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Tese

PRODUÇÃO DE PROTEÍNAS UTILIZANDO LEVEDURAS COMO SISTEMAS DE EXPRESSÃO

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Orientadores:

Prof. Dr. JOSÉ LUIZ DE LIMA FILHO

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Prof. Dr. JOSÉ ANTONIO TEIXEIRA

Recife, fevereiro de 2011.

MARCELA SILVESTRE OUTTES WANDERLEY

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3

"É preciso força para sonhar e perceber
Que a estrada vai além do que se vê."

Macelo Camelo

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Resumo

Leveduras vêm sendo utilizadas como sistemas de expressão para produção de proteínas de interesse biotecnológico. Dentre as diversas aplicações das proteínas heterólogas, as produzidas com finalidade terapêutica e para diagnóstico clínico vêm recebendo grande destaque, como por exemplo, a lectina frutalina e a oncoproteína E7 do Papilomavírus Humano (HPV). A frutalina, lectina extraída das sementes da fruta-pão, tem sido descrita como importante ferramenta no diagnóstico do câncer devido ao seu tropismo com células tumorais. Enquanto, a oncoproteína viral do HPV, E7, por ser encontrada em células tumorais relacionadas à infecção pelo HPV, torna-se um importante alvo para o desenvolvimento de vacinas profiláticas e terapêuticas contra esta infecção. Neste trabalho utilizamos as leveduras *Pichia pastoris* KM71H e *Pichia pastoris* GS115 como sistemas de expressão para a produção das proteínas frutalina e E7 do HPV16, respectivamente. A influência da fonte de carbono e do estresse iônico na produção da enzima β-galactosidase pela *Kluyveromyces lactis* DSM 3795 foi avaliada em diferentes condições de cultivo. Neste trabalho os níveis de frutalina recombinante (13,4 $mg.L^{-1}$), obtidos pelo processo de batelada alimentada, foi 4 vezes maior que em testes com frascos aerados e a suplementação com elementos traços permitiu o aumento de 2.5 vezes na produção da proteína recombinante. Enquanto, o gene de E7 do HPV16 foi corretamente clonado e integrado ao genoma da *P.pastoris* GS115, sendo produzido 218,9$mg.L^{-1}$ da proteína E7 com o peso molecular de 27KDa. A avaliação da influência da fonte de carbono e do estresse iônico na atividade da enzima β-galactosidase pela *K. lactis* DSM 3795 permitiu selecionar a lactose como a fonte de carbono ideal, pois favoreceu maior atividade específica da enzima (271,0 $U.mg^{-1}$). Enquanto que, os cátions Na^{+}, K^{+}, Mg^{2+} potencializaram a atividade da enzima (410,4; 440,1; and 734.71 $U.mg^{-1}$, respectivamente), o Ca^{2+} inibiu parcialmente a produção da β-galactosidase. Por fim, apesar da *P. pastoris* ter se mostrado um sistema eficiente para a produção de proteína heterólogas, ela ainda apresenta algumas limitações. Dessa maneira, o desenvolver estratégias que permitam a otimização de vetores para a produção dessas proteínas, representa um importante alvo no desenvolvimento de produtos para o diagnóstico, prevenção e tratamento do câncer.

Palavras-chave: *Pichia pastoris*, *Kluyveromyces lactis*, Proteína heteróloga, Frutalina, β-galactosidase, oncoproteína E7 e Papilomavírus Humano

Abstract

Yeasts have been related as expression systems for proteins production for biotechnological interest. Heterologous protein production to therapeutic and clinical diagnosis purposes have been received highlighted, such as Human Papillomavirus (HPV) E7 oncoprotein and lectin frutalin. Frutalin, lectin extracted from breadfruit seeds, has been described as an important tool in cancer diagnosis due to its tumoral cells tropism. While HPV E7 oncoprotein is being found in tumor cells, it becomes an important target for the development of prophylactic and therapeutic vaccines against HPV. In this work we used *Pichia pastoris* KM71H and *P. pastoris* GS115 as systems expression for proteins production, frutalin and E7 of HPV16, respectively. Also, Carbon source and ionic stress influence in β-galactosidase activity by *Kluyveromyces lactis* DSM3795 were evaluated. In this study the levels of recombinant frutalin obtained (13.4 $mg.L^{-1}$) by fed-batch process was 4-fold higher than tests on vials and the traces elements supplementation permitted enhance at 2.5-fold in protein production. HPV16 E7 gene was correctly cloning and integrated into *P.pastoris* genome and 218.9 $mg.L^{-1}$ of the HPV16 E7 was produced with molecular weight of 27KDa. Carbon source influence and ionic stress in β-galactosidase activity by *K. lactis* DSM 3795, allowed selecting lactose medium as optimal carbon source to favour higher specific enzyme (271.0 $U.mg^{1}$). The Na^{+}, K^{+}, Mg^{2+} cations acted as activators on β-galactosidase activity (410.4, 440.1, and 734.71 $U.mg^{-1}$, respectively) and Ca^{2+} inhibited partially the enzyme activity. *P. pastoris* had showed an efficient system expression to protein heterologous production, but it still has some limitations. Thus, development of strategies that allow characterize vector to these proteins production, represents an important target in developing products for diagnosis, prevention and treatment of cancer.

Key-words: *Pichia pastoris*, *Kluyveromyces lactis*, heterologous protein, Frutalin, β-galactosidase, E7 oncoprotein e Human Papillomavirus (HPV).

Lista de Figuras

Revisão bibliográfica

Figura 1. Detecção imunohistoquímica de glicoconjugados específicos de frutalina em carcinoma de próstata..... 27
Figura 2. Ilustração de diferentes sistemas para expressão de proteínas heterólogas..... 30
Figura 3. Ilustração das leveduras *P. pastoris* (A) e *Kluyveromyces lactis* (B) obtidas através da microscopia eletrônica de varredura.... 32
Figura 4. Desenho esquemático do metabolismo do metanol pela *P. pastoris*.. ... .35
Figura 5. Atividade específica da Álcool oxidase 1 nas etapas de transição de repressão/derepressão e indução durante cultivo da *P. pastoris* 37
Figura 6. Categorização do secretoma da *Pichia pastoris*. ... .38

Capítulo I

Figura 1. The expression vector used in this study... 64
Figura 2. Agilent 2100 Bioanalyser gel, using DNA 1000 kit. 65
Figura 3. Alignment of DNA sequence of pPICZB/HPV16.E7 clone with the published E7 DNA sequence 66
Figura 4. Agilent 2100 Bioanalyzer total RNA analysis after Trizol isolation. 67
Figura 5. Electropherogram comparing a recombinant protein expression by *P. pastoris* GS115 pPICZB/HPV16.E7 68
Figura 6. Bioanalyzer gel-like image comparing recombinant E7 expression after different extraction methodology. 69

Capítulo II

Figura 1. The fed-batch process to production of recombinant frutalin by *P. pastoris* 82
Figura 2. Elution profile on Sephacryl column of the fed-batch process supernatant.....85

Lista de figuras

Capítulo III

Figura 1. Biomassa production and specific β-galactosidase activity per total protein by *K. lactis* during shaker-flasker cultivation.....100
Figura 2. Influence of the ions Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} in β-galactosidase activity produced by *K. lactis* DSM3795 in shaker-flasker.....102

Lista de tabelas

Revisão bibliográfica

Tabela 1. Análise comparativa de diferentes sistemas de expressão. 29
Tabela 2. Proteínas recombinantes produzidas por diferentes linhagens de *P. pastoris*.....36

Capítulo II

Tabela 1. Biomass production, recombinant frutalin concentration and specific growth-rate obtained in fed-batch operation for *Pichia pastoris* KM71H 80

Capítulo III

Tabela 1. Influence of the carbon source in dry weight production and β-galactosidase activity by *K. lactis* DSM 3795 cultivated in shaker-flask..... ..98

Lista de abreviações

AOX: álcool oxidase

DHA: Dihidroxicetona

DHAP: dihidroxiacetona fosfato

DHAS: dihidroxiacetona sintase

DNA: Ácido desoxirribonucléico

F_6P: frutose-6-fosfato

FBP: frutose bi-bifosfato

FDA: *Food and drug administration*

FLD: Formaldeído desidrogenase

g: força de gravidade

GAP: gliceraldeído 3-fosfato

GAPD: Gliceraldeído-3-fosfato desidrogenase

GRAS: *Generally recognized as safe*

GS: S-formil glutationa

GSH: S-formil glutationa hidrolase

HPV: Papilomavírus Humano

INCA: Instituto Nacional do Câncer

OMS: Organização Mundial de Saúde

OPAS: Organização Panamericana de Saúde

ORF: *Open Reading Frame*

ORI: Origem da replicação

PCR: *Polymerase Chain Reaction*

PHA: Fitohemaglutinina

RE: Retículo endoplasmático

RNA: Ácido Ribonucléico

rpm: rotações por minuto

Xu_5-P: xilulose 5-monofosfato

Sumário

Resumo....11

Abstract....12

Lista de figuras....13

Lista de tabelas....15

Lista de abreviações....16

Introdução....21

Revisão da literatura....24

1-Microrganismo na biotecnologia....24

2-Importância biotecnológica das proteínas heterólogas....25

2.1- Proteínas heterólogas versus Câncer....25

2.1.1- Oncoproteínas....26

2.2.2- Lectinas....27

3-Sistemas de expressão de genes heterólogos....28

3.1-Leveduras como sistemas de expressão....32

3.1.1- Pichia pastoris....33

3.1.2- Kluyveromyces lactis....39

Objetivos....54

Objetivo geral....54

Objetivos específicos....54

Capítulo I....56

Cloning and expression of the E7 gene from Human papillomavirus type 16 in *Pichia pastoris* GS115....56

Capítulo II 74

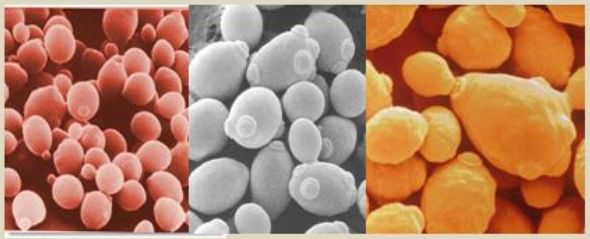
Influence of the traces elements supplementation in recombinant frutalin production by *Pichia pastoris* KM71H through fed-batch process 74

Capítulo III 90

Influence of the carbon source and ionic stress in β-galactosidase activity from *Kluyveromyces lactis* DSM3795 90

Conclusões 116

Anexos ..... **Erro! Indicador não definido.** 118



Introdução

"Qualquer tolo inteligente consegue fazer coisas maiores e mais complexas. É necessário um toque de gênio e muita coragem para ir na direção oposta."
(Albert Einstein)

Introdução

Durante o século XX, aplicações com fins industriais para os microrganismos contribuíram para a expansão da biotecnologia. Através da manipulação genética dos microrganismos, a fim de gerar um produto que normalmente eles não seriam capazes de produzir, a biotecnologia tem se revelado capaz de gerar riqueza e influenciar diversos setores da economia.

A produção de proteína heteróloga consiste na produção de uma proteína que normalmente não é expressa pelo microrganismo, sendo possível através dos processos de clonagem do gene alvo. As aplicações biotecnológicas das proteínas heterólogas vêm despertando grande interesse industrial, principalmente, com finalidade terapêutica, diagnóstica, e reagentes para fins de pesquisa. Entre estas proteínas, podem-se citar as desenvolvidas para a prevenção, diagnóstico e tratamento contra o câncer, como por exemplo, a lectina frutalina e a oncoproteína do Papilomavírus Humano (HPV) E7.

As lectinas, glicoproteínas de origem não imunológica, são encontradas em diversos tipos de organismos e vem despertando interesse terapêutico por atuar como sítos de reconhecimento celular em alguns processos biológicos. A frutalina, lectina ligante da D-galactose extraída das sementes da fruta-pão, tem sido descrita como marcador de células tumorais como potencial aplicação no monitoramento de diversos tipos de câncer. Por outro lado, as oncoproteínas virais do HPV, E6 e E7, são as principais responsáveis pelo desenvolvimento do câncer de colo de útero uma vez que a infecção pelo HPV está relacionada com o avanço da lesão inicial ao câncer. Assim, vacinas terapêuticas e profiláticas vêm sendo desenvolvidas contra os HPVs de alto risco sendo a oncoproteína E7 um importante alvo para o desenvolvimento destas vacinas.

Para a produção de proteínas heterólogas existem sistemas procarióticos e eucarióticos os quais apresentam flexibilidade na expressão de uma variedade de proteínas. Dentre estes sistemas, as leveduras vêm se destacando para a expressão de genes heterólogos de interesse biotecnológico. Estes microrganismos apresentam algumas vantagens em relação aos procariotos, tais como a capacidade de secreção protéica em sua forma biologicamente ativa e de modificações pós-traducionais necessárias para sua ótima atividade e estabilidade.

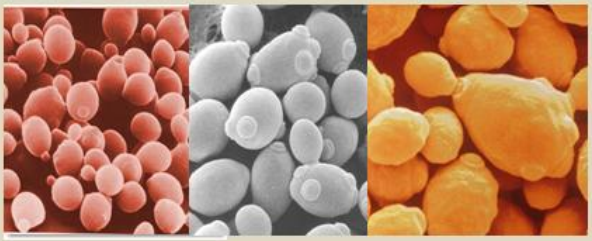
Sacharomyces cerevisiae é uma levedura conhecida desde a antiguidade e apesar de se destacar como um dos sistemas eucariotos mais conhecidos com aspectos biológicos e genéticos bem

caracterizados, apresenta algumas desvantagens como sistema de expressão, tais como a hiperglicosilação das proteínas secretadas e a produção de etanol durante o processo fermentativo. Dessa maneira, outras leveduras têm sido apresentadas como sistemas de expressão alternativos por apresentarem vantagens sobre *S. cerevisiae*. Neste cenário, *Pichia pastoris* e *Kluyveromyces lactis* vêm sendo descritas como sistemas de expressão para a produção de proteína heteróloga.

A levedura metilotrófica *P. pastoris* é um dos principais sistemas para a expressão de proteínas recombinantes. Centenas de proteínas de vírus, bactérias, fungos, animais, plantas e seres humanos têm sido produzidas com sucesso em *P. pastoris*. Essa levedura apresenta vantagens como sistema de expressão, tais como a facilidade para manipulação genética, de sua maquinaria celular permitir um melhor processamento da proteína, e de ser capaz de produzir grande quantidade de proteínas recombinantes. Porém, a expressão de proteínas heterólogas pela *P. pastoris* baseia-se principalmente no seu promotor $_{P}$AOX1 que é fortemente regulado pelo metanol e que em determinadas concentrações pode ser tóxico para a célula.

