.15 h^{-1} in ethanol (Fig. 1A) and from 0.08 h^{-1} to 0.18 h^{-1} in glycerol (Fig. 1B). It has to be mentioned that growth on glucose was not affected by magnesium supplementation (data not shown). Higher growth on ethanol could also be the result of elevated tolerance to this molecule induced by Mg^{II} as previously reported.[2] The increment in growth with glycerol might also be associated with the observed up-regulation of the GUT2 gene (1.8×) under fermentation conditions with Mg^{II}. This gene encodes a mitochondrial glycerol-3-phosphate dehydrogenase involved in glycerol catabolism.[31] This physiogenomic evidence indicates that magnesium turns yeast cells to an oxidative metabolism that allows the efficient assimilation of respirable carbon sources as soon as oxygen or any other electron acceptor is available.

The effect of magnesium on the mitochondrial functions as well as in controlling the fluxes between respiration and fermentation has been reported and can be complemented by the transcriptomics results indicating metabolic changes which are summarized as follows.[8,13,14] The import of acetate, as aforementioned, might result in the production of a succinate pool, which combined with its own transport from the cytoplasm by a shuttle mechanism leads to an increase in its availability to the mitochondria. The accumulation of succinate, in turn, might increase the flux towards the succinate dehydrogenase complex, which triggers increases in both $FADH_2$, used by the respiratory chain for ATP production, and fumarate, which is partially converted to oxaloacetate for the TCA cycle inside the mitochondrion. The remaining fumarate is then exported to the cytosol by the same shuttle mentioned above in order to produce oxaloacetate for gluconeogenesis. These metabolic changes might turn cells prone to a more efficient utilization of respirable carbon sources as indeed we observed for ethanol and glycerol.

Mg^{II} turns on stress responsive genes

Another group of genes induced by Mg^{II} is related to the environmental stress response (ESR). These genes are commonly down-regulated in the presence of glucose in the medium from a concentration that induces glucose catabolite repression (GCR).[32] Six ESR genes, GRE1 (2.6×), ATX1 (1.8×), GAD1 (1.8×), GSH1 (1.5×), AHP1 (1.6×) and HSP150 (1.8×), were identified as differentially expressed in the present work, which are associated with the oxidative stress response. A search for common regulators in the Yeastract database revealed that all of these genes present a binding site for Msn4p, one of the main regulators of the ESR mechanism in yeast. Furthermore, GRE1 is also related to heat shock and osmotic stress responses whilst ATX1 and HSP150 are associated with copper and heavy metal stress response.[33–35]

In a previous work, we discussed the possible antagonistic effect between magnesium and copper on fermentative metabolism of the industrial yeast JP1, as we showed that Cu^{II} reduces ethanol yield while Mg^{II} reverted this negative effect.[4] Cu^{II} is already known as an oxidative stressor agent at a high concentration (>10 mg L^{-1}) in yeast. ATX1 encodes for a cytosolic metallochaperone involved in copper-dependent iron uptake and metal homeostasis, and its over-expression suppresses H_2O_2 sensitivity of SOD mutants.[34] Aerobic growth experiments showed complete inhibition of yeast growth when the synthetic medium contained Cu^{II} at 15 mg L^{-1} and the presence of Mg^{II} at 500 mg L^{-1} partially restored yeast growth (Fig. 2). Therefore, Mg^{II} seems to act as a protective agent against the cellular stress caused by ion metals. As a complement to our previous work that used synthetic medium,[4] fermentation experiments were performed with ion-supplemented industrial

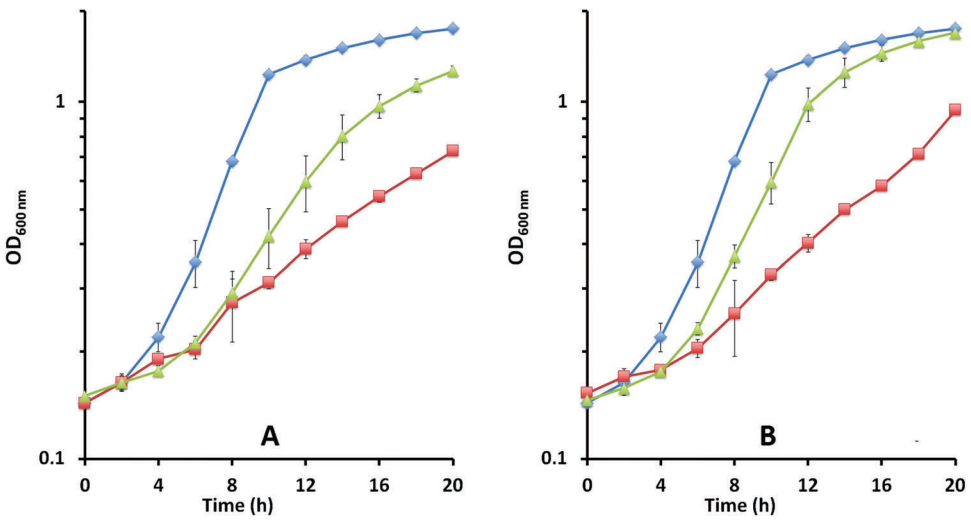

Fig. 1 Effect of Mg^{II} supplementation on *S. cerevisiae* JP1 strain growth using ethanol (panel A) or glycerol (panel B) as a carbon source. YP media containing ethanol or glycerol (square symbol) were supplemented with Mg^{II} at 500 mg L^{-1} (triangle symbol). Medium containing glucose (diamond symbol) was used as the reference.

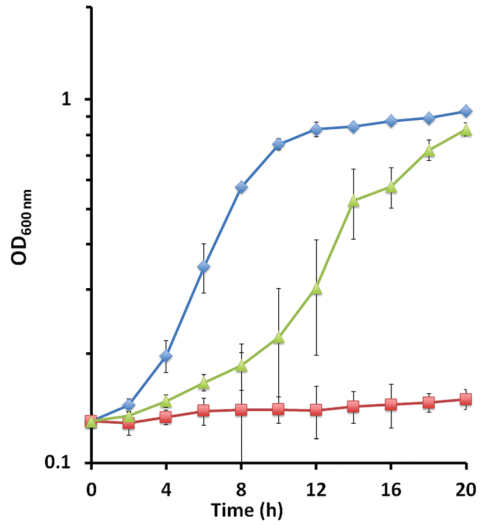

Fig. 2 Effect of Mg^{II} supplementation on the growth repressive effect of Cu^{II} in the *S. cerevisiae* JP1 strain. YNB medium with glucose (20 g L^{-1}) (diamond symbol) was supplemented with Cu^{II} (15 mg L^{-1}) in the absence (square symbol) or in the presence of Mg^{II} (500 mg L^{-1}) (triangle symbol).

