VALDILENE CANAZART DOS SANTOS

ENGENHARIA EVOLUTIVA DA LEVEDURA *Kluyveromyces marxianus* UFV-3 PARA FERMENTAÇÃO DE XILOSE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 19 de dezembro de 2011.

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(Orientadora)

“Comece fazendo o que é necessário, depois o que é possível, e de repente você estará fazendo o impossível.”

(São Francisco de Assis)

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BIOGRAFIA

Valdilene Canazart dos Santos, filha de José dos Santos e Ana Canazartt dos Santos, nasceu em 31 de agosto de 1982, na cidade de João Monlevade, Estado de Minas Gerais.

Em março de 2001, ingressou na Universidade Federal de Viçosa (UFV), graduando-se em Bacharelado em Bioquímica, em 28 de julho de 2005.

Em agosto de 2005, ingressou no curso de mestrado do Programa de Pós-graduação em Microbiologia Agrícola na UFV, com término em agosto de 2007.

Em agosto de 2007, iniciou o curso de doutorado neste mesmo programa, submetendo-se à defesa de tese em 19 de dezembro de 2011.

SUMÁRIO

RESUMO

SANTOS, Valdilene Canazart dos, D.Sc., Universidade Federal de Viçosa, Dezembro de 2011. **Engenharia evolutiva da levedura *Kluyveromyces marxianus* UFV-3 para fermentação de xilose**. Orientadora: Flávia Maria Lopes Passos. Coorientadores: Andreas Karoly Gombert, Luciano Gomes Fietto e Marisa Vieira de Queiroz.

Kluyveromyces marxianus são leveduras promissoras para a fermentação de glicose e xilose, os principais açúcares presente no hidrolisado de bagaço de cana. No presente trabalho, foi investigado o consumo de xilose em presença de glicose bem como a capacidade de *K. marxianus* em fermentar várias combinações desses dois açúcares. Células de *K. marxianus* UFV-3 cultivadas em 20 gL^{-1} glicose e 20 gL^{-1} xilose apresentaram um período de adaptação de 30h para o início do consumo da xilose, após a glicose ter sido totalmente consumida. Entretanto, esse período de adaptação não foi observado quando as células foram cultivadas em 5 gL^{-1} glicose e 20 gL^{-1} xilose. Nessas condições, as células começaram a consumir xilose logo após a glicose ter sido exaurida do meio. Além disso, foi demonstrado que glicose e xilose podem ser consumidas simultaneamente, quando a respiração é bloqueada. A produção de etanol foi maior quando em mistura de glicose e xilose comparado à glicose sozinha. Por outro lado, *K. marxianus* UFV-3 não foi capaz de produzir etanol a partir de xilose nas condições avaliadas. Visando selecionar uma linhagem capaz de fermentar xilose, células de *K. marxianus* UFV-3 foram submetidas à engenharia evolutiva. Mutagênese foi aplicada com o intuito de aumentar a variabilidade genética da população e diminuir o tempo de seleção. Duas técnicas para inserção de mutações aleatórias foram utilizadas: REMI (Integração Mediada por Enzima de Restrição) e radiação ultravioleta (UV). Populações mutantes foram submetidas à seleção em quimiostatos e em bateladas sequenciais. Em quimiostato conduzido sob hipoxia, com uma taxa de diluição de 0.15 h^{-1} e uma mistura de 5 gL^{-1} glicose e 10 gL^{-1} xilose, isolou-se um mutante da população de células submetidas à REMI (KmRhyp). Este isolado produziu 12% mais etanol que a linhagem selvagem, mas com dobro de xilitol. Em batelada sequencial foi isolado um mutante da população irradiada por UV (KmUVsb) capaz de formar etanol de xilose e produzir menor quantidade de xilitol que a linhagem selvagem. Diferenças entre a linhagem selvagem e KmUVsb foram relacionadas a diferenças observadas na atividade de algumas enzimas. A razão entre as atividades específicas da xilitol desidrogenase e da xilose redutase foi maior para a linhagem mutante. As atividades específicas das enzimas da via fermentativa: piruvato descarboxilase e álcool desidrogenase também foram maiores em KmUVsb. Culturas conduzidas em quimiostato limitado por xilose sob condições de aerobiose foram submetidas a um pulso de glicose (50 mmoles.L^{-1}). Nem a linhagem selvagem nem a mutante produziram etanol instantaneamente, confirmando efeito *Crabtree*-negativo. Etanol foi detectado no sobrenadante após 60 minutos, e a produção deste metabólito foi duas vezes maior na linhagem mutante. Além disso, KmUVsb foi capaz de crescer em anaerobiose estrita na presença de xilose como única fonte de carbono.

ABSTRACT

SANTOS, Valdilene Canazart dos, D.Sc., Universidade Federal de Viçosa, December, 2011. **Evolutionary engineering of *Kluyveromyces marxianus* UFV-3 for xylose fermentation**. Adviser: Flávia Maria Lopes Passos. Co-advisers: Andreas Karoly Gombert, Luciano Gomes Fietto and Marisa Vieira de Queiroz.

Kluyveromyces marxianus strains are promising candidates to ferment glucose and xylose, the main sugars in hydrolyzed sugarcane bagasse. In this work, it was investigated the xylose consumption in the presence of glucose and the *K. marxianus* UFV-3 ability to ferment various combinations of these two sugars. *K. marxianus* UFV-3 cultured on 20 gL^{-1} glucose and 20 gL^{-1} xylose presented a lag phase of 30h to start xylose consumption after glucose depletion. However, this period of adaptation was not observed when cells were grown on 5 gL^{-1} glucose and 20 gL^{-1} xylose. Under these conditions, the cells began to consume xylose after glucose had been exhausted. Furthermore, it was demonstrated that glucose and xylose can be consumed simultaneously, when respiratory chain is blocked. The ethanol production was higher in a glucose/ xylose mixture compared to glucose alone. *K. marxianus* UFV-3 was not able to produce ethanol from xylose. In order to select a strain able to ferment xylose, *K. marxianus* UFV-3 were subjected to evolutionary engineering. Mutagenesis was applied to increase the genetic variability of the population and to reduce the selection time. Two methods for random mutations were used: REMI (Restriction Enzyme Mediated Integration) and ultraviolet irradiation (UV). Mutant populations were subjected to selection in chemostats and sequential batches. In chemostats conducted under hypoxia, with a dilution rate of 0.15 h^{-1} in a 5 gL^{-1} glucose and 10 gL^{-1} xylose mixture, a mutant from REMI population was isolated (KmRhyp). The isolate produced 12% more ethanol but with twice xylitol compared to the wild type. In sequential batches, it was isolated a mutant from the UV irradiated population (KmUVsb) which was able to form ethanol from xylose with 2-times less xylitol than wild type strain. The differences between wild type and KmUVsb were related to differences in the activity of some enzymes. The ratio of xylitol dehydrogenase specific activity to xylose reductase specific activity was higher in the mutant strain. The specific activities of fermentative pathway enzymes: pyruvate decarboxylase and alcohol dehydrogenase were also higher in KmUVsb. Cultures conducted in xylose-limited chemostats under aerobiosis were subjected to glucose pulse (50 mmoles/L). Neither the wild type nor the mutant strain formed ethanol instantly, confirming Crabtree-negative effect. Ethanol was detected on the supernatant after 60 minutes and it was twice as high as for mutant strain. In addition, KmUVsb was able to grow under strict anaerobiosis in the presence of xylose as the only carbon source.

1 INTRODUÇÃO GERAL

Existe um crescente interesse por fontes alternativas de energia. Isso se reflete no volume de investimentos que são direcionados para o desenvolvimento de tecnologias sustentáveis. Nesse cenário, os biocombustíveis estão ganhando cada vez mais espaço, especialmente o bioetanol.

O bagaço da cana de açúcar é um dos principais resíduos da indústria sucroalcooleira e sua utilização para aumentar os rendimentos de etanol, sem aumentar área plantada, é vista como promissora. Esse resíduo lignocelulósico contém uma alta concentração de carboidratos, os quais, se fermentados, podem contribuir significativamente para o aumento da produção de etanol (BASTOS, 2007).

Diversos pesquisadores têm concentrado esforços no desenvolvimento de linhagens de leveduras capazes de fermentar eficientemente xilose, a principal pentose presente no hidrolisado de bagaço de cana. Algumas pesquisas têm abordado estratégias envolvendo engenharia metabólica (CHU; LEE, 2007; HAHN-HÄGERDAL *et al.,* 2007; NEVOIGT 2008). Contudo, como faltam informações sobre as redes metabólicas que determinam uma via fermentativa para xilose, essa abordagem não tem alcançado o sucesso pretendido. Em contrapartida, estratégias que utilizam engenharia evolutiva têm apresentado resultados mais promissores. Essa abordagem envolve a seleção de leveduras com um fenótipo de resposta às condições impostas durante um cultivo prolongado (BROWN *et al.,* 1998; FEREA *et al.*, 1999; MASHEGO *et al.*, 2005).

Para obter um organismo melhorado com base em engenharia evolutiva tal organismo precisa dispor de informações genéticas capazes de gerar o fenótipo desejado. Isso se deve ao fato de que, nessa estratégia, pressupõe-se que este fenótipo seja alcançado por meio de mutações que ocorrem durante o cultivo prolongado. Embora essas mutações possam alterar informações presente em um genoma, elas não geram novas informações. Portanto, para utilizar engenharia evolutiva na obtenção de linhagens capazes de fermentar xilose, é imprescindível que o micro-organismo utilizado para tal fim seja capaz de assimilar xilose como fonte de carbono, e que apresente uma via fermentativa eficiente. Um micro-organismo que reúne todas essas características é a levedura *Kluyveromyces marxianus.*

No contexto da produção de etanol, essa levedura não convencional apresenta vantagens em relação à *Saccharomyces cerevisiae*. *K. marxianus* é capaz de crescer e fermentar em temperaturas elevadas, possui taxas elevadas de crescimento, é capaz de utilizar uma diversidade de substratos industrialmente relevantes como a xilose, arabinose, celobiose e lactose (SILVEIRA *et al.,* 2005; FONSECA *et al.,* 2008).

O laboratório de Fisiologia de Micro-organismos da Universidade Federal de Viçosa tem investigado as potencialidades de uma linhagem de *K. marxianus* isolada na Zona da Mata Mineira, denominada *K. marxianus* UFV-3 (SILVEIRA *et al.,* 2005). Essa levedura teve seu potencial fermentativo comprovado e apresentou rendimento de etanol próximo ao teórico quando cultivada em soro de queijo.

Em pesquisas preliminares também foi confirmada a capacidade dessa levedura de utilizar xilose como fonte de carbono, contudo, ela não foi capaz de fermentar essa pentose. Tomando como base o fato de que essa levedura já possui a predisposição genética para utilização de xilose, e também é capaz de fermentar eficientemente outras fontes de carbono, a presente tese de doutorado teve como principal objetivo explorar essas características, e obter uma linhagem de *K. marxianus* capaz de fermentar xilose.

Inicialmente, cultivos em bateladas com diferentes combinações de glicose e xilose foram realizados; além disso, analisou-se a influência da glicose sobre a utilização da xilose. Uma vez que estes açúcares estão presentes ao mesmo tempo no hidrolisado de bagaço de cana, é de interesse que a levedura seja capaz de utilizar ambos os açúcares. Posteriormente, diferentes estratégias de engenharia evolutiva foram utilizadas visando selecionar uma linhagem capaz de fermentar xilose. Como esse processo demanda muito tempo, *K. marxianus* UFV-3 foi submetida a métodos de mutagênese aleatória a fim de aumentar a variabilidade genética inicial da população, bem como diminuir o tempo de seleção em quimiostatos ou bateladas sequenciais. Após o isolamento do mutante, cultivos em quimiostatos foram realizados para confirmar o fenótipo alcançado e, também, elucidar as possíveis causas do fenótipo.

CAPÍTULO 1

2 REVISÃO DE LITERATURA

2.1 Situação do etanol no mundo

O interesse por fontes alternativas de energia vem crescendo mundialmente. Dentro desse contexto, os agrocombustíveis são considerados grandes promessas, especialmente o bioetanol, uma vez que ele pode ser produzido a partir de diversas fontes de biomassa e por meio de diferentes tecnologias (LYND *et al.,* 1991; WYMAN, 1994; SZCZODRAK; FIEDUREK, 1996; JEEWON, 1997; HANSEN *et al.,* 2005).

Segundo Schlesinger (2008), o etanol obtido a partir da cana de açúcar é considerado o mais promissor dos substitutos da gasolina. Ainda de acordo com o autor, as perspectivas de crescente utilização do etanol em diversas partes do mundo, como União Europeia, China, Índia, e Estados Unidos, tem estimulado o aumento de canaviais no Brasil.

Estima-se que o Brasil possa contribuir, de forma significativa, para a substituição da gasolina no cenário mundial. Entretanto, para que isso se torne uma realidade, as áreas destinadas ao plantio da cana de açúcar teriam que ser expandidas (CARVALHO, 2007; SCHLESINGER, 2008). Segundo Carvalho (2007), os canaviais teriam que ocupar 28 milhões de hectares para o país conquistar 50% do mercado mundial.

Embora haja grandes perspectivas do aumento da produção de etanol com a expansão das áreas plantadas, esta representa um dos grandes conflitos na produção de etanol. A produção deste e de outros agrocombustíveis têm levantado diversos debates. De um lado, a preocupação com a poluição ambiental crescente, e de outro, a necessidade de garantia da utilização da terra para produção de alimentos.

Uma forma de contornar esse problema seria a produção do etanol de segunda geração. A implantação dessa tecnologia alternativa permitiria aumentar a oferta do etanol sem aumentar a área plantada. Com base nessas perspectivas, e devido à especificidade da cana em termos de custos e balanço energético positivo, organizações nacionais contemplam o desenvolvimento dessas novas tecnologias de produção de etanol a partir da biomassa lignocelulósica, oriundas de resíduos da indústria sucroalcooleira (BASTOS, 2007).

2.2 Situação do etanol no Brasil

O Brasil é o maior produtor de etanol proveniente da cana de açúcar. É também o único país que integra a produção de açúcar e etanol na mesma planta, reduzindo os custos de ambos os processos. Além disso, a cana de açúcar é considerada uma matéria-prima ideal para as futuras tecnologias, por causa de seu custo relativamente baixo e sua mistura de um terço de sacarose e dois terços de biomassa lignocelulósica (MACEDO, 2007). Portanto, o desenvolvimento integrado da produção de etanol de primeira geração com o de segunda geração poderia ajudar a trazer maior competitividade ao etanol brasileiro.

O aumento da produção de etanol é um imperativo não somente pela demanda mundial crescente, mas também pela demanda nacional. Uma estimativa da UNICA, feita em 2010, previa uma produção de cana de açúcar de aproximadamente 600 milhões de toneladas para a safra 2010/2011, sendo que 56,71% seriam destinadas à produção de etanol, gerando 27,39 bilhões de litros desse combustível (UNICA, 2011). Esse volume de etanol produzido, contudo, não está conseguindo suprir nem mesmo a demanda do mercado nacional. Até o momento, o Brasil produziu menos etanol que em 2010, o que fez aumentar o preço desse combustível. Segundo José Lima Neto, (presidente da BR distribuidora), em entrevista concedida ao jornal *O Estado de São Paulo*, em agosto de 2011, a previsão era de que entrassem no mercado cerca de 3,4 milhões de automóveis contra 3 milhões, que entraram em 2010. Sendo que

95% dessa frota seria de carro *flex*. Dessa forma, o que se observa no cenário nacional é uma demanda crescente pelo etanol combustível enquanto a oferta está estagnada. Adicionalmente, o primeiro levantamento da Safra 2011/2012, realizado em maio de 2011 pela Conab, trouxe estimativas preocupantes. Segundo a Conab, a produção de etanol em 2011/2012 será 1,83% menor em relação à produção da safra 2010/2011 (CONAB, 2011).

Embora a tecnologia para a produção de etanol a partir da sacarose de cana de açúcar já esteja bem estabelecida e atinja rendimentos consideráveis, a produção desse combustível a partir de resíduos da própria indústria sucroalcooleira poderia ser uma solução interessante para se aumentar a oferta, principalmente se associada à indústria já existente, como mencionado anteriormente. Um desses resíduos, que tem atraído o interesse de diversos pesquisadores, é o bagaço da cana (KARR *et al.,* 1998; BANERJEE; PANDEY, 2002; NEUREITER *et al.,* 2002).

Uma vez extraído o caldo da cana – a partir do qual são produzidos o açúcar e o etanol –, o bagaço que sobra é em parte queimado a fim de gerar calor e energia para a própria usina, sendo a outra parte deixada no campo (ARMAS; BIANCHI, 1990). Com o atual processo, apenas um terço da biomassa contida na cana é utilizado para a produção de etanol. O restante do material celulósico, correspondente a dois terços da área plantada, é desperdiçado ou subutilizado (BASTOS, 2007).

Para cada tonelada de cana obtém-se aproximadamente 140 kg de bagaço seco. Esse resíduo é composto basicamente por celulose, hemicelulose e lignina, na proporção aproximada de 40 a 50%, 20 a 30% e 25 a 30%, respectivamente, dependendo da variedade (RODRIGUES, 2007; FUGITA, 2010). Nessa composição, existe uma grande reserva de carboidratos que poderia ser empregada na produção de etanol, aumentando, assim, os rendimentos. De acordo com Rodrigues (2007), se 90% dos açúcares fermentescíveis encontrados no bagaço pudessem ser recuperados e fermentados, o rendimento teórico sob a totalidade dos açúcares fermentescíveis na cana de açúcar (caldo e bagaço) chegaria a 227 kg/ton. Dessa forma, o bagaço contribuiria com um aumento de 50% no etanol produzido. Assim, pode-se pensar no Brasil produzindo uma maior quantidade de etanol por ano, de forma sustentável e a um custo mais baixo do que qualquer outra fonte energética (CAMARGO, 2005).

Tal processo permitiria um aumento na produção de etanol sem que houvesse uma expansão da área plantada, o que evitaria a competição com as áreas produtoras de alimentos ou, até mesmo, o avanço sobre áreas protegidas, como florestas. Apesar de todas essas vantagens, o que se observa é que a utilização da biomassa lignocelulósica ocorre lentamente (FUGITA, 2010).

Além dos conflitos relacionados à expansão da área cultivada, existem outros tipos de problemas ligados à produção de etanol, sejam eles ambientais; relacionados à saúde da população, ou mesmo sociais. Segundo Laschefski e Assis (2006), as queimadas em canaviais, que acontecem em aproximadamente 80% das áreas plantadas, representam o problema ambiental mais visível do setor sucroalcooleiro nacional. De acordo com Schlesinger (2008, p. 30):

> Estas queimadas visam facilitar o corte manual, reduzir custos de transporte e compensar perdas de até 20% na safra. As emissões de gases de efeito estufa daí decorrentes (dióxido e monóxido de carbono, metano, entre outros), em associação com elementos particulados e aerossóis, exercem grande pressão sobre o clima e podem contaminar regiões distantes das áreas de cultivo. Em consequência, as queimadas nos canaviais, além de representarem grave impacto ambiental, estão ocasionando problemas de saúde na população residente nos municípios circunvizinhos às regiões canavieiras.

Atualmente, parte desses problemas está sendo resolvido com a mecanização da colheita (LASCHEFSKI; ASSIS, 2006; SCHLESINGER, 2008).

Como pode ser visto, existem diversos fatores positivos, bem como negativos, referentes à produção de biocombustíveis. De fato, o bioetanol não é a solução para todos os problemas, contudo, dentro do atual cenário mundial, é a que apresenta maiores perspectivas. O desenvolvimento de novas tecnologias, utilizando a biomassa lignocelulósica como matéria-prima, pode oferecer não somente um aumento nos rendimento de etanol, mas, também, contribuir para minimizar alguns dos pontos negativos levantados.

2.3 Biomassa lignocelulósica

A biomassa lignocelulósica é constituída por vários polissacarídeos que formam uma estrutura complexa e compacta, cujas características dependerão do tipo de material a ser processado (bagaço ou palha de cana) (FUGITA, 2010).

O principal componente do material lignocelulósico é a celulose, seguida pela hemicelulose e lignina. A celulose é o polímero mais abundante da Terra. Ela é formada por uma cadeia linear de moléculas de glicose ligadas entre si na posição beta (β)-1,4, formando um material altamente cristalino. A hemicelulose, por sua vez, é um heteropolímero, ou seja, é composta por unidades de diferentes açúcares (pentoses, hexoses, ácidos hexurônicos e deoxi-hexoses). O principal açúcar encontrado nas hemiceluloses é a xilose. Dessa forma, depois da glicose, a xilose é o açúcar mais abundante presente na biomassa (POLIZELI *et al.,* 2005).

Os diferentes açúcares que compõem a hemicelulose formam polímeros de cadeias mais curtas e ramificadas do que na celulose. Essa estrutura ramificada faz com que a energia de ligação entre as moléculas seja menor, tornando-a mais facilmente hidrolisável em relação à celulose (CANETTIRE, 2004).

A lignina, por sua vez, é um polímero formado principalmente de unidades de fenilpropano oxigenado. Durante o processo de hidrólise, ela permanece inalterada, constituindo-se um resíduo sólido que pode ser separado dos açúcares (RODRIGUES, 2007).

Atualmente, existem muitas pesquisas empenhadas no desenvolvimento de um processo eficaz para o pré-tratamento e hidrólise das biomassas lignocelulósicas. Isso se deve ao fato de que essas matérias-primas não podem ser fermentadas diretamente, sendo necessário um processamento prévio para a liberação dos açúcares fermentescíveis (BEGUIN; AUBERT,

1994; SZCZODRAK; FIEDUREK, 1996; LASER *et al.,* 2002; NEUREITER *et al.,* 2002; SUN; CHENG, 2002). Como a biomassa lignocelulósica é uma estrutura complexa, a hidrólise direta com celulase e hemicelulase é praticamente impossível (CHANDRA *et al.,* 2007). Assim, para a conversão completa da hemicelulose e celulose em monossacarídeos, pré-tratamentos químicos e/ou físicos, juntamente com um processo de hidrólise, são obrigatórios. Diferentes métodos já foram abordados e, igualmente, diferentes foram os resultados obtidos em termos de rendimento de monossacarídeos liberados no hidrolisado.

A primeira etapa em qualquer linha de processamento da biomassa lignocelulósica é o pré-tratamento (Figura 1). A função deste é alterar a estrutura lignocelulósica e facilitar o processo de hidrólise (WYMAN, 1994; SZCZODRAK; FIEDUREK, 1996; MOSIER *et al.,* 2005, HENDRIKS; ZEEMAN, 2009). Os processos adotados no pré-tratamento podem atuar de diversas formas: i) pela formação de fragmentos que aumentam a área de exposição dos polímeros; ii) pela hidrólise da fração hemicelulósica, diminuindo os efeitos de proteção; e iii) pela modificação da estrutura rígida da lignina (MOSIER *et al.,* 2005). O aspecto comum em todos os processos é a remoção da proteção em forma de microfibrilas formada pela lignina e, em alguns deles, há também a remoção da hemicelulose.

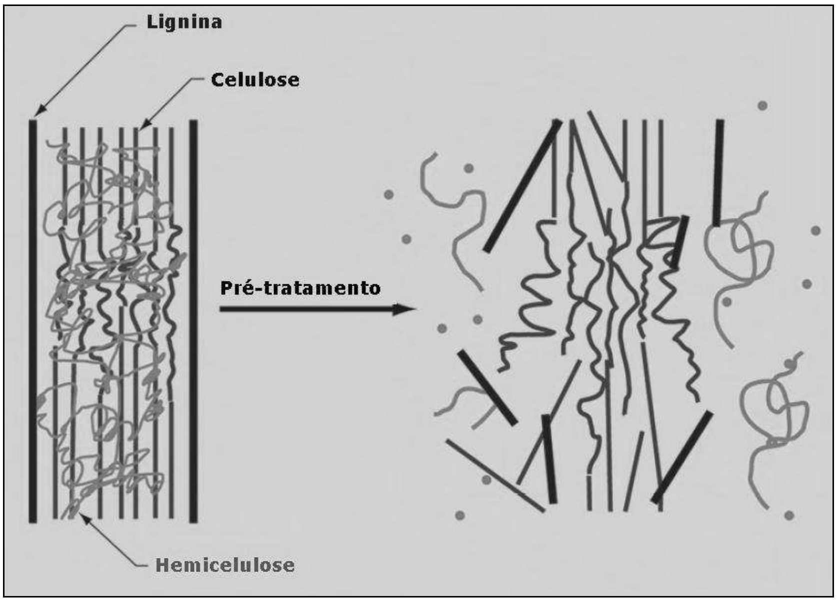

Figura 1 - Esquema da atuação do pré-tratamento sobre a biomassa lignocelulósica. Fonte: Moiser et al., 2005

Os métodos de pré-tratamento podem ser divididos em métodos físicos (picadores, moagem), físico-químicos (auto-hidrólise: descompressão com vapor, com amônia ou com CO_2), químicos (com ozônio, ácidos diluídos ou concentrados, alcalino), com solventes (para dissolver a lignina, como no processo Dedini-Copersucar) ou biológicos (degradação de lignina e hemicelulose por micro-organismos), sendo comum a combinação de alguns deles (MACEDO *et al.,* 2005; FUGITA, 2010).

Cada tipo de pré-tratamento tem um efeito específico sobre a fração de celulose, hemicelulose e lignina. Portanto, os métodos de pré-tratamento e as diferentes condições devem ser escolhidos de acordo com a configuração da linha de processamento adotada, incluindo a hidrólise subsequente e a etapa de fermentação (MOSIER *et al.,* 2005). É desejável que esses processos sejam realizados com a formação mínima de compostos que inibam o crescimento e a atividade fermentativa dos micro-organimos.