K. lactis é uma levedura não convencional cujo habitat é o leite e seus derivados e que apresenta excelentes características fermentativas e notável capacidade de excreção. Essa espécie vem despertando interesse biotecnológico devido às suas características atrativas como a alta taxa de secreção de proteínas, excreção protéica no meio de cultivo e não no espaço periplasmático, além de sua genética molecular acessível. A expressão de proteína heterólogas pela *K. lactis* ocorre através da indução dos promotores LAC por fontes de carbono baratas e de fácil acesso fazendo desse sistema um atrativo hospedeiro para a expressão de genes de interesse biotecnológico

Assim, as leveduras *P. pastoris* e *K. lactis* podem ser utilizadas com sucesso como hospedeiras na produção de proteínas com propósitos terapêuticos, permitindo a sua utilização como um sistema ideal para o desenvolvimento de produtos de interesse biotecnológico. Dessa maneira, o presente trabalho visa contribuir de forma relevante para a ampliação dos conhecimentos a cerca destas proteínas produzidas pela *P. pastoris* e pela *K. lactis*, bem como para o desenvolvimento de produtos com propósitos profilático, diagnóstico e terapêutico.



Revisão da literatura

"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original."
(Albert Einstein)

Revisão da literatura

1-Microrganismo na biotecnologia

A biotecnologia tem-se revelado capaz de gerar riqueza e influenciar todos os setores importantes da economia, como a saúde, o processamento de alimentos, a agricultura e a silvicultura, a proteção ambiental além da produção de materiais e produtos químicos (Gavrilescu e Chisti, 2005). A biotecnologia tradicional ou clássica é constituída por um conjunto de técnicas amplamente difundidas que utilizam seres vivos, ou parte deles para produzir bens e serviços. A biotecnologia moderna engloba os campos como a engenharia genética, a cultura de microrganismos recombinantes, engenharia metabólica, bioeletrônica, nanobiotecnologia, genômica, proteômica e a biosseparação (Gavrilescu e Chisti, 2005). O principal fundamento da biotecnologia é a capacidade que suas ferramentas têm de manipular uma célula para produzir um produto que normalmente ela não seria capaz de produzir. Estes produtos podem ter grande utilidade para indústria biotecnológica como, por exemplo, a produção de proteína heterólogas para o desenvolvimento de novos compostos ou para avaliar sua capacidade de interação com outras células. Vários sistemas procarióticos e eucarióticos estão disponíveis para a expressão de proteínas heterólogas oferecendo flexibilidade para expressão de uma variedade de proteínas (Welch, Villalobos *et al.*, 2009).

Durante o século XX, a biotecnologia se expandiu através do desenvolvimento de diversas aplicações industriais para os microrganismos. Neste último século a clonagem gênica contribuiu para o avanço desta grande área (Romanos, Clare *et al.*, 2001), juntamente com os processos de fermentação, utilizados pela indústria biotecnológica com o propósito de se obter o produto de interesse industrial como consequência da atividade metabólica dos microrganismos (Kingsman e Kingsman, 1988).

Para que os microrganismos sejam utilizados para fins tecnológicos, devem satisfazer alguns critérios específicos, tais como: meio barato para o crescimento; temperatura ótima de crescimento em torno de 30ºC de modo que os custos de resfriamento ou aquecimento do meio devam ser evitados; reação do microrganismo deve ser adequada ao processo utilizado; o microrganismo deve ser estável e deve permitir a manipulação genética; a conversão do substrato a produto deve ocorrer rapidamente e o produto formado deve ser facilmente recuperado do meio de cultura (Welch, Villalobos *et al.*, 2009).

2-Importância biotecnológica das proteínas heterólogas

A produção *in vivo* das proteínas é um processo muito complexo e que envolve modificações pós-traducionais das proteínas que são necessárias para a sua estabilidade e atividade biológica, tais como glicosilação, D-fosforilação e a conformação apropriada da proteína. A produção de uma proteína fora do seu sistema hospedeiro natural é chamada de heteróloga, sendo estas divididas em três grandes grupos: as proteínas usadas como reagentes para fins de pesquisa, as proteínas com várias aplicações industriais e as proteínas terapêuticas ou aquelas usadas para diagnóstico clínico. Entre essas proteínas, as utilizadas para fins terapêuticos constituem uma classe especial com rigorosos padrões de qualidade e que desperta grande interesse industrial (Desai, Shrivastava *et al.*, 2010).

A insulina foi o primeiro produto recombinante aprovado pelo FDA (*Food and drug administration)* para aplicação em seres humanos. Os primeiros sistemas de expressão para a insulina foram desenvolvidos no final da década de 1970 e basearam-se na expressão separadas das cadeias α-e β- as quais foram fundidas às proteínas transportadoras. Para este propósito utilizou-se *Escherichia coli* como vetor de expressão (Gurramkonda, Polez *et al.*, 2010). Nesta mesma época e utilizando o mesmo sistema de expressão foram sintetizados hormônios do crescimento, somatostatina e somatotrofina, com finalidade terapêutica (Ali, Brown *et al.*, 2001).

2.1- Proteínas heterólogas versus câncer

O câncer é uma patologia associada a diferentes fatores e de alta incidência no Brasil e no mundo configurando-se como um grande problema de saúde pública. Mais de 12 milhões de pessoas são diagnosticadas com câncer a cada ano, com 7 milhões de mortes sendo que, 66% destas ocorrem nos países onde os recursos disponíveis para o controle do câncer e os serviços são limitados ou inexistentes (Inca, 2010).

O crescente número de casos de câncer fez com que a biotecnologia recorresse ao desenvolvimento de novos produtos visando à prevenção, o diagnóstico e o tratamento das neoplasias (Pointreau, Bernadou *et al.*, 2010). Neste cenário, a produção heteróloga de lectinas e das oncoproteínas E6 e E7 do HPV vêm sendo descritas de forma relevante para identificação de novos alvos no combate ao câncer.

2.1.1- Oncoproteínas

A infecção pelo papilomavírus humano (HPV) é o fator preponderante no avanço da lesão inicial ao câncer do colo do útero. O surgimento desse tipo de câncer está associado à infecção por um dos 15 tipos oncogênicos do HPV. Mulheres infectadas com tipos de HPV de alto-risco possuem uma probabilidade 100 vezes maior de desenvolver lesões cervicais quando comparadas com mulheres não-infectadas (Wang, Sima *et al.*, 2010).

A família do HPV compreende 30 tipos, sendo dividida em baixo e alto risco de acordo com a sua oncogenicidade. Os sorotipos 16 e 18 são considerados de alto risco e apresentam oncoproteínas E6 e E7 responsáveis pela inativação das proteínas humanas reguladoras do ciclo celular. Os oncogenes E6 e E7 e, consequentemente, as suas oncoproteínas virais são as principais responsáveis pelo desenvolvimento do câncer de colo de útero (Zur Hausen, 2009).

As oncoproteínas E6 e E7 possuem um pequeno tamanho molecular e têm a capacidade de interagir com várias proteínas reguladoras da célula hospedeira e assim levar a imortalização celular e ao desenvolvimento do processo neoplásico. Além disso, quando associadas, desregulam o mecanismo de duplicação dos cromossomos e a segregação durante a mitose e induzem a instabilidade cromossomal (Zur Hausen, 2002; Blakaj, Kattamuri *et al.*, 2006).

O oncogene E7 codifica uma proteína de 98-100 aminoácidos e com peso molecular de 11-13 KDa (Moody e Laimins, 2010). O potencial oncogênico da E7 é atribuído à sua capacidade de ligação com as proteínas reguladoras do ciclo celular, da família retinoblastoma. A afinidade de ligação entre o HPV e a proteína retinoblastoma (Rb) varia de acordo com o tipo de tipo viral, E7 de HPV de alto risco (16 e 18) apresenta maior afinidade de ligação com a Rb em relação à E7 de HPV de baixo risco (6 e 11) (Piccioli, Mckee *et al.*, 2010). A oncoproteína E7 do HPV 16 tem sido produzida por sistemas de expressão de proteína heterólogas, como *Escherichia coli*, células de inseto, células de mamíferos e pela levedura *Schizosaccharomyces pombe* (Braspenning, Manetti *et al.*, 1997; Fernando, Murray *et al.*, 1999; Fiedler, Campofernandez *et al.*, 2006; Mirecka, Rudolph *et al.*, 2006).

O principal método de diagnóstico das lesões iniciais do tecido do colo uterino é o teste de Papanicolau, desenvolvido pelo patologista George Papanicolaou em 1949. Este teste tem uma especificidade alta, mas uma baixa sensibilidade (Stanley, 2006). Por este motivo, ainda é grande o número de mulheres infectadas pelo HPV que evoluem da lesão precursora para o câncer cervical.

Assim, vacinas terapêuticas e profiláticas vêm sendo desenvolvidas contra os HPVs de alto risco a fim de reduzir as taxas de mortalidade do câncer de colo de útero. As oncoproteínas E6 e E7 são alvos da maioria dessas vacinas, uma vez que são proteínas consistentemente expressas em células de tumores associados ao papilomavírus (Yan, Reichenbach *et al.,* 2009). Desta forma, essas duas proteínas oncogênicas representam potenciais antígenos para o desenvolvimento de uma vacina contra o HPV.

2.2.2- Lectinas

Lectinas são glicoproteínas que apresentam afinidade por carboidratos específicos. As lectinas podem ser encontradas em todos os tipos de organismos, desde organismos complexos como humanos até os vírus. Esta glicoproteína pode atuar como sítio de reconhecimento celular em muitos processos biológicos (Nasi, Picariello *et al.*, 2009).

Atualmente, as lectinas vêm despertando interesse como ferramentas nas áreas da pesquisa biológica, médica e biotecnológica (Lei e Chang, 2009). Algumas lectinas, tais como a fitohemaglutinina e a concanavalina A (ConA), vêm sendo citadas em estudos relacionados a descorberta de moléculas com potencial antineoplásico (Pryme, Pusztaib *et al.*, 1994; Liu, Li *et al.*, 2009). Outras lectinas, como a frutalina, são descritas como marcadores de células tumorais (Oliveira, Teixeira *et al.*, 2009).

Diante da diversidade de lectinas disponíveis na natureza encontra-se a frutalina que é uma lectina purificada das sementes da fruta-pão (*Artocarpusin tegrifolia*) e que apresenta afinidade pela D-galactose. Sua denominação surgiu devido à sua semelhança estrutural com uma outra lectina, a jacalina. Porém estudos físico-químicos e biológicos descrevem particularidades da frutalina em relação à jacalina, como por exemplo uma maior atividade hemaglutinante. A especificidade da frutalina com a D-galactose é determinada pelo tamanho e pela conformação do sítio de ligação da lectina com o referido carboidrato (Campana, Moraes *et al.*, 2002). A interação com neutrófilos induzindo a "quimiotaxia", a ação imunomoduladora e a capacidade de estimular linfócitos humanos são características biológica da frutalina (Brandolima, Saldanhagama *et al.*, 2006).

Recentemente, estudo imunohistoquímico em amostra de carcinoma de próstata descreveu a formação de glicoconjugados entre a frutalina e as células cancerígenas (figura 1). Este relato confirmou a importância da frutalina como marcador de células tumorais (Brandolima, Saldanhagama *et al.*, 2006; Oliveira, Teixeira *et al.*, 2009).

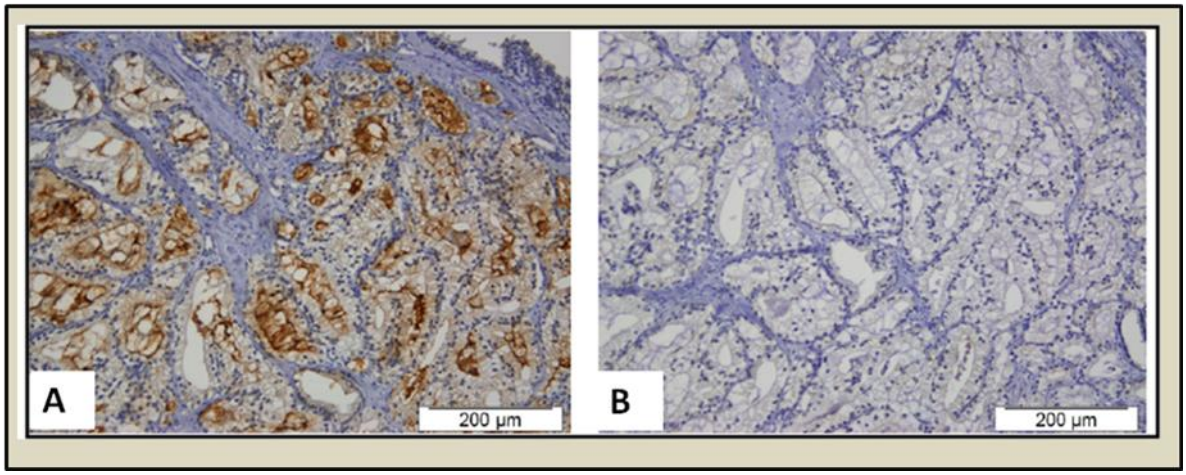


Figura 1. Detecção imunohistoquímica de glicoconjugados específicos de frutalina em carcinoma de próstata: (A) tecido imunomarcado com a frutalina, (B) controle negativo. Imagem modificada de: (Oliveira, Teixeira *et al.*, 2009).

Apesar do seu grande valor biotecnológico, as lectinas são proteínas que apresentam baixo nível de expressão durante sua extração a partir do organismo de origem (até 30mg da lectina Nictaba/1kg de folha de tabaco) (Lannoo, Vervecken *et al.*, 2007). Dessa maneira, algumas lectinas estão sendo produzidas utilizando microrganismos como sistemas de expressão: *Pleurotus cornucopiae*, fitohemaglutinina (PHA), aglutinina do *Galanthus nivalis* (GNA), lectina C-tipo do receptores, a lectina do *Polygonatum* e a frutalina (Raemaekers, De Muro *et al.*, 1999; Sumisa, Iijima *et al.*, 2004; Oliveira, Felix *et al.*, 2008; Soanes, Ewart *et al.*, 2008; Oliveira, Costa *et al.*, 2009; Li, Sun *et al.*, 2010).