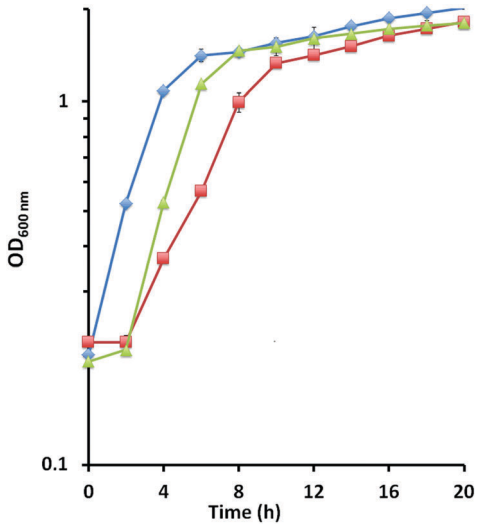

Fig. 4 Effect of Mg^{II} supplementation on the growth inhibitory effect of sulphuric acid in the *S. cerevisiae* JP1 strain. The YPD medium was adjusted to pH 2.5 with sulphuric acid in the absence (square symbol) or in the presence of Mg^{II} (500 mg L^{-1}) (triangle symbol). Unadjusted YPD medium (diamond symbol) was used as the reference.

sugarcane juice (SCJ) and sugarcane molasses (SCM). The results showed that, relative to sugarcane juice, the presence of Mg^{II} increased the efficiency of fermentation by 8% whilst Cu^{II} reduced it by 12% (Fig. 3). The fermentation efficiency with molasses, which is normally lower than that in sugarcane juice, decreased by 8% in the presence of magnesium and by 19% with copper. However, in both substrates the negative effect of copper was partially overcome by the presence of magnesium (Fig. 4), confirming that Mg^{II} suppressed the oxidative stress triggered by Cu^{II}.

Oxidative stress is also faced by yeast cells when exposed to an acidic environment caused by an inorganic acid, such as sulphuric acid.[18] In order to survive this stress, yeast cells have to reduce the activity of the Ras-cAMP/PKA mechanism and de-repress ESR genes and genes for oxidative metabolism from the effects of GCR.[18] Thus, one might suggest that the de-repressive action of Mg^{II} could protect, to some extent, yeast cells from the acid-induced damages. In order to test this hypothesis, yeast cells were submitted to aerobic growth in synthetic medium adjusted to pH 2.5 with sulphuric acid. In this condition, growth rate was calculated as 0.25 h^{-1} compared to the 0.35 h^{-1} calculated for unadjusted medium. Addition of Mg^{II} completely restored the yeast growth rate in acidic medium to 0.35 h^{-1} (Fig. 4). Tolerance to acid stress is associated with the oxidative stress response and also to the induction of PKC-dependent expression of cell wall integrity (CWI) genes.[18] Among those CWI genes, we previously reported the up-regulation of PIR2/HSP150 (1.2×) and PIR3 (6.9×) upon inorganic acid treatment.[18,36] These genes encoded for *O*-mannosylated and *O*-glycosylated cell wall attached proteins, respectively, involved in the stability and repair of the cell wall. In the present work, up-regulation of PIR2 (1.8×) was observed when Mg^{II} was present in the medium. Altogether, these results show that Mg^{II} releases the expression of some genes involved in ESR as a putative consequence of GCR alleviation.

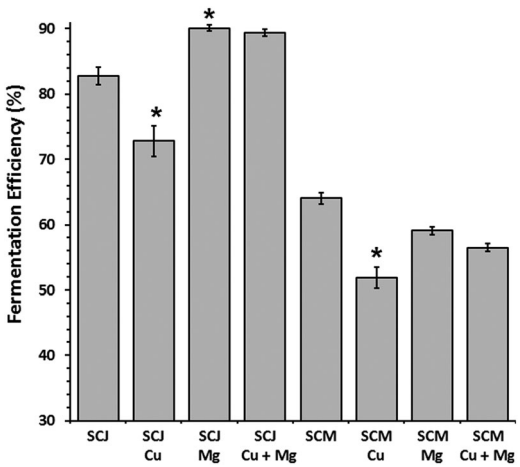

Fig. 3 Effect of Mg^{II} (500 mg L^{-1}) and/or Cu^{II} (15 mg L^{-1}) supplementation on the efficiency of ethanol production by the industrial *S. cerevisiae* JP1 strain using sugarcane juice (SCJ) or sugarcane molasses (SCM) as industrial substrates. (*) values show statistical differences ($p < 0.05$).

Mg^{II} releases yeast genes from glucose repression

It is noteworthy that the expression of most of the aforementioned up-regulated genes is negatively regulated by the presence of glucose in the medium, as a consequence of the GCR mechanism. Thus, given the presence of large amounts of residual glucose in the medium (50 g L^{-1}) at the time of sampling for RNA extraction, these genes should be transcribed at the same level as under the reference conditions. Their up-regulation indicates that the GCR mechanism is at least partially released when Mg^{II} is present in the medium. In previous works, we have shown that yeast cells reduce the activity of the Ras/cAMP-PKA cascade involved in GCR when

exposed to acid stress caused by sulphuric acid, resulting in a decrease of protein synthesis and growth rate, as well as inducing genes involved in general stress response and oxidative metabolism.[18,36] In this case, Mg^{II} ions could turn yeast cells more tolerant to the stressful conditions imposed by the industrial environment.