Diferentes processos de pré-tratamento já foram investigados utilizando uma extensa variedade de matérias-primas. Existem diversos artigos recentes de revisão que proporcionam uma visão geral sobre o assunto (TAHERZADEH; KARIMI, 2008; YANG; WYMAN, 2008; HENDRIKS; ZEEMAN, 2009; ALVIRA *et al.,* 2010). No presente capítulo, será apresentada uma visão sumária de alguns deles.

Os métodos de pré-tratamento que utilizam bases, como a explosão de fibra por amônia (AFEX), podem reduzir eficientemente o teor de lignina e remover a hemicelulose. Constitui um método caro, devido aos elevados custos da amônia e também do processo de recuperação da mesma (CHANDRA *et al.,* 2007). Métodos utilizando hidróxido de cálcio (ou sódio) podem ser uma alternativa. Estes últimos têm menor custo e permitem utilizar temperaturas mais baixas, contudo, são mais demorados.

Os processos de pré-tratamentos que utilizam ácido são os mais utilizados, por serem eficazes em diferentes substratos lingnocelulósicos (MOSIER *et al.,* 2005). Segundo os autores supracitados, esse tipo de pré-tratamento pode alcançar excelentes rendimentos de açúcares provenientes da hemicelulose, além de permitir alta acessibilidade das enzimas à celulose. O ácido mais amplamente utilizado é o ácido sulfúrico diluído (MOSIER *et al.,* 2005). O ácido nítrico (RODRIGUEZ-CHONG *et al.,* 2004), ácido clorídrico

(GOLDSTEIN *et al.,* 1983), e ácido fosfórico (ISRAILIDES *et al.,* 1978) também já foram testados.

Em outro processo, o de explosão a vapor, ocorre uma rápida evaporação da água e um cisalhamento provocado pelo deslocamento do material do reator para o ciclone, o que promove grandes modificações na estrutura dos materiais lignocelulósicos. Durante esse processo, pode ocorrer degradação dos açúcares liberados, e formação de compostos tóxicos ao metabolismo microbiano, como furfural, hidroximetilfurfural e compostos fenólicos (CANETTIERE, 2004).

Segundo Sun e Cheng (2002), o ozônio é um oxidante que também pode ser empregado em processos de pré-tratamento, uma vez que apresenta alta eficiência na remoção de lignina. Ainda de acordo com os autores, uma desvantagem importante que deve ser considerada é a grande quantidade de ozônio necessária, tornando o processo economicamente inviável.

Pré-tratamentos biológicos também têm sido investigados (SÁNCHEZ; CARDONA, 2008). Nesses tratamentos, micro-organismos, principalmente fungos, capazes de degradar tanto a lignina quanto a hemicelulose, são inoculados para decompor a estrutura lignocelulósica, como é comum ocorrer na natureza. Entretanto, esse método apresenta uma desvantagem. Os micro-organismos utilizados podem consumir os açúcares liberados, diminuindo os rendimentos de processos subsequentes de fermentação.

Uma vez concluída a fase de pré-tratamento, a celulose fica livre e pronta para ser hidrolisada. Existem diferentes tipos de métodos de hidrólise e cada um possui vantagens e desvantagens. Um desses métodos é a hidrólise alcalina. Esse processo tem a desvantagem de proporcionar baixos rendimentos na liberação de açúcares, além de gerar um grande volume de efluentes quando comparado com os outros processos de hidrólise (CANETTIRE, 2004).

A hidrólise também pode ser feita com catálise ácida. A hidrólise ácida é eficiente, sendo que a quantidade de açúcares fermentescíveis encontrada no bagaço pode ser recuperada com 90% de eficiência após duas etapas de tratamento ácido. Por outro lado, ela pode gerar produtos capazes de inibir a fermentação, além do risco de degradar os açúcares, devido ao tempo de hidrólise (CANETTIRE, 2004).

A hidrólise enzimática de biomassa lignocelulósica, por sua vez, apresenta diversas vantagens, a saber: especificidade da reação; ausência de reações secundárias; ausência de formação de produtos secundários que poderiam inibir a fermentação; e reação em condições suaves, que não requerem altas pressões e temperaturas ou ambientes corrosivos para os equipamentos (BASTOS, 2007). Entretanto, esse processo também apresenta desvantagens. Atualmente, ele ainda é oneroso devido à quantidade de enzimas requeridas (CANETTIERE, 2004).

Em termos gerais, a fração hemicelulósica pode ser quase que totalmente extraída do complexo lignocelulósico durante o pré-tratamento da matéria-prima, alcançando rendimentos de ordem de 90%, dos quais a maior fração é de xilose (SUN; CHENG, 2002; SÁNCHEZ; CARDONA, 2008). Embora ofereça maior resistência e exija etapas adicionais de processamento, a fração celulósica também pode ser hidrolisada, liberando glicose (JEFFRIES; KURTZMAN, 1994). Portanto, o hidrolisado obtido a partir de materiais lignocelulósicos é rico em glicose e em xilose.

Nesse ponto, outro gargalo no processo de utilização da biomassa lignocelulósica para produção de etanol é a fermentação dos açúcares liberados. Os processos biotecnológicos industriais, que vêm utilizando as matérias-primas lignocelulósicas, têm utilizado somente a glicose, oriunda do componente celulósico. Entretanto, a partir de todos os dados apresentados, verifica-se que a alta proporção de pentoses – particularmente xilose – na fração hemicelulósica, aliada a sua maior facilidade de extração do complexo lignocelulósico, dita que essa fração deve ser utilizada para efetiva exploração dessas matérias-primas. O grande desafio é encontrar um micro-organismo capaz de, eficientemente, fermentar tanto a glicose quanto a xilose, de modo a obter uma produção industrial sustentável de bioetanol.

2.4 Fermentação de glicose/ xilose e seus desafios

A falta de um micro-organismo robusto para co-fermentação de glicose e xilose constitui uma das principais barreiras à implantação de processos de produção de etanol a partir de biomassa lignocelulósica. A levedura predominantemente utilizada na produção de etanol, a *Saccharomyces cerevisiae*, é capaz de, naturalmente, converter glicose em etanol com altos rendimentos, contudo, é incapaz de metabolizar xilose (JEFFRIES; JIN, 2004; LIN; TANAKA, 2006). Por outro lado, os micro-organismos capazes de metabolizar xilose não conseguem convertê-la a etanol com altos rendimentos ou são difíceis de serem cultivados (LIN; TANAKA, 2006; SERVICE, 2007). Dessa forma, pesquisadores no mundo todo têm adotado diferentes estratégias visando solucionar esse problema.

Substanciais esforços de engenharia metabólica têm sido dirigidos para o desenvolvimento de cepas de leveduras capazes de fermentar tanto as hexoses quanto as pentoses (CHU; LEE, 2007; HAHN-HÄGERDAL *et al.,* 2007; NEVOIGT, 2008). Até o presente momento, nenhum resultado efetivo foi alcançado. O insucesso dessas tentativas se deve, em parte, a problemas relacionados ao transporte da xilose por meio da membrana celular, e também ao desequilíbrio no balanço redox gerado pela via de assimilação desse açúcar.

Em leveduras que naturalmente metabolizam xilose, o transporte desse açúcar tem sido relacionado a dois sistemas proteicos distintos. Um deles seria um sistema de baixa afinidade, que também é utilizado para o transporte de glicose; e o outro, de alta afinidade, específico para xilose (HAHN-HÄGERDAL *et al.,* 2001).

A levedura mais extensivamente estudada capaz de utilizar xilose é a *Scheffersomyces* (*Pichia*) *stipitis*. Embora seu genoma revele uma série de genes putativos para transportadores de xilose (JEFFRIES *et al.,* 2007), eles ainda não foram totalmente caracterizados bioquimicamente. Em *Candida intermedia* PYCC4715, o rápido crescimento em xilose foi atribuído a um transportador de glicose/ xilose ligado ao simporte de prótons (GARDONYI *et al.,* 2003; LEANDRO *et al.,* 2006).

Acredita-se que, em linhagens de *S. cerevisiae* recombinantes (geneticamente modificadas para metabolizar xilose), a xilose seja transportada pelo mesmo sistema de transporte de glicose, com afinidade de aproximadamente 200 vezes menor para xilose do que para glicose (KÖTTER;

CIRIACY, 1993). Dessa forma, o transporte de xilose pode ser inibido competitivamente pela glicose (Figura 2) (OLOFSSO *et al.,* 2008).

Segundo Hamacher *et al.* (2002), os transportadores de glicose de alta e intermediária afinidade (Hxt4, Hxt5, Hxt7 e Gal2) seriam os mais importantes envolvidos no transporte de xilose. Se por um lado uma alta concentração de glicose poderia inibir, por competição, o transporte da xilose, por outro, uma baixa concentração seria necessária para um transporte eficiente de xilose (LAWFORD; ROUSSEAU, 1999; OLOFSSO *et al.,* 2008; SOUTO-MAIOR *et al.,* 2009).

Segundo Olofsso *et al.* (2008), a presença de glicose também seria importante para aumentar a expressão de algumas enzimas glicolíticas, e na geração de intermediários metabólicos importantes nas etapas iniciais do metabolismo de xilose e na via das pentoses fosfato. A presença da glicose, portanto, não é o maior problema, nem para o transporte nem para o metabolismo da xilose, dependendo, certamente, da concentração desses.

Uma vez no citoplasma, a xilose é inicialmente reduzida a xilitol pela enzima xilose redutase (XR). Em seguida, xilitol desidrogenase (XDH) converte xilitol em xilulose, a qual é convertida em xilulose-5-fosfato pela enzima xilulose quinase (XK). Xilulose-5P entra na via das pentoses-fosfato, a partir da qual alimenta as vias metabólicas centrais (Figura 2) (HAHN-HÄGERDAL *et al.,* 1994). A coenzima para XR, em algumas espécies de leveduras, é NADPH e, em outras espécies, pode ser tanto NADPH quanto NADH. A XR de *S. stipitis* pode utilizar tanto uma coenzima quanto a outra (VERDUYN *et al.,* 1985; RIZZI *et al.,* 1988). Em geral, as outras XR de fungos descritas na literatura podem utilizar somente o NADPH como coenzima. XDH utiliza, sempre, o NAD^+ como coenzima (HAHN-HÄGERDAL *et al.,* 1994). Como as duas reações de oxidação-redução nas etapas iniciais da via de utilização de xilose apresentam especificidades diferentes em relação às coenzimas requeridas, isso gera um desequilíbrio na concentração de NADPH e NADH na célula, o que torna o metabolismo de xilose dependente das concentrações dessas coenzimas, causando grandes efeitos no metabolismo.

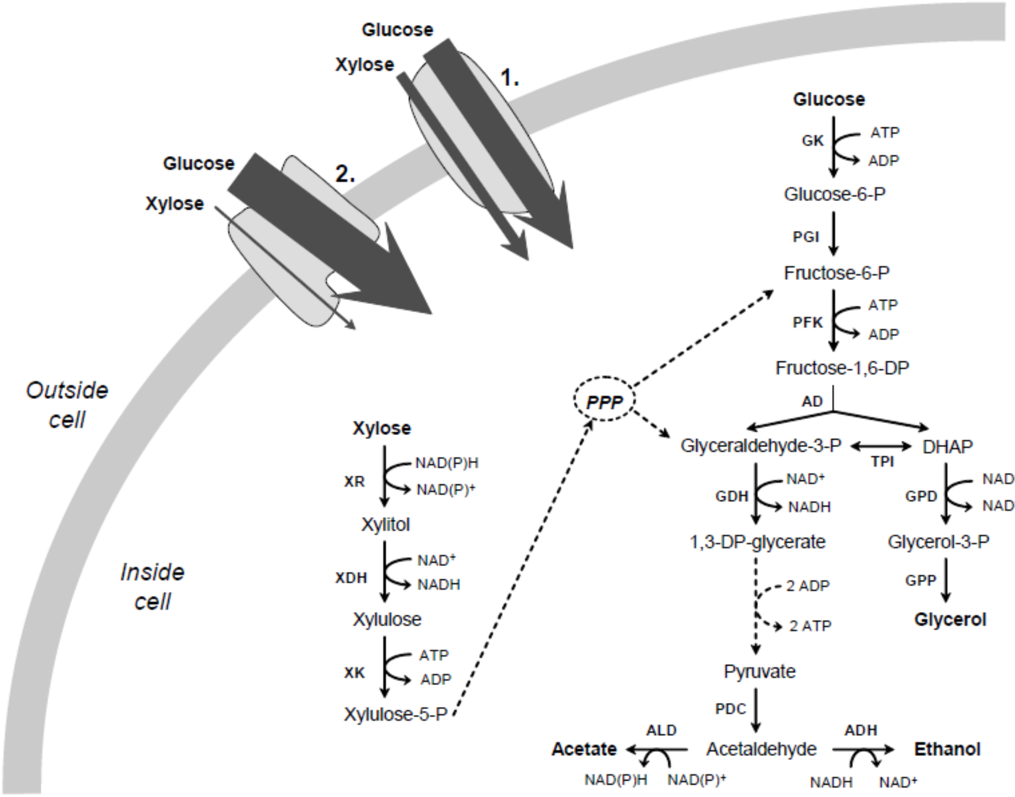

Figura 2 – Esquema simplificado do transporte e do metabolismo de glicose e xilose em *S. cerevisiae* recombinante. 1.Transportadores de baixa e intermediária afinidade por hexose. 2. Transportadores de alta afinidade hexose. (Abreviações: PPP, via das pentose fosfato; XR, xilose redutase; XDH, xilitol desidrogenase; XK, xiluloquinase; GK, glucoquinase; PGI, fosfoglicose isomerase; PFK, fosfofrutoquinase; AD, aldolase; TPI, triose fosfato isomerase; GDH, gliceraldeído-3-P desidrogenase; GPD, glicerol-3-P desidrogenase; GPP, glicerol-3-fosfatase; PDC, piruvato descarboxilase; ALD, acetaldeído desidrogenase, ADH, álcool desidrogenase).

Fonte: Olofsson et al., 2008.

O NADPH é gerado principalmente na parte oxidativa da via das pentoses-fosfato, onde a redução de $NADP^+$ é acoplada à geração de CO_2 pela enzima glicose-6-fosfato desidrogenase (G6PDH) codificada pelo gene *ZWF1* (ALEXANDER *et al.,* 1988; ARISTIDOU *et al.,* 1999). A geração de NADPH por essa via conduz à perda de carbono (1 mol de dióxido de carbono por 2 moles de NADPH). Dessa forma, uma fração considerável de carbono é perdida na geração de NADPH para a reação XR (VERHO *et al.,* 2003).

De acordo com Verho *et al.* (2003), os efeitos, para o metabolismo, da perda desse carbono pode ser analisado considerando as vias metabólicas em duas seções: uma parte superior, levando em consideração as pentoses e hexoses, e, a outra parte, inferior, após as trioses fosfato e ácidos orgânicos. Segundo os autores supracitados, a formação de NADPH por si só não constitui um problema para a célula, mas causa um problema nas vias subsequentes, uma vez que a principal rota para regenerar NADH começa no piruvato, e a perda de carbono na parte superior da rede metabólica reduz o fluxo de carbono após o piruvato. Dessa forma, o fluxo de carbono é preferencialmente utilizado na formação de NADPH, deixando uma menor quantidade para a formação de NAD^+, o que torna a formação de NAD^+ o fator limitante para a fermentação de xilose.

A regeneração do NADH produzido durante a oxidação de xilitol a xilulose pode ser realizada na cadeia transportadora de elétrons ou em reações redox catalisadas pelas enzimas glicerol-3-fosfato desidrogenase (GPD1) ou álcool desidrogenase (ADH) (Figura 2) (ARISTIDOU *et al.,* 1999). Sob condições de aerobiose, o NADH é oxidado a NAD^+ pela cadeia transportadora de elétrons na mitocôndria. Sob condições de anaerobiose, leveduras produtoras de xilitol não conseguem regenerar totalmente o NADH produzido, ocasionando o acúmulo dessa coenzima reduzida na célula, e impedindo, consequentemente, a oxidação de xilitol, que também acumula. Por outro lado, as poucas leveduras que fermentam xilose possuem uma XR que apresenta especificidade também para NADH; logo, elas podem regenerar o NADH tanto pelas vias fermentativas, produzindo etanol, quanto pela reação catalisada por XR. Contudo, esse não deve ser o único meio de balancear o potencial redox da célula crescendo em xilose, uma vez que linhagens recombinantes, capazes de expressar esse tipo de XR, não conseguem fermentar xilose (KUYPER *et al.,* 2003; WAHLBOM *et al.,* 2003; VERHO *et al.,* 2003). Diversas modificações genéticas foram empreendidas visando contornar esses problemas e aumentar os rendimentos de etanol.

2.5 Engenharia Metabólica versus Engenharia Evolutiva

Almejando contornar o desequilíbrio no balanço redox, gerado nas etapas iniciais do metabolismo de xilose, e também aumentar os rendimentos de etanol a partir desse açúcar, pesquisadores têm investigado diferentes estratégias envolvendo engenharia metabólica (LEE *et al.,* 2003; JEFFRIES, 2006; BETTIGA *et al.,* 2009; HUGHES *et al.,* 2009; XU *et al.,* 2009). Essas pesquisas vêm explorando desde modificações em mecanismos de transporte da xilose até modificações na afinidade das enzimas pelas suas respectivas coenzimas.

Atualmente, com os avanços nas técnicas de biologia molecular, a inserção de modificações no genoma de um organismo não é o maior desafio enfrentado pelos pesquisadores que desejam alterar uma via metabólica. O desafio está em alcançar o fenótipo desejado. Raramente a modificação genética produz um único efeito na célula e, nem sempre, são efeitos desejados.

Em geral, as pesquisas realizadas somente com engenharia genética, visando o aumento do rendimento de etanol a partir de xilose, resultaram em linhagens com um limitado crescimento em xilose e baixos rendimentos de etanol (KUYPER *et al.,* 2003; 2004; 2005a). Além disso, a inserção de genes heterólogos tem demonstrado causar grande instabilidade genética nas células recombinantes, que exibem algum tipo de deficiência no crescimento ou instabilidade com relação fenótipo alcançado (MEINANDES; HAHN-HÄGERDAL, 1997). De fato, engenharia metabólica frequentemente interfere na resposta fisiológica global da célula. Nesse contexto, muitos pesquisadores têm adotado a “engenharia evolutiva” no lugar da engenharia metabólica.

O termo engenharia evolutiva foi empregado na década de 1990 para descrever um processo utilizado na seleção de micro-organismos com fenótipos melhorados por meio de mutações espontâneas e seleção em cultivo seletivo (BUTLER *et al.,* 1996; PETRI; SCHMIDT, 2004). De acordo com Horsch (2008, tradução nossa):

> Esta metodologia tem como vantagem o fato de que não é necessário nenhum conhecimento prévio da natureza molecular e da regulação de um determinado fenótipo. Este aspecto é de particular relevância quando se considera a complexidade das interações das vias metabólicas e regulatórias que são responsáveis pela expressão de fenótipos

> industrialmente relevantes. Por essa razão, engenharia evolutiva é, frequentemente, a estratégia preferida em comparação à engenharia metabólica.

Uma ferramenta útil adotada nessa estratégia é a cultura contínua. Na literatura existem diversos relatos da utilização de cultura contínua em processos de engenharia evolutiva. Alguns exemplos incluem a seleção de linhagens contendo modificações em mecanismos de transporte de substratos (TSEN *et al.,* 1996; MANCHE *et al.,* 1999); outros, alterações na via preferencial de utilização de determinados nutrientes (FEREA *et al.,* 1999); alguns, ainda, apresentam a seleção de linhagens com maior tolerância a toxinas e outros inibidores (LANE *et al.,* 1999); e, por fim, há relatos do melhoramento da utilização de xilose (SONDEREGGER; SAUER 2003; WAHLBOM *et al.,* 2003).

A cultura contínua sempre foi conhecida como um sistema de forte pressão seletiva. Esse sistema foi desenvolvido a partir dos trabalhos de Monod (1942) e tem como base o fato de que a taxa específica de crescimento, relativa ao seu máximo teórico, é governada pela concentração de substrato externo limitante. Na cultura contínua, a entrada de meio esterilizado, a partir de um reservatório, é balanceada com a saída do meio contendo células, permitindo que o crescimento de novas células seja equilibrado com a saída das mesmas (NOVICK; SZILARD, 1950).

Contudo, a forma como conduzir uma cultura contínua em experimentos de “evolução” não é somente uma função de combinações de condições químicas e físicas, as quais induzem a um fenótipo desejado, mas está inevitavelmente ligada à presença de linhagens diferentes dentro da cultura. Dessa forma, a seleção por cultura contínua é determinada pela capacidade de competição entre linhagens diferentes que estão presentes em um dado momento sobre as condições aplicadas. Essas linhagens diferentes podem surgir ao longo de um cultivo prolongado, durante o qual o fenótipo desejado é alcançado pela ocorrência de múltiplos eventos adaptativos (HORSCH, 2008).

Segundo Ferea *et al.*(1999), culturas em quimiostatos – isto é culturas contínuas governadas pela concentração de substrato –, estendidas por mais de 20 gerações, podem introduzir um elemento significativo de adaptação evolutiva por mutações espontânea. Contudo, é preciso ter em mente que não é possível isolar um mutante com determinado fenótipo de interesse sem que a linhagem original não possua precedentes genéticos para isso. Dessa forma, o cultivo empregando quimiostato permite a obtenção de linhagens melhoradas, tomando como base os potenciais genéticos e, por conseguinte, metabólicos, pré-existentes e passíveis de manipulação (DYKHUIZEN; HARTL, 1983).

Uma característica importante da cultura contínua é que ela aumenta as possibilidades de obtenção de mutantes espontâneos pelo fato de ser um sistema que mantém as células se dividindo continuamente. Em uma população, mutações ocorrem espontaneamente a uma frequência baixa, em torno de 10^{-5} a 10^{-10} modificações por nucleotídeo replicado por geração (HORSCH, 2008). Em cultura contínua, a célula está sob contínua divisão, de modo que novas células são formadas a uma taxa de 10^{10} a 10^{14} células por dia. Nessas condições, o número de mutações que podem ser geradas e acumuladas aumenta. Uma mutação que ofereça vantagens em relação à linhagem selvagem pode favorecer o mutante e, assim, esse poderá prevalecer dentro do quimiostato (DYKHUIZEN; HARTL, 1983; HOSKISSON; HOBBS, 2005). Além disso, a remoção de células pelo fluxo de saída em uma cultura contínua é aleatória, tornando-se, assim, uma função seletiva, com a taxa de crescimento como o principal fator determinante de sobrevivência e permanência no meio.

Em particular, cultivos em condições de limitação por nutriente impõem uma pressão seletiva positiva sobre mutantes que possuem uma maior afinidade pelo nutriente limitante. Essa afinidade pode ser melhor compreendida segundo a equação proposta por Monod (1942),

$$\mu = \frac{\mu max . [S]}{K_s + [S]}$$

onde μ é a velocidade específica de crescimento, [S] é a concentração do substrato limitante, e K_s corresponde à constante de afinidade da célula pelo substrato limitante, sendo a constante de saturação. Dessa forma, mutantes com um aumento na afinidade por um substrato limitante podem alcançar a taxa específica de crescimento ditada pelo sistema em menores concentrações residuais do substrato, do que a população original, podendo, assim, sobressair e dominar a cultura.

Uma maior afinidade pelo substrato limitante no quimiostato pode ser resultado de um sistema mais eficiente de transporte por meio da membrana celular. Um exemplo desse tipo de seleção foi reportado por Collins *et al.* (1976). A mutação envolvia o sistema de transporte de serina-glicina-alanina, o qual é associado com o transporte de prótons, sistema de co-transporte simporte. Após dois meses de cultivo em cultura contínua, mutantes com diferentes estequiometrias na troca de prótons e aminoácidos foram isolados. Estes apresentaram um valor 20 vezes menor de K_s para o aminoácido. Tal experimento também contribuiu para realçar a importância dos mecanismos de transporte dos substratos que limitam crescimento (COLLINS *et al.,* 1976).

Em outros estudos, linhagens de *S. cerevisiae* isoladas após 500 gerações, em cultura contínua sob limitação por glicose, apresentaram alterações no material genético (número de cópias do DNA). Isso foi resultado de rearranjos cromossômicos (translocações e duplicações) na região do cromossomo IV, que inclui a expressão de genes para transportadores de hexose (DUNHAM *et al.,* 2002). Esses estudos mostraram que o cultivo prolongado de *S. cerevisiae* em cultura contínua, sob condições limitantes de glicose, promove a redução da concentração de glicose residual no meio e alterações no metabolismo primário da levedura (BROWN *et al.,* 1998; FEREA *et al.,* 1999; MASHEGO *et al.,* 2005).

Uma abordagem diferente foi apresentada por Sonderegger e Sauer (2003). Esses pesquisadores demonstraram a aplicação da engenharia evolutiva em complementação à engenharia metabólica para a seleção de linhagens melhoradas. A partir de uma linhagem recombinante de *S. cerevisiae*, capaz de expressar as enzimas xilose redutase e xilitol desidrogenase, foi obtida uma linhagem melhorada capaz de crescer em condições de limitação de oxigênio utilizando xilose como fonte de carbono. Essa linhagem melhorada foi obtida após 260 gerações de seleção em cultura contínua.