Considerando o grande potencial na aplicação biotecnológica destas proteínas é importante desenvolver estratégias que aumentem sua produção. Avanços da engenharia genética têm permitido ampliar as utilizações de leveduras manipuladas para a produção em alta-escala de proteínas heterólogas podendo ser aplicada para o desenvolvimento de medicamentos e vacinas terapêuticas contra algumas patologias (Rubio-Texeira, 2005).

3-Sistemas de expressão de genes heterólogos

Ferramentas da biologia molecular têm auxiliado na produção de proteínas de interesse biotecnológico nas mais diversas espécies de microrganismos. As ferramentas essenciais para a produção de proteínas heterólogas são: um gene ou DNA que codifica a proteína desejada, um vetor adequado e um sistema biológico (sistema de expressão) que pode transcrever e traduzir o gene na proteína desejada. Assim um sistema de expressão deve: (i) ser capaz de produzir proteínas, (ii) ter

boa produtividade, (iii) ser de fácil manuseio e manutenção, (iv) ser seguro e econômico, e (v) apresentar fácil processo de purificação (Desai, Shrivastava *et al.*, 2010). Para obtenção de um sistema de expressão ideal, este precisa apresentar vetores adequados, com sequências reguladoras transcricionais e pós-traducionais específicas, com alto rendimento protéico e o microrganismo deve apresentar um perfil fermentativo que atenda as condições industriais (Hunt, 2005).

Tabela 1. Análise comparativa de diferentes sistemas de expressão. Fonte: (Tamas e Shewry, 2006).

	Bactéria	**Levedura**		**Baculovírus**
	E. coli	***P.pastoris***	***S.cerevisiae***	
Vantagens	• Baixo custo, rapidez e facilidade de manipulação • Utilização para uma variedade de vetores e sistemas de expressão • Elevada produção proteíca	• Facilidade de manipulação • Boa capacidade secretória • Elevada produção • Uma variedade de vetores e linhagens disponíveis • A integração do vetor em sítios específicos do genoma é estavél • O escalonamento de frascos para reatores são relativamente simples • Baixo risco de contaminação • Glicosilação e processamento proteolítico • Processamento e dobramento proteíco ocorre no RE	• Glicosilação e processamento proteolítico • Processamento e dobramento proteíco ocorre no RE • Autêntico processamento proteíco	• Elevada produção • Glicosilação e processamento proteolítico • Processamento e dobramento proteíco ocorre no RE
Desvantagens	• Proteína precipta em corpos de inclusão • A formação de ligações S–S ocorre apenas em linhagens específicas • Não há modificações pos-trasducionais	• Toxicidade celular causada pelo acúmulo de metanol • Concentração óptima de metanol deve ser determinada para cada proteína expressa	• Produtividade baixa • Baixa capacidade secretória	• Custo elevado

Sistemas de expressão de genes heterólogos apresentam vantagens e desvantagens inerentes (tabela 1). Não há um sistema de expressão "universal" que garanta elevada produção da proteína recombinante. Porém, para selecionar um sistema ideal de expressão a produtividade, a

bioatividade, o propósito e as características físico-químicas da proteína de interesse devem ser levados em consideração, juntamente com o custo, com a conveniência e a segurança do próprio sistema (Yin, Li *et al.*, 2007).

A clonagem de genes em sistemas de expressão para produção de proteínas recombinantes é uma estratégia biotecnológica para potencializar a produção de uma proteína de interesse. Geralmente o gene de interesse é amplificado através de uma reação de *Polymerase Chain Reaction* (PCR) e clonado em um plasmídeo (vetor de expressão) que na maioria dos casos é selecionado e replicado em *Escherichia coli*. Então, o plasmídeo clonado pode ser usado para expressão da proteína de interesse em um dos diversos sistemas de expressão (Freuler, Stettler *et al.*, 2008).

Para construção de um sistema de expressão ideal é importante selecionar alguns parâmetros. Primeiro a expressão é normalmente induzida a partir de um plasmídeo protegido por um sistema com *background* genético compatível. Os elementos genéticos do plasmídeo de expressão incluem origem da replicação (ORI), um marcador de resistência a antibióticos, promotores transcricionais, regiões iniciadoras da tradução bem como os terminadores transcricionais e traducionais (Sorensen e Mortensen, 2005).

Na última década, pesquisas em genômica e metagenômica levaram à identificação de vários genes que codificam polipeptídeos com funções desconhecidas. Assim, a expressão de genes heterólogos é necessária, como método fundamental para a síntese de novas proteínas, a fim de estudar suas estruturas, interações celulares e funções fisiológicas (Katzke, Arvani *et al.*, 2010). Por isso, muitos sistemas de vetores recombinantes foram desenvolvidos para expressar funcionalmente proteínas heterólogas em organismos hospedeiros diferentes, incluindo as bactérias, *Escherichia coli* e *Bacillus subtilis*; microrganismos eucarióticos como a levedura *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces sp.*, bem como fungos filamentosos, insetos e células de mamíferos (Yin, Li *et al.*, 2007; Sun, Frearson *et al.*, 2008; Potvin, Ahmad *et al.*, 2010) (figura 2). As plantas também vêm sendo estudadas como promissores sistemas de expressão para a produção de proteínas heterólogas, especialmente proteínas com finalidade terapêutica. A utilização de plantas e animais transgênicos como sistema de expressão, apesar de apresentar baixo custo de produção, apresenta baixos níveis de expressão (Corrado e Karali, 2009; Desai, Shrivastava *et al.*, 2010).

E. coli tem sido abordada como o procarioto de escolha para a expressão de proteínas heterólogas, devido a sua facilidade de manipulação genética, disponibilidade de ferramentas

genéticas eficientes, alta eficiência de transformação e seu rápido crescimento em relação à outros microrganismos. Porém, algumas desvantagens são observadas em sistemas procariotos, tais como: ausência de modificações pós-trasducionais e formação dos corpos de inclusão (Shapiro, 2009). Adotar microrganismos eucariotos como sistemas de expressão pode contornar estes inconvenientes, uma vez que estes microrganismos permitem a formação de ligação dissulfeto, maturação proteolítica, N- e O-Glicosilações, além de outras modificações pós-traducionais (Yin, Li *et al.*, 2007).

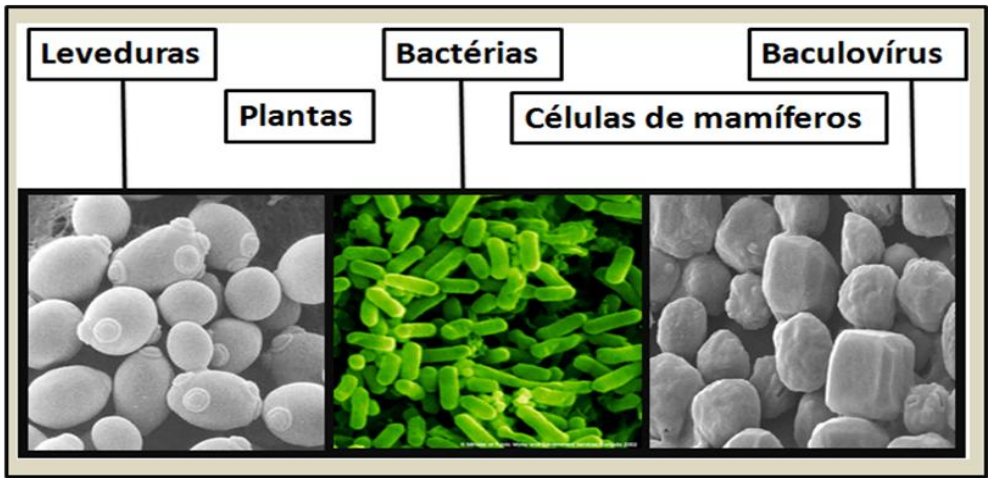


Figura 2. Ilustração de diferentes sistemas para expressão de proteínas heterólogas. (www.chemistryland.com; blog.poolcenter.com e www.vir.wur.nl/UK, acessado em setembro de 2010).

O Baculovírus é um sistema de expressão universalmente reconhecido como uma maneira versátil de produzir grandes quantidades de proteínas recombinantes, pois apresenta facilidade de manuseio, alto nível de expressão e capacidade de incorporar modificações pós-traducionais (Futatsumori-Sugai e Tsumoto, 2010). Porém, cada ciclo de síntese da proteína de interesse requer infecção em novas células de inseto. Portanto, esse sistema é limitante em relação aos sistemas procarióticos e as leveduras em termos de sua capacidade para fermentação contínua (Kato, Kageshima *et al.*, 2008).

Células de mamíferos também têm sido utilizadas como sistemas de expressão por apresentar vantagens que se assemelham às dos eucariotos, como o processamento da proteína de interesse, apesar de ser uma tecnologia cara e de difícil manuseio (Durocher e Butler, 2009).

3.1-Leveduras como sistemas de expressão

As leveduras são microrganismos unicelulares pertencentes à classe *Ascomycetes* ou *Basidiomycetes* (Ingold e Hutchínson, 1969) e têm sido exploradas pelo homem há milhares de anos. A manipulação destes microrganismos causou um grande impacto nas indústrias alimentícia (produção de pão, queijo, iogurte, vinho e cerveja), química e farmacêutica (produção de bioativos como enzimas, antibióticos e vacinas) repercutindo no desenvolvimento socioeconômico da humanidade (Torres e Moraes, 2002).

Pelo fato das leveduras compartilharem algumas características moleculares, genéticas e bioquímicas com os eucariotos superiores e por apresentarem perfil fermentativo que favorece ao processo em alta-escala, as leveduras demonstram grande potencial comercial como sistema de expressão de proteínas heterólogas. Atualmente, *Saccharomyces cerevisiae* e *Pichia pastoris* (figura 3) são as leveduras mais utilizadas como sistemas de expressão. Porém, leveduras não-convencionais vêm se estabelecendo como sistemas de expressão, a exemplo de *Kluyveromyces lactis* (figura 3), *Hansenula polymorph*a e *Yarrowia lipolytica (Yin, Li et al., 2007).*

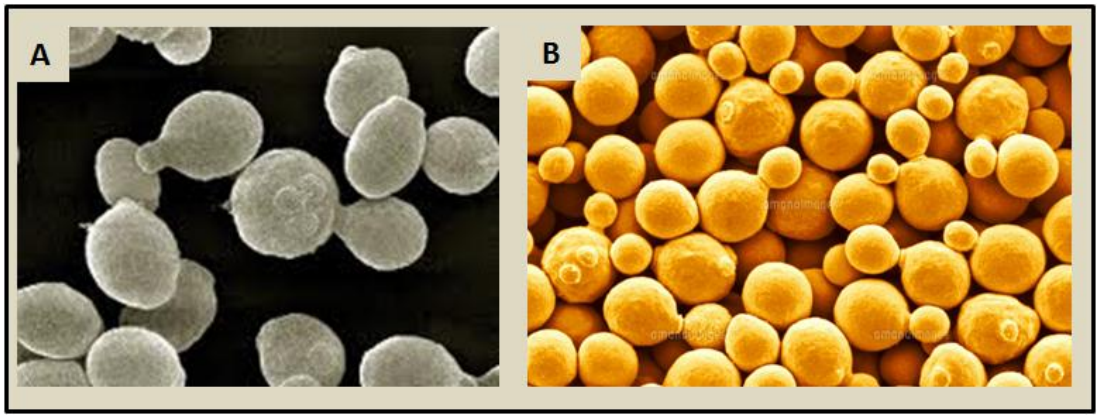


Figura 3. Ilustração das leveduras *P. pastoris* (A) e *Kluyveromyces lactis* (B) obtidas através da microscopia eletrônica de varredura. (http://www.visualsunlimited.com, acessado em 03 de fevereiro de 2011).

As leveduras também são mais facilmente cultivadas e manipuladas geneticamente em relação às células de mamíferos. As leveduras podem potencialmente produzir proteínas solúveis e realizar um eficiente processamento das proteínas recombinantes que foram submetidas a modificações pós-traducionais. Além das vantagens de cultivo simples, rápido crescimento e baixo custo, a maquinaria celular das leveduras permite que proteínas sintetizadas intracelularmente sejam secretadas para o meio extracelular. A segurança desses sistemas também garante a ausência de endotoxinas e oncogenes (Idiris, Tohda *et al.*, 2010).

No campo da pesquisa aplicada, as leveduras vêm se destacando como um forte candidato para a expressão de genes heterólogos de interesse biotecnológico. Esses microrganismos apresentam algumas vantagens para a produção de proteínas heterólogas, tais como a capacidade de secreção protéica em sua forma biologicamente ativa e modificações pós-traducionais necessárias para sua ótima atividade e estabilidade (Torres e Moraes, 2002).

Dentre as leveduras, *S. cerevisiae* destaca-se tanto pela sua grande aplicação industrial como também como um dos sistemas eucariotes mais conhecidos com aspectos biológicos e genéticos bem caracterizados, inclusive com genoma totalmente seqüenciado (Pérez-Martínez, De León-Rodríguez *et al.*, 2007). *S. cerevisiae* tem sido utilizada como sistema de expressão há mais de 25 anos, sendo classificada pelo FDA como microrganismo *Generally Recognized As Safe* (GRAS) e atendendo a um importante critério técnico para aplicações alimentícias e produção de biofármacos por engenharia genética. Esta levedura apresenta papel importante na preparação de pão, vinho, queijo e cerveja, sendo capaz de utilizar uma grande variedade de açúcares. Porém não é capaz de metabolizar a lactose, devido à ausência da enzima β-galactosidase e da lactose permease (Murphy e Kavanagh, 1999; Cebollero, Gonzalez-Ramos *et al.*, 2007).

A hiperglicosilação das proteínas secretadas e a produção de etanol durante o processo fermentativo são desvantagens encontradas na aplicação de *S. cerevisi*ae como sistema de expressão (Yin, Li *et al.*, 2007). A hiperglicosilação da proteína leva a imunogenicidade e a modificação conformacional da molécula inviabilizando sua aplicação como produto terapêutico. Enquanto que, a produção de etanol durante a produção da proteína de interesse pode ser tóxico para as células repercutindo na diminuição ou não formação do produto (Meta, Nakatake *et al.*, 2009).