This GCR-releasing mechanism seems to be the consequence of the observed up-regulation of the REG2 gene (1.7×), encoding the regulatory subunit of the Glc7p phosphatase. It is well-known that the Reg2p–Glc7p complex interacts with the targets of Snf1p kinase for the release of GCR-controlled genes.[37] The expression of REG2 during the diauxic shift is dependent on the action of Cat8p, a transcription factor responsible for the reprogramming of carbon metabolism in yeast towards oxidative rather than fermentative metabolism, as well as for the use of ethanol during respiration.[38] However, the up-regulation of the CAT8 gene was not observed. In addition, we observed the up-regulation of the IMP2 gene (2.7×) that encodes an important transcriptional factor involved in the de-repression of genes for the utilisation of ethanol, galactose, raffinose and maltose,[39] as well as in ion homeostasis.[40] Thus, it can be hypothesised that Mg^{II} could alleviate the repression over some GCR-controlled genes by regulating the activity of Cat8p even in the presence of glucose leading to an increased expression of the IMP2 and REG2 genes. This hypothesis is supported by the up-regulation of the tight glucose-regulated gluconeogenesis genes PCK1 (2.7×) and FBP1 (2.9×), as well as the glyoxylate cycle gene ICL1 (2.4×). PCK1 encodes a phosphoenolpyruvate carboxykinase that converts oxaloacetate to phosphoenolpyruvate.[41] Above, we proposed that the combined action of Sdh1p of the TCA cycle with the Sfc1p malate-succinate shuttle would fulfil the requirement for oxaloacetate by Pck1p. FBP1 encodes the key regulatory gluconeogenesis fructose-1,6-bisphosphatase that allows yeast cells to grow on ethanol and glycerol.[42] Both PCK1 and FBP1 genes are co-repressed by Mig1p as part of Ras/cAMP-PKA activation of GCR,[43] and are also co-induced by Cat8p during glucose starvation or the diauxic shift.[42] The gene ICL1 encodes the isocitrate lyase that breaks isocitrate into glyoxylate and succinate in the cytoplasm.[44] The succinate could then be transported into the mitochondria *via* the Sfc1p malate-succinate shuttle mentioned above. This gene is repressed by glucose and induced by growth on ethanol,[44] and also by Mg^{II} as described in the present work. Therefore, these results confirm the genetic evidence that Mg^{II} indeed antagonises the GCR mechanism.

In order to confirm this de-repressive effect we carried out a growth assay adding 2-deoxyglucose (2DG) to synthetic medium containing glucose (20 g L^{-1}) and supplemented with Mg^{II}. 2DG is a glucose analogue and potent inducer of GCR that is phosphorylated to 2DG-6P, but not further metabolised.[45] The presence of 2DG reduced yeast growth relative to the reference medium, whilst the presence of Mg^{II} in the medium partially released cell growth from such inhibition (Fig. 5). This result confirms that Mg^{II} promotes a reprogramming of the yeast metabolism that allows cells to deactivate the GCR mechanism. However, this effect presents constraints as the complete

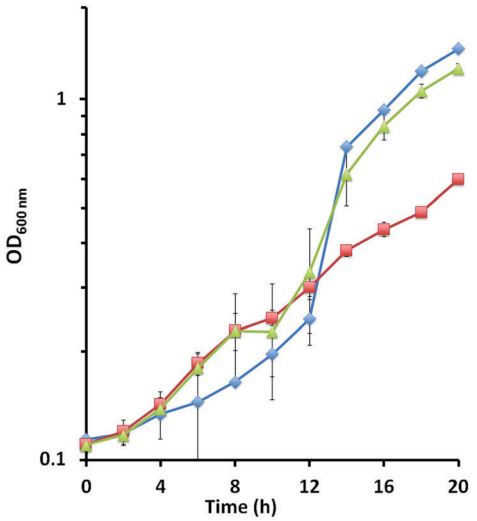

Fig. 5 Effect of Mg^{II} supplementation on the growth repressive effect of 2-deoxyglucose (2DG) in the *S. cerevisiae* JP1 strain. YNB medium with glucose (20 g L^{-1}) (diamond symbol) was supplemented with 2-DG (1 mmol L^{-1}) in the absence (square symbol) or in the presence of Mg^{II} (500 mg L^{-1}) (triangle symbol).

impairment of growth on ethanol by 2DG was not surpassed by Mg^{II} (data not shown).

In summary, Mg^{II} seems to trigger a relief effect that de-represses gluconeogenic genes *via* the transcriptional activation of REG2 and IMP2, with the participation of Cat8p. Starting with Icl1p, yeast cells could produce succinate from isocitrate in the cytosol, which could be further carried into the mitochondria by the Scf1p shuttle. Once inside, succinate is oxidised to malate by Sdh1p producing $FADH_2$ for respiration and malate that is also partially carried out by the Scf1p shuttle. Once outside, malate is converted to oxaloacetate, which is used for gluconeogenesis by Pck1p and Fbp1p. Altogether, this reprogramming activity could make possible the utilization of respirable sources of carbon even in the presence of glucose. Once again, it might represent the preparation of cells for higher respiration activity once oxygen, or any alternative electron acceptor, such as acetoin or acetaldehyde, becomes available in the environment. This hypothesis is corroborated by the coordinated Mg^{II}-induced up-regulation of the JEN1 (2.0×) and ADY2 (2.3×) genes even in the presence of glucose. The first encodes a cell membrane protein involved in the proton symport of lactate, acetate and pyruvate, whose transcriptional activity is tightly repressed by glucose and released by a Cat8p-dependent mechanism.[38] The second gene encodes the essential cell membrane acetate transporter and is transcribed when cells are transferred from glucose to acetate medium.[46] Therefore, the cells seem to be prepared for full oxidation of such carbon sources.

Regulatory subnetworks involved in the response to the supplementation of Mg^{II}

We used computational analysis to propose the network of interactions that responds to the supplementation of Mg^{II} by promoting differential gene expression. First, the set of 92 down-regulated genes were uploaded to the Yeastract database in order

to identify the common repressive transcription factors, which resulted in the following proteins: Gcr1p, Sko1p and Yap6p (Fig. 6). Gcr1p is involved in the repression of glucose-repressible genes;[47] Sko1p and Yap6p are involved in the de-repression of glucose-repressible genes and carbohydrate metabolism genes, as well as in the HOG-dependent induction of stress-responsive genes.[48–50] It is worth noting that Sko1p represses the expression of Cdc28p, whose genes were down-regulated by Mg^{II} as shown above. Since none of these factors had the expression of their genes altered in the present work, we can assume that Mg^{II} might work as a positive effector of these repressor proteins, or induce the upstream transduction cascade that activates those proteins. All these transcription factor encoding genes were uploaded to Yeastract resulting in the identification of Rap1p as the common regulator of this network (Fig. 6). Rap1p works in a broad spectrum of metabolic responses, acting as an inducer or a repressor for more than 185 genes, ranging from ribosomal protein-coding genes and glycolysis to telomere maintenance. Therefore, Rap1p appears as the key protein in the subnetwork responsible for the down-regulation of these 92 genes (Fig. 6).