Kuyper *et al.*(2004) também adotaram engenharia metabólica associada à engenharia evolutiva para obtenção de uma linhagem de *S. cerevisiae* capaz de fermentar xilose. Esses pesquisadores trabalharam com uma linhagem de *S. cerevisiae* capaz de expressar a enzima xilose isomerase. Essa linhagem foi submetida a bateladas sequenciais, em meio contendo xilose como única fonte de carbono. O resultado obtido foi um aumento na velocidade específica máxima de crescimento de 0,005 h^{-1} para 0,18 h^{-1}. A linhagem selecionada, contudo, não foi capaz de crescer em anaerobiose plena e uma nova seleção em regime de bateladas sequenciais foi realizada. Dessa vez, a pressão de seleção aplicada foi uma crescente limitação de oxigênio até o ponto de anaerobiose plena. Um mutante capaz de crescer em xilose (0,03 h^{-1}), sob condições estritas de anaerobiose foi obtido.

Em pesquisas posteriores, houve uma tentativa de super expressar alguns genes da via das pentoses fosfato nesta linhagem evoluída, contudo, a linhagem resultante apresentou baixa velocidade específica de consumo de xilose quando cultivada em meio contendo uma mistura de xilose e glicose (KUYPER *et al.,* 2005a). Com o intuito de melhorar este fenótipo, essa linhagem foi submetida a um cultivo em quimiostato em meio limitado por xilose e em anaerobiose, seguido de uma série de cultivos em regime de bateladas sequenciais, em meio contendo glicose e xilose. Uma nova linhagem, capaz de crescer sob condições de anaerobiose, em meio formado pela mistura destes dois açucares, foi obtida (KUYPER *et al.,* 2005b).

Nesses exemplos foi citada outra ferramenta também bastante utilizada na engenharia evolutiva: a batelada sequencial. Culturas em bateladas apresentam diferenças marcantes em relação a culturas contínuas em quimiostatos. Essa diferença ocorre devido ao fato de que, na cultura em batelada, as condições variam constantemente. Nutrientes estão disponíveis em excesso no início e se esgotam de forma contínua ao longo do crecimento. Em quimiostatos, por sua vez, as condições de cultura são muito bem definidas, e mantidas em *steady-state* estritamente reguladas ao longo do tempo (SAUER, 2001).

A seleção em bateladas sequenciais é caracterizada pelo fato de que a população é submetida, por repetidas vezes, às diferentes fases do ciclo de crescimento, incluindo uma fase de latência, seguida por uma fase exponencial de crescimento e, por fim, uma fase estacionária. Cada uma dessas fases e condições exercem diferentes tipos de pressões de seleção sobre a população. Pode-se, portanto, esperar melhorias evolutivas para cada fase do cultivo (SAUER, 2001; HORSCH, 2008).

Tanto a seleção em quimiostato quanto em bateladas sequenciais partem do pressuposto de que mutações espontâneas podem ocorrer ao longo do cultivo. Entretanto, como a taxa de mutação espontânea é baixa, como mencionado anteriormente, observa-se que o tempo requerido para a evolução das linhagens é muito longo, em média 200 gerações no mínimo, mesmo para culturas conduzidas em quimiostatos.

Nesse contexto, uma exposição prévia a agentes mutagênicos (físicos, químicos ou biológicos) poderia fornecer um material de partida com maior diversidade, e diminuir o tempo de seleção. Em trabalho realizado com *S. cerevisiae* adotando tal estratégia, foi possível reduzir o tempo de obtenção de uma linhagem com fenótipo desejado de 350 gerações para 150 gerações, utilizando batelada sequencial (MC BRYDE, 2006; HORSCH, 2008). No trabalho desenvolvido por Horsch (2008) foram utilizados dois métodos mutagênicos: um químico (EMS – etilmetano sulfonado) e outro biológico (hibridização). Assim como esses, existem diversos outros métodos que podem ser utilizados para inserir mutações aleatórias, cada um com suas vantagens e desvantagens. No presente capítulo serão abordados somente dois: radiação ultravioleta (UV) e Integração Mediada por Enzima de Restrição (REMI).

A exposição à radiação UV pode causar dois grandes tipos de lesões aos ácidos nucléicos: os danos diretos e os danos indiretos. Os danos indiretos são aqueles mediados por radicais livres e espécies reativas de oxigênio (COLLINS; HORVATHOVA, 2001). A mais conhecida ação da radiação UV no DNA é a indução de dímeros de pirimidina. Trata-se do favorecimento de ligações carbono-carbono entre pirimidinas adjacentes, sendo mais comum com a timina. Essa estrutura, resultante da saturação das ligações duplas entre os carbonos 5’ e 6’, é chamada de dímero de pirimidina ou dipirimidina ciclobutano (CPD) (FRIEDBERG *et al.,* 1995). Dependendo da forma isomérica em que esses dímeros se encontram, podem ocorrer grandes distorções na hélice de DNA, o que acarreta parada obrigatória da replicação. Outro tipo de lesão no DNA provocado por UV é o fotoproduto 6-4, que causa grande distorção na dupla hélice. Esse tipo de fotoproduto é mais abundante entre as citosinas do que entre as timinas (FRIEDBERG *et al.,* 1995). A radiação UV também está diretamente ligada à produção de radicais livres, os quais, assim como as espécies reativas de oxigênio, produzem uma série de lesões no DNA (SAFFI; HENRIQUES, 2003). Em geral, UV não causa mutação direta. A mutação é gerada quando a célula tenta corrigir os danos causados.

As mutações induzidas pela luz UV, devido aos danos gerados na molécula de DNA, são mais comumente substituições de bases, algumas vezes afetando as duas bases consecutivas complementares do dímero. Mutações pelo deslocamento do quadro de leitura e deleções também podem ocorrer. A relação entre a taxa de mutação e a dose de UV é variável, dependendo do tipo de mutação, do organismo e das condições empregadas (FRIEDBERG *et al.,* 1995). No laboratório, mutagênese por UV é uma técnica bastante utilizada por ser rápida, fácil e econômica, contudo, é muito difícil identificar a posição da mutação.

Outro método que também pode ser utilizado para inserir mutações aleatórias é baseado em integrações ectópicas (não homólogas) de fragmentos de DNA no genoma do organismo. Um desses métodos que tem sido utilizado com sucesso para mutagênese em diversos organismos é denominado Integração Mediada por Enzima de Restrição (REMI) (RIGGLE; KUMAMOTO, 1998). REMI baseia-se na transformação do organismo em estudo com um DNA linearizado por uma enzima de restrição que é adicionada concomitantemente à reação de transformação. A enzima de restrição entra no núcleo da célula juntamente com o DNA linearizado. Em seguida, ela cliva o DNA cromossomal da linhagem hospedeira, gerando sítios específicos complementares aos sítios presentes no DNA linearizado. Estes se ligam *in vivo* e geram uma integração não homóloga (RIGGLE; KUMAMOTO, 1998). Este método pode ser aplicado em qualquer micro-organismo, mesmo naqueles que possuem baixa taxa de recombinação homóloga, pois não depende de homologia para que a integração ocorra. Mas a grande vantagem é que a mutação pode ser facilmente rastreada, uma vez que a sequência do DNA inserido é conhecida.

2.6 *Kluyveromyces marxianus*: uma linhagem promissora

Como mencionado, um dos grandes desafios da produção de etanol de segunda geração é encontrar um micro-organismo capaz de fermentar os diferentes açúcares presentes no hidrolisado do bagaço de cana, especialmente glicose e xilose. As pesquisas que são direcionadas para solucionar este problema normalmente visam a levedura *S. cerevisiae*. De fato, esta linhagem é capaz de produzir etanol, a partir de glicose, com rendimento próximo ao teórico. Contudo, ela apresenta diversas limitações que comprometem sua utilização no processo de produção de etanol a partir de biomassa lignocelulósica. Nesse cenário, *Kluyveromyces marxianus* apresenta-se como uma candidata promissora, tanto para o processo de hidrólise quanto para o de fermentação. Essa levedura apresenta diversas vantagens que faltam à *S. cerevisiae*

K. marxianus é uma espécie de levedura termotolerante capaz de produzir etanol em altas temperaturas com rendimentos que chegam próximos do teórico (considerando-se 0,53 g/g o rendimento teórico de etanol a partir de lactose e 0,51g/g a partir de glicose) quando cultivada em altas concentrações de substrato e baixas concentrações de oxigênio (SILVEIRA *et al.,* 2005; FONSECA *et al.,* 2008).

A capacidade de *K. marxianus* de fermentar a 45 ^{o}C agrega vantagens ao processo de fermentação com a possibilidade de se conjugar a hidrólise enzimática da biomassa lignocelulósica, que requer altas temperaturas, com o processo fermentativo. Esse processo, no qual a hidrólise enzimática e a fermentação ocorrem simultaneamente, é denominado SSF (Simultânea Sacarificação e Fermentação) (SOUTH *et al.,* 1995; SUN; CHENG, 2002) e possui a vantagem de reduzir a inibição das celulases por glicose, uma vez que este açúcar é consumido durante a fermentação (STENBERG *et al.,* 2000; XIAO *et al.,* 2004). Outra vantagem é que a xilose liberada no pré-tratamento pode ser consumida sem a repressão por altas concentrações de glicose, uma vez que essa é liberada do complexo celulolítico de forma gradual.

Genes de enzimas do complexo lignocelulolítico, tais como endo-β-1,4-glucanase, celobiohidrolase e β-glucosidase, foram clonados e eficientemente expressados em linhagens de *K. marxianus* (HONG *et al.,* 2007). Genes que codificam para xilanases também foram clonados em linhagens de *K. marxianus* (Patente EP0316023).

Além da capacidade de hidrolisar o material lignocelulósico, é de interesse que as linhagens de *K. marxianus* também sejam capazes de fermentar os açúcares gerados para que o processo de SSF seja eficiente. Em contraste com *S. cerevisiae*, *K. marxianus* é capaz de utilizar, naturalmente, xilose, arabinose e glicerol (NONKLANG *et al.,* 2008). Ao mesmo tempo, ela é capaz de produzir quantidades de etanol equiparáveis à *S. cerevisiae* utilizando glicose como fonte de carbono (RODRUSSAMEE *et al.,* 2011).

Nonklang *et al.* (2008) estudaram diferentes linhagens de *K. marxianus* no intuito de verificar a produção de etanol a partir de xilose e muito pouco etanol foi detectado. Ainda nesse mesmo estudo, *K. marxianus* DMKU3-1042 foi testada quanto a sua capacidade de crescer em anaerobiose na presença de xilose e não houve crescimento, mostrando que fermentação de xilose sob condições de anaerobiose não é natural em *K. marxianus.* (NONKLANG *et al.,* 2008). Contudo, relatos anteriores mencionam a direta conversão de xilose a etanol por linhagens de *K. marxianus* (MARGARITIS; BAJPAI, 1998; WILKINS *et al.,* 2008). Como *K. marxianus* é uma levedura altamente polimórfica (FONSECA *et al.,* 2008), não é improvável que existam linhagens capazes de fermentar xilose enquanto outras não.

Wilkins *et al.* (2008) trabalharam com diferentes linhagens de *K. marxianus* (IBM2, IBM4, IBM5) isoladas na Índia. Eles utilizaram meio mínimo suplementado com extrato de levedura e obtiveram um baixo rendimento de etanol (0,15 g etanol/ g xilose). Concluíram o trabalho com perspectivas negativas referentes à utilização dessas linhagens em fermentação de xilose sob anaerobiose. Outra pesquisa realizada com uma *Kluyveromyces* sp. IIPE453 alcançou o mesmo rendimento de etanol em um meio mínimo também suplementado com extrato de levedura (KUMAR *et al.,* 2009). Em estudos mais recentes, uma produção de 2,5 gL^{-1} de etanol a partir de 20 gL^{-1} de xilose foi observada utilizando a *K. marxianus* DMKU3-1042 (RODRUSSAMEE *et al.*, 2011). Esses pesquisadores utilizaram para as análises um meio complexo, contendo peptona e extrato de levedura. Até o momento, não foram encontrados relatos de *K. marxianus* produzindo etanol a partir de xilose em meio mínimo sem a suplementação de extrato de levedura.

Ensaios preliminares realizados com *K. marxianus* UFV-3, uma linhagem pertencente à coleção de micro-organismos do Laboratório de Fisiologia de Micro-organismos da Universidade Federal de Viçosa, demonstraram que essa levedura é capaz de assimilar xilose. Contudo, ela também não apresentou grandes rendimentos de etanol a partir desse açúcar. As mesmas limitações impostas ao metabolismo de outros micro-organismos, que fermentam xilose produzindo xilitol em vez de etanol, podem estar presentes no metabolismo de *K. marxianus* UFV-3. Entretanto, não é possível tirar maiores conclusões, já que informações sobre o metabolismo de xilose em *K. marxianus* ainda são escassas.

Nesse contexto, visando obter uma linhagem melhorada de *K. marxianus* quanto à fermentação de xilose, a engenharia evolutiva pode ser utilizada sem a necessidade de uma prévia engenharia metabólica, uma vez que esta levedura já possui uma rota metabólica para o consumo de xilose e, ao mesmo tempo, também apresenta uma grande capacidade fermentativa. A predisposição genética de *K. marxianus* UFV-3 em assimilar xilose e possuir uma via fermentativa ativa é de extrema importância, pois como foi discutido anteriormente, a engenharia evolutiva permite que as informações genéticas sejam modificadas, mas não é capaz de gerar novas informações. Portanto, a combinação de métodos de mutagênese aleatória, seja por mutagênese insercional utilizando REMI, ou por radiação UV, com seleção em culturas contínuas ou bateladas sequenciais, constituem estratégias que merecem ser avaliadas no melhoramento de linhagens de *K. marxianus* UFV-3, visando a efetiva conversão de xilose em etanol.

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CAPÍTULO 2

Kinetic of growth and ethanol formation from glucose/ xylose mixture by *Kluyveromyces marxianus* UFV-3

ABSTRACT

The hydrolyzed of sugarcane bagasse contains two major monosaccharides: glucose and xylose. Fermentation of both sugars is important to maximize ethanol yield from lignocellulosic biomass. This study analyzed the possibilities of various combinations of glucose and xylose concentrations being fermented by the yeast *K. marxianus* UFV-3. In almost all analyzed conditions, glucose repressed the xylose assimilation. In some growth conditions, xylose utilization began after glucose had been exhausted. The exception was when Antimycin A was added to the culture medium. In this condition, glucose and xylose were used simultaneously. Another remarkable difference was the absence of adaptation period to start xylose consumption after glucose depletion, when 5 gL^{-1} glucose was used. This period was of 30 h when 20 gL^{-1} glucose was used. The ethanol formation was always higher in the mixture of the two sugars than in just glucose. The highest concentration of ethanol (8.65 gL^{-1}) was achieved in 8 hours of fermentation in a mixture of 20 gL^{-1} glucose and 20 gL^{-1} xylose. The biomass at 24 h was 3.5 ± 0.1 gL^{-1}. In contrast, the maximum of 5.6 gL^{-1} of ethanol was formed from glucose (20 gL^{-1}) with about the same biomass as mentioned (3.6 ± 0.1 gL^{-1}) at 24 hours. An equation for yield optimization was obtained from glucose and xylose combinations.

Keywords: fermentation, glucose, xylose, *Kluyveromyces marxianus,* bioethanol

1 INTRODUCTION

The search for alternative energy sources is growing worldwide and many governments are investing in sustainable technologies for second-generation ethanol. Lignocellulosic biomass has been investigated as a source of substrate for the ethanol fermentative production. It is composed by cellulose, hemicelluloses and lignin. Cellulose constitutes 35–50% dry weight in lignocellulosic biomass and is a linear polymer of glucose. Hemicelluloses constitute 20–35% dry weight in lignocellulosic biomass. In contrast to cellulose, hemicelluloses are heterogeneous polymers of xylose, arabinose, mannose, glucose, galactose and sugar acids, with xylose as the major sugar (Hahn-Hägerdal et al., 2006; Hector et al., 2008; Leandro et al., 2008; Polizeli et al., 2005). Hence xylose is the most abundant monosaccharide in renewable biomass after glucose (Aristidou and Penttila, 2000).

Lignocellulose is often hydrolyzed by acid treatment (Sun and Cheng, 2002). Glucose and xylose are the two dominant sugars in lignocellulosic hydrolysates and, microorganisms should be required to efficiently ferment these sugars in order to guarantee a successful industrial production of bioethanol (Katahira et al., 2006). However, the lack of industrially robust microbes for co-fermentation of glucose and xylose has been a major barrier (Jeffries and Jin, 2004; Lin and Tanaka, 2006; Service RF, 2007).

There are many searches with different approaches in order to develop strains capable of fermenting both the glucose and xylose (Amore et al., 1991; Bothast et al., 1999; Chu and Lee, 2007; Hahn-Hägerdal et al., 2007; Nevoigt, 2008). Up to date, no satisfactory results have been achieved. The performance of recombinant strains is still inferior to that of native xylose-fermenting yeasts. One of the problems in xylose utilization by the recombinant strains is the limitation of xylose uptake into the cell. Since the xylose uptake is mediated by the same transport system as for glucose (Hamacher et al., 2002), the limitation may be due to the lower affinity of the transport system by xylose (Han et al., 2011; Kötter and Ciriacy, 1993), and also due to catabolic repression by glucose, at concentrations used in fermentation processes (van Zyl et al., 1999).

However, the mechanism of glucose repression on xylose metabolism in yeast is still not totally clear. Previous searches have shown that glucose can repress the induction of the genes which codify to xylose reductase (XR) and xylitol dehydrogenase (XDH) even in the natural xylose-fermenting yeasts as *Pachysolen tannophilus* and *Sheffersormyces* (*Pichia*) *stipitis* (Bicho et al., 1988; Bolen and Detroy, 1985; Ditzelmueller et al., 1984; Slininger et al., 1987). Further, in recent searches, it was observed that ethanol concentrations, around 50 gL^{-1}, fully repressed enzyme induction of xylose metabolism (Slininger et al., 2011).

Despite these negative effects of glucose over xylose metabolism, there are many studies showing that is possible co-ferment glucose and xylose. Agbogbo and co-workers (2006), working with *S. stipitis*, evaluated the fermentative ability of this yeast in a sugar mixture. The highest ethanol yield was obtained at 25% glucose with 75% xylose fraction (Agbogbo et al., 2006). Other researchers have observed that xylose uptake was enhanced at low concentrations of glucose compared to identical reaction conditions lacking glucose (Meinander et al., 1999; Pitkänen et al., 2003; Souto-Maior et al., 2009). Thus, there is a condition in which is possible the co-fermentation of glucose and xylose, and the challenge is to find and optimize it.

The focus of this work was to find this condition for *K. marxianus* UFV-3, a fermentative yeast that naturally is able to metabolize both glucose and xylose. We present the behavior of *K. marxianus* UFV-3 in various glucose/ xylose mixtures and the effects of this mixture on cell growth kinetic, sugar consumption and ethanol formation aiming to subsidize future fermentation process modeling for hydrolyzed sugarcane bagasse.

2 MATERIAL AND METHODS

2.1 Microorganism and maintenance

The yeast strain used in this work is designated as *Kluyveromyces marxianus* UFV-3. It was isolated from a Brazilian southeast region dairy industry, and taxonomically identified by the Centraalbureau voor Schimmelcultures [Utrecht, The Netherlands] as *Candida kefir* (Beijerinck) Van Uden & Buckey [non-ascospore forming state of *Kluyveromyces marxianus* (Hansen) Van der Walt]. Some other strains, as *K. marxianus* ATCC8554, *Debaryomyces hansenii* UFV-170 and *Sheffersormyces* (*Pichia*) *stipitis*, were also used for comparison. The yeast strain was maintained on YPX plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose, 2% (w/v) agar]. For storage of the long-term, cells were maintained frozen at -80 °C with 20% (v/v) glycerol.

2.2 Culture media and inoculum preparation

Bioconversions were carried out in a medium containing 0.67% (w/v) YNB (Yeast Nitrogen Base); 0.06 % (w/v) yeast extract and carbon source. Some treatments were conducted in the presence of respiratory chain inhibitors: 5 µM Antimycin A or 2 mM malonate. Solutions of D-xylose, D-glucose and yeast extract were sterilized separately by autoclaving at 121 °C for 20 min. For pre-cultures, cells frozen at -80 °C were transferred to 125 mL Erlenmeyer flasks containing 50 mL of the above medium. The flasks were maintained at 37 °C under agitation in an orbital shaker at 180 rpm. The *S. stipitis* and *D. hansenii* were incubated at 30 °C. After 18-20 h incubation, cells were collected by centrifugation at 4,000 x *g* for 5 min, then washed twice with sterile water and used for further inoculations.

2.3 Shake-flask experiments

In order to investigate the influence of carbon concentration on glucose/ xylose ethanol conversion, a set of 17 batches tests was carried out. It was used 125 mL-Erlenmeyer flasks containing 50 mL of the same cultivation medium used for pre-cultivations, but having initial concentration of glucose and xylose varying between 5 gL^{-1} and 40 gL^{-1}. To evaluate the effect of initial biomass concentration on the process, an additional set of 12 batches runs was performed in the range of Abs_{600} from 0.1 and 1.0. All tests were performed in triplicate and the cultures incubated at 37 °C under 180 rpm. The kinetic parameters (K_s and μ_{max}) and yield were calculated. Ethanol yield was calculated over consumed glucose ($Y_{E/S,}$ gg^{-1}); biomass yield was calculated per total sugar consumed ($Y_{X/S,}$ gg^{-1}); specific growth rate (μ, h^{-1}) was determined by linear regression of the plot Ln Abs_{600} unit *versus* time (h), at the exponential growth phase. The volumetric productivity (Q_P, $gL^{-1}h^{-1}$) was calculated as the ratio between maximum formation of ethanol (P_m, gL^{-1}) and the time required for its achievement.

2.4 Analytical methods

Aliquots of the culture were periodically sampled, centrifuged at 5,000 x *g* for 10 min, and the supernatants analyzed for ethanol, xylose, and glucose by HPLC, using an Aminex HPX 87H, 300mm x 7.8 ion exchange column with a 30 x 4.6 mm guard column (Bio-Rad Laboratories Inc., Hercules, CA, USA) on an HP 1050 Series HPLC system equipped with a refractive index detector (HP 1047A). Samples (20 µL) were injected onto a heated column (60 °C) and eluted at a rate of 0.7 mL min^{-1} using 5 mM H_2SO_4 as mobile phase. For cell concentration, the absorbance (Abs) was determined at 600 nm. A calibration curve was used to relate Abs_{600} with cell dry weight (1 Abs_{600} = 0.507 gL^{-1} for *K. marxianus* UFV-3).

2.5 Statistical analysis

A 2^3 full-factorial experimental design was used to point out the relationship between the response functions and process variables as well as to determine those conditions to maximize ethanol formation process. The starting xylose concentration (Xyl_0) and the starting glucose concentration (Glu_0), as the initial cell mass concentration (X_0), were selected as the independent variables, due to their influence on sugar-to-ethanol bioconversion. The ethanol yield was chosen as the dependent variable or response. The Fisher's test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The "STATGRAPHICS *plus*" software [version 5] was employed for the regression analysis and graphical optimization (Cornell, 1990; Myers and Montgomery, 1995; Polhemus, 1999).

3 RESULTS AND DISCUSSION

3.1 Kinetics of growth and ethanol formation from xylose and glucose by naturally xylose metabolizing yeasts

The kinetic of growth and ethanol formation of different yeast strains, including two different *K. marxianus* strains, on xylose and glucose, were evaluated. The maximum specific growth rate of *K. marxianus* UFV-3 in xylose was close to that of *S. stipitis* NRRLY-7124 and to *D. hansenii* UFV-170, and higher than for *K. marxianus* ATCC8554 (Table 1). In glucose, *K. marxianus* UFV-3 exhibited the highest growth rate, followed by *K. marxianus* ATCC8554, *D. hansenii* UFV-170 and *S. stipitis* NRRLY-7124. In general, *K. marxianus* UFV-3 showed the highest growth rates in both glucose and xylose.

The kinetic of growth was also evaluated in the presence of Antimycin A, a respiratory chain inhibitor at cytochrome bc1 complex. In medium containing xylose and Antimycin A, the *K. marxianus* UFV-3 and ATCC8554 strains, as well as the *D. hansenii* UFV-170, grew at very low rates (around 0.05 h^{-1}). In this condition, *S. stipitis* exhibited the highest growth rate, around 0.25 h^{-1}. Opposite results were found when Antimycin A was added to glucose medium. In this case, *K. marxianus* UFV-3 achieved the highest growth rate.

In medium containing xylose, the ethanol yield ($Y_{E/S}$) was the highest in the *S. stipitis* culture. For this strain, the $Y_{E/S}$ was close to maximal theoretical yield ($Y_{E/S}$ = 0.51 g ethanol/ g xylose) when Antimycin A was used. In contrast, both *K. marxianus* strains and *D. hansenii* UFV-170 did not produce ethanol from xylose. This was verified on xylose with and without Antimycin A. On the other hand, on glucose with Antimycin A, the ethanol yield was higher for both *K. marxianus* strains compared the other yeasts strains. In medium without the inhibitor, the ethanol yield was 2-fold higher in *K. marxianus* UFV-3 culture compared to other three strains, including the *K. marxianus* ATCC 8554. In this condition, the $Y_{E/S}$ achieved for *K. marxianus* UFV-3 was around 50% of the maximal theoretical yield, while that for other strains was around 25% (considering 0.51 gg^{-1} as maximal theoretical yield on glucose). This result indicates a higher fermentative potential of *K. marxianus* UFV-3 compared to other yeasts evaluated.