Ao longo dos últimos anos, outras leveduras têm sido apresentadas como sistemas alternativos de expressão por apresentarem vantagens sobre a *S. cerevisiae*. Entre esses novos sistemas destacam-se *Pichia pastoris* e *Kluyveromyces sp*.

3.1.1- Pichia pastoris

A levedura metilotrófica *P. pastoris* é um dos principais sistemas para a expressão de proteínas recombinantes dentre uma variedade de procarióticos e eucarióticos. Até o ano 2000, mais de 200 proteínas de vírus, bactérias, fungos, animais, plantas e seres humanos foram expressos com sucesso em *P. pastoris* (Tabela 2). Esta levedura vem sendo descrita como um dos mais atrativos sistemas de expressão para a produção de proteínas recombinantes. Este fato está relacionado a algumas de suas particularidades, tais como facilidade para manipulação genética, pois é um

sistema similar a *E. coli* e a *S. cerevisiae*. No entanto permite melhor processamento da proteína, como a glicosilação, a formação de pontes dissulfeto e o processamento proteolítico; além de ser capaz de produzir grande quantidade de proteínas recombinantes, seja no interior da célula ou secretada para o meio extracelular (Cregg, Cereghino *et al.*, 2000).

Leveduras metilotróficas são aquelas capazes de utilizar o metanol como fonte de carbono. Estes microrganismos apresentam vias metabólicas interessantes às quais vêm sendo extensivamente estudadas para o desenvolvimento de vetores para expressão protéica. Dentre as leveduras metilotróficas dois gêneros merecem destaque: *Pichia spp*. e *Candida spp* (Houard, 2002).

A via metabólica para utilização do metanol parece ser a mesma para todas as leveduras metilotróficas e envolve a ativação de um único grupo de enzimas (Cereghino, Cereghino *et al.*, 2002). A álcool oxidase, presente no interior dos peroxissomos, é a primeira enzima dessa via metabólica sendo responsável pela oxidação do metanol a formaldeído gerando peróxido de hidrogênio. Dentro dos peroxissomos, o peróxido de hidrogênio, altamente citotóxico, é convertido a H_2O e O_2 pelas catalases peroxissomais. Porém, parte do formaldeído escapa dos peroxissomos para ser oxidado por duas desidrogenases citoplasmáticas resultando na síntese de formato e CO_2. O formaldeído restante é assimilado para formar componentes celulares por meio de uma via cíclica iniciada pela sua condensação com a xilulose 5-monofosfato (Xu_5-P), uma reação catalisada pela di-hidroxicetona sintase. Essa reação tem como produto o gliceraldeído 3-fosfato (GAP) e a dihidroxicetona (DHA), que, ao saírem do peroxissomo, entram na via citoplasmática que regenera a xilulose 5-monofosfato (Figura 4) (Cereghino e Cregg, 2000; Céreghino, 2006).

Na década de 80, a companhia *Phillips Petroleum*, juntamente com a *Salk Institute Biotechnology/Industrial Inc*. (SIBIA; La Jolla USA), avaliaram o potencial de *Pichia pastoris* como sistema de expressão para a produção de proteínas heterólogas e desenvolveram métodos de fermentação em meio definido contendo metanol para a manipulação da *P. pastoris* em culturas contínuas de alta densidade celular. Desde então, esta levedura vem sendo amplamente utilizada para produção de proteínas recombinante de interesse biotecnológico e acadêmico (Cereghino e Cregg, 2000).

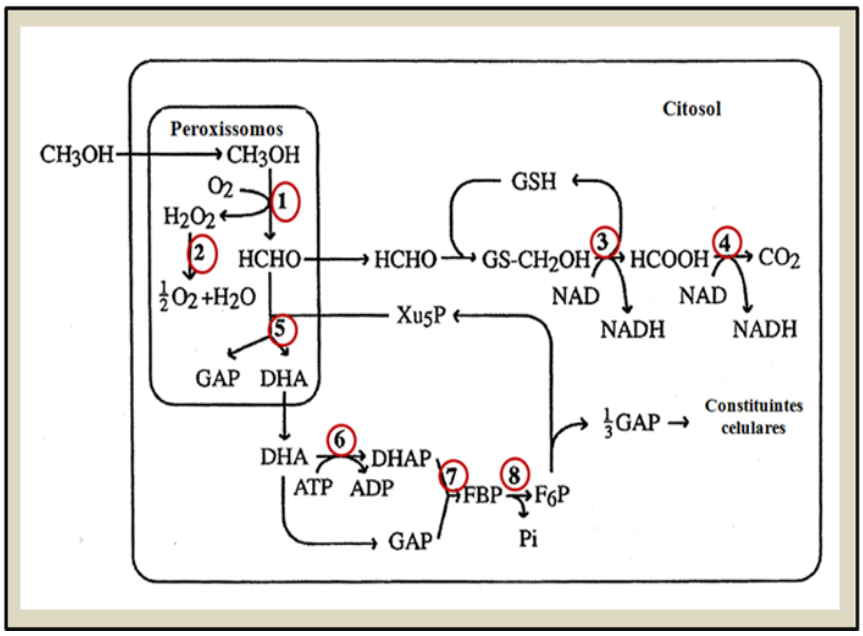


Figura 4. Desenho esquemático do metabolismo do metanol pela *P. pastoris*: 1) álcool oxidase; 2) catalase; 3) formaldeído deidrogenase; 4) formato desidrogenase, 5) di-hidroxiacetona sintase; 6) di-hidroxiacetona quinase; 7) frutose 1,6-bi-fosfato aldolase; 8) frutose 1,6-bisfosfatase. DHAP (di-hidroxiacetona fosfato), FBP (frutose bi-bifosfato), F_6P (frutose-6-fosfato), GS (S-formil glutationa), GSH (S-formil glutationa hidrolase) e DHA (di-hidroxiacetona). Fonte: Modificada de (Cereghino e Cregg, 2000).

As linhagens de *P. pastoris* podem ser divididas em quatro grupos: as selvagens (X-33, Y-11430), os mutantes auxotróficos que apresentam deficiência na enzima histidinol desidrogenase (GS115), os mutantes que são deficientes em genes envolvidos na utilização do metanol (KM71, MC 100-3) e os deficientes em proteases (SMD1163, SMD1165, SMD1168) (Jahic, Veide *et al.*, 2006).

P. pastoris é um sistema de expressão que apresenta promotores indutivos e constitutivos. Dentre os promotores que precisam de uma substância indutora para produzir a proteína recombinante, destacam-se o formaldeído desidrogenase ($_P$FLD1), álcool oxidase ($_P$AOX1) e a di-hidroxiacetona sintase ($_P$DHAS). O $_P$FLD1 é induzido pelo metanol e pela metilamina (fonte de nitrogênio), enquanto que $_P$AOX1e $_P$DHAS são induzidos pelo metanol. Gliceraldeído-3-fosfato desidrogenase (pGAP) e o fosfoglicerato quinase ($_P$PGK1) são promotores constitutivos no qual o gene de interesse é expresso tanto quando as células crescem em metanol, como em glicose ou glicerol. Nestes sistemas, ao contrário dos sistemas indutivos a fase de produção de biomassa e de expressão da proteína ocorre simultaneamente (Potvin, Ahmad *et al.*, 2010).

Tabela 2. Proteínas recombinantes produzidas por diferentes linhagens de *P. pastoris*.

Linhagem	Fenótipo (*)	Proteína recombinante	Tipo de expressão	Referência
P. pastoris SMD1168	Mut^+	E1e E2 do vírus da hepatite C	Intracelular	(Cai, Su *et al.*, 2010)
P. pastoris GS115	Mut^+	rhGCSF	Extracelular	(Apte-Deshpande, Rewanwar *et al.*, 2009)
P. pastoris GS115	Mut^+	Poligalacturonato liase alcalina	Intracelular	(Wang, Xu *et al.*, 2009)
P.pastoris X-33	Mut^+	Insulina	Extracelular	(Gurramkonda, Polez *et al.*, 2010)
P.pastoris X-33	Mut^+	Homeobox-1pancreática	Extracelular	(Li, Sun *et al.*, 2010)
P.pastoris X-33	Mut^+	Oncostatina M	Extracelular	(Kong, Mu *et al.*, 2009)
P. pastoris GS115	Mut^S	HBsAg	Intracelular	(Gurramkonda, Adnan *et al.*, 2009)
P. pastoris KM71H	Mut^S	Domínio III do envelope do vírus da dengue tipo 2	Extracelular	(Batra, Gurramkonda *et al.*, 2010)
P.pastoris KM71H	Mut^S	Lipase	Extracelular	(Arnau, Ramon *et al.*, 2010)

(*) Fenótipo de acordo com a utilização do metanol: Mut^S (lenta) e Mut^+ (positiva).

O sistema de expressão de proteínas heterólogas pela *P. pastoris* baseia-se principalmente no seu promotor $_P$AOX1 que é fortemente regulado pelo metanol. A álcool oxidase (AOX) (E.C.1.1.3.13) é a enzima mais importante envolvida no metabolismo do metanol nas leveduras metilotróficas. Sua atividade é fortemente regulada pela fonte de carbono tendo a glicose e o glicerol como repressores do sistema AOX enquanto que o metanol é um forte indutor. O sorbitol, o manitol, a alanina, ácido láctico e a trealose são relatados como não-repressores do sistema em questão (Xie, Zhou *et al.*, 2005).

Em *P. pastoris* existem dois genes que codificam a álcool oxidase: o AOX1 e o AOX2. O gene AOX1 é o responsável pela maior parte da atividade da álcool oxidase na célula. A expressão deste gene é controlada em nível transcricional e a presença do metanol é essencial para induzir os seus elevados níveis. Já o gene AOX2 é expresso em baixos níveis (Cereghino e Cregg, 2000). Em células cultivas em metanol aproximadamente 5% do RNA é do gene AOX1. A regulação desse

gene ocorre em duas etapas: 1) Repressão/derepressão: fase de formação de biomassa; 2) Indução: indução transcricional do AOX1 com a adição de metanol (figura 5).

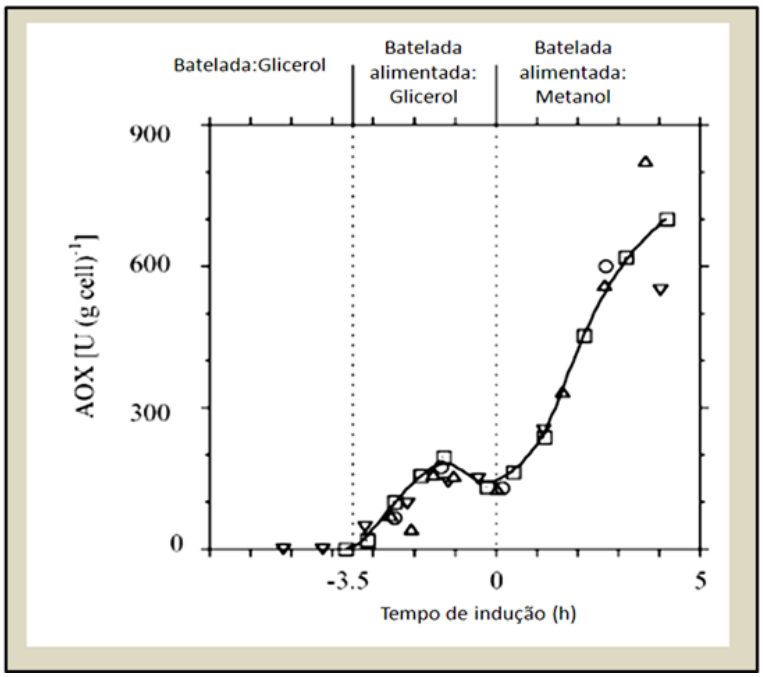


Figura 5. Atividade específica da Álcool oxidase 1 nas etapas de transição de repressão/derepressão e indução durante cultivo da *P. pastoris* (Jahic, Veide *et al.*, 2006).

P. pastoris, de acordo com sua capacidade de metabolizar o metanol, pode apresentar três fenótipos. No fenótipo Mut^{+} (Methanol Utilization Positive) o transformante possui os genes AOX1 e AOX2 funcionais e crescem em metanol em níveis similares ao selvagem. Enquanto que o fenótipo Mut^{S} (Methanol Utilization Slow) o metabolismo do metanol é dependente da transcrição do gene AOX2 pois o promotor AOX1 está reprimido, levando à crescimento mais lento em metanol. No fenótipo Mut^{-} (Methanol Utilization Negative) os genes AOX1 e AOX2 estão inativos, resultando na não metabolização do metanol por esta linhagem (Chauhan, Arora *et al.*, 1999). Adicionalmente, diversas metodologias de indução do $_{P}$AOX1 vêm sendo aplicadas para a otimização da produção de proteína heteróloga por *P. pastoris*. O $_{P}$AOX1 pode ser usado para aumentar os níveis de expressão da proteína recombinante. Além disto, ele pode ser "desligado" usando o glicerol ou a glicose como fonte de carbono. Assim estas fontes de carbono podem ser utilizadas durante a formação de biomassa (Daly e Hearn, 2005). Adicionalmente, através da engenharia metabólica e da bioinformática, o genoma e o metaboloma de *P. pastoris* vem sendo estudado, demonstrando que *P. pastoris* GS115 apresenta um genoma de 9,43 Mb, 4 cromossomos e 5313 ORF (Mattanovich, Graf *et al.*, 2009).