The set of 130 up-regulated genes were also uploaded to Yeastract in order to identify the common inductive transcription factors, resulting in the following proteins: Adr1p, Met32p, Gis1p and Cat8p (Fig. 6). Adr1p is involved in the activation of genes required for the utilisation of ethanol, glycerol and fatty acids.[51] We observed that ADR1 (1.5×), as well as SNF1 (1.4×), was up-regulated in the supplemented medium when alleviating the parameters from the combined *B*-value together with the adjusted *p*-value to the adjusted *p*-value only. Additionally, our analysis identified the presence of a Snf1p binding site at the promoter of the ADR1 gene, as an indication of the genetic interaction of both genes in the expression of GCR-controlled genes (Fig. 6). Interestingly, both SNF1 and ADR1 genes appear to be regulated by Rap1p as indicated by the presence of the Rap1-binding site in their promoter regions. We found that Met32p acts as a coregulating gene involved in copper and iron metabolism and it regulates GSH1 expression in response to cadmium.[52,53] We did not observe up-regulation of the MET32 gene in any statistic parameter applied. However, we were able to identify in its promoter a binding motif, Yap1p, a protein required for oxidative stress tolerance and also involved in response to cadmium.[54,55] Gis1p is involved in the transcriptional de-repression of genes subjected to the Ras-cAMP dependent GCR mechanism in response to nutrient limitation,[56] whereas Cat8p is involved in the activation of genes required for cell growth in non-fermentable carbon sources.[57] This includes all those genes with mitochondrial functions described above. Again, we did not observe the up-regulation of the GIS1 and CAT8 genes by Mg^{II}. Furthermore, computational analysis revealed that the SNF1, ADR1, GIS1 and CAT8 genes contain the binding motif for Abf1p, a broad-range transcription factor for induction of genes involved in different cellular processes,[58] including respiration.[59] Therefore, Abf1p appears to be a key protein in the subnetwork responsible for the up-regulation of these 130 genes (Fig. 6).

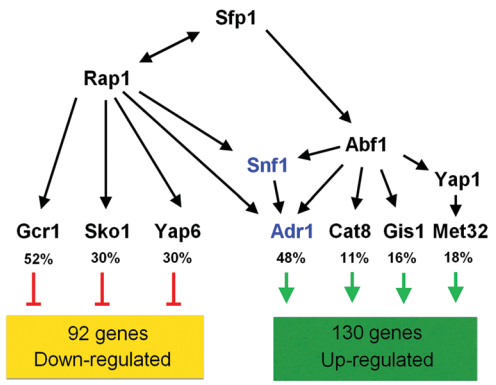

Fig. 6 Proposed model for the regulatory network that differentially regulates the expression of *Saccharomyces cerevisiae* genes in response to the presence of Mg^{II} (500 mg L^{-1}) in the fermentation medium. Up- or down-regulated genes were uploaded to the Yeastract database to search for the binding motifs for transcriptional factors (TF) in their promoter sequences. Names in black represent the TFs found in the computational search, while names in blue represent TFs that were also up-regulated in transcriptomic analyses. The values below the tfs represent the percentage of genes containing a binding motif for each TF. Red lines with a bar at one end represent down-regulation by the TFs with experimental evidence in the literature. Black arrows represent the interactions among the TFs with experimental evidence in the literature. Green arrows represent up-regulation by the TF.

Upstream of these regulatory cascades, we identified Sfp1p as the regulator of the RAP1 and ABF1 genes (Fig. 6). This is a zinc-finger protein that has an even broader activity as it regulates biological processes such as biogenesis, nutrient and stress responses, and cell cycle progression, as well as regulating the cell size according to nutrient availability.[60,61] The SFP1 gene was not induced by Mg^{II} in our analysis, indicating that this ion acts as an effector molecule of this regulatory activity. Therefore, Mg^{II} could act on Sfp1p as a master regulator of the downstream cascades controlled on the one hand by Rap1p and on the other hand by Abf1p, leading to the de-repression of respiratory, gluconeogenic and stress resistance genes in yeast.

It has been well-known since the 1970s that micro-aeration is very stimulatory of the fermentation process. The so far accepted explanation is that the stimulation of respiration increases the glycolytic flux and releases cell metabolism from some energy and redox bottlenecks imposed by the absence of oxygen. Also, the induction of (at least residual) lipid synthesis by oxidative metabolism is beneficial to fermentative metabolism. Thus, Mg^{II} could be mimicking this metabolic situation in anaerobiosis, despite the lack of functioning of the respiratory chain, by releasing the respiratory genes from glucose catabolite repression.

Conclusions

In view of the positive role of magnesium supplementation in the fermentation capacity of yeast cells, surpassing the inhibitory effect of supplementation of minerals in the substrate, we looked for the molecular processes influenced by this ion that could explain its biological action. The global view of the results leads to the conclusion that Mg^{II} primarily alleviates glucose

catabolite repression, releasing genes involved in oxidative metabolism, respiration, gluconeogenesis and stress response, with a positive consequence in ethanol yield when in anaerobiosis or increased ethanol catabolism when in aerobiosis. In an industrial context, the use of Mg^{II}-enriched biomass would result in processes with higher yields and productivity due to two main aspects: the cells would be more metabolically efficient in converting sugar to ethanol as well as becoming more tolerant to inhibitors, which keeps the population viable for longer periods of recycling. We are taking into consideration that these positive effects are applicable to other industrial processes beyond fuel ethanol production, such as the production of beverages and the biomass (baker's yeast) itself. Further work will investigate the effect of Mg^{II} on the phosphorylation profile of regulatory proteins such as those we have modelled in the present study.

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6.3 Anexo III

Yeast genes regulated by copper under fermentation condition

Rafael Barros de Souza, Rayssa Karla Silva, Sérgio de Sá Leitão Paiva Junior, Will de Barros Pita e Marcos Antonio de Morais Junior

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ARTICLE

Yeast genes regulated by copper under fermentation condition

Rafael Barros de Souza[a†], Rayssa Karla Silva[a], Sérgio de Sá Leitão Paiva Junior[d], Will de Barros Pita[a,c] and Marcos Antonio de Morais Junior[a,b]

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The mineral composition of the sugarcane juice, especially magnesium and copper ions, has been pointed out as showing significant impact on yeast metabolism, as well as in the ethanol production. In spite of being involved in several biological processes, both metals can present antagonistic effects on yeast metabolism. While magnesium increase ethanol yield and stress tolerance, copper can be toxic for the cell and negatively impact the ethanol production. Recently, we have shown the means by which magnesium regulates yeast metabolism under fermentation conditions, by using DNA microarray technology. In the present work, we pointed our attention to investigate the response of the industrial yeast *Saccharomyces cerevisiae* JP1 to copper excess under fuel-ethanol fermentation condition. First, we performed a growth assay to determine the toxic level for copper in the industrial strain and the results showed that 10 mg L^{-1} was enough to inhibit yeast growth. This concentration was, therefore, used to perform fermentation assays and also negatively impacted yeast metabolism by decreasing glucose consumption and ethanol yield by amost 20% and glycerol production by 42%. From this fermentation assay, transcriptome-wide expression analysis was carried out by DNA microarray. The results showed that DNA replication process is sensitive and highly damaged by copper, since several proteins involved in response to DNA replication stress presented their genes up-regulated. Moreover, the analyses of down-regulated genes showed that the translation process is repressed by decreasing ribosomal and rRNA production. In conclusion, our results showed that high copper concentration is toxic for the yeast under fermentation conditions, due to its potential as a DNA damage agent, causing mutagenesis or alteration in the chromatin conformation.