K. marxianus UFV-3 has showed advantages over the native xylose-fermenting yeast *S. stipitis* since it grows faster on glucose, although growth rate in xylose it is similar. In addition, although *K. marxianus* UFV-3 is not able to ferment xylose, it is able to grow on this sugar. Thus, it is not necessary to use metabolic engineering in this strain to introduce any pathway for xylose metabolism like in *S. cerevisiae*. Therefore, *K. marxianus* UFV-3 can be a potential strain to fermenting a glucose/ xylose mixture present in lignocellulosic biomass.

Table 1 - Physiological parameters of yeast (*K. marxianus* UFV-3, *K. marxianus* ATCC8554, *D. hansenii* UFV-170 and *S. stipitis* NRRLY-7124) in batch cultures with 20 gL^{-1} initial carbon source.

Strain	**Maximum Growth rate (μ, h^{-1})**				**Yield ($Y_{E/S}$) gg^{-1}**			
	Xyl	**Xyl + Ant A**	**Glu**	**Glu + Ant A**	**Xyl**	**Xyl + AntA**	**Glu**	**Glu + Ant A**
K. marxianus UFV-3	0.35 ± 0.00	0.05 ± 0.00	0.63 ± 0.00	0.53 ± 0.01	0.00	n.d.	0.25 ± 0.01	0.35 ± 0.01
K. marxianus ATCC8554	0.23 ± 0.00	0.05 ± 0.00	0.55 ± 0.00	0.46 ± 0.11	0.00	n.d.	0.13 ± 0.03	0.35 ± 0.01
D. hansenii UFV-170	0.36 ± 0.01	0.05 ± 0.00	0.56 ± 0.00	0.23 ± 0.00	0.00	n.d.	0.14± 0.01	0.11 ± 0.01
S. stipitis NRRLY-7124	0.32 ± 0.01	0.25 ± 0.00	0.40 ± 0.00	0.26 ± 0.01	0.15 ± 0.02	0.55 ± 0.05	0.13 ± 0.01	0.21 ± 0.00

$Y_{E/S}$: ethanol yield on substrate*;* Xyl: Xylose; Ant A: Antimycin A; Glu: Glucose; n.d.: not detectable. Data are the average ± standard deviation of the mean of the analyses in triplicate.

3.2 Fermentation on various combinations of glucose and xylose mixtures

In practice, the acid hydrolyzed lignocellulosic biomass may have different ratios of glucose/xylose due to different hydrolysis conditions and different substrates. Sendelius and co-workers (2005), performing a pre-treatment of sugarcane bagasse at 190°C for 5 min with 2% of SO_2 , obtained 96.3% solubilized glucose from the total biomass while 66.6% xylose was obtained at the same conditions but 10 ºC lower (180°C). Rodriguez-Chong and co-workers (2004), treating 100 g sugarcane bagasse with 6% nitric acid for 9.3 min at 122° C, obtained 18.6 gL^{-1} xylose, 2.87 gL^{-1}glucose and 2.04 gL^{-1} arabinose. As mentioned, the sugar proportion in hydrolyzed bagasse varies, being necessary the study of yeast behavior in different concentrations.

Initially, it was evaluated the influence of three factors over ethanol formation by *K. marxianus*: initial cell mass concentration (X_0), starting glucose concentration (Glu_0) and starting xylose concentration (Xyl_0). Initial cell mass concentration did not have effect over ethanol yield (data not shown), while both glucose and xylose strongly influenced ethanol yield and final ethanol concentration.

The ethanol production by *K. marxianus* UFV-3 from diverse ratios of glucose/ xylose revealed differences (Figure 1). In all cases, ethanol yield was higher when both glucose and xylose were present in the medium. The productivity was substantially higher on 40 gL^{-1} glucose/ 20 gL^{-1} xylose while the highest ethanol yield was achieved on 20 gL^{-1} glucose/ 20 gL^{-1} xylose (Figure 1). On the other hand, major variations on ethanol formation were observed in low glucose concentration. Under these conditions, small changes in sugars concentration were able to produce large changes on ethanol yield.

Statistic analyses were performed in order to verify the effect of glucose/xylose mixture over ethanol formation. Tukey test was applied at 5% significance level and it was observed difference among the treatments with and without the mixture. In fact, the ethanol yield was higher when glucose/ xylose mixture was used compared to glucose alone (Figure 2a). The effect on fermentation was higher in lower glucose concentration (5 to 10 gL^{-1}) (Figure 2b and 2c) as previously observed (Figure 1). Probably, glucose at low concentration (around 5gL^{-1}) assists xylose consumption and in high concentrations (above 20 gL^{-1}), the effect can be opposite. For this reason, a simultaneous optimization of the two responses was considered to be necessary to select the best overall conditions for the fermentation process.

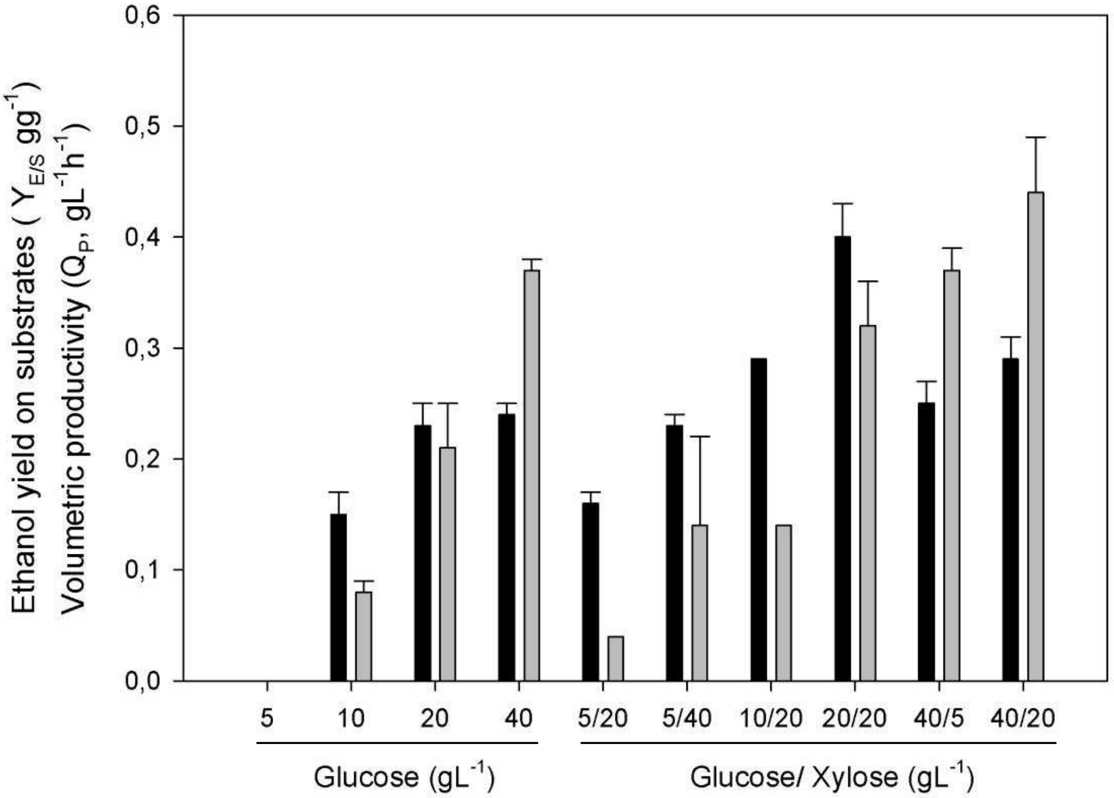


Figure 1 - Fermentation parameters in different concentrations of glucose and mixtures of glucose/xylose. Individual values represent the initial glucose concentration in gL^{-1}. Double values are mixture of glucose/xylose - the first value corresponds to the initial glucose concentration and the value after the bar the initial xylose concentration in gL^{-1}. Black columns are ethanol yield and gray columns are volumetric productivity. In all experiments was used an initial biomass of 0.05 gL^{-1}.

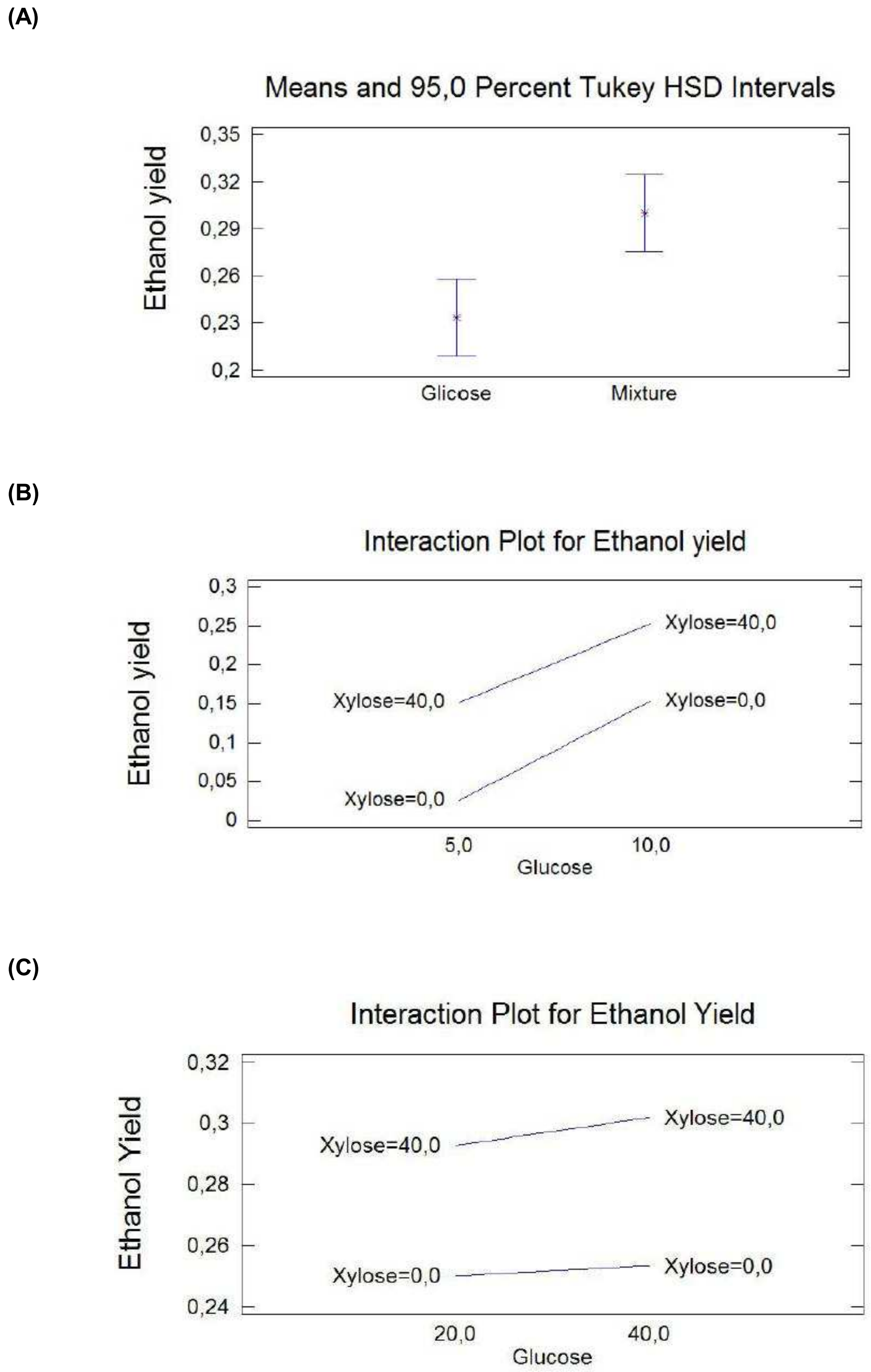


Figure 2 - Ethanol yield in function of sugars mixture. (A) Comparison between treatments with glucose/ xylose mixture versus glucose only; (B) Comparison performed with lower glucose concentration and (C) Comparison performed with higher glucose concentration. Glucose and xylose concentration are expressed as gL^{-1}. Ethanol yield is expressed as g ethanol/ g glucose. Plots were configured by 'STATGRAPHICS plus" software.

Thus, the response surface model was expressed in terms of coded values of both independent variables, and neglecting the statistically insignificant terms. It was analyzed the interaction under low glucose concentrations. The response surface to predict ethanol yield within region under investigation is shown in Figure 3. A plot optimization was conducted by means of design expert software. The equation of the fitted model is:

(**Eq.1**)

$$Y_{E/S} = -\ 0.1475 + 0.0294444{*}Glu + 0.0139167{*}Xyl - 0.000216667{*}Glu{*}Xyl - 0.000222917{*}Xyl^{\wedge}2$$

where the values of the variables are specified in their original units, $Y_{E/S}$ corresponds to ethanol yield on total sugar, Glu and Xyl mean glucose and xylose, respectively.

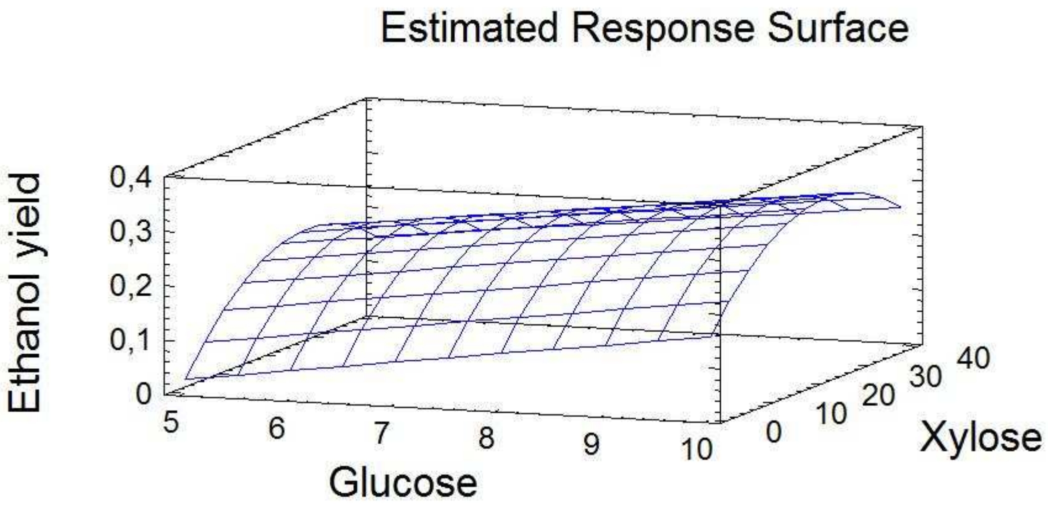


Figure 3 - Response surface of glucose and xylose mixture on ethanol yield. Glucose and xylose concentration are expressed as gL^{-1}. Ethanol yield is expressed as g ethanol/ g glucose. Plot was configured by 'STATGRAPHICS plus" software.

The above equation fitted the experimental data with good determination coefficient (R^2 = 0.9834), demonstrating a good correlation between the independent variables and indicating that the model can explain 98% of total variation. The results of F-test for analysis of variance (data not shown) revealed that this regression is statistically significant (p= 0.009) at 95% confidence level.

From equation 1, it can be observed that both linear terms are positive. The fitted surface increases as both Glu_0 and Xyl_0 increase. Thus, an increase in the initial substrate concentration, in fact, implies a simultaneous increase in ethanol levels. The same was not observed with sugars interaction. Once the interaction term is negative, the fitted surface decrease as both Glu_0 and Xyl_0 increase. This result indicates that the increased of glucose concentration can inhibit the xylose consumption, and consequently decreases the ethanol yield, in the assumed conditions.

Experiments in shake-flasks were performed in order to validate the equation. The experimental value (0.27 gg^{-1}) agreed with the predicted one (0.29 gg^{-1}), indicating the suitability of the model employed and convenience of response surface methodology in optimizing the conditions of ethanol formation from glucose/xylose mixture. From the equation, the optimum combination for $Y_{E/S}$ 0.33 is 10 gL^{-1} glucose with 24 gL^{-1}xylose.

Nothing is known about the glucose effect on xylose utilization in *K. marxianus* UFV-3. The cell growth as well as substrates consumption and products formation were accompanied during the *K. marxianus* UFV-3 cultivation in medium containing glucose and xylose. In medium with 0.5% (w/v) glucose and 2% (w/v) xylose, the maximum ethanol concentration achieved was 1.5 gL^{-1} at the 10th h. After, it had been felt slightly (Figure 4a). The maximum cell mass achieved was 4.46 gL^{-1} at 55 h. Glucose was consumed quickly, and immediately after, the xylose started to be consumed. It has been documented that small amounts of glucose stimulate xylose uptake in naturally xylose-fermenting yeasts as well as in recombinant xylose-utilizing *S. cerevisiae* (Jeffries et al., 1985; Karhumaa et al., 2007; Meinander and Hahn-Hägerdal, 1997). Lee and co-workers (2002), for example, observed that when the extracellular glucose concentration was low [0.05% (w/v)] there was an 85% increase in the maximum xylose transport rate.

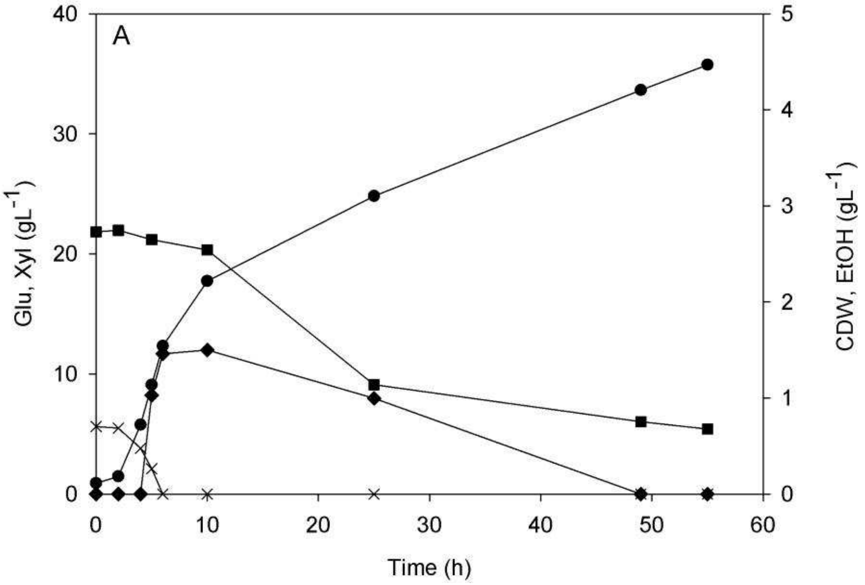
It is also known that under glucose limitation, high-affinity glucose transporters are induced. Some of these transporters, like HxT4p, Hxt5p, Hxt7p and Gal2p, also have been shown to be able to transport pentoses (Liang and Gaber, 1996; Pitkänen et al., 2003). More specifically, Hamacher and co-workers (2002) have reported that HxT4p and Hxt7p have exhibited highest activity for uptaking xylose into the yeast and concluded that efficient xylose utilization is possible by increasing these hexose transporters via supplementation with glucose. However, the stimulus of xylose consumption by glucose presence has a limit. Rodrussamee and co-workers (2011) observed that 20 gL^{-1} glucose inhibits xylose consumption by *K. marxianus* DMKU3-1042.

The time course of *K. marxianus* UFV-3 cultured in 20 gL^{-1} glucose and 20 gL^{-1} xylose was analyzed. The glucose was firstly consumed at 37°C within 8 h (Figure 4b). There was an adaptive period for *K. marxianus* UFV-3 to consummate xylose after glucose had been depleted from the medium. The xylose utilization began at 40 h cultivation, after glucose depletion. Ethanol concentration and biomass increased quickly at the beginning of fermentation. After glucose had been exhausted, there was not ethanol formation and the biomass increased slowly, once *K. marxianus* UFV-3 had a low growth rate in xylose in this condition. Final biomass was 4.42 gL^{-1} at 74 h fermentation. The maximum ethanol concentration, 8.65 gL^{-1}, was achieved at 8 h and felt slowly at the 70th h, even though xylose was still present.

High glucose concentration may have inhibited the xylose transport. Some researchers observed that, in some yeasts, the range of glucose to stimulate xylose consumption is between 0.8 and 2% (w/v) and above 4% glucose, the xylose utilization is retarded due to competitive inhibition of xylose uptake by glucose (Lawford and Rousseau, 1999).

Besides competing for the same carrier, glucose can also repress the genes coding for other carbon sources metabolism. Glucose can repress XR and XDH induction in *S. stipitis* and *P. tannophilus*, for example (Bicho et al., 1988; Bolen and Detroy, 1985). There are many studies about glucose repression in *S. cerevisiae* and *Kluyveromyces lactis*. However, they are mainly related with sucrose, lactose, or galactose utilization (Dong and Dickson, 1997; Gancedo, 1998; Venkat et al., 2010). In *K. marxianus,* it has been reported that some symporters appear to be sensitive to carbon catabolic inactivation (De

Bruijne et al., 1988); nevertheless no detailed report on xylose transport systems nor in gene expression is available.



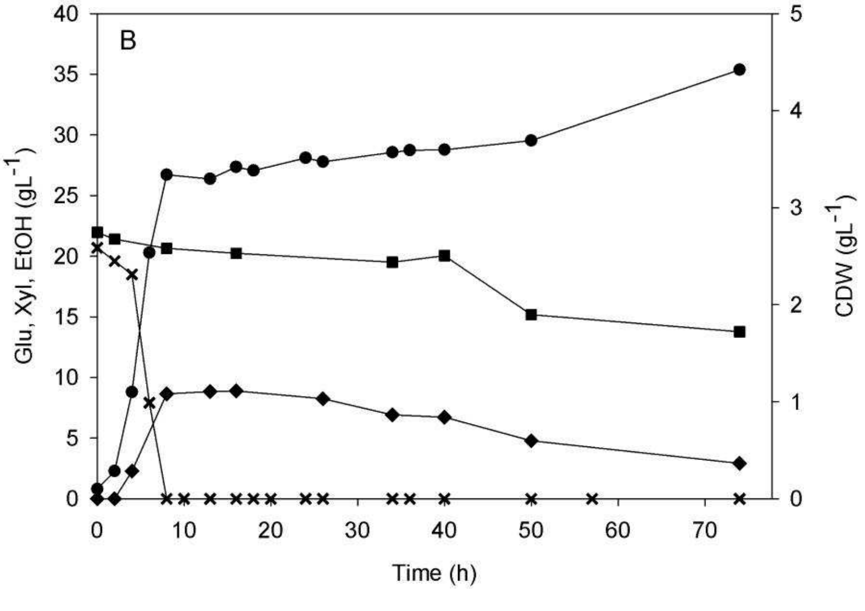
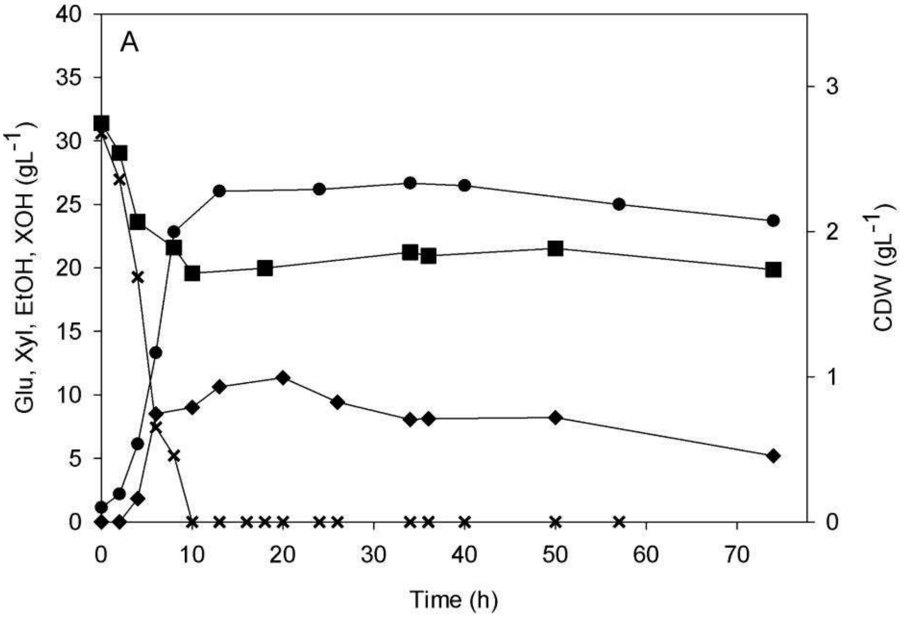


Figure 4 - Growth kinetic and product formation of *K. marxianus* UFV-3 in a mix substrate of glucose and xylose. (A) 5 gL^{-1} Glucose and 20 gL^{-1} Xylose; (B) 20 gL^{-1} Glucose and 20 gL^{-1} Xylose. Symbols: ● Cell Dry weight (CDW); × Glucose; ■ Xylose; ♦ Ethanol.

When cells were cultured only on xylose and Antimycin A, they grew very slowly and did not produce ethanol (Table 1). This result suggested that efficient utilization of xylose requires respiratory activity to sustain cofactor balance. Surprisingly, when cells were cultivated on glucose/ xylose mixture in the presence of Antimycin A, xylose was consumed, and simultaneously with the glucose (Figure 5a). The physiological basis for co-consumption of xylose and glucose when Antimycin A is present is not entirely clear. One possible explanation is that glucose would have allowed xylose consumption by offset the redox balance of the cell. This assumption can be reinforced by the fact that, after glucose has been depleted, xylose could not be further consumed (Figure 5a). The maximum ethanol formation (10.64 gL^{-1}) and the maximum cell mass concentration (2.41 gL^{-1}) were achieved at 13 h. When malonate, another respiratory chain inhibitor, was used (Figure 5b), the maximum ethanol concentration achieved was 9.6 gL^{-1} at 8 h, however the maximum cell mass concentration was 3.54 gL^{-1} in 50 h of fermentation. These results revealed that even in the presence of high glucose concentration (30 gL^{-1}) it is possible the co-utilization of glucose and xylose if the respiratory chain is blocked.