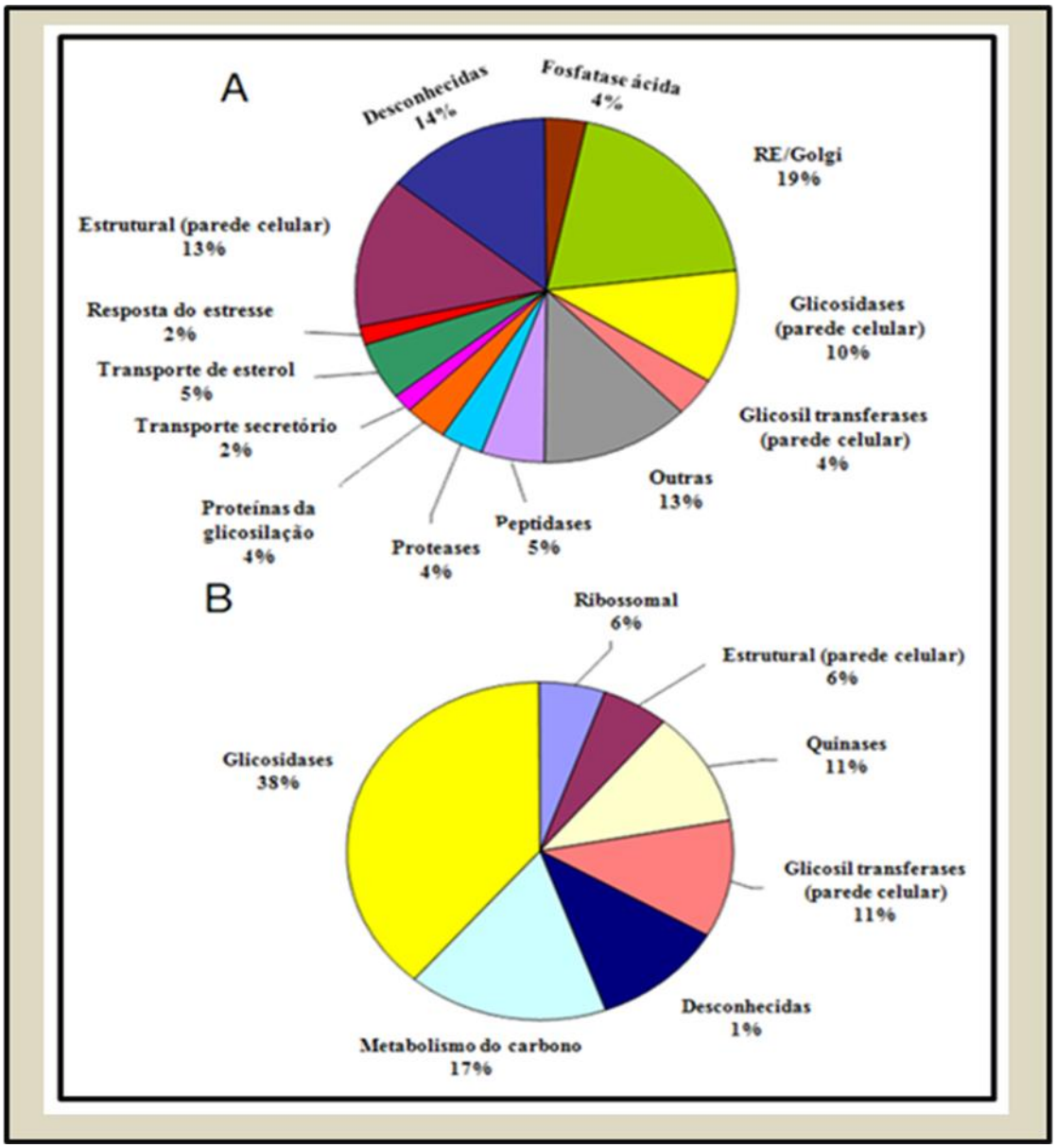


Figura 6. Categorização do secretoma de *Pichia pastoris*: (A)Predito e (B)Secretoma detectado. Proteínas sem homologia com a *S. cerevisiae* estão classificadas como desconhecidas (Mattanovich, Callewaert *et al.*, 2009).

Estudos computacionais predizem a secreção de aproximadamente 200 proteínas pela *P. pastoris*. Porém, estas predições apresentam algumas limitações, como o meio em que a levedura está sendo cultivada. Dependendo do meio de crescimento algumas proteínas ficam retidas em compartimentos extracelulares, não sendo secretadas (Sohn, Graf *et al.*, 2010). Ou seja, a composição do secretoma depende das condições de crescimento do microrganismo. Mattanovich e colaboradores (2010) estudaram o perfil secretômico da *P. pastoris* e, através de uma avaliação *in silico*, correlacionaram os dados usando ferramentas da bioinformática. Conforme ilustrado na Figura 6, os resultados acerca do perfil secretômico da *P. pastoris* compartilham com a comunidade

científica o conhecimento sobre a excreção protéica desta levedura em condições de crescimento específicas.

3.1.2- Kluyveromyces lactis

Kluyveromyces lactis foi inicialmente chamada de *Saccharomyces lactis,* e vem sendo estudada desde 1960 (Herman e Halvorson, 1963). O gênero *Kluyveromyces* é composto por espécies de leveduras unicelulares, nucleadas e sem motilidade. Classificam-se como *Ascomycetos* e podem se reproduzir sexuadamente por esporulação e assexuadamente através de brotamento ou fissão (Bottger, Barnett *et al.*, 2000). Podem ser encontrados em uma grande variedade de queijos, insetos e no solo (Barba, Beolchini *et al.*, 2001; Alvarez-Martin, Floreza *et al.*, 2007). Dentre as espécies desse gênero a *K. lactis, K. marxianus* e *K. fragilis* destacam-se como leveduras não-convencionais que têm despertado grande interesse industrial e biotecnológico (Becerra, Baroli *et al.*, 2000; Merico, 2004; Pecota, Rajgarhia *et al.*, 2007).

O gênero *Kluyveromyces* compartilha da mesma subfamília que *S. cerevisiae*, família *Saccharomycetoideae*. A capacidade de assimilar uma variedade de fontes de carbono e a sua classificação como leveduras fermentadoras facultativas do tipo "Crabtree-negativa", são particularidades fisiológicas de *Kluyveromyces* não encontradas em *S. cerevisiae* (Bussereau, Casaregola *et al.*, 2006). A *Kluyveromyces* apresenta a vantagem de não produzir etanol em aerobiose, o que possibilita grande aplicabilidade, em escala industrial, para produção de proteína heteróloga (Breunig, Bolotin–Fukuharab *et al.*, 2000).

K. lactis é uma das espécies mais estudadas do gênero *Kluyveromyces*. Tem como seu habitat o leite e seus derivados (Lodi, 2004), utiliza a via pentose fosfato para metabolizar a glicose (Blank, Lehmbeck *et al.*, 2005) e apresenta excelentes características fermentativas, notável capacidade secretória e disponibilidade para sistemas vetoriais (Becerra, Gonzalezsiso *et al.*, 2006). Estima-se que seu genoma apresenta um tamanho de 12Mb com apenas seis cromossomos e DNA mitocondrial circular (Breunig, Bolotin–Fukuharab *et al.*, 2000).

Os estudos com *K. lactis* antecederam a década de 60 (Breunig, Bolotin–Fukuharab *et al.*, 2000), mas apenas em 1990 foi possível identificar essa levedura como fonte primária de β-galactosidase utilizando-a para a transformação enzimática do soro do leite rico em lactose (Castillo, 1990). Assim, observou-se que essa espécie utiliza a lactose como fonte de carbono e de energia (Becerra, Gonzalezsiso *et al.*, 2006). Essa espécie vem recebendo maior interesse biotecnológico devido às suas características atrativas como a alta taxa de secreção de proteínas

(interleucina 1β, proquimosina, galactosidase, albumina sérica humana e a glicoamilase), excreção protéica no meio de cultivo e não no espaço periplasmático, seus "status" GRAS, sua genética molecular acessível (estabilidade como sistema vetorial) e sua excelente característica fermentativa (Schaffrath, 2000; Merico, 2004; Martínez-Villaluenga, Cardelle-Cobas *et al.*, 2008). Dessa maneira o uso da *K. lactis* vem competindo com a da *S. cerevisiae* como hospedeiro para produção de proteína heteróloga (Alteriis, Silvestro *et al.*, 2004).

Em 2004, Becerra e colaboradores realizaram uma análise comparativa do perfil transcricional da *S. cerevisiae* e da *K. lactis* ambas cultivadas em mesmo meio de cultivo. Este estudo revelou grande correlação entre os genes relacionados com a mitose e a biogênese da membrana celular em ambas as leveduras. Uma discreta correlação foi observada entre os genes envolvidos no estresse oxidativo e a maior diferença foi verificada nos genes envolvidos com o metabolismo dos carboidratos e com as funções respiratórias.

A habilidade de usar a lactose como fonte de carbono distingue a *K. lactis* das demais leveduras (Schaffrath, 2000). O metabolismo da lactose pela *K. lactis* está relacionado à presença dos genes LAC4 e LAC12 os quais codificam a β-galactosidase e a lactase permease, respectivamente (Breunig, Bolotin–Fukuharab *et al.*, 2000). O transporte de lactose intracelular pela levedura depende de alguns fatores, como afinidade da membrana celular, temperatura e pH. Nessa espécie, não há alta afinidade pela lactose e o transporte de açúcar é induzido pela galactose. A atividade da β-galactosidase não é reprimida pela glicose, consequentemente a afinidade da membrana pela lactose aumenta quando a lactose for hidrolisada em galactose e glicose (Becerra, Baroli *et al.*, 2000). Devido à facilidade de indução e repressão dos promotores LAC por fontes de carbono baratas e de fácil acesso, este sistema tem sido usado para produção de proteínas heterólogas, e *K. lactis* como hospedeiras dos genes de interesse biotecnológico (Rubio-Texeira, 2005).

K. lactis apresenta metabolismo oxidativo suportado pela glicólise, pela via das pentoses-fosfato e pela respiração mitocondrial (Gonzalez-Siso, Ramil *et al.*, 1996). Apesar da *K. lactis* ser capaz de fermentar a glicose, ela não consegue crescer em condições restritas de oxigênio (Fontanesi, Viola *et al.*, 2006).

O sistema álcool desidrogenase de *K. lactis* também representa um sistema de expressão com potencial utilização, sendo composto por quatro genes estruturais, dos quais dois codificam

isoenzimas citoplasmáticas (K1ADH1 e K1ADH2) e dois isoenzimas mitocôndriais (K1ADH3 e K1ADH4). As isoenzimas citoplasmáticas, além de apresentarem atividade semelhante à isoenzima álcool desidrogenase de *S. cerevisiae* (SADHI), têm perfil fermentativo e seus genes são expressos em células cultivadas em meio contendo glicose. Por outro lado, os genes das isoenzimas mitocondriais são regulados através da concentração de etanol no meio de cultura (Breunig, Bolotin–Fukuharab *et al.*, 2000; Brisdelli, Saliola *et al.*, 2004). O controle das condições extracelulares e a modificação de diferentes parâmetros no meio de cultura influenciam significativamente a produção de proteínas heterólogas, possibilitando a utilização deste sistema de forma mais eficiente (Rubio-Texeira, 2005).

Dentre diversas aplicações de *K. lactis* destacam-se a sua utilização na produção industrial de enzimas homólogas e produção de leite com menor concentração de lactose; na biorremediação; conversão de resíduos da indústria de queijo em produto viável; e na produção de galactooligossacarídeos pela indústria alimentícia (Merico, 2004; Rubio-Texeira, 2005). Avanços da engenharia genética têm permitido ampliar as utilizações dessa levedura sendo, também, manipuladas para a produção em alta-escala de proteínas heterólogas (Gellissen e Hollenberg, 1997; Rubio-Texeira, 2005), podendo ser aplicada para o desenvolvimento de medicamentos e vacinas terapêuticas contra algumas patologias.

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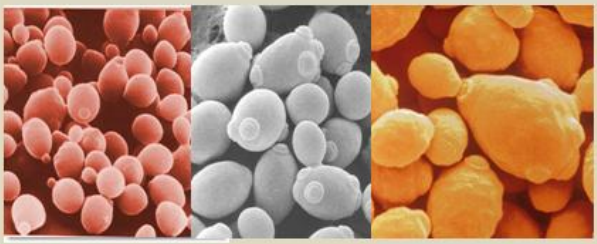


Objetivos

"Há uma força motriz mais poderosa que o vapor, a eletricidade e a energia atômica: a vontade. " (Albert Einstein)

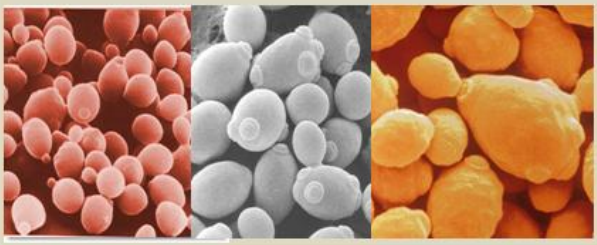
Objetivos

Objetivos geral

Utilizar as leveduras *Pichia pastoris* e *Kluyveromyces lactis* como sistemas de expressão visando produção de proteínas de interesse médico e biotecnológico.

Objetivos específicos

- Avaliar a influência da suplementação com elementos traços na produção da frutalina recombinante pela *P. pastoris* KM71H através do processo de batelada alimentada;
- Clonar e expressar o gene E7 do HPV16 em *P. pastoris* GS115;
- Avaliar a influência da fonte de carbono e do estresse iônico na atividade da enzima β-galactosidase pela *K. lactis* DSM3795.



Capítulo I

"Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos." (Isaac Newton)

Capítulo I

Submetido para Journal of Biotechnology

Cloning and expression of the E7 gene from Human papillomavirus type 16 in Pichia pastoris GS115

Cloning and expression of the E7 gene from Human papillomavirus type 16 in *Pichia pastoris GS115*

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Absctract

Pichia pastoris is a methylotrophic yeast that can be genetically engineered to express proteins for industrial use, as well as for medical purposes. Human Papillomavirus (HPV) is the main etiological cause of cervical neoplasm. Biological malignancy is produced by expression of E6 and E7 oncogenes, but the mechanism of viral protein interaction with human proteins is poorly understood. In this way, due to the difficult to maintain HPV in tissue culture, cloning and expression of these genes represents a possibility of advances in virus studies. This work reports the cloning and functional expression of E7 gene from HPV16 in *P. pastoris* GS115 using Agilent 2100 Bioanalyzer lab-on-a-chip. E7 gene was correctly cloned in yeast vector and integrated into yeast genome, being able to produce 218.9 $mg.L^{-1}$ of recombinant protein from HPV16 with molecular weight of 27KDa.

Keywords: Human Papillomavirus, E7 oncoprotein, *Pichia pastoris*, Agilent 2100 Bioanalyzer.

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1.Introduction

Human Papillomavirus (HPV) is a small DNA virus that shows a tropism for squamous epithelium. Over 100 types of HPV have been identified and around one-third of these infect the

squamous epithelia of the genital tract. Approximately 70–75% of genital warts are caused by HPV16 and 18 types (Piccioli, Mckee *et al.*, 2010) ant their persistence in the infection increase the presence of these types to 99.7% of carcinoma of the cervix (Zur Hausen, 2002) .