Significance to Metallomics statement

The toxic effect of copper on cell metabolism is regarding to the overproduction of hydroxyl radical provoking oxidative stress. So far, the available literature deals with information on yeast response to this stress when cultivated under aerobic environment. Following our recent findings about metal influences on yeast fermentation, the present work provides evidences for the first time on how yeast cell respond to copper at toxic level under fermentation condition by combining gene-wide and physiological data. The results indicated the occurrence of chromosome and DNA damages that may have a drastic consequence to cell viability and industrial fuel ethanol production.

[a.] *Interdepartmental Research Group in Metabolic Engineering.*
[b.] *Department of Genetic and*
[c.] *Department of Antibiotics, Federal University of Pernambuco. Avenida Moraes Rego, Nº 1235, Recife, PE 50760-901, Brasil.*
[d.] *Laboratory of Bioinformatics and Evolutionary Biology, Federal Rural University Pernambuco. Rua Dom Manoel de Medeiros, s/n, Recife, PE 52171-900, Brasil.*
† Autor for correspondence: Rafael Barros de Souza, Departamento de Genética – Universidade Federal de Pernambuco, A.v. Moraes rego, Nº 1235, Cidade Universitária 50670-901 Recife PE Brasil. E-mail: barrosrs87@gmail.com. Phone: 55-81-21267817 Fax: 55-81-21268622
Electronic Supplementary Information (ESI) available: Supplementary Material. List of all *S. cerevisiae* genes up-regulaed (positive LogFC) and down-regulated (negative LogFC) under copper stress with LogFC value equal or above 0.5. See DOI: 10.1039/x0xx00000x

Introduction

The mineral composition of the sugarcane juice in Brazilian northeast distilleries has been studied in order to evaluate the availability of minerals and its effect on yeast physiology under growth and fermentation conditions.[1] The results have shown that the mineral concentration in sugarcane juice can largely change according to the farm where the plant was harvested and with the type of sugarcane variety cultivated.[1] Moreover, the mineral composition can impact the fermentation metabolism, especially when considering the concentration of magnesium and copper ions.[1] These two metals showed an antagonistic effect on yeast physiology. While magnesium increases ethanol yield and stress tolerance, copper decreases the ethanol production and can be toxic for the cell at high concentrations.[1,2]

Several roles for magnesium on yeast physiology have been described in the literature, especially due to its importance as cofactor of many enzymes, its concentration control inside the cell and its impact on yeast growth and metabolite production.[3] Recently, we showed through global gene expression analyses that magnesium also affects carbohydrate metabolism regulation. It partially releases genes regulated by glucose catabolite repression (GCR) and as consequence it unblocks the expression of genes related to stress response.[2]

Copper ions are required as cofactor of enzymes involved in oxidative growth, such as the cytochrome oxidase complex.[4]

However, above a threshold concentration, copper can be toxic to the cell. In order to maintain an adequate copper cellular level, two transcription factors (TF) Mac1 and Ace1 are operational. Mac1 encodes a TF that induces the expression of two high-affinity copper transporters, Ctr1p and Ctr3p, when in low copper concentration.[4,5,6] On the other hand, the Ace1p, also known as Cup2p, is required in response to excess of copper and activates the expression of genes such as CUP1, CRS5 and SOD1.[7,8] CUP1 and CRS5 encode metallothioneins that can bind copper and remove it from the cell.[9,10] CUP1 is the main gene in response to copper tolerance and is present in two copies, CUP1-1 and CUP1-2, in the genome of different *Saccharomyces cerevisiae* strains.[11] SOD1 encodes a cytosolic copper-zinc superoxide dismutase and is required for oxygen radical detoxification and copper homeostasis.[12] In addition, ATX1 is also required in excess of copper by encoding a cytosolic metallochaperone responsible to deliver copper from Ctr1p to Ccc2p on post-Golgi vesicle and controlling the intracellular trafficking of this complex.[13]

High level of copper is toxic for the cell because it can catalyse the production of hydroxyl radical (OH*) from hydrogen peroxide (H_2O_2) causing oxidative damages in essential molecules such as DNA.[14] Copper-dependent DNA damages are associated to reactive oxygen species (ROS) production that oxidize nitrogen bases, produce single and double strand breaks, and as consequence telomere shortening.[15] However, copper can also directly cause DNA damage, binding itself to DNA bases in chromatin, altering the integrity of DNA and affecting both replication and transcription.[16]

Effect of copper ions on yeast aerobic metabolism seems well established in the literature. On the other hand, it is still unclear how this metal, when in excess, could affect yeast cells under oxygen depletion condition. Therefore, in the present work we carried out physiological experiments associated with global transcriptional response of *S. cerevisiae* JP1 industrial strain with high copper concentration. The results showed that, for the *S. cerevisiae* JP1, copper is toxic at 10 mg L^{-1} (157 µM). This concentration was able to extend the lag phase on aerobic growth, also to decrease the ethanol yield under fermentation conditions and to change the expression of 171 genes. The transcriptional response herein provides genetic evidences that excess of copper can cause DNA damage in fermentation conditions even in anaerobiosis. The implications of these physiological results to ethanol industry and the functions and regulations of the genes are discussed herein.

Methods

Yeast strain and media for growth and fermentation assays

Industrial strain *S. cerevisiae* JP1, which is employed as fermenting strain in many distilleries of Northeast Brazil, was used in the present study. Cells were maintained and cultivated in YPD medium (yeast extract: 10 g L^{-1}; glucose: 20 g L^{-1}; bacteriological peptone: 20 g L^{-1}) at 32°C. Solid medium contained agar to 20 g L^{-1}. The reference synthetic medium (RSM) for the growth and fermentation assay was prepared with glucose at 20 g L^{-1} and 140 g L^{-1}, respectively. The nutritional composition (nitrogen source and minerals) was based on our previous work.[2] The effect of the copper supplementation was evaluated increasing its concentration to 1, 5, 10 and 15 mg L^{-1} in the media. In the RSM, copper concentration was 0.2 mg L^{-1}. The salt was provided to the medium as a sulphate salt ($CuSO_4*5H_2O$). All the media were filtered sterilized prior use (Millipore membrane 0.22 µm).