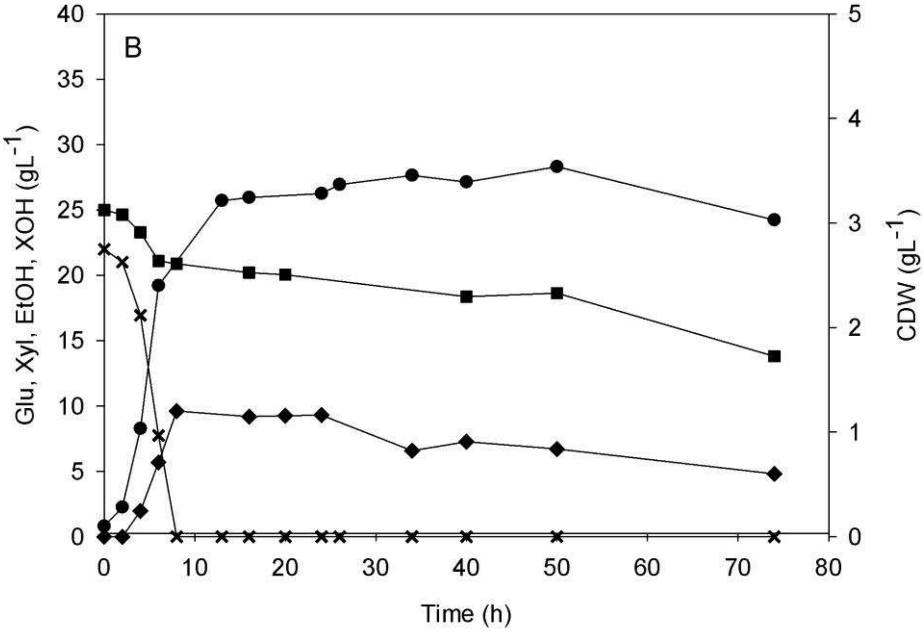


Figure 5 - Growth kinetic and product formation of *K. marxianus* UFV-3 in a mix substrate of glucose and xylose in the presence of inhibitors of the electron transport chain. (A) 30 gL^{-1} Glucose and 30 gL^{-1} Xylose, supplemented with Antimycin A and (B) 25 gL^{-1} Glucose and 25 gL^{-1} Xylose, supplemented with malonate. Symbols: ● Cell Dry weight (CDW); × Glucose; ■ Xylose; ♦ Ethanol.

4 CONCLUSION

The glucose and xylose co-utilization is essential for the process of ethanol production from lignocellulosic biomass. This paper analyzed the conditions that would allow co-utilization of these sugars by *K. marxianus* UFV-3. It was clear that the initial concentration of both sugars is the essential factor. It was found that glucose can repress the xylose consumption, but this effect can be higher or lesser depending on the initial glucose concentrations. The most promising result was observed when the respiratory chain had been blocked someway. In this condition, it was proven that the co-consumption of glucose and xylose it is possible in *K. marxianus* UFV-3. The results obtained in the present work reinforced the potential *K. marxianus* UFV-3 as an alternative ethanol producer. In addition, it defines an optimization equation for ethanol production from mixture of glucose and xylose present in hydrolyzed sugarcane bagasse.

5 ACKNOWLEDGEMENTS

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CAPÍTULO 3

Mutagenic methods associated with pressure selection: a promising strategy to select improved xylose-fermenting yeast strains

ABSTRACT

Bioethanol production from sugarcane bagasse is a demanding technology. However, xylose fermentation, the most abundant pentose in lignocelulosic biomass after glucose, has not been established yet. This study explores evolutionary engineering approaches to improve the xylose fermentation in the presence of glucose by the yeast *Kluyveromyces marxianus* UFV-3. Random mutagenesis methods - Restriction Enzyme Mediated Integration (REMI) and Ultraviolet irradiation (UV) – were assayed. From REMI it was generated some mutants, that had been selected in continuous culture (chemostat) under hypoxic condition in a mixture of glucose/ xylose with glucose-limiting and 0.15 h^{-1} dilution rate. One isolated mutant was able to produce 12% more ethanol although it also produced twice xylitol compared to wild type in 5 gL^{-1} glucose and 10 gL^{-1} xylose. In sequential batches, some mutants were isolated from UV irradiated population. One isolated mutant was able to produce around 0.08 g ethanol/ g xylose and 2-times less xylitol than the wild type strain. The time required for selection decreased from 300 generations to 200 generations in sequential batches. This study has revealed the greater potential of combination of mutagenesis and continuous culture or sequential batches to evolve yeast cells with desired phenotypes in a shorter time.

Keywords: evolutionary engineering, mutagenic methods, bioethanol, xylose fermentation, *Kluyeromyces marxianus*

1 INTRODUCTION

There is an increased interest in producing ethanol from lignocellulosic materials. Different crop residues can be used for ethanol conversion (Lynd et al., 1991; Rabinovich, 2006; Wiselogel et al., 1996). In Brazil, the most relevant agricultural residue for ethanol production is the sugarcane bagasse. Nowadays, the bagasse is burned to generate heat and power for the industry itself or is left in the field. According to some studies, the sugarcane bagasse may contribute with higher amount of energy than the current one. It is estimated that for each ton of cane is possible to get about 140 kg of dry bagasse, and if 90% of fermentable sugars present in the bagasse were converted to ethanol, would increase in 50% the ethanol production (Camargo, 2005; Macedo, 2006).

To make this possible, it is necessary that the lignocelulosic biomass be deconstructed, hydrolyzed and fermented, and that these processes be coordinated for higher ethanol productivities. In this sense, it is also interesting that the two mainly sugars released from the hydrolysis process, i.e. glucose and xylose, be converted to ethanol. Many efforts have been made all around the world to develop this technology and several researchers have focused in the selection of the microorganisms appropriate to consolidate the process.

Nowadays, only the hexoses are fermented to ethanol and many approaches have been attempted to select and improve yeast strains which can also ferment xylose. Among these approaches, the evolutionary engineering stands out (Becker and Boles, 2003; Kuyper et al., 2004; Sonderegger and Sauer, 2003; Wisselink et al., 2007).

The continuous culture has been considered a promising system to pressure the selection of a robust improved strain. A continuous culture, conducted for longer period, can introduce a significant element of evolutionary adaptation by spontaneous mutation (Ferea et al., 1999). In this system a culture grows continuously limited by the low nutrient concentration which is maintained constant in the medium (Novick and Szilard, 1950). Thus, chemostat cultivation selects for cells with an increased affinity, i.e. cells that can achieve a higher specific growth rate at a given suboptimal concentration of the growth-limiting nutrient.

Another system used in evolutionary engineering is sequential batches. Cultivation in batch differs from continuous cultivation in chemostats, once in the first the conditions are variable. Nutrients are available in excess at the beginning and are depleted continuously over time. The cell population goes through various periods of growth - a lag phase, followed by exponential and stationary phase. From the repeated exposure of the cell population to these different conditions, it is possible obtain cells with evolutionary improvements for every phase of cultivation (Sauer, 2001).

Evolutionary engineering using batches and chemostat cultures has been assayed to improve the xylose and arabinose fermentation by genetically engineered *Saccharomyces cerevisiae* (Kuyper et al., 2005; Sonderegger and Sauer, 2003; Wisselink et al., 2009). However, this type of strategy has proved the need of a prolonged cultivation in order to support success, since the desired phenotype is determined by the occurrence of multiple adaptive events that can occur in long-term chemostat or sequential batches. In fact, long-term carbon-limited growth, around 250-550 generations, has been used to select strains with improved affinity for carbon source (Adams et al., 1985; Ferea et al., 1999; Jansen et al., 2005).

In this work, the proposal was obtain yeast with improved mixed-sugar utilization and higher fermentative ability, using evolutionary engineering. In order to reduce the time for selection, initially, it was induced random mutations into the yeast population expecting to generate multiple mutations in each individual genome, and thus providing more diverse starting material for selection. The investigated yeast cell was a natural xylose respiring but not fermenting *Kluyveromyces marxianus*.

2 MATERIAL AND METHODS

2.1 Strains and maintenance

A xylose-respiring but not xylose-fermenting *Kluyveromyces marxianus* UFV-3 was isolated in the Southeast of Brazil and belongs to Culture Collection of the Microbiology Department/ BIOAGRO of the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil). The yeast strain was monthly maintained on YPX plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose, 2% (w/v) agar]. It was activated at 37° C in shake flasks on a synthetic medium YNB 0.67% w/v (Yeast Nitrogen Base, Sigma-Aldrich St. Louis, MO, USA) supplemented with 2% (w/v) xylose and 0.06% (w/v) yeast extract. For storage of cell population generated during selection processes, samples were periodically collected, centrifuged, resuspended in synthetic medium with 2% (w/v) carbon source and 20% (v/v) sterile glycerol and stored at – 80° C for further characterizations. *Escherichia coli* DH5α was used for maintenance and amplification of plasmid DNA. The wild-type and recombinant *E. coli* cells were sub-cultured regularly in Luria-Bertani (LB) medium at 37 °C, supplemented with ampicilin (50 µg ml^{-1}) as appropriate.

2.2 Culture conditions and media

All *K. marxianus* strains (wild type and selected mutants) were pre-cultured in YPX medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose]. YPX agar containing 200 µg mL^{-1} of Geneticin (G418, SIGMA®) was used to select pUG6 transformants (YPXG). Screening plates containing 0.67% (w/v) YNB (without amino acids and with ammonium sulfate), 2% (w/v) carbon source and 2% (w/v) agar were used to isolate auxotrophic mutants. The same medium, supplemented with specific amino acids or base (adenine, uracil, methionine, threonine, isoleucine, histidine, tryptophan, phenylalanine, tyrosine, valine, leucine or arginine), were also used for characterization of auxotrophic mutants. In order to evaluate the growth rate and ethanol yield, shake-flask cultures were performed at 37° C and 180 rpm on YNB containing 2% (w/v) xylose or 2% (w/v) glucose, 0.06% (w/v) yeast extract, with or without Antimycin A (inhibitor of electron transport chain) (SIGMA®). The fermentative ability of the selected strains was also analyzed at low pH (3.4) which was maintained by buffer potassium phosphate. These experiments were performed in YNB medium supplement with 4% (w/v) of xylose.

2.3 Mutagenesis by Restriction Enzyme Mediated Integration (REMI)

The plasmid pUG6 containing the *kanMX* gene from Tn903, that confers resistance to the aminoglycosid antibiotic G418 (Güldener et al., 1996), was used for transformed yeast. The Wizard miniprep DNA purification system (Promega) was used for plasmid DNA preparation. The plasmid was linearized with four different enzymes: *Bgl*II, *Eco*RV, *Xba*I and *Xho*I. For REMI (Restriction Enzyme Mediated Integration) experiments, each restriction enzyme was added to the transformation mixtures as shown in Table 1. For each transformation 3 to 5 µg of plasmid DNA with 50 µg of single-stranded carrier DNA was used per 100 µL of solution in each reaction tube.

Yeast transformation was performed by electroporation as described by Kooistra et al. (2004) with few modifications. A pulse of 1 KV, 25 µF and 400 Ohm was applied and 1 mL of YPX was added immediately at room temperature. The cells were transferred to sterile tube and incubated under shaking at 37° C for 3 hours. The cells were collected by centrifugation, washed with sterile water, plated on selection medium and incubated at 37° C or transferred to chemostat or flask cultures for selection.

Table 1 – DNA form, amount of enzyme and conditions used on transformation mixture for mutagenesis experiments by REMI.

Symbols	DNA used on the transformation mixture and condition
C	Circular pUG6 (experiment control) without enzyme.
C+Enz	Circular pUG6 + 20 U enzyme.
L	Linear pUG6 + Enzyme. Linearization with 20 U enzyme (37° C / 2 h) and addition of cleavage reaction in the transformation mixture without residual enzyme inactivation.
L+Enz	Linear pUG6 + Enzyme. Linearization with 20 U enzyme (37° C / 2 h) and addition of cleavage reaction (without residual enzyme inactivation) plus 20 U enzyme in the transformation mixture.
L+Inb	Linear pUG6. Linearization with 20 U enzyme (37° C / 2 h) and subsequent residual enzyme inactivation.
L+Inb+Enz	Linear pUG6 + Enzyme. Linearization with 20 U enzyme (37° C / 2 h), residual enzyme inactivation and addition of 20 U enzyme in the transformation mixture together with linear DNA.

2.4 Mutagenesis by Ultraviolet irradiation (UV)

Pre-established conditions were adopted for UV mutagenesis (James and Kilbey, 1977). Cells were cultivated until log phase and 2.63 x 10^7 cells/mL was transferred for plastic petri dishes. It was stirred with a magnetic stirrer and exposed to UV light at distance of 13 cm, for different times, in order to obtain a survive curve. The time of 6 min, which correspond to around 10% of survival, was chosen for mutagenesis experiments. For selection, surviving yeast cells were transferred to chemostat or flask culture after exposition to UV. Both chemostat and flask culture with UV irradiated cells were maintained in the dark for 3 days to avoid activity of photoreactivation repair system.

2.5 Selection in continuous culture (chemostat) using a mixture of glucose and xylose

The REMI transformants and the UV irradiated yeast cells were transferred to individual bioreactors. Aerobic chemostat cultivation was performed at successive dilution rate (*D*) of 0.30, 0.42 and 0.48 h^{-1}, at 37 ° C in 0.5 L laboratory fermenters (Wheaton Bioreactor Flask, USA) with working volume of 0.1 L. Minimal medium YNB with different combination of glucose and xylose mixture was used in all selection experiments in chemostat. Initially, a set of conditions with D=0.30 h^{-1}, a mixture of 10 gL^{-1} glucose and 16 gL^{-1} xylose and 5 µM Antimycin A was applied. After the steady state being reached, the feed had been switched to D=0.42 h^{-1} with 10 gL^{-1} glucose with 5 µM Antimycin A, followed by D=0.48 h^{-1} with 10 gL^{-1} glucose and 5 µM Antimycin A in the medium. The working volume of the cultures was kept on 0.1 L by a peristaltic effluent pump coupled. The exact working volume was measured after the experiment. Chemostat cultures were routinely checked for potential bacterial and fungal infection by microscopy. After each steady state, samples were collected and plated. Isolated colonies were storaged for further analyzes.

Another setup for selection on chemostat did include hypoxia in bioreactors (Wheaton Bioreactor Flask, USA) at 37°C with a dilution rate of 0.15 h^{-1}. The working volume of the reactors was 0.1 L. Hypoxia was maintained by sparging nitrogen (White Martins, Praxair Technology Inc). Anti-foam (SIGMA®) was included in the feed at a concentration of 0.5 ml/liter. Fatty acids as 0.42 gL^{-1} Tween 80 (SIGMA®) and 10 mgL^{-1} ergosterol (SIGMA®) were added to the culture. The medium was supplemented with a mixture of 5 gL^{-1} glucose and 10 gL^{-1} xylose as carbon sources. After steady state (~10 generations), samples were storaged for further analyzes.

2.6 Selection in sequential batches

The transformants obtained by REMI and the cell population irradiated by UV were transferred to individual flasks containing YNB with different mixture of glucose and xylose and incubated under shaking at 37° C. After growth, 2.0% of culture was centrifuged and the cells resupended in fresh medium. Four different treatments were tested with three different media. In the first treatment, the cells were transferred to medium containing 5 gL^{-1} glucose and 10 gL^{-1} xylose (M1). After 24 hours, 2.0% of that culture was centrifuged and resupended in a fresh M1 medium. This sequential batch was repeated every 24 hours for 20 times. The second treatment was the same except that the medium used contained 1 gL^{-1} glucose and 10 gL^{-1} xylose (M2). In the third treatment two media were used, the M1 described above and the medium containing just 10 gL^{-1} xylose (M3). In this third treatment, the culture was conducted alternating these media. Initially cells were cultured on medium M1 up to 24 hours, then 2.0% of culture was centrifuged and resupended in fresh M3 medium. After 24 hours, 2.0% of culture was centrifuged again and the cells resupended in fresh M1 medium, starting a new cycle. Finally, the fourth was performed similarly to the third but alternating medium M2 and medium M3.

2.7 Analytical methods

Ethanol, xylitol, xylose, and glucose concentrations were determined by HPLC using an Aminex HPX 87H, 300mm x 7.8 ion exchange column with a 30 x 4.6 mm guard column (Bio-Rad Laboratories Inc., Hercules, CA, USA) on an HP 1050 Series HPLC system equipped with a refractive index detector (HP 1047A). Samples (20 µL) were injected into a heated column (60°C) and eluted at a rate of 0.7 mL/min using 5 mM H_2SO_4 as mobile phase.

The optical density (OD) was determined at 600 nm. Cell dry weight (CDW) was measured by filtering a known volume of the culture through a pre-dried and pre-weighed 0.45-µm pore size nitrocellulose filter. The filters were washed with water, dried for 10 min in a microwave, and weighed again.

2.8 Fermentation parameters in batch culture

The growth rate of the selected yeasts was determined by linear regression of the plot ln OD_{600nm} unit *versus* time (h), at the exponential growth phase on minimal medium YNB containing only one carbon source. Yield parameters were determined according to Silveira et al. (2005).

3 RESULTS AND DISCUSSION

3.1 Mutagenesis of *K. marxianus* UFV-3

The first mutagenesis strategy adopted in this work, in order to promote random mutations in *K. marxianus* UFV-3, was the REMI (Restriction Enzyme Mediated Integration). This method presents many advantages and one of them is the possibility of mutation identification. Because this was the first time that a genome of *K. marxianus* UFV-3 was submitted to REMI, it was necessary to standardize and to characterize the process for that yeast. It was investigated the influence of the physical state of the plasmid and the presence of restriction enzyme on the transformation efficiency. The plasmid used in the assays was the pUG6 (Güldener et al., 1996) which confers resistance to Geneticin (G418) for the transformed cells.

Transformed colonies of *K. marxianus* UFV-3 resistant to G418 were screened up to 5 days of incubation. As expected, the physical state of pUG6 vector and the presence of restriction enzyme in the transformation mixture did influence the transformation efficiency of *K. marxianus* UFV-3 (Table 2).

Integration requires the plasmid linearization. No integrations were found when circular plasmids were transformed in the absence of enzyme. On the other hand, transformation of covalently closed circular plasmids in the presence of a restriction enzyme with a site in the plasmid yields transformation with some efficiency (Table 2). This can be attributed to hydrolysis on both chromosomal and plasmid restriction enzyme sites during transformation events.

In addition to the linear state of the vector, the presence of the restriction enzyme increased transformation efficiency. Few transformants were obtained when linear vector had been used without enzymes. The use of linear vector pUG6 together with additional enzymes (without residual enzyme inactivation) was the best condition for all enzymes used. In any other conditions, the efficiency of transformation depended on the type of enzyme (Table 2).

It has been recognized that restriction enzymes can mediate vector integration by different ways. Restriction enzymes can mediate integrations into their respective genomic sites (Schiestl and Petes, 1991). Alternatively, occasional DNA double-strand breaks could change the conformation of chromosomal DNA favoring integration events. Furthermore, by binding nonspecific, even though weakly to transforming DNA, restriction enzymes could enhance uptake of DNA and indirectly increase transformation and integration efficiencies (Xu and Schildkraut, 1991).

Table 2 – Transformation efficiency of *K. marxianus* UFV-3 using the vector pUG6. The yeast cells were transformed by electoporation and was used the REMI method for mutagenesis.

	Transformation Efficiency (transformants/ µg DNA)					
Digesting Enzyme	**C**	**C+Enz**	**L**	**L+Enz**	**L+Inb**	**L+Inb+Enz**
*Bgl*II	0	0	0	58	0.44	0.44
*Eco*RV	0	1	10.66	142	0	0
*Xba*I	0	3.3	16.66	125	0.33	150
*Xho*I	0	22	1	117	0	22

More details see the text section 2.3 and the Table 1.

In any case, restriction enzyme may cause chromosomal double-strand breaks, cell death, chromosomal aberrations, translocations, and gene mutations (Obe et al., 1992). Some authors suggest that enzymes producing blunt ends are more active in mutagenesis than enzymes producing cohesive ends (Bryant, 1984). Although others affirm that restriction enzymes which produce blunt-ended fragments do not work very well for REMI (Manivasakam and Schiestl, 1998). Experiments with *EcoR*V were performed to evaluate if restriction enzymes producing blunt ends would mediate REMI events in *K. marxianus* UFV-3. Surprisingly, transformants were obtained (Table 2). According to these results, it is possible to use restriction enzymes producing blunt ends and blunt-ended fragments for REMI in *K. marxianus* UFV-3. Considering the interest in inducing detectable mutations in *K. marxianus* UFV-

3 and the fact that enzymes producing blunt ends are more active in mutagenesis, this result with REMI method is promising. However the high efficiency transformation with *Eco*RV was achieved only in one condition analyzed (L+Enz, see Table 1and 2).

For other restriction enzymes used, the efficiency of transformation was higher with *Xba*I and *Xho*I comparing to *Bgl*II. This result suggests that *Bgl*II might possess a low activity to mediate integrations in the conditions analyzed. There are some possible explanations for this including: the enzyme may not enter the cell; even if it does, it may not be active inside the nucleus or it may be degraded upon entry (Manivasakam and Schiestl, 1998).

The transformants obtained with all enzymes were analyzed for auxotrophy. Colonies grown on YPXG plates were replica plated on YNB without amino acid and YPX medium. Colonies that failed to grow on YNB were selected. Eight auxotrophic mutants were obtained, including Ade$^-$, Ura$^-$, Met$^-$, Thr$^-$, Iso$^-$, His$^-$, Phe$^-$, Tyr$^-$, Leu$^-$ and Arg$^-$. Some mutants presented two or more auxotrophic markers (data not shown). Most of auxotrophic mutants were obtained from REMI events with *Eco*RV. These results strengthen the assumption that enzymes producing blunt ends could generate more mutants.

The second strategy adopted to induce random mutations in *K. marxianus* UFV-3 was exposure to UV irradiation. Ultraviolet radiation damages cells in a number of ways. UV raises rate of DNA alterations, including transitions, transversions, as well as small additions and deletions (Hashimoto et al., 2005; James and Kilbey, 1977). For some cases, this type of mutagenesis can be most interesting than mutagenesis by integration of a vector. When the interest is to obtain mutants with higher or lower activity of some enzymes, for example, the UV mutagenesis offers better chances. It can generate punctual alterations on several important DNA regions, such as the gene codifying the target enzyme, DNA regions that control gene expression or genes coding transcription factors resulting in increasing or decreasing its interactions to their targets.

The decision of expose the *K. marxianus* UFV-3 population to UV for 6 minutes was made after results of a UV survival curve had indicated that 90% of the yeast cells were killed between 6 and 7 min (data not shown). Determination of the dose and time of incubation to be applied is a critical step. The UV irradiation can lead to a population with lower genetic diversity if the dose is suboptimal. On the other hand, it can kill the cells if the dose is too high. In addition, a slightly too high dose can create many mutations; many of which unfavorable and that can mask potentially advantageous mutations (Sauer, 2001).

3.2 Selection of improved xylose-fermenting mutants in chemostat

After REMI transformation or UV exposure, selection pressure was applied on potential mutant population. Initially a continuous culture conducted in nutritional limitation (chemostat) was used for selection. Earlier Harder and Kuenen (1976) have considered that selection carried out at low dilution rates (low concentration of the limiting nutrient source) selects microorganisms with a low maximum specific growth rate but also with a high affinity for the substrate. On the other hand, selection carried out at high dilution rates (high concentration of the limiting nutrient source) leads to selection of microorganisms with a higher growth rate and also higher affinity by the substrate. Thus, chemostat cultivation can be employed for the enrichment of strains exhibiting an improved phenotype according the interest. As the aim of the present work to select cells with higher specific growth rate and also higher affinity for the limiting substrates, aerobic chemostat cultivation was performed at ascendant successive dilution rate (*D*): 0.30, 0.42 and 0.48 h^{-1} (see section 2.5). The culture was also conducted in the presence of Antimycin A to block the electron transports chain and to select strain with higher fermentative ability.

Both, UV-irradiated *K. marxianus* UFV-3 (KmUV) and *K. marxianus* UFV-3 REMI transformed strains (KmR), were subjected to chemostat at D=0.30 h^{-1}. KmUV strains were washed-out of the system and only cells of KmR were able to grow. KmR selected cells isolated in each steady state were cultured in batches with 20 gL^{-1} initial carbon source (Table 3). When the KmR mutant cells selected on first chemostat (D=0.30 h^{-1}) were cultured on xylose, it was observed an increase of around 17% in maximum growth rate compared with wild type (KmWT). The same cells cultured on just glucose, exhibited a decrease of 12% in maximum growth rate in this carbon source. The ethanol yield remained the same even on the presence of Antimycin A.

Table 3 – Fermentation parameters of wild-type *K. marxianus* UFV-3 (KmWT) and chemostat selected mutants (KmR). Aaerobic chemostat cultivation was performed at successive dilution rate (*D*) and in each steady state cells were selected and cultivated in batch cultures with 20 gL^{-1} initial carbon source.