The oncogenic effect, however, is dependent on E6 and E7 genes of the high-risk HPV types, and their continuous expression seems to be necessary for transformation and maintenance of a neoplastic or dysplastic phenotype of the cells (Bodily e Laimins, 2011). E6 and E7 proteins from HPV16/18 have been identified as responsible for oncogenic transformation of cells including human keratinocytes after transfection of viral DNA (Wang, Sima *et al.*, 2010). These proteins are consistently expressed in HPV-associated cervical cancers and interact with cellular proteins involved in cell cycle control and DNA repair. E7 exerts its oncogenic function by modulating cellular proliferation and apoptosis regulating pathways, mediated by its interaction with several nuclear and cytoplasmic regulatory proteins, resulting in functional inactivation (Zur Hausen, 2009).

Despite the complex biology of HPV, substantial progress has been made in vaccine development, mainly for prophylaxis (Bonanni, Boccalini *et al.*, 2009). E6 and E7 proteins are considered key targets in the development of new diagnostic and therapeutic modalities for this disease (Mabruk, 2008). However, the maintenance of HPV *in vitro* has been reported as difficult and increased the interest of cloning HPV genes into expression systems (Chinami, Sasaki *et al.*, 1994).

Pichia pastoris is a methylotrophic yeast that can be genetically engineered to express proteins for industrial use (Zhang, Wang *et al.*, 2008). Viral proteins, Hepatitis B core and dengue type II antigens have been expressed in *P. pastoris* (Rolland, Gauthier *et al.*, 2001; Batra, Gurramkonda *et al.*, 2010) that uses the methanol-induced alcohol oxidase (AOX1) promoter to control the expression of alcohol oxidase. This enzyme is involved in the first step of methanol metabolism, being strongly induced by methanol (Files, Ogaw *et al.*, 2001). Methylotrophic yeasts

are now widely recognized as efficient expression systems for many heterologous proteins that combine easy genetic manipulation and convenient scale-up with the ability of eukaryotic protein processing, folding, and post-translational modifications (Potvin, Ahmad *et al.*, 2010).

The expression of HPV16 E7 gene has so far been successfully reported in *E. coli* and in insect cell expression systems (Fernando, Murray *et al.*, 1999; Fiedler, Campofernandez *et al.*, 2006). However, both expression hosts show their limitations uncorrected folding and low expression levels, high production cost, respectively. The yeast *Schizosaccharomyces pombe* was used to produce HPV16 E7, but phosphorylation, a post-translational modification, was detected on protein structure (Braspenning, Manetti *et al.*, 1997).

Conventional technology to cloning and expression genes steps analysis requires elevate manipulation time, higher sample concentration, higher medium volume and consequently elevate cost. Recently, a new technology has been related to nucleic acids and protein analyses, lab-on-a-chip. Many advantages including improve data precision and reproducibility, short analysis times and minimal sample consumption make it an attractive tool to systems expression study (Apte-Deshpande, Somani *et al.*, 2009).

This is the first study that reports the successful cloning and expression of the HPV16 E7 gene by *P. pastoris* GS115 strain using a lab-on-a-chip system for steps certification.

2.Materials and Methods

2.1. Microorganisms

Escherichia coli DH5α was used as host as well as *Pichia pastoris* GS115 (*his4*) strain, purchased by Invitrogen (San Diego, CA). pGEM-T Easy Vector System (Promega, USA) was used as cloning vector and pPICZB (Invitrogen - San Diego, CA) was used to compose the expression system. Bacterial growth was performed in LB broth (10g/l tryptone, 5g/l yeast extract, 10 g/l NaCl) and low-salt LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). *P. pastoris* GS115 strain was

cultivated in YPD (1% yeast extract, 2% peptone and 2% dextrose) or BMGH (0.1M potassium phosphate, 4 x 10^{-5}% biotin, 1.34% YNB, and 1% glycerol, pH 6.0).

2.2. E7 gene cloning procedures

The PCR reaction was made using 20 ng DNA (pET28a containing E7 gene from HPV16); 1µl specific primers (10 pmol) forward primer E7 *EcoR*I - *GAA TTC* ATG CAT GGA GAT ACA CC and reverse primer E7 *Not*I - *GCG GCC* GCT GGT TTC TGA GA; 0.5 µl Platimun Taq (Invitrogen); 1.5 µl $MgCl_2$ 50mM, 1 µl Taq buffer and ultrapure water for 10 µl total reaction. Amplifications were performed in Rotor Gene 3000 (Applied Biosystems, USA) with the following settings: 95°C for 1 min, followed by 40 cycles at 95°C for 30 s, 66°C for 20s and 72°C for 1 min, and ending with 72°C for 1 min. The amplified products were observed and quantified using Agilent 2100 Bioanalyzer. PCR product was purified with QIAquick PCR Purification Kit (QIAgen, Hilden, Germany) and subsequently cloned into pPICZB, using T_4 DNA ligase (Invitrogen), following the manufactory instructions. The vector was transformed in *E. coli* DH-5α competent cells by heat shock and incubated over night in low-salt LB media containing 25µg/mL zeocin, under orbital shaker at 37^{o}C. Recombinant colonies were selected by zeocin-resistance. The plasmids were extracted for alkaline lysis according to (Sambrook, Fried *et al.*, 1989). *EcoRI* e *NotI* enzymes were used to release E7 gene from pPICZB-E7 to follow the cloning steps.

2.3. P. pastoris integration assay

Midi-prep plasmid protocol was used to obtain high amount of pPICZB/HPV16 E7. The plasmid was digested with *Sac*I, restriction enzyme that has unique restriction site in pPICZB, allowing vector linearization for efficient integration into *P. pastoris* strain. The recombinants plasmids extracted and linearized were run on Agilent 2100 Bioanalyzer. Digested plasmid was purified to remove enzymes and enzymatic buffer using MinElute Reaction Cleanup Kit (QIagen, USA). LiCl transformation method (Sambrook, Fried *et al.*, 1989) was used to transform *P. pastoris* cells with shuttle vector pPICZB. Recombinant colonies were selected on YPD plates containing

Zeocin (100µg/ml) after 2 days incubation at 30ºC. To confirm the integration, a colony PCR was performed in Gradient Eppendorf Mastercycle (USA) using a single colony ressuspend in water and treated with lyticase enzyme (Invitrogen). AOX1 primers were used in the PCR reaction, programmed for 40 amplification cycles running with 95ºC denaturation step for 1 min, 55ºC annealing step for 1 min and 72ºC extension step for 1 min, including an initial denaturation step of 2 min and final extension step of 7 min. The amplicons were analyzed by microchip electrophoresis on Agilent 2100 BioAnalyzer with DNA 7500 assays.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolation using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was verified in Agilent 2100 BioAnalyzer. Amplicons from total RNA and E7 cDNA fragments were analyzed by microchip electrophoresis.

RNA was reverse transcribed into cDNA using Superscript II (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified by PCR using E7 forward primer 5'- ATG CAT GGA GAT ACA CCT ACA - 3' and reverse primer 5' – TTA TGG TTT CTG AGA ACA GAT G- 3'. The PCR reaction was made using 20ng cDNA; 1µl specific primers (10 pmol) primer, GoTaq buffer (Promega) and ultrapure water for 12.5µl total reaction. Amplifications were performed in Gradient Eppendorf Mastercycle (USA) with the following settings: 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30s and 72°C for 30s, and ending with 72°C for 5 min.

2.5. DNA sequencing

Determination of E7 gene sequence was achieved using a fluorescent-based dideoxy sequencing method using DYEnamic™ ET Dye Terminators in automated DNA sequencing system (MegaBACE 750, GE, Life Science – USA).

The E7 gene was amplified by PCR using forward primer AOX1 (5'-GACTGGTTCAATTGACAAGC-3') and Reverse primer AOX1 (5'-GCAAATGGCATTCTGACATCC-3'). The PCR reaction was made using 20ng cDNA; 1µl specific primers (10 pmol) primer, GoTaq buffer (Promega) and ultrapure water for 12.5µl total reaction. Amplifications were performed in Gradient Eppendorf Mastercycle (USA) with the following settings: 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 55°C for 30s and 72°C for 30s, and ending with 72°C for 5 min.

The sequences of the PCR-amplified products were treated with Exonuclease I (EXO) and Shrimp Alkaline Phosphatase (SAP) enzymes and submitted to dideoxy sequencing in MegaBACE 750 (GE, Life Science), following the manufacturer instructions. The basecalling raw data was analyzed by Sequence Analyzer (GE, Life Science) and submitted to BLASTN (NCBI) to compare the similarity of E7 gene cloned and NCBI databank.

2.6. Protein expression

HPV16 E7 expression was evaluated in baffled-flask, by inoculating a single colony into 50 ml of BMGH to grow at 30°C in a shaking incubator (250rpm) during 24 hours. The cells were harvest by centrifugation at 3000 x g for 15 minutes at 4ºC and ressuspend to an OD_{600} of 1.0 in BMMH (0.1M potassium phosphate, 4 x 10^{-5}% biotin, 13.4% YNB, and 0.1% methanol, pH 6.0) medium to induce expression. To overcome oxygen limitations, induction was carried out in 500 ml baffled-flasks filled with 50 ml of culture medium and covered with two layers of sterile gauze. Every 24 hours, 100% methanol to a final concentration of 1.0 % methanol (v/v) was added to maintain induction. Induction phase occurred during 72 hours at 25°C.

To optimization the heterologous protein extraction chemical and mechanical break methodology were performed. Chemical lysis was carried out by Trizol reagent (Invitrogen) according to the manufacturer's instructions. In mechanical break the cells were lysates using breaking buffer (50 mM sodium phosphate, 1 mM EDTA, 1mM PMSF and 5% glycerol). Equal

volume of acid-washed glass beads (425-600 mm, Sigma) were added before the beginning of 10 cycles of the vortex (40 seconds) and ice (20 seconds). The lysates were centrifuged at maximum speed for 10 minutes at +4°C and the clear supernatant was filtered through 0.22µm pore size filters. Then, the soluble protein was analysed. To detect the insoluble recombinant protein, urea 10M was added to the pellet and incubated for 30 minutes at 27°C. The mixed solution was centrifuged at maximum speed for 10 minutes at +4°C. Proteins extracted were analysed by microchip electrophoresis.

2.7. Electrophoresis on Agilent 2100 Bioanalyzer platform

Total RNA quality was analyzed by determining UV 260/280 absorbance ratios and RNA size distribution was determined by RNA Nano LabChips 6000 (Agilent Technologies, Santa Clara, CA, USA) using Agilent 2100 Bioanalyzer. The software total RNA electrophoresis generates the RNA integrity number (RIN). It has been designed to provide unambiguous assessment of RNA integrity. Then, for samples analyzed, a RIN was generated for each Bioanalyzer trace using 2100 Expert Software, which the maximum RIN score is 10.

Agilent DNA 1000 kit was designed for the sizing and quantization of double-stranded DNA fragments from 25 to 1000 base pairs. Intracellular protein detection was performed by Agilent Protein 80 kit, which enables the separation of proteins in the 5 - 80 kDa range. All samples were prepared and analyzed using manufacturer's protocol. The data was represented in an electropherogram by plotting fluorescence intensity units (FU) versus retention time in seconds.

3. Results and Discussion

3.1. E7gene cloning

HPV16 E7 gene was cloned in frame with the C-terminal region encoding the polyhistidine tag into pPICZB expression plasmid. *P. pastoris* cells were transformed with the vector constructed

(Figure 1) and the coding sequence was maintained under the control of the AOX1 promoter, which is tightly repressed under glucose growth conditions but can be induced over 1000-fold under methanol growth conditions (Cregg, Cereghino *et al.*, 2000).

The colonies were selected on YPD medium containing Zeocin. Several colonies were re-streaked for purification and analyzed by colony PCR to confirm the presence of the E7 gene in the yeast genome. E7 gene amplification was performed using specific primers to obtain an amplicon of 306 bp as PCR product (Figure 2). Additionally, AOX1 primers were used in the PCR reaction, to confirm the integration of E7 gene into *P. pastoris* genome. The figure 2 shows a DNA fragment of 520 bp obtained as sum of gene size and 214 bases from AOX1 gene. The amplicon obtained demonstrates the E7 gene integrated into yeast genome.

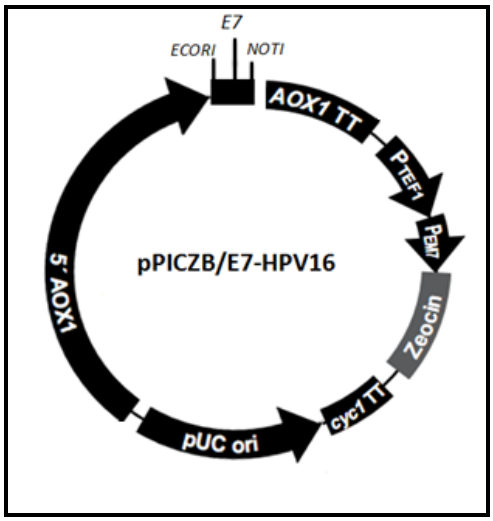

Figure 1. The expression vector used in this study: 5' AOX1 promoter region, ECORI restriction enzyme site, HPV16 E7 gene, NotI restriction enzyme site, 3' AOX1 transcription terminator region, fragment containing TEF1 promoter, EM7 promoter, Zeocin resistance gene, CYC1 transcription termination region and pUC origin.

3.2. DNA sequencing

Recombinant clones of pPICZB/HPV16.E7 were confirmed by DNA sequencing. The sequence analysis showed that the cloned gene completely matches with the known DNA sequence of the E7

gene of the HPV16 (GENE ID: 1489079 E7, PubMed database) as depicted in Figure 3A. The nucleotides sequence of the pPICZB/HPV16.E7 was translated in protein using CLC Main workbench 5.1 Software for further analysis. The amino acid sequence obtained was aligned with HPV16 E7 protein (PROTEIN ID: 1489079) in CLC Main workbench 5.1 Software using Basic Local Alignment Search Tool (BLAST), NCBI database. The alignment showed 98 amino acids and 100% of the homology of the pPICZB/HPV16.E7 with native protein confirming the HPV16 E7 gene was correctly cloned into *P. pastoris* genome.