Fermentation and Growth Experiments

All the fermentations were carried out on a single-batch in 250 ml flasks containing a total of 200 ml of medium and yeast cells. Cells were pre-grown in YNB medium to the beginning of stationary growth phase, collected by centrifugation and suspended to 10^8 cells mL^{-1} in the fermentation media supplemented with Cu^{II}. The fermentations were carried out for 7 h at 32°C without agitation.[1] These fermentation conditions were used in order to mimic the conditions find in the Brazilian ethanol industry, which is used high initial sugar concentration, high cell density, short fermentation time and without aeration.[1] At the end of the fermentations, samples were withdrawn, centrifuged and the supernatant taken for metabolite analysis by HLPC. Samples were taken to determine biomass production by cell dry weight and to evaluate cells viability by direct microscopic count on a Neubauer chamber after dying with methylene blue. In addition, cells collected from Cu^{II}-supplemented synthetic media were immediately used for RNA extraction as described below. The measurement of carbohydrates, glycerol, acetate and ethanol was carried out by HPLC (Waters Co., USA) using an Aminex HPX-87H column (BioRad, USA) heated at 60°C. Sulphuric acid at 5 mM was used as mobile phase at a flux of 0.06 l min^{-1}. Fermentation experiments were carried out in triplicate.

The growth experiments followed strictly the protocol described in our previous work,[2] in which yeast cells were inoculated in the reference synthetic medium and copper-supplemented media to initial cell density (OD600) of 0.2 in sterile microtiter plates (96-well) for total volume of 200 µL with specific media. The growth assays were performed in Synergy HT device (BioTek, Swissland) at 32°C and with absorbance measurement every 10 minutes for 20 h. Growth curves were prepared from the average of three biological experiments and the growth rate was calculated from the slope of exponential growth phase.

Transcriptome-wide expression microarray analyses

Collected cells were centrifuged and re-suspended in 400 µL AE Buffer (50 mM Sodium Acetate, 10 mM EDTA pH 5.3) and 80 µL of 10% SDS solution, mixed by vortex and incubated at 65°C for 10 min. Total RNA in the lysates was purified by using RNAspin Mini RNA Isolation Kit (GE HealthCare, USA) and quantified in NanoDrop ND-2000 UV-Vis spectrophotometer (ThermoFisher Scientifics, USA). Synthesis of cDNA, cRNA and labelling were performed by Two-Color Low Input Quick Amp Labelling Kit (Agilent, USA) according to manufacturer instructions, with Two-Color RNA spike-in kit as internal control (Agilent, USA). cRNA from cells taken from fermentation experiment supplemented with copper was labelled in red with Cy-5 dye and assigned as target samples, while cRNA from cells taken from fermentation experiment in reference medium was labelled in green with Cy-3 and assigned as reference sample. Target and reference cRNA samples were pooled and used to hybridise yeast gene expression 8x15K spots slides (Agilent,

USA) at 65°C for 17 hours at 10 r.p.m. in the microarray hybridization oven (Agilent, USA). Fluorescent data from 3 µm-resolution microarray scanner (Agilent, USA) were extracted as .txt files using the software Feature Extraction. Groups of genes with stringent statistical significance (concomitant Adj.p < 0.05 and B ≥ 3) were utilized and their logFC values used to classify up- (≥0.5LogFC) and down-regulated (≤-0.5LogFC) genes, a terminology meaning the abundance of transcripts in test condition relative to reference condition.[2,17] Microarray data was submitted to Gene Expression Omnibus (GSE75803). The *Saccharomyces* Genome Database (SGD), Gene Ontology Slim Mapper and YeastMine databases were used for identification of encoded proteins and GO clustering of the genes. The analyses of transcription factor binding sites were carried out with the Yeastract database.

Results and discussion

Growth and fermentation parameters altered by copper

The toxic effect of copper in yeast metabolism is well known, however, toxic concentration of copper can change according to yeast strain and experimental condition. In order to determine the copper toxic concentration in industrial strain *S. cerevisiae* JP1, we carried out a growth experiment with YNB medium with copper concentration at 0.2 (reference medium), 5, 10 and 15 mg L^{-1} (Figure 1). There was no alteration in the growth profile in the medium supplemented with 5 mg L^{-1}. On the other hand, with 10 mg L^{-1} the lag phase was elongated for additional 6 hours compared to reference medium, nevertheless the growth rate and the final biomass were not changed. The concentration of 15 mg L^{-1} impaired yeast growth for 20 hours (Fig. 1), as showed in our previous work.[2] However, it did not mean that cells were not viable, as some growth was observed after 40 hours of cultivation (data not shown). The lag phase is characterized as a period of cell adaptation to a different condition and its length is dependent on environmental conditions such as nutritional availability.[18]

Copper concentration of 10 mg L^{-1} was enough halt yeast growth for some time without affecting growth rate after the adaptation period, indicating a non-killing toxic effect. It is worth noting that this copper concentration can be found in the sugarcane juice.[1] Therefore, this concentration was chosen to carry out the fermentation assay in order to determine the impact of this metal on the fermentative parameters and the global gene expression of the yeast cell.

In the fermentation assay the main parameters of the industrial process such as glucose consumption, ethanol production and cell viability were measured. Glycerol production was also measured as indicative of cell anabolic state. The results showed that copper at 10 mg L^{-1} negatively affected the fermentation productivity by decreasing glucose assimilation and ethanol yield by almost 20% and glycerol production by 42% (Table 1). Biomass production was slightly decreased in the medium supplemented with copper (Table 1) and despite the toxic effect of this metal, no cell death was observed, as cell viability remained near 100% (data not shown), as previously reported.[1]

It is worth noting that glucose was not completely consumed in both conditions in the 7 hours of fermentation assay, however residual glucose in copper medium was higher. A possibly copper effect can be explained by the changes of the gene regulation that will ahead described. Moreover, a nutritionally poor medium can lead to an incomplete fermentation as observed in sugarcane juice composition.[20] Therefore, it seems that synthetic YNB medium used in this work is, indeed, nutritionally poor for the yeast and can be used to simulate the sugarcane juice. In the context of the ethanol industry, slower fermentations and low ethanol production retard the whole industrial flow decreasing the overall productivity. Low productivity on the fermentation is normally associated to bacterial and/or yeast contamination or death of yeast cell caused by stress conditions such as acid stress induced by sulphuric acid in the biomass treatment used to eliminate bacteria.[21,22,23] Once again, we present the copper negative effect on ethanol production, reaffirming the participation of copper in eventual episodes of drops of industrial productivity associate to metals as shown in our previously work[1].