Strain	**Maximum Growth Rate (μ, h^{-1})**				**Yield ($Y_{E/S}$) gg^{-1}**			
	Xyl	**Xyl + AntA**	**Glu**	**Glu + AntA**	**Xyl**	**Xyl + AntA**	**Glu**	**Glu + AntA**
KmWT	0.35 ± 0.00	0.05	0.63 ± 0.00	0.53	0	0.01	0.25	0.35
KmR D=0.30h^{-1} [a]	0.41 ± 0.01	0.00	0.56 ± 0.00	0.53	nd	0.02	nd	0.34
KmR D=0.42h^{-1} [b]	0.30 ± 0.00	0.00	0.62 ± 0.00	0.53	nd	0.01	nd	0.33
KmR D=0.48h^{-1} [b]	0.26± 0.00	0.00	0.52± 0.00	0.52	nd	nd	0.18	0.34

(a) Selected cells in aerobic chemostat culture with 10 gL^{-1} glucose and 16 gL^{-1} xilose mixture in the presence of 5 μM Antimycin A and G418; *(b)* Selected cells in aerobic chemostat culture with 10 gL^{-1} glucose in the presence of 5 μM Antimycin A; $Y_{E/S}$: ethanol yield on substrate*;* Xyl: Xylose; Ant A: Antimycin A; Glu: Glucose; n.d.: not determinate. Data are the average ± standard deviation of the mean of the analyses in triplicate.

The condition used for the second round of selection was changed. As growth rate on glucose decreased, it was decided to use just this sugar and Antimycin A on feeding medium in order to obtain more robust strains able to assimilate and ferment this carbon source with higher rate. In steady state at *D*=0.42 h^{-1}, selected cells exhibited lower growth rate on xylose and the same growth rate for glucose, compared to the KmWT strains in batch culture (Table 3).

For the last round of selection, it was maintained the same medium but at the dilution rate *D*=0.48 h^{-1}. Unexpectedly, the selected cells exhibited less grow rates than wild type strain, in all carbon sources when analyzed in batch cultures.

As for the ethanol yields, they were the same for all selected cells, when the Antimycin A was used. In contrast, in absence of this inhibitor, the ethanol yield was lower for the KmR selected in chemostat with higher dilution rate (Table 3). It was reported that prolonged carbon-limited cultivation (around 200-

450 generations) led to a reproducible, strong decrease of the fermentative capacity (Ferea et al., 1999; Jansen et al., 2005; Kovarova-Kovar and Egli, 1998). In these studies, the decrease of the fermentative capacity of selected strain were due to the decrease of glycolytic enzyme levels and to the expression increased of genes encoding the enzymes of the tricarboxylic acid cycle, ATP generation, and oxidative phosphorylation (Ferea et al., 1999; Jansen et al., 2005).

Selection in chemostat was also conducted under hypoxia with a mixture of xylose and glucose without Antimycin A at dilution rate of 0.15 h^{-1}. The *K. marxianus* UFV-3 wild type was subjected to a new round of mutagenesis with REMI and UV, and both surviving populations, KmR and KmUV respectively, were continuously cultured. Again, the UV-irradiated surviving population was completely washed-out. The KmR population, in opposite, was able to grow under hypoxic conditions. Selected culture samples that have been taken from the steady state were stored at – 80° C for 3 weeks.

New chemostats under hypoxic condition were performed with selected KmRhyp strain and KmWT for comparison (Table 4 and 5). Both carbon sources were used, although xylose in lower concentration. Glucose was completely depleted from the medium vessel (Table 4). There were some alterations on specific sugar-consumption (*q*) and product-formation rates (*q*) in KmRhyp culture compared to KmWT (Table 5). While *q*Xylose was the same for both strains, *q*Glucose for mutant KmRhyp was higher than for KmWT. As the cells are subject to glucose limitation in the chemostat, probably those with mutations, which facilitate uptake and metabolism of this carbon, can multiply faster and dominate the system (Dykhuizen and Hartl, 1983).

The ethanol yield was 12% higher for KmRhyp compared with KmWT. This outcome points to increase of fermentation ability of the selected mutant. The ethanol specific formation (*q*Ethanol) was also higher for mutant agreeing with the increase of ethanol yield, corroborating with the suggestion of an increasing fermentative capacity of chemostat selected mutant KmRhyp. Also for this mutant, xylitol specific formation rate was twice compared to wild type. Mutations that induce increase in some enzyme activities such as pyruvate decarboxylase and alcohol dehydrogenase, could explain the increase in ethanol formation. A metabolic flux analysis should further be carried out to look for changes on biochemical routes for glucose/ xylose metabolism by mutant *K. marxianus* UFV-3.

Table 4 - Sugar consumption, ethanol yield on glucose and on total sugar, biomass yield on glucose by wild-type *K. marxinaus* UFV-3 (KmWT) and REMI mutant *K. marxianus* UFV-3 (KmRhyp) in chemostat cultures conducted under hypoxia at a dilution rate of 0.15 h^{-1}.

Initial Sugar in YNB medium (gL^{-1})	Glucose in (mM)	Xylose in (mM)	Xylose out (mM)	Ethanol out (mM)	Biomass Yield on glucose (gg^{-1})	Ethanol Yield on glucose (gg^{-1})	Ethanol Yield on total sugars (gg^{-1})	Ethanol Yield on biomass (gg^{-1})
Km WT 5 glucose + 10 xylose	34.19	85.79	78.78	51.96	0.15	0.39	0.33	2.57
KmRhyp 5 glucose + 10 xylose	30.339	72.52	67.25	53.59	0.14	0.45	0.39	3.03

Table 5 - Specific sugar-consumption and product-formation rates by steady-state hypoxic continuous cultures of wild-type *K. marxinaus* UFV-3 (KmWT) and REMI mutant *K. marxianus* UFV-3 (KmRhyp). Chemostats was performed in minimal media YNB with glucose-limited on glucose/xylose mixtures. The dilution rate of 0.15 h^{-1} was used.

Initial Sugar in YNB medium (gL-1)	*q*Glucose	*q* Xylose	*q* Ethanol	*q* Glycerol	*q* Xylitol
Km WT 5 glucose + 10 xylose	5.51	1.13	8.54	0.38	0.35
KmRhyp 5 glucose + 10 xylose	5.76	1.00	9.94	0.41	0.70

Specific rates (*q*) are expressed as mmol substrate (g biomass)$^{-1}$ h^{-1}.

3.3 Selection of improved xylose-fermenting mutants in sequential batches

Another selection strategy adopted in this work was to subject UV-irradiated and REMI *K. marxianus* UFV-3 mutants to a serial of sequential batches (SB) as described in section 2.6 during 20 days. Along the cultivation, the glucose and xylose consumption and ethanol and xylitol formation were analyzed. When a mixture of 5 gL^{-1} glucose and 10 gL^{-1} xylose (M1) was used, no change was observed for both KmUV and KmR potential mutant population (data not shown). Initial glucose concentration in the medium was reduced to 1 gL^{-1} and xylose concentration was maintained (M2), in order to increase exposure time in xylose and also to reduce repression by glucose. Selected strains exhibited higher xylose consumption and higher cell mass formation, but no change in ethanol formation was observed for either KmUV or KmR strains (data not shown).

It was attempted an additional approach. The culture media (M1 and M2) were alternated with another medium containing just xylose (10 gL^{-1}) as carbon source (M3) (see section 2.6). When the M1-M3 combination was used, no change was observed. However, when the M2-M3 combination was used, cells with an increase on ethanol formation and decrease on xylitol formation were selected among the KmUV strains (Figure 1a). For KmR strains and KmWT (wild type strain was also submitted to sequential batches), no modifications were observed even with alternating medium (data not shown).

The simultaneous presence of the two sugars at different concentrations in the sequential batches strategy can impute selective pressure for rapid subsequent or even partially simultaneous use of the substrates (Wisselink et al., 2009). As the two carbon sources are present at the same time on the medium, mutant cells that can use more efficiently both sugars, can dominate the system and be selected. Indeed, the residual xylose concentration found in the last cycle was about twice lower than that found on the first cycle (Figure 1b).

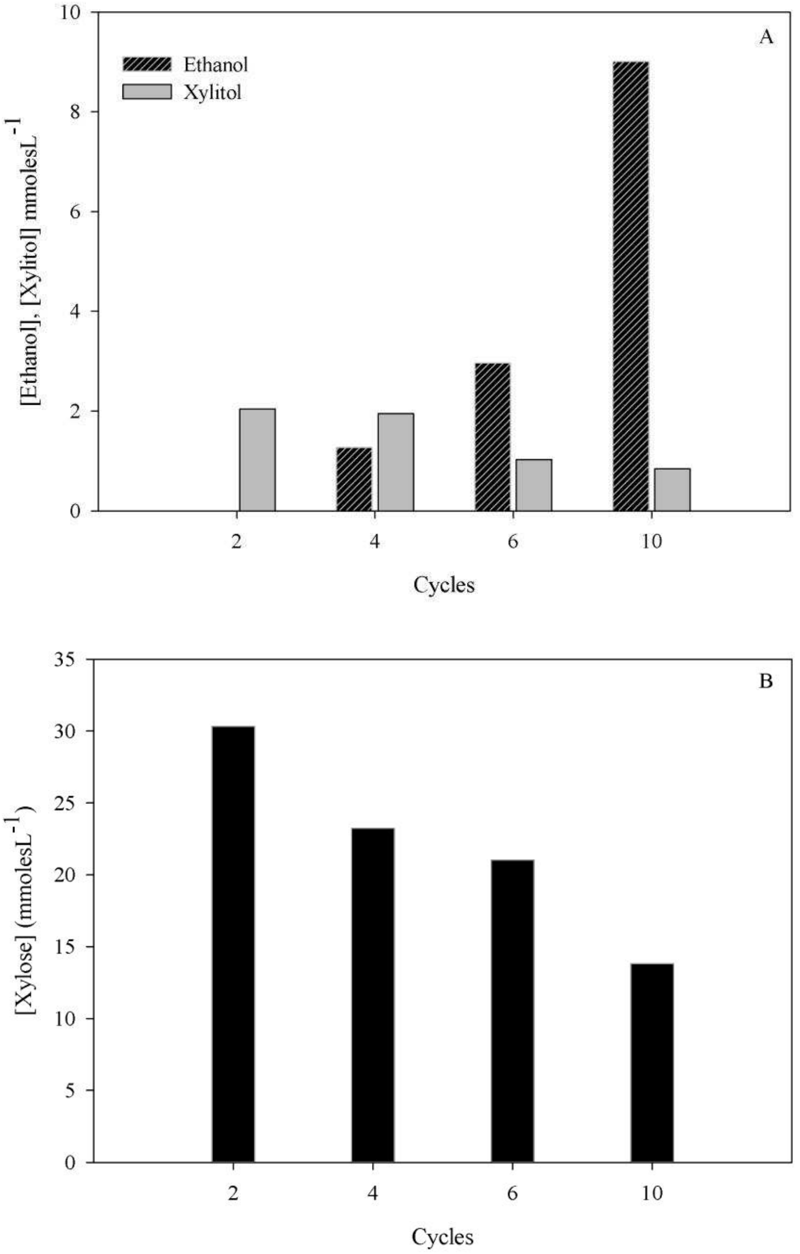

Figure 1 – Selection in sequential batch (SB) cultivation of UV-irradiated *K. marxianus* UFV-3 (KmUVsb). The mutant cells were subjected to sequential batch alternating the medium. Each cycle correspond to two days of cultivation: first day in M2 medium (1 gL^{-1} glucose and 10 gL^{-1} xylose) and second day in M3 medium (10 gL^{-1} xylose). The results in the figure represent the ethanol and xylitol concentration (A) and the xylose residual (B) in the M3 medium.

Selected culture samples taken from the SB were stored at – 80° C for 3 weeks and subsequently pre-cultured in shake flasks. KmUVsb produced more ethanol. The ethanol yield was 3-fold higher while the xylitol was 2-fold lesser in the KmUVsb batch culture compared to KmWTsb and KmRsb cultures in 2% and 4% (w/v) xylose (Figure 2a and b). In addition, KmWTsb and KmUVsb were cultivated in a mixture of 5gL^{-1} glucose and 10 gL^{-1} xylose. KmUVsb produced 0.62 gL^{-1} ethanol against 0.18 gL^{-1} produced by KmWT at 3 h of cultivation.

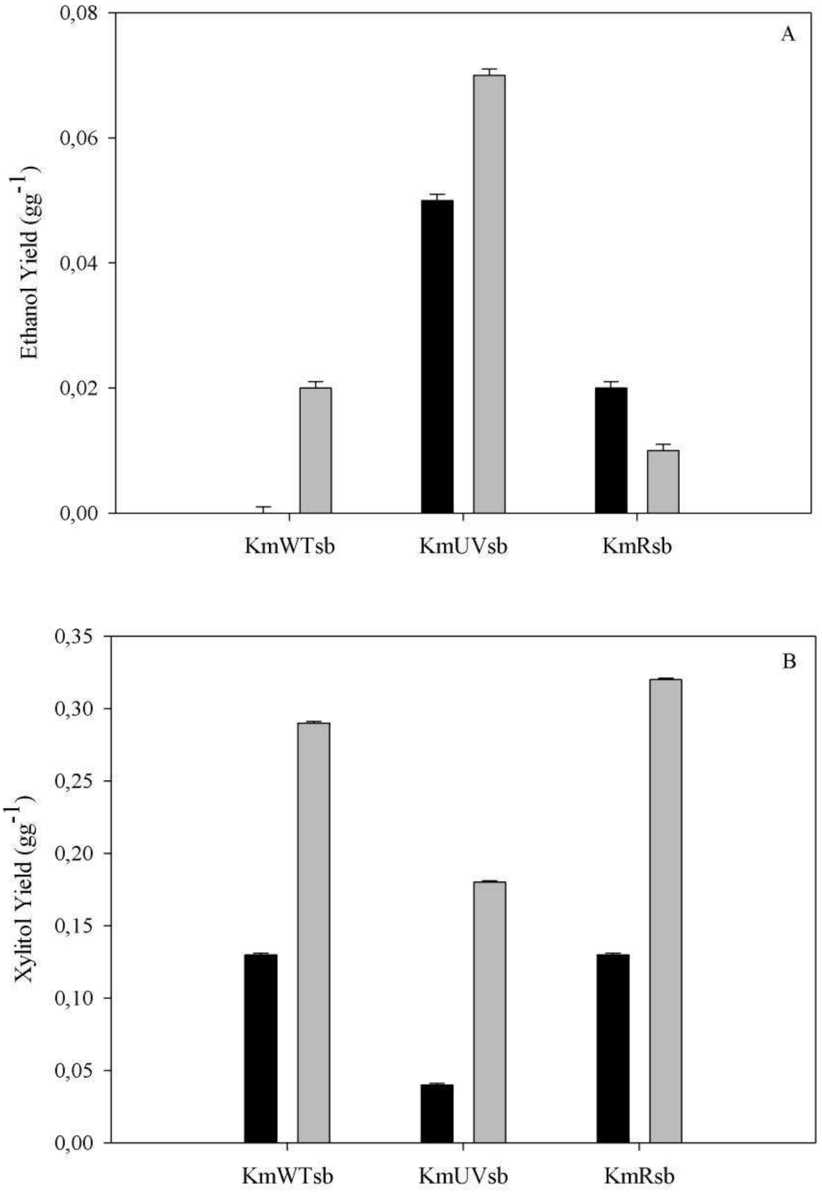

Figure 2 - Ethanol yield (A) and xylitol yield (B) from batch cultivations of wild-type *K. marxianus* UFV-3 (KmWTsb), UV mutant *K. marxianus* UFV-3 (KmUVsb) and REMI mutant *K. marxianus* UFV-3 (KmRsb) strains. These strains were selected and isolated after cultivation in sequential batches. Black bars: 2% (w/v) xylose and Gray bars: 4% (w/v) xylose.

Finally, in order to explore acid tolerance of the mutants obtained, they were cultured in buffer minimal medium at low pH (3.4). Acid tolerant yeast is crucial for bioethanol production from acid hydrolysis of lignocellulosic biomass. Under such pH, all mutants and wild type strain were able to grow and the ethanol yield of KmUVsb was about 7-fold higher than those of KmWTsb and KmRsb (Figure 3). Selection involving growth may reveal mutations affecting plasmatic membrane which may in addition confer tolerance to lower pH. Interesting is that KmUVsb did show changes in its morphology. It was more elongated than original cell (data not shown).

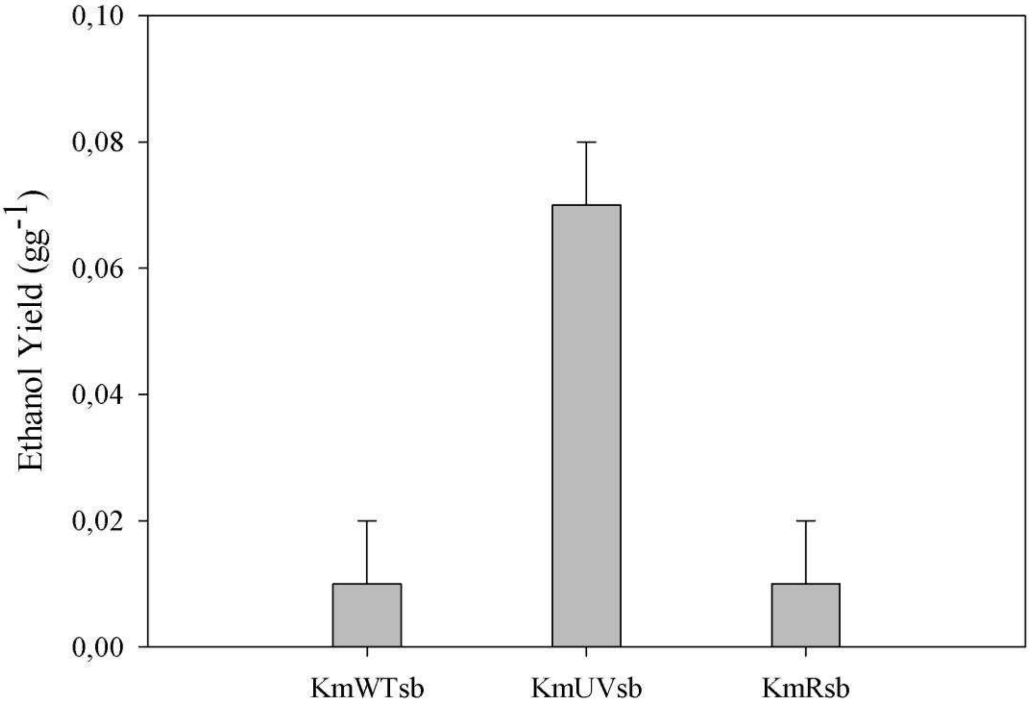

Figure 3 – Ethanol yield of wild-type *K. marxianus* UFV-3 (KmWTsb) and two mutants: *K. marxianus* UFV-3-UV (KmUVsb) and *K. marxianus* UFV-3-REMI (KmRsb) selected by sequential batch (see text for details). The cultures were conducted under low pH (3.4) with initial 4% (w/v) xylose.

From all mutants selected and isolated, a promising one was the KmUVsb for its ability to produce ethanol from xylose with less xylitol. The present study confirms the significant potential of combining mutagenesis with continuous culture as a strategy to evolve specific phenotypes. This strategy allowed the selection of mutant cells that was able to multiply faster under non limiting-carbon source producing more ethanol and less xylitol.

The time for select a mutant with desired phenotype was decreased from around 300 generations to 10 in chemostat, and to 200 generations for sequential batches with mutagenic agent application. Since the yeast populations in sequential batches were maintained for longer time in the selective environment, the contribution of initial mutagenesis to the specific phenotypes of the strains selected could be questioned. In the present work, the cells population that was not submitted to mutagenesis was also cultivated in sequential batches at the same time and significant alterations on phenotype were not observed. Therefore, the mutagenesis was accountable for the improved phenotypes.

4 CONCLUSIVE REMARKS

Ethanol formation from xylose is interesting to increase bioethanol yield from lignocellulosic biomass. In this study, an improved *K. marxianus* UFV-3 strain was isolated from UV-mutagenesis associated with selection in sequential batches. The selected mutant strain produced ethanol from xylose and seems suitable for use on fermentation of carbon mixture source common on hydrolyzed lignocelulosic biomass. For scientific advances, the availability of a mutant yeast strain able to ferment xylose with less xylitol production could be useful for the elucidation of the factors determining this specific phenotype.

5 ACKNOWLEDGEMENTS

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CAPÍTULO 4

Physiological characterization of improved strains of *Kluyveromyces marxianus* able to ferment xylose

ABSTRACT

The yeast *Kluyveromyces marxianus* grow in xylose but do not ferment this pentose. In previous study, *K. marxianus* UFV-3 was subjected to mutagenesis and selected in chemostat under hypoxia and sequential batches using medium with glucose/ xylose mixture. It was evolved an improved strain able to convert xylose to ethanol. However, the extension of effects of this modification on the metabolism to set the basis for further application remains uncertain. In the present work, the improved strain was cultured in xylose-limited chemostats under aerobiosis, hypoxia and anaerobiosis, with dilution rate 0.05 h^{-1}. The specific ethanol production rate was found to be 0.15 mmol/g biomass/ h under hypoxia. The specific xylose-consumption rate (1.03 mmol/g biomass/ h) was similar to wild type (1.06 mmol/g biomass/ h). The results indicate that the improved phenotype is not associated with xylose transport into the yeast. On the other hand, alterations on activities of some enzyme were detected. The ratio of XDH/XR activities was higher for mutant strain as well as the PDC and ADH activities, suggesting that activity modulation of these enzymes is important for efficient ethanol fermentation from xylose by *K. marxianus*. A glucose pulse was applied in a xylose-limited chemostat under aerobiosis and the mutant strain produced 2-fold more ethanol (1.3 gL^{-1}) than wild type strain (0.7 gL^{-1}). Surprisingly, the mutant strain was also able to grow under anaerobiosis in medium containing xylose as the only carbon source.

Keywords: *Kluyveromyces marxianus* UFV-3, xylose metabolism, fermentation, chemostat

1 **INTRODUCTION**

A major challenge in the biofuel industry is to improve yeast metabolism in order to increase ethanol yields from potential diverse substrates. There are many investigations to develop yeast strains that can efficiently ferment the different sugars present in the lignocellulosic biomass, mainly glucose and xylose (Kuyper et al., 2003; Sonderegger and Sauer, 2003; Walfridsson et al., 1997). The yeast *Kluyveromyces marxianus* appear to be particularly promising. It is able to ferment at high temperature, to metabolize a wide variety of lignocelulosic relevant sugars such as xylose, arabinose, and cellobiose and also to grow with a high growth rate. In addition, the glucose repression is not as strong as in *Saccharomyces cerevisiae* (Fonseca et al., 2008; Rodrussamee et al., 2011).

In our laboratory, dairy industry isolated strain of *K. marxianus* designated UFV-3 has a strong fermentative metabolism with ethanol yield close to theoretical (Silveira et al., 2005). Ethanol production from glucose generated from lignocellulosic biomass hydrolysis is not the problem for *K. marxianus* UFV-3. The main challenge is xylose fermentation. Although the *K. marxianus* UFV-3 is able to assimilate xylose, it is not able to convert this sugar to ethanol. Under oxygen-limited conditions, the main secreted metabolite is xylitol. Informations about xylose metabolism in *K.marxianus* strains are scarce (Kumar et al., 2009; Wilkins et al., 2008), and the possible electron and carbon balance allowing conversion of xylose to ethanol is not totally clear.

At the xylose metabolic pathway in yeast, the two first oxido-reductive reactions are catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XDH) which use different coenzymes NADH and NADPH. This can generate redox imbalance, depending on oxygen level. Some yeasts are able to circumvent this situation and produce ethanol from xylose, but the way to solve this imbalance is not completely known. Researchers have correlated this property with the presence of XR enzymes able to use both coenzymes (Jeffries and Shi, 1999; Sonderegger and Sauer, 2003; Walfridsson et al., 1997). However, this is not too simple. Recombinant yeasts for xylose assimilation did not produce ethanol just expressing the genes of those enzymes. In fact, the main metabolite produced by most of them is xylitol (Eliasson et al., 2000; Jin et al., 2004). Probably, there are other ways to circumvent the redox imbalance which are specific for the xylose-fermenting yeasts. Some additional possibilities were raised. The activities ratio of first enzymes has been considered important to avoid the xylitol accumulation (Eliasson et al., 2001). In addition, it has been considered that the xylulokinase activity is also important (Matsushika and Sawayama, 2008). Some reports have shown the importance of the oxidative part of pentose phosphate pathway (Verho et al., 2003).

In a previous work, different improved xylose-fermenting strains *K. marxianus* UFV-3 were selected (Chapter 3). One mutant from REMI mutagenesis was selected in continuous culture (chemostat) under hypoxic condition in a medium containing glucose/ xylose mixture and 0.15 h^{-1} dilution rate. This mutant strain was able to produce 12% more ethanol although it also had produced twice xylitol compared to wild type in a mixture of 5 gL^{-1} glucose and 10 gL^{-1} xylose. Other mutant strain was selected in sequential batches after UV mutagenesis. This mutant was able to form ethanol from xylose and had produced 2-fold less xylitol compared to wild type strain. This last one also revealed to produce 7-fold more ethanol than wild type under low pH (3.4). These potential mutants offer an opportunity for further studies about the xylose fermentation pathway in *K. marxianus.*

Therefore, we propose to investigate the improved xylose-fermenting *K. marxianus* UFV-3 mutants along with its wild type strains concerning their ability to grow on and to produce ethanol from xylose under defined physiological conditions. Chemostat cultivation under aerobic, hypoxic and anaerobic atmosphere, as well as the influence of glucose pulse over cells growing in xylose-limit chemostat under aerobiosis, was evaluated. The information collected can be useful to further improve the xylose fermentation in this already promising strain *K. marxianus* UFV-3.