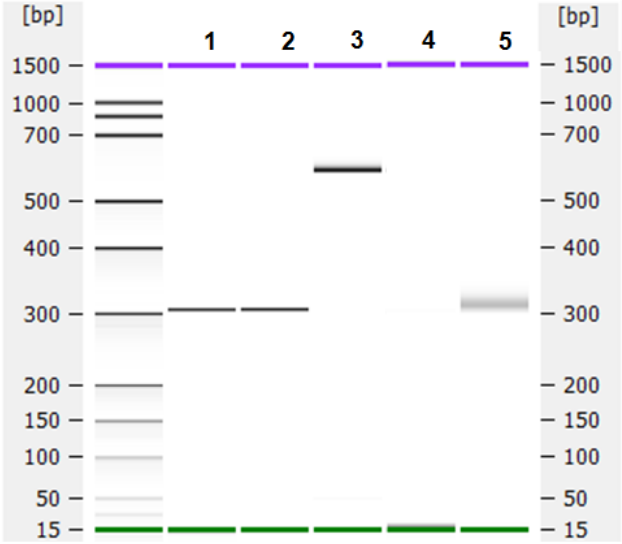

Figure 2. Agilent 2100 Bioanalyser gel, using DNA 1000 kit. Lanes: 1)E7-HPV16 PCR colonies, 2) cDNA E7-HPV16, 3) 5'AOX-E7 3'primers amplification after cDNA synthesis , 4 and 5) controls negative and positive, respectively.

3.3. RNA isolation and cDNA synthesis

Positive clones were grown in 50 mL of BMGH medium to accumulate biomass and then shifted to BMMH medium containing methanol as a sole carbon source. The methanol in this medium also functions as inducer, driving expression of the E7 protein under the AOX1 promoter. After growth in methanol-based medium, total RNA was isolated by Trizol reagent and its integrity was analyzed as an important step for transcriptome study (Harrington, Winther *et al.*, 2009).

Agilent 2100 Bioanalyzer together with RNA Nano LabChips were used in order to standardize the process of RNA integrity interpretation as high quality tool for RNA quality assessment.

Figure 4 shows the electropherogram of *P. pastoris* pPICZB/HPV16.E7 total RNA. Electropherogram indicated 3.4 ng/µL of total RNA in the sample and indicating the effectiveness of total RNA quality control step with RIN score at 7.6. This result reveals a good integrity in total RNA samples with minimum degradation. Microarray expression profiling study using Agilent 2100 Bioanalyzer referred that RIN scores lower than 7.0 are related to degrade RNA samples (Harrington, Winther *et al.*, 2009). After the total RNA integrity was confirmed, cDNA synthesis was conducted.

```
(A)
1489079 E7          1  TCATGCATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAGACAA 60
pPCIZB/HPV16 E7     1  TCATGCATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAGACAA 60

1489079 E7         61  CTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGAGGATGAAATAGATG 120
pPCIZB/HPV16 E7    61  CTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGAGGATGAAATAGATG 120

1489079 E7        121 GTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAATATTGTAACCTTTTGTTGCA 180
pPCIZB/HPV16 E7   121 GTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAATATTGTAACCTTTTGTTGCA 180

1489079 E7        181 AGTGTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTCGTACTTTGG 240
pPCIZB/HPV16 E7   181 AGTGTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTCGTACTTTGG 240

1489079 E7        241 AAGACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCA 296
pPCIZB/HPV16 E7   241 AAGACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCA 296

(B)
1489079 E7          1 MHGDTPTLHEYMLDLQPETTDLYCYEQLSDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCK 60
pPCIZB/HPV16 E7    1 MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCK 60

1489079 E7         61 CDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP 98
pPCIZB/HPV16 E7    61 CDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP 98
```

Figure 3. (a) Alignment of DNA sequence of pPICZB/HPV16.E7 clone with the published E7 DNA sequence (GENE ID: 1489079 E7, PubMed database). (b) Alignment of the amino acid sequence of the pPICZB/HPV16.E7construct with published E7 amino acids sequence using CLC Main workbench 5.1 Software.

cDNA synthesis is one of the fundamental methodology in molecular biology, and most of our knowledge about transcripts and specific proteins expression has been provided following this

analysis (Harbers, 2008). In this study the cDNA synthesis obtained from total RNA samples was detected in lab-on-a-chip system (Figure 2, lane 2). Agilent 2100 Bioanalyser gel shows a fragment of 306 bp related to cloned HPV16 E7 cDNA, demonstrating the effectiveness transcription of the target gene by *P. pastoris*.

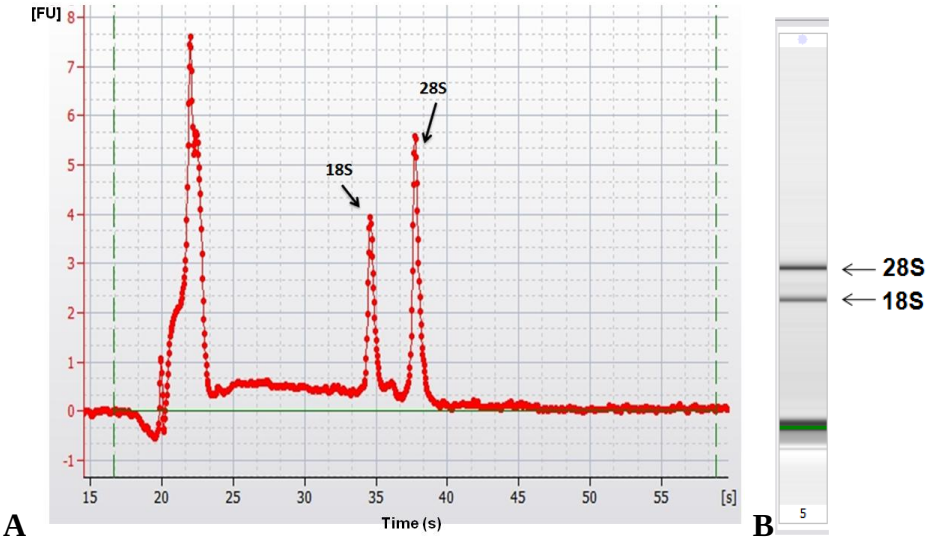

Figure 4. Agilent 2100 Bioanalyzer total RNA analysis after Trizol isolation. The data was represented in an electropherogram by plotting fluorescence intensity units (FU) versus retention time in seconds: A) Electropherogram shows the lower marked, 18S and 28S ribosomal peaks, B) Agilent 2100 Bioanalyzer gel with 18S and 28S ribosomal bands underline.

3.4. Protein expression

To examine the intracellular recombinant protein expressed by *P. pastoris* GS115 pPICZB/HPV16.E7, this strain was grown in BMMH medium at 25ºC during 96 hours. The behaviour of recombinant strain was similar to the control strain *P. pastoris* GS115 grown in the same conditions (data not shown).

In our study, electropherogram analysis (figure 5) shows a peak at 27 seconds in migration time, which refers to 27kDa size. This peak was visualized only in recombinant clones cultivation, suggesting that E7 from HPV16 is being expressed. The molecular weight calculated for E7 protein

based on the amino acid sequence (98 residues) is around 12 kDa. However, the literature has shown that different expression systems can express the same protein in different size. *E.coli* has been related to express E7 protein from HPV16 with apparent molecular weight range of the 16-20 kDa (Braspenning, Manetti *et al.*, 1997; Fiedler, Campofernandez *et al.*, 2006). Whereas, in human osteosarcoma cells, the HPV16 E7 protein was detected with 19 kDa (Fiedler, Campofernandez *et al.*, 2006).

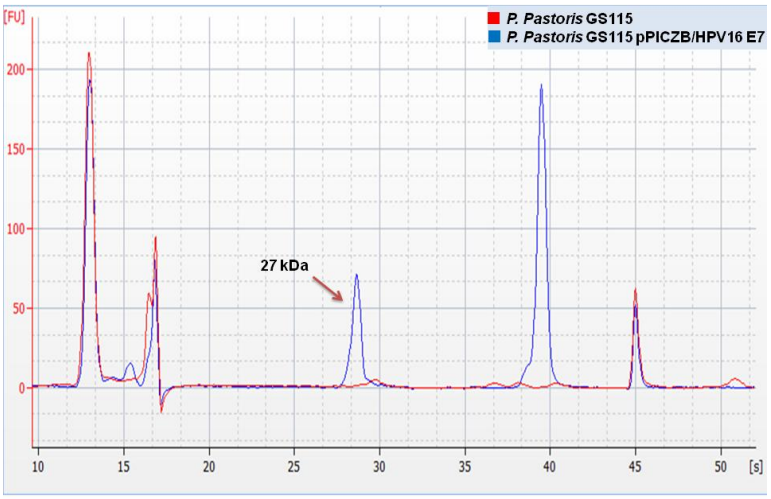

Figure 5. Electropherogram comparing a recombinant protein expression by *P. pastoris* GS115 pPICZB/HPV16.E7 (blue) with yeast negative control (red) after Methanol (1%) induction at 25°C in shaker-flakers. Fluorescence units (FU) versus migration time in seconds(s).

Otherwise, utilization of *P. pastoris* strains as heterologous expression system has been cited to produce recombinant protein with larger molecular mass than native protein (Boraston et al 2001). This behaviour could be attributed to three points:

1) yeast ability to perform many post-translational modifications (Cereghino e Cregg, 2000): *N*-acetylatilation effect, as observed for Hepatitis B core antigen (HBcAg) expressed in *P. pastoris* (Rolland, Gauthier *et al.*, 2001) and phosphorylation events observed for HPV16 E7 produced in *S. pombe* causing molecular weight changes; 2) Homodimeres formation: *P. pastoris* is able to

perform dimerization during protein processing while in *E. coli* protein folding processing may occurred incompletely. Comparative study between both systems producing the same proteins showed that proteins until 20 KDa produced by *Pichia* can be larger than expressed by *E. coli* (Lueking, Holz *et al.*, 2000); 3) Profile migration in Agilent Bioanalyzer run: Glycoproteins analyzed by Agilent 2100 Bioanalyzer migrate at different known molecular weight, probably due to the glycan attachment that can keep the protein assuming a rod shape, also changing the charge-to-mass ratio (Kelly e Barthmaier, 2003). In our study, further analyses are needed to evaluate the structures of the recombinant E7 from HPV16 to understand post-translational modifications that occurred on the target protein.

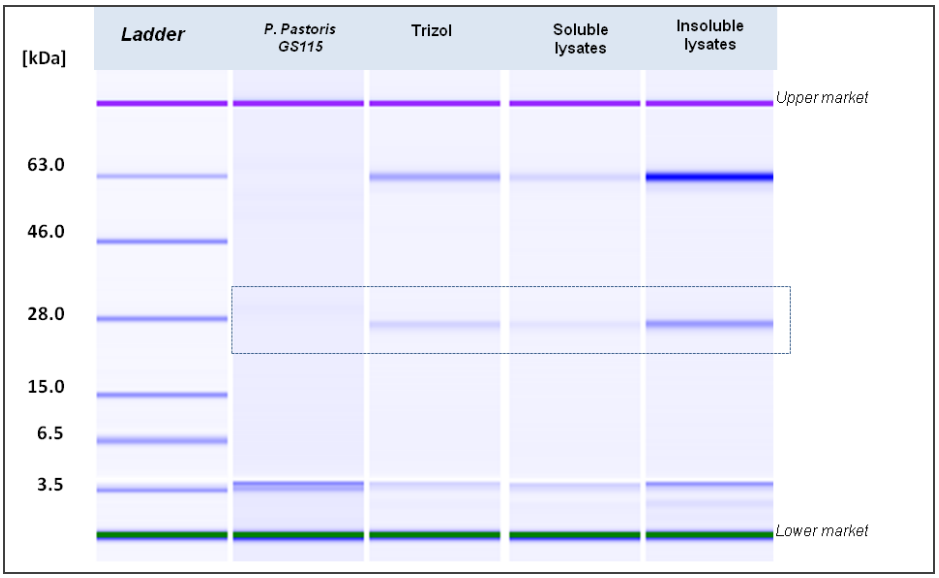

Figure 6. Bioanalyzer gel-like image comparing recombinant E7 expression after different extraction methodology. Lane 1: ladder; Lane 2: negative control; Lane 3: recombinant protein after chemical extraction; Lane 4 and 5: recombinant protein after mechanical extraction, soluble and insoluble (urea 10M) fractions, respectively. The molecular weight sizing scale (kDa) is provided on the sides of the gel image.

Recombinant HPV16 E7 intracellular expression was study in cellular lysates. To optimization the heterologous protein extraction two methodologies were carried out chemical (trizol reagent) and mechanical break (breaking buffer and glass beads). During mechanical break

urea 10M was added in the pellet to evaluate the insoluble protein aggregation (insoluble fraction). Virtual gel-like image (figure 6) illustrates the presence of bands at 27 KDa in all clones samples indicating that both methods were able to extract the recombinant protein. The highest intensity band was detected in insoluble extract while the lowest intensity band was visualized in soluble extract. This behaviour is confirmed with protein specific dosage by Agilent 2100 Bioanalyzer software. Insoluble fraction obtained using mechanical breaking was able to extracted 2.5-folds higher recombinant protein (218.9 $mg.L^{-1}$) than chemical method (84.4 $mg.L^{-1}$). In soluble extract, a low concentration of recombinant E7 (42.0 $mg.L^{-1}$) were obtained. Probably an insoluble form of this protein was formed due to the precipitation during the extraction. Descriptions of the same protein in *E. coli* (125 $mg.l^{-1}$) and *S. pombe* (0.8 $mg.l^{-1}$) showed the insoluble form produced by refolding of inclusion body (Pahel, Aulabaugh *et al.*, 1993; Braspenning, Manetti *et al.*, 1997; Fernando, Murray *et al.*, 1999).

4. Conclusions

This work demonstrates the successful cloning and expression of HPV16 E7 gene in *P. pastoris* GS115 using a lab-on-chip system for analysis. *P. pastoris* expression system was able to produce higher amount of the target protein, being more efficient than the previous systems described for E7 expression. Protein expression of the high-risk HPV 16 type is an important strategie for studying the biological functions of E7 oncoprotein in human body as well as for developing an effective prevention and therapy options against cervical cancer.

5. Acknowledgments

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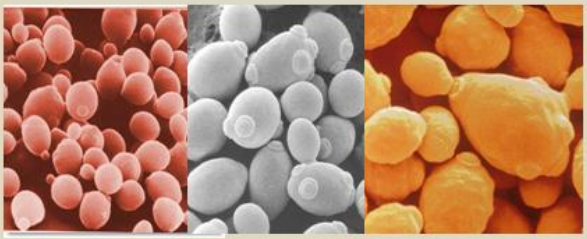

Capítulo II

"A coisa mais bela que podemos experimentar é o mistério. Essa é a fonte de toda a arte e ciência verdadeira."