Overview of the transcriptome-wide response under copper supplementation

Significant alterations in the transcriptomic profiles of yeast cells was observed between the reference medium and in the medium enriched with copper at 10 mg L^{-1}. After statistical analysis of the 6335 ORFs printed on the Agilent arrays, one hundred seventy-one (171) genes were classified as up-regulated or down-regulated, a denomination that represents the amount of transcript in the test medium compared to reference medium.

Eighty-five out of 171 genes were up-regulated and eighty-six genes were down-regulated (for detailed list of genes see Supplementary Material). Among them, the well-known genes responsible for copper detoxifications were up-regulated, such as CUP1-1 (2.9-fold), CUP1-2 (2.8-fold), CRS5 (1.9-fold) and ATX1 (1.8-fold). In turn, it was observed the down-regulation of CTR3 gene (-1.9-fold). Previous works also using DNA microarray technology showed similar profile of gene regulation of this work in response to high level of cooper.[4, 24, 25] In contrast, we did not observe the upregulation of SOD1 gene that was previously indicated as very important for copper detoxification.[25] A possibly explanation for this difference could be related to the experimental condition of this work (anaerobiosis) and that previous work (aerobiosis). Therefore, since its protein function to detoxify oxygen radical ,[12] we propose that the overexpression SOD1 may be also dependent on the oxygen availability. Thus, in view of the importance of SOD1 gene in the copper detoxification, absence of its up-regulation in the scenario of very high copper level may have led the cell to more severe damages, such damage to DNA as explained below.

Therefore, to better understand what the 171 differentially expressed genes really means for the cellular copper stress response, all genes were grouped by gene ontology (GO) analysis and were also analysed according to their descriptions in *Saccharomyces* Genome Database (SGD). Moreover, analyses of transcription factor (TF) binding sites in the genes were carried out in the Yeastract database with the aim to find a regulatory network induced by copper. These *S. cerevisiae* databases have demonstrated to be effective in the integration

of transcriptome and physiological data.[2,17] The analyses of the genes by these platforms indicate that copper in excess can causes DNA damage and revealed how the cells can overcome the copper toxic effect. The up-regulated genes formed only a consistent biological process, according to GO analysis, involved in telomere maintenance via recombination, however several genes are associated to DNA replication stress. The down-regulated genes were significantly related to the translation process. In the following sections, we will discuss in detail each biological process and the respective regulated genes.

Genes up-regulated by high copper level under fermentation

Despite of low fold-change, several up-regulated genes found in the present work encode isoforms of DNA helicases known as Y'-Help1 (Y'Helicase protein 1) or proteins with unknown functions similar to and dependent of helicases (Table 2). YRF1-1, YRF1-3, YRF1-6 and YRF1-7 genes are found in telomeric Y'elements in the subtelomeric regions of the yeast chromosomes IV, VII, XIV and XVI, respectively. These genes are induced in strains deficient for telomerase activity and promotes homologous DNA recombination among Y' elements, thereby preventing shortening of the telomeres.[26] Telomere shortening leads to loss of DNA sequences at the ends of replicated chromosomes and can stop the cell division and lead to cell death.[27] Actually, telomere shortening is a natural process of cell aging and the native Y' elements are not expressed in wide-type cells due to the telomeric silencing effect.[28] Thus, the induction of YRF1 genes from shortened telomeres seems to be regulated also by telomerase-independent mechanisms.[26]

Moreover, high intracellular level of copper seems to induce DNA damage due to the overproduction of ROS that can oxidise the DNA and lead to a telomere shortening.[15] In this scenario, we expected to observe a strong signal of oxidative stress by the up-regulation of the most representative genes. However, only the genes STF2 (2.0-fold), AHP1 (2.0-fold), TRX2 (1.6-fold) and HSP12 (1.6-fold) were up-regulated as part of the oxidative stress response, as previously characterised.[29,30,31,32] A reference gene for oxidative stress induced by copper, SOD1, was not regulated in the present work. Therefore, in order to understand the induction of these oxidative stress genes, we performed a TF analyses in the Yeastract database. We identified that Msn2p, Msn4p, Cin5p and Fkh1p transcriptionally activate these four genes. Msn2p/Msn4p are stress-responsive transcriptional activator involved in response of various stress conditions and the Cin5p mediates pleiotropic drug resistance and is involved in chromosome instability.[33,34,35] In turn, Fkh1p is a member of a forkhead (FOX) family of TFs implicated in cell cycle regulation and it is suggested that acts in chromatin architecture and organization.[36] Thus, from the transcriptional profile and the *S. cerevisiae* database analysis, and taken in consideration that the yeast cells were in fermentation condition (no oxygenation), the results indicate that DNA replication seems to be the main molecular target for copper ions.

Furthermore, about 12% of the 85 up-regulated genes encode proteins that are more abundant in the cell in response to DNA replication stress, despite of these genes belong to different biological process (Table 3). TF analyses revealed that these genes are activated by transcriptional factor Hsf1p, excepted STF1 and YJR085C. Hsf1p regulates transcription in response to several stresses, activating genes involved in protein folding, detoxification, energy generation, carbohydrate metabolism, cell wall organization and cell cycle progression.[37,38,39,40] It is activated by hyperphosphorylation under stress condition, however mutation in this gene can also activate it.[41] Therefore, copper metal could activate Hsf1 in the both situations, inducing stress, as mentioned early, and also provoking mutation since copper is a mutagenic agent.[42]

Finally, corroborating with the described above, the induction of the genes TFB5 (1.6-fold), RDH54 (1.5-fold) and RAD59 (1.5-fold) definitely confirm the DNA damage provoked by copper. The TFB5 gene is component of RNA polymerase II general transcription factor that is involved in transcription initiation and DNA repair.[43] In turn, the RDH54 encodes a DNA recombination/repair translocase involved in recombinational repair of DNA double-strand breaks during mitosis and meiosis.[44] Lastly, the Rad59p is a yeast recombination factor also involved in the DNA double-strand break repair.[45] This gene acts associated to RAD52 to determine the structure of the genome after acute exposure to DNA damaging agents.[46]

In short, besides copper is harmful for DNA replication due to its association with ROS production, in this work, copper stress is caused due to its capacity of binding itself to DNA and its mutagenic effect. In turn, the cells trigger DNA helicases genes, general stress mechanisms and recombination factors to avoid DNA-strand break and as consequence telomere shortening.