2 MATERIAL AND METHODS

2.1 Strains and maintenance

Kluyveromyces marxianus UFV-3 was used in this study. This strain was isolated in Brazil and it belongs to the Culture Collection of the Microbiology Department of the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil). In a previous study (Chapter 3) the wild type *K. marxianus* UFV-3 was subjected to UV-irradiation and REMI in order to obtain random mutations. Subsequently, cells were subjected to chemostat and sequential batch selection in order to obtain an improved xylose-fermenting strain. Single isolated cells were recovered by plating culture samples on a solid medium containing xylose followed by re-streaking isolated colonies on the same solid medium. Some mutants were recovered from these selection cultures, among which KmUVsb, KmRsb and KmRhyp were subject of the further studies.

The yeast strains were maintained on YPX plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose, 2% (w/v) agar]. For long term storage, yeast samples were grown at 37° C in shake flasks, centrifuged and re-suspended in synthetic medium [0.67% (w/v) Yeast Nitrogen Base-YNB] supplemented with 2% (w/v) xylose and 20% (v/v) sterile glycerol and stored at – 80° C.

2.2 Shake-flask cultivations

Cells were grown in synthetic medium (SM) as described by Verduyn et al. (1992), containing (gL^{-1}): $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; and trace elements (mgL^{-1}): EDTA, 15, $ZnSO_4$. $7H_2O$, 4.5, $MnCl_2$. $2H_2O$, 0.84; $CoCl_2$ .6 H_2O, 0.3; $CuSO_4$. $5H_2O$, 0.3; Na_2MoO_4. $2H_2O$, 0.4; $CaCl_2$. $2H_2O$, 4.5, $FeSO_4$. $7H_2O$, 3.0, H_3BO_3, 1.0, KI, 0.1. A solution containing vitamins was filter-sterilized and added to the synthetic medium to a final concentration of (mgL^{-1}): d-biotin, 0.05, calcium pantothenate, 1.0, nicotinic acid, 1.0, myo-inositol, 25, thiamine HCl, 1.0; pyridoxine HCl, 1.0, and para-aminobenzoic acid, 0.20.

Xylose solution (autoclaved separately) was added to a final concentration of 20 gL^{-1} and the initial pH was adjusted to 5.0 by 4 M KOH. Shake-flask cultures were performed in 0.5L Erlenmeyer flasks containing 0.1L medium and incubated at 39°C at 100 rpm in an orbital shaker (New Brunswick Scientific, Edison, NJ).

2.3 Continuous culture (chemostat) and glucose pulses

Pre-cultures were prepared by inoculating 0.1 L synthetic medium (as described above) containing 20 gL^{-1} of the appropriate sugar (xylose or glucose) in 0.5 L shake flask with a frozen stock culture. After 2–3 days of incubation at 37° C in an orbital shaker (180 rpm), this culture was used to inoculate bioreactors. Chemostat experiments were performed in a BioFlo III bioreactor (New Brunswick Scientific), as described elsewhere (Basso et al., 2010), using the synthetic medium described above supplemented with 0.2 gL^{-1} anti-foam Emulsion C (Sigma, St. Louis, USA) and the carbon source. Cultures were run under the following conditions: working volume of 1 L, pH 5.0 controlled by automatic addition of 2 M KOH, at 37 °C and agitation set at 700 rpm. After xylose had been exhausted, cultures were switched to continuous mode at a dilution rate (*D*) of 0.05 h^{-1}. For fully aerobic cultures, 7.5 gL^{-1} xylose was used as the carbon source and the airflow was set to 0.5 $L.min^{-1}$, which maintained the dissolved O_2 concentration above 60% of air saturation as indicated by an O_2 electrode. Under hypoxia, xylose concentration was increased to 12.5 gL^{-1} and a mixture of air and nitrogen gas was designed to result in an oxygen molar fraction of 1% in the inflow gas. For anaerobic cultures, airflow was replaced by nitrogen gas (99.995% purity). The medium containing 25 gL^{-1} carbon source (xylose or glucose) was supplemented with anaerobic growth factors ergosterol (10 mgL^{-1}) and Tween 80 (0.42 gL^{-1}) dissolved in ethanol, resulting in ~12 $mmoles.L^{-1}$ ethanol in the medium. To minimize the O_2 diffusion into the system, fermentors were equipped with Norprene tubing (Cole Parmer Instrument Company, Vernon Hills, IL) and viton “O”-rings, and nitrogen gas was sparged into the feeding medium vessel continuously. Cultures were assumed to be in steady-state after at least five residence times had passed since the last changes in the culture conditions. At this moment, the culture dry weight and the specific carbon dioxide formation rate should vary less than 2% over 2 volume changes, in order for the culture to be considered at steady state. The exhaust gas from the bioreactor was cooled in a condenser (2°C) to minimize ethanol evaporation and subsequently dried in silica-filled flasks, before entering the off-gas analyzer, which detected the molar fractions of O_2 and CO_2 (Beckman 755 paramagnetic analyzer, and Fuji infrared detector, respectively). Glucose pulses were performed during aerobic xylose-limited chemostat cultures by injection of a sterile concentrated glucose solution in the bioreactor to result in a final concentration of 50 mmoles.L^{-1} sugar. During pulse experiments, both feeding and removal pumps remained on.

2.4 Enzymatic assays

Samples for *in vitro* enzyme activity assays were taken from chemostats. Approximately 20 mL samples (2.5 gL^{-1} cell mass) were harvested from steady state cultures and immediately centrifuged (5,000 × g at 4 °C for 10 min), after which the pellets were resuspended in 4 mL ice-cold freeze-buffer (10 mM potassium phosphate buffer pH 7.5 supplemented with 2 mM EDTA) and frozen at −20 °C. Prior to analysis, cells were harvested by centrifugation and, after a washing step, resuspended in a cell lysis buffer (100 mM potassium phosphate buffer, 2 mM $MgCl_2$, 1 mM DTT, pH 7.4) and mixed with glass beads (0.5 mm diameter). The suspension was vortexed (5 times: 1 min bursts, 1 min cooling interval, at 4 °C). Cell debris and glass beads were separated by centrifugation (20,000 × g, 5 min, 4 °C). The remaining supernatant (the cell extract) was used for enzyme activity measurements, which was carried out using a Spectrophotometer (Beckman DU serial 600) at 340 nm and 37 °C. *In vitro* activities of Xylose Reductase (XR) with NADPH and NADH, Xylitol Dehydrogenase (XDH) with NADH were determined as previously described (Ikeuchi et al., 2000; Yokoyama et al., 1995). Pyruvate decarboxylase (PDC) activity was determined as described in Hoppner and Doelle (1982). Alcohol dehydrogenase (ADH) activity was measured according to Blandino et al.

(1997). Protein concentrations were measured according to Bradford (1976), with bovine serum albumin as a standard. One unit (U) of enzyme activity was defined as 1.0 µmol reduced or oxidized NAD(P)H per minute under the assay conditions. Specific activities are expressed as U mg protein^{-1}.

2.5 Analytical methods

Culture supernatants obtained by centrifugation were used for sugar (glucose and xylose), organic acids, ethanol, glycerol and xylitol determination. In the specific case of residual sugar concentration analysis, rapid sampling with cold-steel beads was employed, as described by Mashego et al. (2003). Compounds were separated by HPLC using a HPX-87H ion exchange column (BioRad, USA) at 60°C, with 5 mM H_2SO_4 as the mobile phase at 0.6 mL min^{-1}. Glucose, xylose, xylitol, ethanol, glycerol, succinate and lactate were detected by a Waters 2414 refractive index detector; pyruvate and acetate were detected by a Waters 2487 UV detector at 214 nm. Biomass concentration was determined indirectly by absorbance measurements at 600 nm (Thermo Scientific Genesys 20, West Palm Beach, FL) and/ or directly by dry weight measurements, which was performed according to Olsson et al. (1997).

2.6 Maximum specific growth rate and fermentation parameters in batch cultures

The maximum specific growth rate was determined by linear regression of the plot ln Abs_{600nm} *versus* time (h), using points in the exponential growth phase on minimal medium containing only one carbon source. The fermentative parameters were determined as described in Silveira et al. (2005) at 24 hours fermentation.

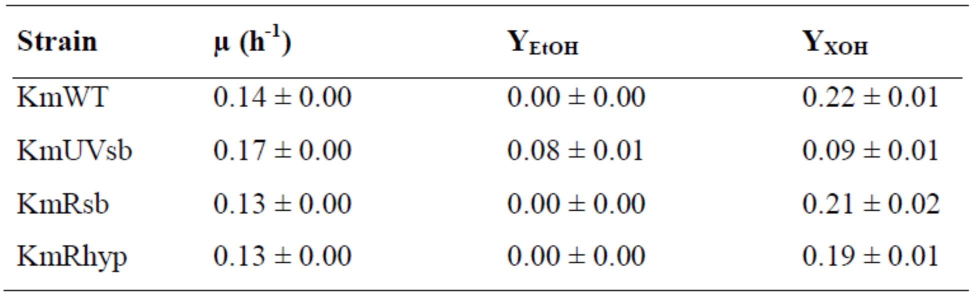
3 RESULTS

3.1 Growth and extracellular product formation during batch cultivations

In a previous work, three mutants were obtained using different mutagenesis and selection strategies: KmRhyp, KmRsb and KmUVsb. KmRhyp was obtained using a combination of REMI and subsequent selection in chemostat under hypoxic condition; KmRsb was also obtained using REMI method but the selection was in sequential batches; KmUVsb was obtained using a combination of UV irradiation and selection in sequential batches. These strains were further studied in the present work.

Growth kinetics of the mutant strains and their corresponding wild type in defined mineral medium with 20 gL^{-1} initial xylose were compared. It was the first time that *K. marxianus* UFV-3 was cultured in a completely defined medium, without any yeast extract supplementation and with xylose as the only carbon and energy source. The maximum specific growth rate to KmUVsb was higher (0.17 ± 0.00 h^{-1}) than to Km WT (0.14 ± 0.00 h^{-1}) (Table 1).

Table 1 - Maximum specific growth rate and ethanol and xylitol yields on xylose from *K. marxianus* UFV-3 wild type (KmWT) and the analyzed mutants (KmUVsb, KmRsb and KmRhyp) during growth in minimal medium containing 20 gL^{-1} initial xylose in shake-flasks.



Strain	**μ (h^{-1})**	**Y_{EtOH}**	**Y_{XOH}**
KmWT	0.14 ± 0.00	0.00 ± 0.00	0.22 ± 0.01
KmUVsb	0.17 ± 0.00	0.08 ± 0.01	0.09 ± 0.01
KmRsb	0.13 ± 0.00	0.00 ± 0.00	0.21 ± 0.02
KmRhyp	0.13 ± 0.00	0.00 ± 0.00	0.19 ± 0.01

μ = maximum specific growth rate; Y_{EtOH} = ethanol yield on xylose*;* Y_{XOH} = xylitol yield on xylose. Yields (Y) are expressed as g g^{-1}. Data refer to 24h of fermentation.

This growth was associated with higher xylose consumption by KmUVsb compared to the other mutants (Figure 1a). Additionally, cell dry weight (CDW) at the end of the cultivation was higher in this mutant when compared to the wild type in this condition (Figure 1b).

The xylitol yield was 2-fold lower in KmUVsb compared to KmWT, whereas the other mutants, KmRhyp and KmRsb, presented a xylitol yield close to that achieved by the original strain (Table 1). The only strain able to produce ethanol from xylose was the KmUVsb with a yield of 0.08 ± 0.01 (g g^{-1}). Detectable amounts of ethanol were not observed in the culture of other strains and also in the first eight hours of KmUVsb cultivation (Figure 1b). Ethanol titers were maximal at 48h cultivation by strain KmUVsb (1.05 gL^{-1}). Curiously, acetate, which was found at 30h with a concentration of 0.37 gL^{-1} in the supernatant of the KmUVsb culture (data not shown), was not found in other strains cultures. This clearly indicates that the xylose metabolism in this strain was modified, concerning to that of the parental wild-type UFV-3 strain. KmUVsb strain presented an increased metabolic flux towards ethanol formation and a decreased flux towards xylitol accumulation, and therefore, it was chosen for additional studies.

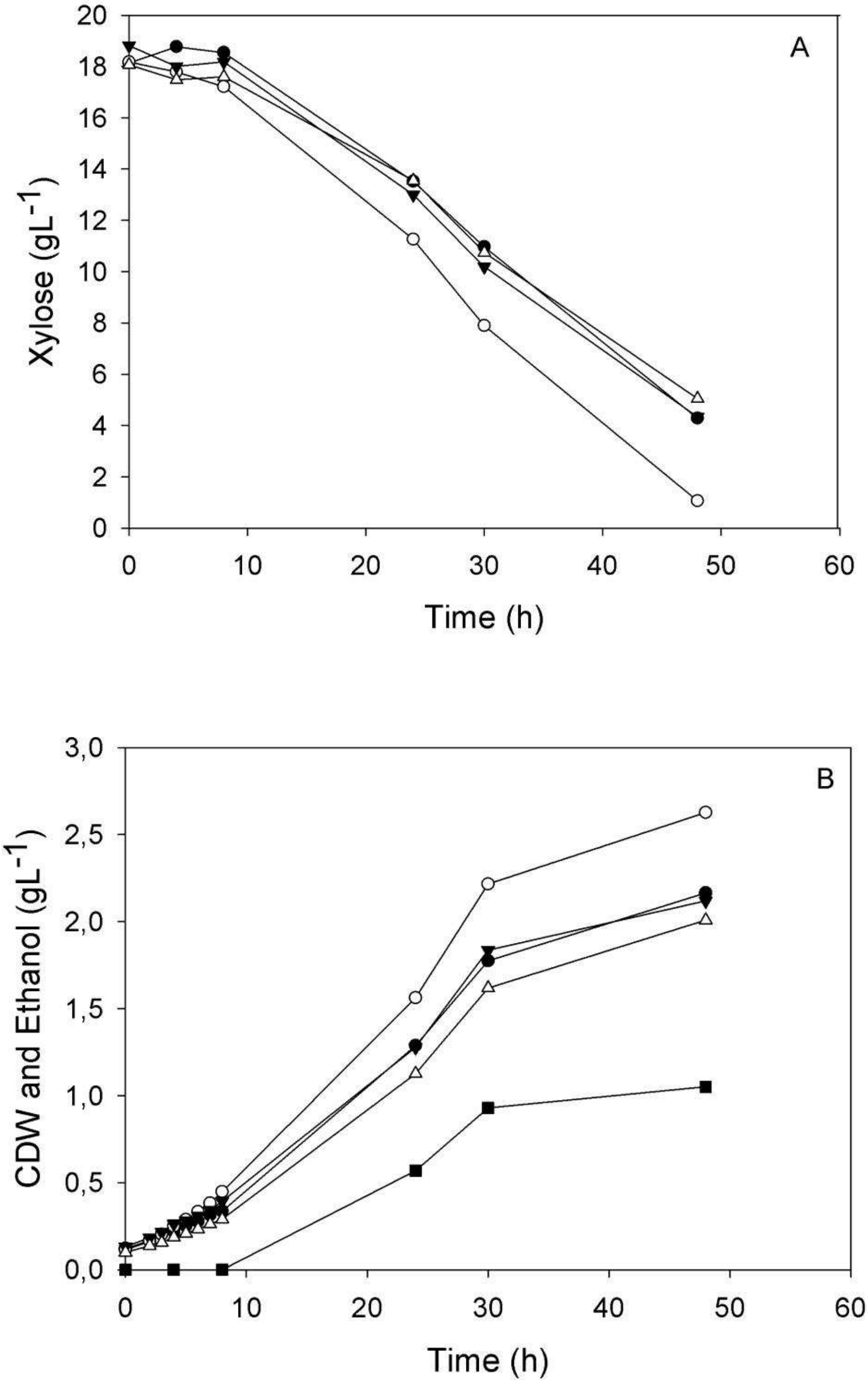


Figure 1 – Shake-flask cultures of wild type and mutant strains of *K. marxianus* UFV-3 on 20gL^{-1} initial xylose. The profiles of xylose consumption (A) as well as cell dry weight (CDW) and ethanol formation (B) are presented. KmWT (black circles), KmUVsb (white circles), KmRsb (black triangles), KmRhyp (white triangles), ethanol production by KmUVsb (black square).

3.2 Physiology of the *K. marxianus* UFV-3 – wild type and KmUVsb mutant – during chemostat cultivations under xylose limitation and different oxygen availabilities

Continuous cultures (*D*=0.05 h^{-1}) of KmUVsb on xylose limitation under aerobiosis and hypoxia were set up. A control culture with KmWT was conducted in parallel for comparison. Specific rates of substrate consumption, extracellular metabolite production and carbon recovery are presented in Table 2. For all steady states achieved, carbon recoveries were close to 100%. The concentrations (mmolesL^{-1}) of xylose and ethanol at steady-state, as well as the biomass and ethanol yields obtained during the aerobic and the hypoxic steady states are presented in Table 3.

Table 2 – Specific substrate-consumption and product-formation rates during steady states of xylose-limited aerobic and hypoxic chemostat cultures of *K. marxianus* UFV-3 strains (KmWT and KmUVsb).

	qXylose	qEthanol	qAcetate	qXylitol	qCO_2	Carbon recovery %
KmWT						
aerobiosis	0.95 ± 0.01	0.00	0.00	0.00	2.46 ± 0.04	104.17 ± 0.68
hypoxia	1.06	0.00	0.00	0.09	2.39	96.76
KmUVsb						
aerobiosis	0.86 ± 0.04	0.00	0.00	0.00	2.33 ± 0.01	105.29 ± 1.58
hypoxia	1.03	0.15	0.08	0.05	2.63	104.10

Aerobiosis data are the average of two independent steady-state cultures. Hypoxia data correspond to one experiment. Specific rates (*q*) are expressed as mmol (g biomass)$^{-1}$ h^{-1}.

The culture of both KmWT and KmUVsb were started fully aerobically on 8.02 gL^{-1} and 7.89 gL^{-1} xylose, respectively, at a dilution rate of 0.05 h^{-1}. A steady state was reached at a cell density of 2.93 gL^{-1} CDW for KmWT and 2.96 gL^{-1} CDW for KmUVsb. Subsequently, without interrupting the feed, conditions were switched to hypoxia by injecting a mixture of air and nitrogen, in such a way to provide the inlet gas with ~1% molar fraction of oxygen.

Additionally, the feeding medium was changed and the new xylose concentration was increased to 12.5 gL^{-1}, since a lower biomass yield on substrate was expected under hypoxia, when compared to full aerobiosis. Steady-state at hypoxia was reached at a cell density of 2.50 gL^{-1} CDW for KmWT and 2.82 gL^{-1} CDW for KmUVsb.

Table 3 – Residual sugar concentration, ethanol formation, biomass yield on xylose and ethanol yield per biomass of *K. marxianus* UFV-3 (KmWT and KmUVsb) grown in aerobic or hypoxic xylose-limited continuous culture (*D*=0.05 h^{-1}).

	Xylose in ($mmolesL^{-1}$)	**Xylose out ($mmolesL^{-1}$)**	**Ethanol out ($mmolesL^{-1}$)**	**Biomass yield on Xylose (gg^{-1})**	**Ethanol yield per biomass**
KmWT					
aerobiosis	53.09 ± 0.05	1.17 ± 0.01	0.00	0.37 ± 0.00	0.00
hypoxia	91.59	40.57	0.00	0.33	0.00
KmUVsb					
aerobiosis	52.57 ± 0.03	2.80 ± 0.01	0.00	0.41 ± 0.02	0.00
hypoxia	88.75	32.47	5.43	0.35	0.13

Aerobiosis data are the average of two independent steady-state cultures. Hypoxia data correspond to one experiment.

Under aerobiosis, the specific substrate consumption rate (*q*Xylose) was higher in the wild type strain culture (Table 2), but the biomass yield was the same for both strains (Table 3). Thus, KmUVsb did use the xylose more efficiently than KmWT, under aerobiosis. In fact, the mutant strain was able to achieve a similar biomass yield as that achieved by wild type strain, using less sugar. In addition, the residual xylose concentration in the wild type culture was around 2-fold lower than in mutant culture (Table 3).

Under hypoxia, there was more residual xylose in the wild type (40.57 $mmolesL^{-1}$) than in the mutant culture (32.47 $mmolesL^{-1}$) (Table 3). Although there was a difference on residual xylose concentration over the wild type, the *q*Xylose was the same in both cultures under hypoxia, and it was higher under aerobiosis. When conditions changed from aerobiosis to hypoxia, *q*Xylose increased 10% for KmWT and 16.5% for KmUVsb. Furthermore, the biomass yield per xylose decreased under hypoxia for both strains as expected. This result indicates that the efficiency of carbon conversion to biomass was lower than under aerobiosis, i.e. less C-xylose was incorporated into biomass and more carbon was released as fermentation products or in CO_2 form (Nobre et al., 2002). In fact, under oxygen-limited conditions, lesser ATP amount is produced per mol of consumed substrate and also lesser biomass is formed.

Under aerobiosis, the KmWT specific oxygen utilization rate was 2.46 mmol/h g on xylose and for KmUVsb it was 2.58 mmol/h g. The respective carbon dioxide evolution rates were 2.46 and 2.33 mmol/h g. Therefore, the respiratory quotients (RQ) were fairly close to unity under aerobiosis (1.00 and 0.90, respectively).

Under hypoxia, the specific carbon dioxide evolution rates were 2.39 and 2.63 mmol/h g for KmWT and KmUVsb, respectively. There was an increase on qCO_2 at the KmUVsb culture when compared with the aerobic condition (Table 2), probably as a result of respiro-fermentative growth observed under this condition.

Both strains did not produce xylitol under aerobiosis, but produced under hypoxia (Table 2). This result was expected since oxygen-limiting conditions favor xylitol formation (Roseiro et al., 1991). The specific accumulation rate of xylitol was about 2-fold higher to KmWT than to KmUVsb. Acetate was produced in vestigial amounts but only by KmUVsb, under hypoxia. The same was observed for ethanol, i.e. only KmUVsb under hypoxia was able to form ethanol. The KmUVsb strain produced 0.04 g ethanol/ g xylose, which is about 9% of the maximal theoretical yield (0.51 g g^{-1}).

3.3 Effect of xylose and oxygen availability on enzymatic activities in cell extracts of *K. marxianus* during xylose-limited chemostat cultivations

Activities of relevant enzymes were measured in cell-free extracts of steady-state culture samples from xylose-limited chemostats under different oxygen availabilities. The specific enzyme activities for both control and mutant are summarized in Table 4.

Table 4 – Enzyme activities (U.mg^{-1}) of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), xylose reductase (XR) and xylitol dehydrogenase (XDH) of *K. marxianus* UFV-3 wild type (KmWT) and *K. marxianus* UFV-3 mutant (KmUVsb) in cell extracts during steady state of xylose-limited chemostat cultures under aerobiosis and hypoxia.

	XR	**XDH**	**PDC**	**ADH**
KmWT				
Aerobiosis	0.30 ± 0.01	0.47 ± 0.01	2.33 ± 0.29	11.15 ± 0.19
Hypoxia	nd	0.20 ± 0.00	2.82 ± 0.43	6.86 ± 0.48
KmUVsb				
Aerobiosis	0.13 ± 0.02	0.33 ± 0.05	6.38 ± 0.58	23.22 ± 0.27
Hypoxia	nd	nd	5.86 ± 0.83	11.79 ± 0.00

n.d.: not determinate.

Under aerobiosis, the XR activity was around 2-fold higher in the KmWT. As for XR, XDH activity was also higher in the control strain. However, the XDH/XR ratio was higher for KmUVsb. Although the reason for this remains unclear, higher ratio of XDH/XR is desirable for xylose fermentation. As pointed out previously (Matsushika et al., 2009) the reaction catalyzed by XDH becomes the limiting step in fermentation pathway from xylitol to ethanol if the XDH/XR ratio is very low.

The fermentative enzymes (PDC and ADH) activities were higher for KmUVsb under aerobiosis as well as under hypoxia. This result may assist to explain the production of ethanol in the mutant strain. Mutations could have occurred on the genes encoding PDC and ADH resulting in higher activity of these enzymes in the KmUVsb favoring ethanol formation from xylose.

3.4 Glucose pulse over aerobic xylose-limited continuous culture

Even under full aerobiosis, some yeasts instantaneously produce ethanol when expose to excess of glucose, a phenomenon known as the 'short-term Crabtree effect' (Rieger et al., 1983; van Urk et al., 1990). To check how the mutant strain reacts to glucose excess under aerobiosis, around 50 mmoles L^{-1} glucose pulse was added directly to steady-state xylose-limited chemostat cultures. Similarly as the control strain (KmWT), the mutant KmUVsb did not exhibit instantaneous ethanol production after the glucose pulse (Figure 2) had confirmed the Crabtree-negative character of *K. marxianus* UFV-3.

Ethanol production was delayed about 60 min but it was around 2-fold higher in mutant culture compared to KmWT culture. Distinctively, the maximum Abs_{600nm} achieved was higher to the original strain. Thus, the mutant cells showed higher fermentative capacity. For both strains, the biomass returned for the initial concentration after had achieved the new steady state under aerobiosis.