Translation process repressed by high copper concentration

Among the 86 down-regulated genes, beside the copper transporter CTR3 as previously cited, two other genes that encodes metal transporters were also down-regulated, IZH1 (-1.9-fold) and IZH2 (-2.0-fold). IZH1 and IZH2 expression are induced in zinc deficiency and repressed in excess of zinc and their proteins are part of a protein family characterized by the presence of four highly conserved motifs rich in metal-binding amino acids.[47] Thus, copper could replace zinc and simulate an environment zinc-replete, since both are divalent metals.

Moreover, two important genes of the glycolytic and gluconeogenesis pathway were also down-regulated, ENO1 (-2.0-fold) and ENO2 (-1.5-fold). Both genes encode phosphopyruvate hydratase, which catalyses conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis.[48] So, the repression of these genes can slow down the glucose assimilation, as observed in the fermentation (Table 1).

The most of the down-regulated genes are related in general to translation process. GO analysis of all the down-regulated genes formed four consistent biological processes such as rRNA processing, cytoplasmic translation, ribosomal large subunit biogenesis and translational elongations (Table 4). The highest repressed gene in the rRNA processing and cytoplasmic translation processes was SSB1 (-1.9-fold). This gene encodes a chaperone localized to the ribosome as part of the ribosome-associated complex and its expression is also reduced upon heat shock.[49,50] From the ribosomal large subunit biogenesis process, the highest down-regulated gene was BRX1 (-2.4-fold). Actually, this gene was one of the most repressed among all down-regulated genes in this work. It encodes a nucleolar protein constituent of 66S pre-ribosomal particles and needed to assembly of large ribosomal subunits.[51] Regarding to translational elongation process, the three genes

TEF1 (-2.0-fold), TEF2 (-2.0-fold) and EFT1 (-1.4-fold) are fundamentals to elongation step flux. TEF and TEF2 encodes a transcriptional elongation factor EF-1 alpha that act binding to and delivering aminoacylated tRNA to the A-site of ribosomes for elongation of nascent polypetides.[52] The gene EFT1 encode an elongation factor 2 in which catalyses ribosomal translocation during protein synthesis.[53]

From the repression of these biological processes, it is clear that cells are diminishing the global protein production by decreasing ribosomal and rRNA production and consequently the elongation process. During amino acid starvation, *S. cerevisiae* also repress the ribosomal and rRNA production.[54] Indeed, eukaryotic cells have evolved mechanisms to cope with stress conditions and the response of the yeast decreasing the translation process upon copper stress seems to be a consequence of the DNA replication damage.

Conclusions

As well known, copper ion can be harmful for the cells in certain concentrations, however, most studies in the literature describe copper toxic effect associated mainly to ROS production in aerobic growth conditions. Therefore, in the present work, we provide evidences of how the industrial strain *S. cerevisiae* JP1 respond to toxic copper level (10 mg L^{-1}) under fermentation conditions. Our findings revealed that the yeast is under DNA replication stress and, as response, it induces genes involved in DNA repair, genes encoding DNA helicases and abundant protein associated to DNA replication stress. As consequence, cells repress the translation process, specifically, through the down-regulation of translational elongation factor, ribosomal large subunit biogenesis and rRNA processing. This genetic landscape suggests that copper is damaging directly the DNA by changing the DNA chromatin conformation and/or causing direct mutation. Therefore, in the context of fuel-ethanol production with cell recycling, constant high copper content in sugarcane juice can lead to an irreversible damage in the yeast cells, decreasing its fermentation performance and consequently causing industrial losses.

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Metallomics Template

ARTICLE

Tables

Table 1 Fermentation assay carried out with *S. cerevisiae* JP1 in YNB medium supplemented to 10 mg L^{-1} of copper at 30ºC during seven hours. Yield values showed statistical differences ($p<0.05$).

	Concentration [g L^{-1}]		Yield [g g^{-1}]	
	RSM*	Copper	RSM	Copper
Glucose consumed	98.27 ±3.5	82.15 ±1.8		
Ethanol produced	39.09 ±1.2	26.64 ±1.0	0.4	0.32
Glycerol produced	6.21 ±0.02	3.61 ±0.02	0.06	0.04
Biomass produced	3.12 ±0.2	2.7 ±0.03	0.04	0.03

* Reference synthetic medium

Table 2 Helicases genes of *S. cerevisiae* JP1 strain up-regulated by copper stress under fermentation condition.

Genes	Fold change	SGD description
YPR204W	1.8	DNA helicase encoded within the telomeric Y' element
YLL066C	1.8	Putative Y' element ATP-dependent helicase
YHR219W	1.7	Putative protein of unknown function with similarity to helicases
YJL225C	1.7	Putative Y element ATP-dependent helicase
YIL177C	1.6	Putative Y element ATP-dependent helicase
YRF1-1 YRF1-3 YRF1-6 YRF1-7	1.7 – 1.6	Helicase encoded by the Y' element of subtelomeric regions
SLH1	1.5	Putative RNA helicase

Table 3 Biological process, based on GO annotation, of the up-regulated genes, in which the proteins are abundant in DNA replication stress.

Genes	Fold change	Biological process
STF2	2.0	Cellular response to desiccation
FES1	1.8	Cytoplasmic translation
YJR085C	1.7	Unknow
HCH1	1.7	Cellular response to heat and protein folding
SOL4	1.6	Pentose-phosphate shunt, oxidative branch
HSP12	1.6	Plasma membrane organization.
STF1	1.6	Negative regulation of ATPase activity
CPR6	1.5	Protein folding
MBF1	1.5	Positive regulation of transcription from RNA polymerase II promoter
GLC3	1.4	Glycogen biosynthetic process

Table 4 Gene ontology analyses of the down-regulated genes of the industrial strain *S. cerevisiae* JP1 submitted to 10 mg L^{-1} of copper under fermentation condition.

GO Biological process	Down-regulated genes
rRNA processing	RPL7, DBP8, SSB1, SSF1, RPS2, RPF2, RRP8, NHP2, DBP3, RPS1A, RPL8A, MRM1, NIP7, IPI3, KRR1, PUS7, NOC4, PSP1
Cytoplasmic translation	RPL7, RPL12A, SSB1, SSA1, RPL20B, RPL16B, SRO9, RPS1A, RPL8A, RPL26B, RPL2A, RPL20A, RPL12B, RPL2B, RPL5
Ribosomal large subunit biogenesis	RPL7, RPL12A, BRX1, SSF1, RPF2, RRP8, DBP3, RPL8A, RPL26B, NIP7, RPL12B, IPI3, RPL5
Translational elongation	TEF1, TEF2, EFB1, SSB1, EFT1, HYP2, NAM7