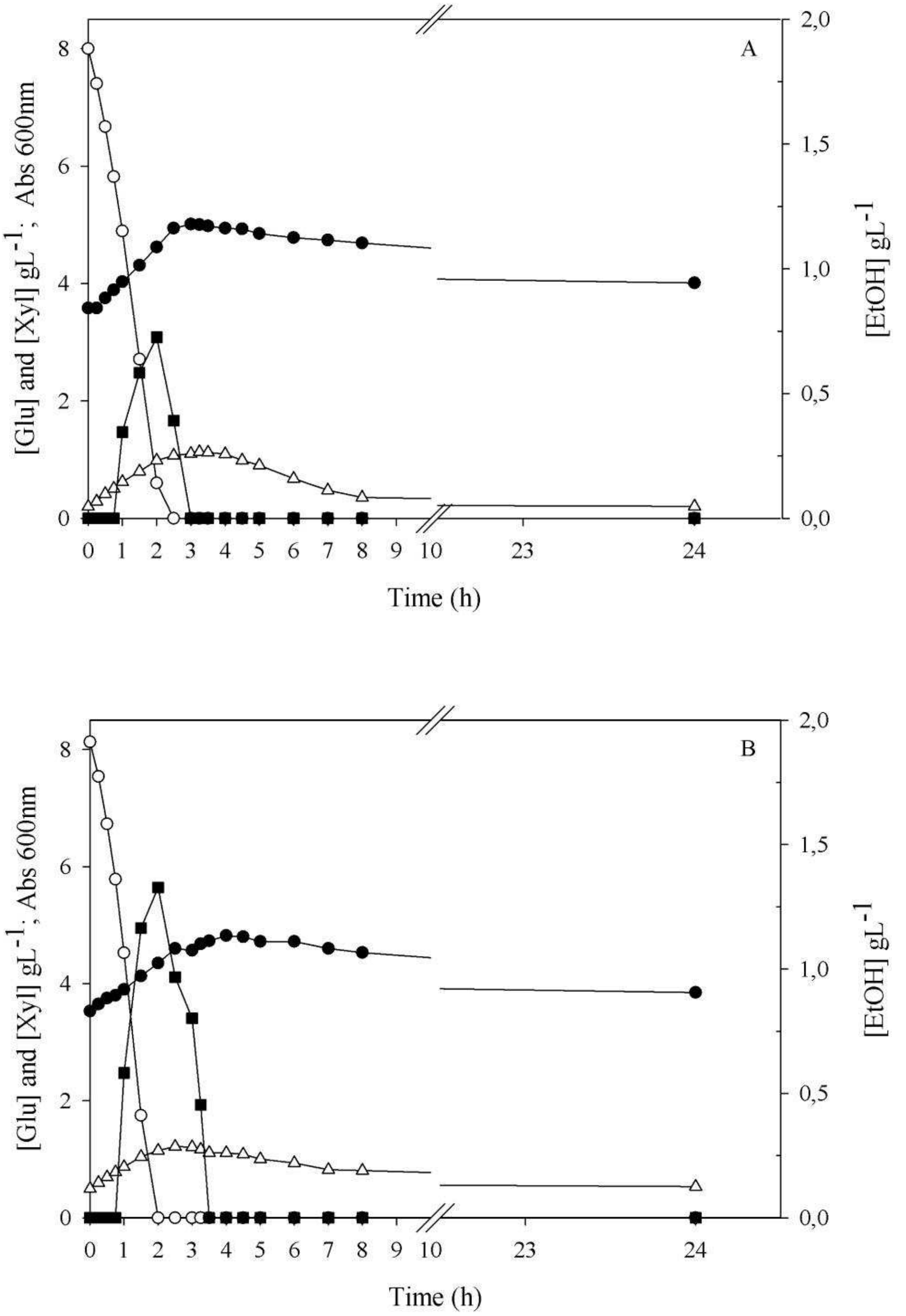
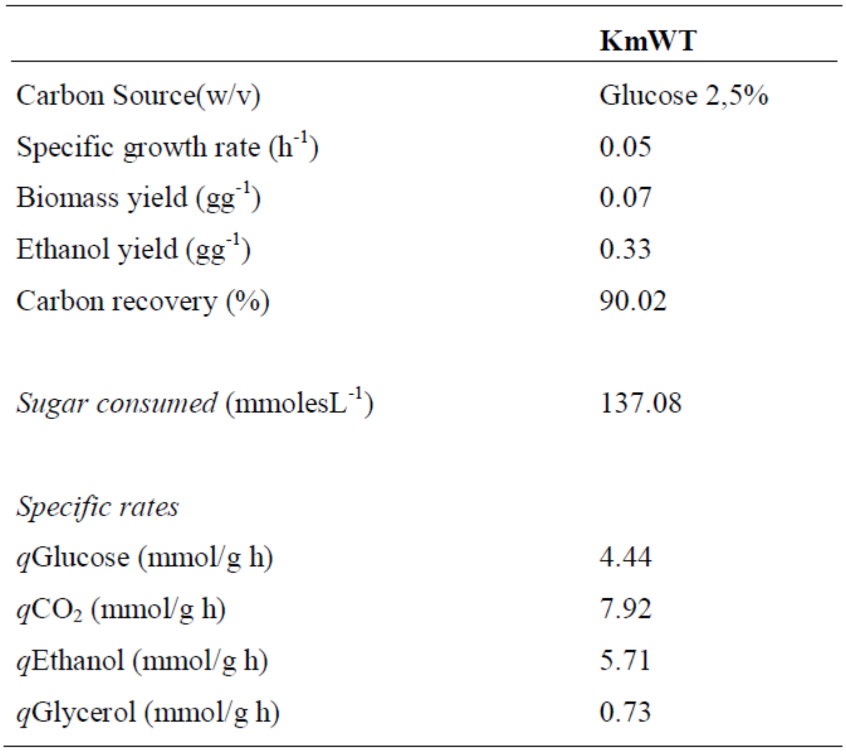


Figure 2 - Glucose pulse over *K. marxianus* UFV-3 wild type (A) and mutant KmUVsb (B) cultures. For both strains, glucose was added to aerobic xylose-limited continuous culture (D=0.05 h^{-1}) in steady state at 0 time. Data for glucose (open circles), xylose (open triangles), ethanol (filled squares) concentrations, and Abs 600nm (filled circles) during a glucose pulse experiment are plotted against elapsed time after glucose injection.

3.5 Physiology of *K. marxianus* UFV-3 during fully anaerobic glucose-limited and xylose-limited chemostat cultivations

Although it has been reported that *Kluyveromyces* yeasts do not grow in the absence of oxygen (Snoek and Steensma, 2006), anaerobic chemostat cultures were also performed in the present study. *K. marxianus* UFV-3 was cultivated under full anaerobic conditions in a glucose-limited chemostat. This wild type strain was able to grow under strict anaerobiosis, as shown in Table 5, and the culture achieved steady-state. In this condition, KmWT produced 8 g ethanol/L, which is around 60% of the theoretical maximal concentration of ethanol produced from 25 g glucose/L, assuming an ethanol yield of 0.51 g g^{-1}.

Table 5 – Growth parameters, sugar consumption and product formation by *K. marxianus* UFV-3 wild-type during steady state of an anaerobic glucose-limited continuous culture.



	KmWT
Carbon Source(w/v)	Glucose 2,5%
Specific growth rate (h^{-1})	0.05
Biomass yield (gg^{-1})	0.07
Ethanol yield (gg^{-1})	0.33
Carbon recovery (%)	90.02
Sugar consumed (mmolesL^{-1})	137.08
Specific rates	
*q*Glucose (mmol/g h)	4.44
qCO_2 (mmol/g h)	7.92
*q*Ethanol (mmol/g h)	5.71
*q*Glycerol (mmol/g h)	0.73

The wild type and the mutant strains were also cultivated in fully anaerobic xylose-limited chemostats. Without stopping the feed at the steady state under hypoxia (as described above), pure nitrogen gas was injected into the reactor and the condition was switched to anaerobiosis. Although the KmWT cultivation under anaerobiosis was performed just until 20h, it is possible to observe that the experimental washed out curve of this strain follow the theoretical curve (Figure 3a). Therefore, it was assumed that the *K. marxianus* UFV-3 wild type is not able to grow in xylose-limited chemostat cultivations under strict anaerobiosis. On the other hand, the mutant strain (KmUVsb) was not washed out of the system (Figure 3b). The biomass stabilized at 40h of cultivation in 1.27 gL^{-1}.

For KmWT, it was observed lesser amount of xylitol (0.45 gL^{-1}) after 10h under anaerobic xylose-limited chemostat than under hypoxia (0.7 gL^{-1}). This result confirmed the wash out system observed. In contrast, for KmUVsb, it was detected about 2-fold higher xylitol (0.75 gL^{-1}) under anaerobiosis than under hypoxic condition (0.40 gL^{-1}). Acetate and ethanol were not detected in the KmWT culture. On the other hand, 0.3 gL^{-1} of both were detected in the KmUVsb culture, after 10h under anaerobic cultivation. This value is very similar to the one found for this strain at the steady state under hypoxic condition. Thus, the KmUVsb was able to ferment xylose under anaerobic condition.

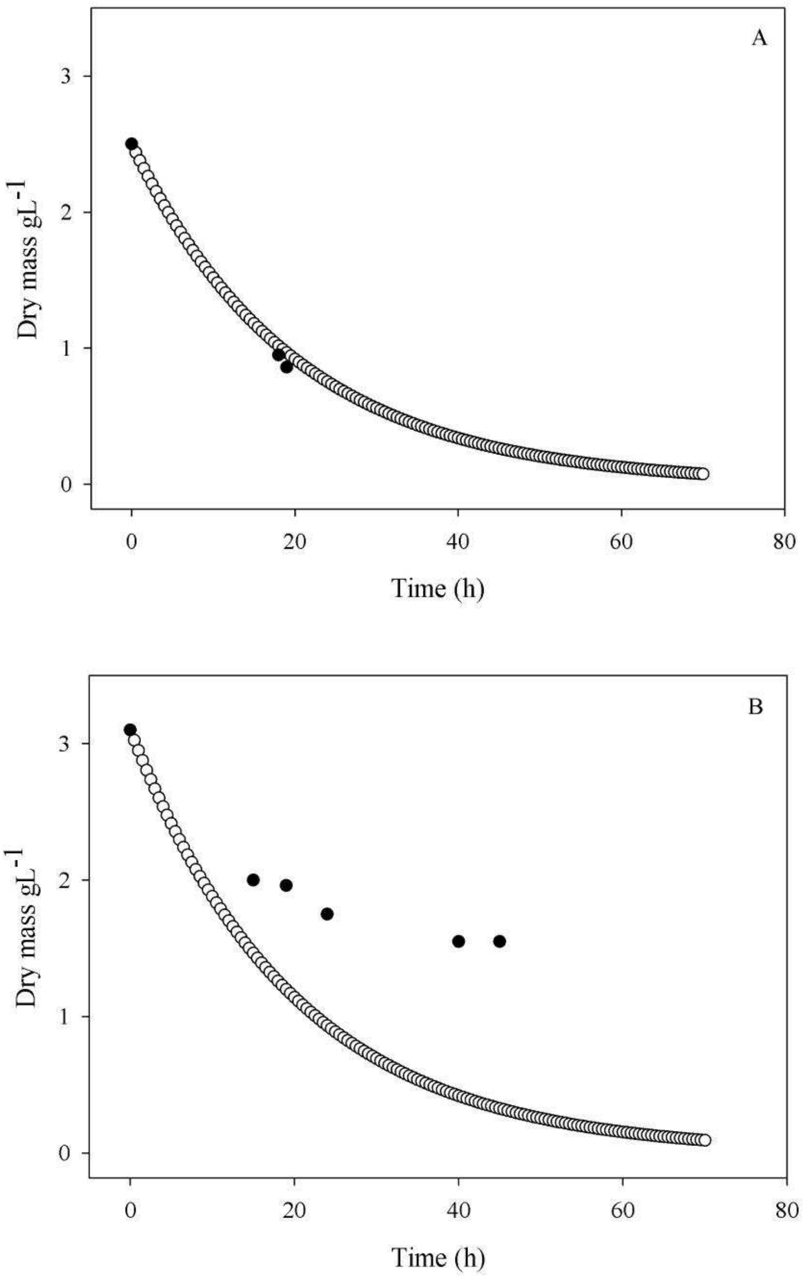


Figure 3 – Wash out curve of *K. marxianus* UFV-3 strains cultured in an anaerobic xylose-limited chemostat culture. (A) KmWT (wild-type strain). (B) UV selected in sequential batches (KmUVsb). Nitrogen was injected to a continuous culture (D=0.05 h^{-1}) in steady state, and dry mass was measured throughout time. Symbols: ○ theoretical wash-out curve, ● experimental values.

4 DISCUSSION

In this work, *K. marxianus* UFV-3 and three different mutants derived from this strain were investigated in terms of their ability to grow on and to produce ethanol from xylose, which is the main pentose constituent of hemicellulose. For this purpose, different cultivations strategies were employed (shake-flask and bioreactor cultivations, batch and continuous cultures) using different conditions of oxygen supply. The glucose effect on xylose consumption was also examined, as a way of evaluating how the fermentation of both monosaccharides would occur in a mixture of glucose and xylose, which is common in hydrolyzed biomass. Finally, we decided to verify whether some of the strains can grow under complete anaerobiosis, which is a possible but rare phenomenon among yeasts (Rodrigues and Ludovico et al., 2006; Snoek and Steensma, 2006), and the condition under which industrial fermentations normally occur (Basso et al., 2008).

In batch culture, the KmUVsb was the single strain able to produce ethanol, and xylitol production rate was twice lower compared with the other strains. It was observed that, although the ethanol accumulation began after 10h of cultivation, its production accompanied the cell growth. Therefore, ethanol formation was associated to growth, when xylose was used as carbon source.

The KmUVsb mutant was studied with more details in xylose-limited chemostat cultivations under different oxygenation conditions, and the results were compared to those obtained with the wild type strain. Under aerobic xylose-limited continuous culture, neither ethanol nor xylitol were detected on supernatant of both *K. marxianus* UFV-3 wild type and mutant cultures. This indicates that all xylose was consumed through oxidative pathways towards biomass synthesis and CO_2 production. Similar behavior was observed for other xylose-assimilating yeasts, i.e. *Debaryomyces hansenii*, *Candida shehatae* and *Scheffersomyces* (*Pichia*) *stipitis* (Nobre et al., 2002; van Dijken and Scheffers, 1986). The absence of xylitol was expected since the production of this metabolite is generally associated with intracellular redox imbalances which occur when conditions is oxygen-limited or when catabolism/anabolism uncoupling occurs (Nobre et al., 2002).

Under hypoxia, less xylose was incorporated into cell mass by the two strains and different extracellular metabolites were observed. In the KmWT culture, the only extracellular metabolite detected was xylitol, of which the yield was higher than KmUVsb. On the other hand, beside of xylitol it was detected acetate and ethanol in KmUVsb culture. The difference on extracellular metabolite suggests difference on metabolism and/or on enzyme activities.

It is known that different dissolved oxygen levels may influence the activity of xylose reductase (XR) and xylitol dehydrogenase (XDH) (Girio et al., 1989; van Dijken and Scheffers, 1986, Wandeska et al., 1995; Yablochkova et al., 2004). XR, the enzyme responsible by the first enzymatic reaction on xylose metabolism, and XDH, enzyme that catalyze the second reaction, possess different specificities for the pyridine nucleotide coenzymes, which are involved in many biochemical redox processes (Yablochkova et al., 2004). In some yeasts, XR is dependent on NAD(P)H as a cofactor, while XDH depends on NAD^+ (Jeffries and Jin, 2004).

In the xylitol-producing yeasts, $NADP^+$ and NADH are regenerated independently. The $NADP^+$ that is formed during xylitol formation can be reduced in the pentose phosphate cycle reactions (Yablochkova et al., 2004, 2003). The NADPH generated via that route leads to a loss of 1 mole of carbon dioxide per 2 moles of NADPH produced. The loss of carbon at the upper part of the metabolic pathway reduces carbon to flux toward lower glycolysis leading to pyruvate. Thus, part of carbon is lost in NADPH-generation for the XR reaction (Pitkanen et al., 2003).

Jeppsson et al. (2002) demonstrated that disruption of either GND1 (one of the isogenes of 6-phosphogluconate dehydrogenase), or ZWF1 (gene for glucose 6-phosphate dehydrogenase), resulted in higher ethanol yields and lower xylitol yields, in addition of lower xylose consumption rate.

The above observation is similar to the findings in the present work. Thus, the increase in the ethanol yields associated with lower xylitol production and decrease in xylose consumption rate observed in the KmUVsb culture could be explained by the loss of the NADPH- generating futile cycle of the oxidative pentose phosphate pathway and also by lesser XR activity. Further studies including activity analysis of others enzymes and also analysis of the expression of genes coding for mainly enzymes active in this part of the pathway are needed to confirm this hypothesis.

There is another possibility. NADPH can also have been formed in a reaction catalyzed by acetaldehyde dehydrogenase (Blank et al., 2005). Once acetate was detected in supernatant of KmUVsb cultures, $NADP^+$ dependent acetaldehyde dehydrogenase can also have contributed to $NADP^+$ reduction.

The reoxidation of NADH generated during conversion of xylitol to D-xylulose requires an electron acceptor (Lighthelm et al., 1989). Under aerobiosis, the electron transfer chain in KmWT and KmUVsb was able to convert NADH to NAD^+ providing the NAD^+ for the XDH reaction. For this, there was not xylitol accumulation.

On the other hand, the electron transfer chain does not work under hypoxia, which affects the whole cellular cofactor balance (Pitkanen et al., 2003). Therefore, the xylitol accumulation observed when KmWT and KmUVsb were cultivated in this condition is due to increase of NADH intracellular pool. Once the reoxidation of NADH in electron transfer chain was not possible, there was not sufficient NAD^+ for the XDH-reaction wich blocked xylose catabolism at the stage of xylitol production.

One other way to NADH reoxidation is in fermentative pathways resulting in an increase of ethanol and glycerol production (Pitkanen et al., 2003). At this point there is an outstanding difference between the KmWT and KmUVsb. It was observed ethanol and acetate formation in KmUVsb culture but not in KmWT. Some activation of the fermentative pathway was made possible into KmUVsb but not into KmWT in the analyzed conditions.

Such as in NADPH regeneration, some carbon is also lost as carbon CO_2 in NAD^+ regeneration. This is further evidence that the fermentative pathway was active in KmUVsb, once the CO_2 formation rate was higher for this strain under hypoxia compared to KmWT. This carbon lost as CO_2 will also never reach ethanol (Pitkanen et al., 2003) which can explain the low ethanol yields compared with other xylose fermentative strains.

In fact, ethanol-producing yeasts are able to ferment xylose with higher yields and reduced amount of xylitol in part due to a particularity of their XR enzymes. In these yeasts, the NADH can also be partially regenerated by NADH-linked XR. This additional regeneration cycle reduces the imbalance between NADH and NAD^+ allowing a higher ethanol yield (Lighthelm et al., 1989). *S. stipitis* and *C. shehatae* are examples of ethanol-producers yeasts. These yeasts have XR enzymes with relatively high affinity for NADH and are able to produce ethanol from xylose with yields of 0.40 g ethanol/ g xylose (Yablochkova et al., 2003).

The activity of XR in UFV-3 strains was performed in order to establish their dependence on NADH and NAD(P)H. It was observed that *K. marxianus* UFV-3 has exclusively NADPH-linked XR activity (results not shown). In contrast of these results, it was reported a *K. marxianus* strain denominated Y-488 that has a little NADH-linked XR activity (Wilkins et al., 2008).

In general, the xylitol presence means less carbon being oxidized through the pentose phosphate pathway, consequently less ethanol being produced. However, the presence of acetic acid and the ethanol in the KmUVsb culture indicates oxidation of xylitol since both metabolites are produced from pyruvate after glycolysis. In the present work, the main difference observed at the level of the pyruvate node was the activities of fermentative enzymes (PDC and ADH) which proved to be higher in the mutant KmUVsb. As presupposed, the fermentative pathway in this strain was more active, which explains the ethanol formation.

The *K. marxianus* wild type and mutant were also cultured under anaerobiosis. According to some researchers, the majority of *K. marxianus* strains described in the literature cannot grow under strict anaerobiosis and that the ethanol formation is almost exclusively linked to oxygen limitation (Fonseca et al., 2008; Snoek and Steensma, 2006). Against this statement, the present work did prove that the *K. marxianus* UFV-3 strain is able to grow under anaerobiosis using glucose as the only carbon source.

The inability of yeasts to grow under anaerobic condition using pentose as carbon source has been reported since 1980 (Wang and Schneider, 1980). The first work reporting the ethanol production from xylose under anaerobiosis was just in 2000 (Eliasson et al., 2000). These researchers worked with a recombinant *S. cerevisiae* strain. However, this yeast grew on xylose only in the presence of glucose. In studies carried out more recently, researchers obtained a strain able to grow under anaerobiosis after an extensive selection procedure. This evolved strain presents a growth rate of 0.05 h^{-1} (Kuyper et al., 2004).

The *K. marxianus* UFV-3 was also not able to grow under anaerobisis using xylose as the only carbon source. Surprisingly, the mutant, KmUVsb, was able to grow. The reasons that explain this fact remain unclear. A gene expression analyses (real time PCR, for example) should be carried out to look for different biochemical routes for xylose metabolism by the selected mutant *K. marxianus* UFV-3. This will be the subject of further search**.**

5 CONCLUSION

In our previous work (Chapter 3) we had reported the improvement of a xylose-assimilating strain of *K. marxianus* UFV-3, enabling xylose to be converted to ethanol with less xylitol and glycerol as by-products. In the present work, this strain was characterized using minimal medium containing xylose as the sole carbon source and continuous cultivations under different oxygen levels.

Compared with the wild type strain, KmUVsb had clearly improved xylose utilization rates, reflected by the higher growth rates on xylose, higher ethanol formation rates and higher ethanol yields. It also exhibited lower xylitol formation rates and lower xylitol yields. These improvements were accompanied by higher XDH/XR ratio and higher PDC and ADH activities. One additional advantage of the selected mutant is that anaerobic xylose fermentation is very promising. Besides revealing metabolic differences between wild type and mutant strains, this study also extended the physiological knowledge on the metabolism in *K. marxianus* UFV-3.

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CONCLUSÕES GERAIS

A levedura *Kluyveromyces marxianus* UFV-3 é um micro-organismo promissor no contexto da produção de etanol a partir de hidrolisado de bagaço de cana de açúcar. Existem alguns relatos sobre o potencial dessa levedura em fermentar outras fontes de carbono tais como lactose, mas esta é a primeira vez que um estudo voltado para a co-fermentação de glicose e xilose é realizado. De fato, poucas informações estão disponíveis sobre o metabolismo de xilose em *K. marxianus*, bem como sobre a influência da glicose na assimilação dessa pentose.

No presente trabalho, foi demonstrado que altas concentrações de glicose inibem a assimilação de xilose e retardam o início de sua utilização pela levedura *K. marxianus* UFV-3. Por outro lado, o consumo de xilose foi praticamente instantâneo após a depleção de glicose do meio, quando uma baixa concentração inicial dessa hexose (5 gL^{-1}) foi utilizada. Mediante esses resultados é possível inferir que uma concentração baixa de glicose seja importante para o metabolismo de xilose, o que já foi observado em outras linhagens de leveduras.

Um resultado surpreendente foi obtido a partir do cultivo de *K. marxianus* em mistura de iguais concentrações de glicose e xilose na presença de inibidores da cadeia de transporte de elétrons. Nesta condição, houve um consumo simultâneo de glicose e xilose. Portanto, o co-consumo desses açúcares é possível em *K. marxianus* UFV-3.

A influência de diversas combinações de glicose/ xilose sobre a produção de etanol também foi analisada. A produção desse metabólito foi superior na presença dos dois açúcares. Contudo, o etanol produzido foi oriundo somente da fermentação da glicose, enquanto a xilose foi utilizada para a produção de biomassa e xilitol.

Visando obter uma linhagem melhorada quanto à fermentação de xilose, *K. marxianus* UFV-3 foi submetida à engenharia evolutiva. A estratégia de engenharia evolutiva aplicada baseou-se na associação de métodos mutagênicos com seleção em culturas contínuas e bateladas sequenciais. O potencial dessa estratégia para obtenção de linhagens melhoradas foi confirmado. O método de mutagênese aleatória adotado - REMI (Integração Mediada por Enzima de Restrição) - foi eficiente na geração de mutantes de *K. marxianus* UFV-3. Diversos mutantes auxotróficos foram isolados. Quanto aos sistemas de seleção, ambos, quimiostato e batelada sequencial, foram eficientes na seleção de linhagens mutantes.

Dentre os mutantes selecionados, um apresentou especial fenótipo de interesse: foi capaz de produzir etanol a partir de xilose, e também produziu menor quantidade de xilitol, se comparado com a linhagem selvagem. Esse fenótipo foi correlacionado com modificações na atividade de algumas enzimas. A linhagem mutante, denominada KmUVsb, apresentou maior atividade de piruvato descarboxilase e também de álcool desidrogenase. Além disso, a razão entre a atividade específica das duas primeiras enzimas da via de assimilação de xilose – xilose redutase e xilitol desidrogenase – também foi maior para a linhagem mutante. Pode-se concluir, portanto, que as atividades dessas enzimas são importantes para a fermentação de xilose, e constituem alvos interessantes para engenharia metabólica.

O presente trabalho confirmou que *K. marxianus* UFV-3 é capaz de crescer sob condições estritas de anaerobiose, utilizando glicose como fonte de carbono. Além disso, verificou-se que a linhagem mutante – KmUVsb –, ao contrário da linhagem selvagem, é capaz de crescer naquelas condições, utilizando xilose como única fonte de carbono e energia .

O conjunto de resultados aqui apresentados traz perspectivas quanto à utilização de *K. marxianus* UFV-3 em fermentações industriais, onde prevalecem baixa oxigenação, bem como na fermentação de misturas de glicose e xilose.