(Albert Einstein)

Capítulo II

A ser submetido para Process Biochemistry

Influence of the traces elements supplementation in recombinant frutalin production by Pichia pastoris KM71H through fed-batch process

Influence of the traces elements supplementation in recombinant frutalin production by *Pichia pastoris* KM71H through fed-batch process

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Abstract

Frutalin, a galactose-specific lectin reported as a tumor marker, is a protein with low expression level in breadfruit. So, fed-batch fermentation was used to enhance protein production by a recombinant *P. pastoris* KM71H, obtaining an improvement of 4-fold of recombinant frutalin compared to shaker-flasks. Traces elements (PTM) supplementation was used as alternative to stimulate the production of recombinant. High cell density (124.1 $g.L^{-1}$ dry weight) was obtained during the fed-batch process. The addition of PTM to the minimum medium allowed recombinant protein production to be attained (13.4 $mg.L^{-1}$), being 2.5-fold higher than observed in the culture without PTM. Development of strategies to improve the production of recombinant frutalin may broaden its application in cancer diagnostic.

Keywords: *Pichia pastoris, Fed-batch process, Frutalin, lectin and traces elements supplementation*

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Introduction

Lectins are proteins found in all kingdoms of life ranging from viruses, fungi and bacteria to animals and plants (Lannoo, Vervecken *et al.*, 2007). These proteins are able to bind reversibly to high specific mono- or oligosaccharides (Peumans, Roy *et al.*, 1998), being related to biological recognition phenomena, mainly cell recognition, interaction, and adhesion (Peumans e Van Damme, 1998).

Frutalin is a homotetrameric α-D-galatose lectin belonging to the jacalin family isolated from *Artocarpus* incise. Functionally, this lectin is a potent lymphocyte stimulator (Abdul Rahman, Anuar Karsani *et al.*, 2002), able to induces chemotaxis and rearrangement of actin cytoskeleton in human neutrophils and it has been successfully used as a cancer diagnostic tool (Brandolima, Saldanhagama *et al.*, 2005). However, the use of frutalin isolated from their natural sources presents several disadvantages like heterogeneity of the sample due to the presence of lectin isoforms, which may lead to results variability (Brandolima, Saldanhagama *et al.*, 2006).

To overcome this problem, lectin expression in a heterologous system allows the obtention of pure protein samples in high levels. *Pichia pastoris* is currently a high effective and versatile system for the expression of heterologous proteins (Macauley-Patrick, Fazenda *et al.*, 2005). This metilothrofic yeast has been successfully used for lectin expression from lower organisms such as *Aleuria aurantia* (Amano, Takase *et al.*, 2003) and *Pleurotus cornucopiae* (Sumisa, Iijima *et al.*, 2004) as well as from plant, such as *Phaseolus vulgaris agglutinin* (PHA), *Galanthus nivalis agglutinin* (GNA) (Raemaekers, Muro *et al.*, 1999), *Canavalia brasiliensis* (ConBr) (Bezerra, Carvalho *et al.*, 2006). Synthetic frutalin was also cloned and expressed in *P. pastoris* strain under batch conditions (Oliveira, Felix *et al.*, 2008).

The production of recombinant proteins by *P. pastoris* has been performed in fed-batch operation that exhibits advantage compared to batch cultivation, such as reduction of the toxic effect of methanol induction, increase of protein production without biomass gain and possibility of extracellular expression with low quantity of homologous proteins (Taherzadeh, Adler *et al.*, 2002). Some chemical were been used during fed-batch process, trace elements (PTM) supplementation, allowing higher specific protein production and an improved its final concentration (Boze, Céline *et al.*, 2001). In recombinant protein production phase, there are nutrients starvations by the cells resulting in an increase on protease concentration. Then, PTM is added and, by substrate competition, proteolytic activity is reduced following an increase of the recombinant protein stability and productivity (Macauley-Patrick, Fazenda *et al.*, 2005).

This work aimed to analyse the effect of PTM supplementation in fed-batch process to increase the production of synthetic frutalin by *P. pastoris* KM71H.

Materials and methods

Yeast culture media

*Pichia pastoris KM71H/*pPICZaA/recombinant frutalin, previously constructed (Oliveira, Felix *et al.*, 2008) was grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or BMGH (0.1M potassium phosphate, 4×10^{-5}% biotin, 1.34% YNB, and 1% glycerol, pH 6.0). Trace elements (PTM) solution contained ($g.L^{-1}$) 1.0 $FeSO_4\times7H_2O$, 0.005 $CuSO_4\times5H_2O$, 0.01 H_3BO_3, 0.01 $MnSO_4\times H_2O$, 0.07 $ZnSO_4\times7H_2O$ and 0.01 $Na_2MoO_4\times2H_2O$. All solutions were either autoclaved (121 ºC, 20 min) or filter sterilized (0.22 µm pore size, Millipore®) prior to use.

Fed-batch fermentation

All fermentation cultures were carried out in fed-batch mode in a 1.6-L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Wald, Switzerland), containing 1L fermentation medium (working volume). The vessel was jacketed and the water bath temperature was kept constant at 30ºC, while the pH was maintained at 5.0. NaOH (1.0 M) was used to compensate for pH changes and 100µL defoaming agent (Antifoam A, Sigma) was used to control excessive foaming. The dissolved oxygen (DO) level was maintained over 30% of air saturation by a cascaded control of agitation rate (320–550 rpm) and aeration rate (3–5 $L.min^{-1}$). The following parameters: temperature, DO, pH, agitation, aeration, antifoam, and carbon source were monitored throughout the fermentation period.

The fed-batch fermentation process consisted of three distinctive phases: glycerol batch for initial cell growth, glycerol fed-batch for alcohol oxidase derepression and high cell density, and induction for expression of recombinant frutalin. In the first step, *P. pastoris* KM71H was growth in BMGH medium (1 L) for 24 h. After that, the glycerol fed-batch phase started, being the feed medium (30 mL of 50% glycerol solution supplied or not with 12 $ml.L^{-1}$ trace metal solution) fed in a flow rate of 1.5 or 6 $ml.h^{-1}.L^{-1}$. In the induction phase, the induction medium (Methanol 100% supplied or not with 12 $ml.L^{-1}$ trace metal solution) was fed in a flow rate of 6 $mL.min^{-1}$. In this phase, the temperature was kept at 28ºC.

Analytical methods

Cell concentration (dry weight per volume) was determined by measuring the optical densities (OD) of the yeast culture samples at 660 nm, which was correlated to a calibration curve (dry weight ×OD). Glycerol and methanol concentrations were measured by high-performance

liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector, a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) a 60°C, 0.005 M sulfuric acid as eluent in a flow rate of 0.7 $mL.min^{-1}$ according to manufacturer's instructions. The total protein concentration in the samples was determined by the Bradford protein assay using bovine serum albumin as the concentration standard. Protein production was analyzed by SDS–PAGE performed using a 12% gel and stained with Coomassie brilliant blue, as described by Oliveira et al. (2008). All analytical analysis was carried in triplicate.

Recombinant protein purification

The supernatants from fed-batch fermentation were separated from the yeast cells by centrifugation (3000 x*g*, 4 ºC, and 10 min). The salts were precipitated by the pH increase to 7.5 through addition of 10N NaOH, being removed by centrifugation (3000g, 4ºC, and 10 min). Then, the supernatants were filtered through 0.22 µm pore size filters, concentrated and washed with PBS buffer (8 $g.L^{-1}$ NaCl, 0.2 $g.L^{-1}$ KCl, 2.68 $g.L^{-1}$ $Na_2HPO_4 \times 7H_2O$ and 0.24 $g.L^{-1}$ KH_2PO_4, pH 7.4), to a final volume of 1–1.5 ml in 10 kDa Amicon tubes (Millipore). Treated supernatants were analysed by SDS–PAGE using a Sephacryl S-100 HR column (HiPrep 16/60, Pharmacia Biotechnology), and a FPLC system (Pharmacia Biotechnology), which had been previously washed with distilled water and equilibrated with PBS buffer at a flow rate of 1 $mL.min^{-1}$. The frutalin purity was confirmed through SDS–PAGE followed by silver nitrate staining. The pure recombinant frutalin concentration was determined by measuring the optical densities at 280 nm.

Hemagglutination assays

Recombinant frutalin samples were prepared by doing 1:2 serial dilutions from a starting 1:1 until 1:128, and 100 µl were incubated with 100 µl of rabbit erythrocytes (2% (v/v) in 0.15 M

NaCl) at 37 °C for 30 min (Oliveira, Felix *et al.*, 2008). The degree of agglutination was monitored visually after the tubes had been left at 37°C for 30 min and subsequently left at room temperature for another 30 min. Samples that yielded no visible agglutination activity after these incubation steps were regarded as negative.

Results and discussion

Recombinant frutalin production in fed-batch

KM-71H recombinant was grown in minimal medium with glycerol and the frutalin production was induced with methanol, once it is inducer and the carbon source for *P. pastoris* during the fermentation, being a key factor to protein (Cereghino e Cregg, 2000). The induction step was initiated after glycerol consumption; recombinant protein expression was induced by the addition of 0.8% methanol to the culture medium.

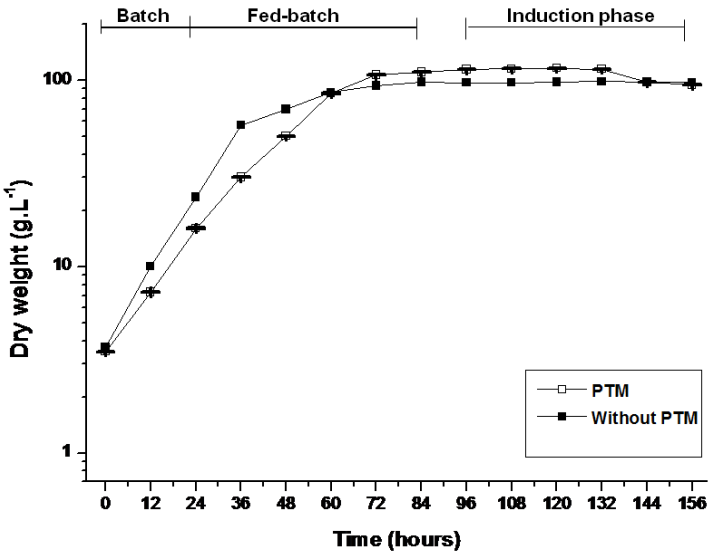
In this study, fed-batch showed biomass production of 122.3 $g.L^{-1}$ and the final pure frutalin concentration was 5.23 $mg.L^{-1}$, being higher than in Erlenmeyer-flasks that showed 2.1 $mg.L^{-1}$. The levels of protein obtained from shaker-flasks are generally lower than in bioreactors, once cell density is lower and the efficiency in aeration is often limited (Daly e Hearn, 2005). Recombinant lectins have been expressed in *P. pastoris* under flasks condition (30°C and 300 rpm) like fruiting-body lectins of *Pleurotus cornucopiae* (60 $mg.L^{-1}$) (Iijima, Amano *et al.*, 2003) and *N. tabacum* lectin (6 $mg.L^{-1}$) (Lannoo, Vervecken *et al.*, 2007); beyond the lectins produced under fed-batch process like *Amaryllidaceae* snowdrop agglutinin (80.0 $mg.L^{-1}$) (Baumgartner, Harper *et al.*, 2003) and *Phaseolus vulgaris* phytohemagglutinin E-form (100 $mg.L^{-1}$) (Baumgartner, Raemaekers *et al.*, 2002).

Table 1. Biomass production, recombinant frutalin concentration and specific growth-rate obtained in fed-batch operation for *Pichia pastoris* KM71H.

Induction conditions	**Protein concentration ($mg.L^{-1}$)**	**$\mu^{(*)}(h^{-1})$**	**Dry weight ($g.L^{-1}$)**
PTM supplementation	13.4	0.060	124.1
Without PTM supplementation	5.23	0.059	122.3

(*) μ was acquired by linear regression of the observed specific growth rate during the glycerol feeding.

The frutalin concentration in fed-batch was very low compared to other recombinant proteins expressed by *P. pastoris*, demonstrating that this system was not efficient to produce high level of frutalin. However, the electrophoretic characteristics of the cloned frutalin are similar to native one, despite no hemagglutinating activity. This synthetic frutalin was previously cloned and expressed in *P. pastoris KM71H* using shaker-flaskers (Oliveira, Felix *et al.*, 2008). The authors showed that native and recombinant frutalin had similar sugar-binding specificity, but significant difference in carbohydrate-binding affinity. The native protein carbohydrate-binding demonstrated to be stronger than recombinant protein affinity (Oliveira, Felix *et al.*, 2008). Although, no loss in the ability of identifying prostatic neoplasic cells was detected (Oliveira, Teixeira *et al.*, 2009). Behaviour like this can be attributed to the glycosylation pattern of *P. pastoris* also observed for another fruiting-body lectin (Iijima, Amano *et al.*, 2003).



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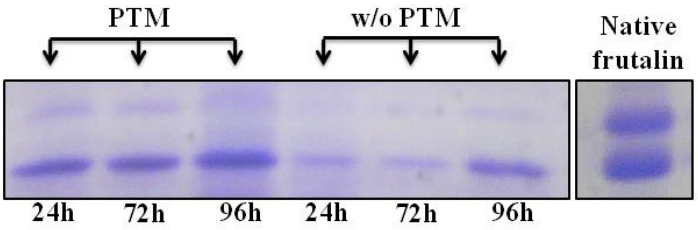

B

Figure 1. The fed-batch process to production of recombinant frutalin by *P. pastoris* KM71H: A) Comparative profile of yeast growth with and without PTM supplementation and time course of batch, fed-batch and induction phase, B) Recombinant frutalin migration in SDS–PAGE gel followed by Coomassie brilliant blue staining at 24, 72 and 96hours of the methanol induction. Influence of the PTM supplementation in recombinant frutalin production.

Influence of PTM in frutalin production

To verify the influence of the supplementation with PTM on frutalin production, assays were performed in shaker-flasks and fed-batch bioreactor, once the limitation of basal salts during high cell density growth period may prevent the use of carbon source. The need for nutrients supplementation in the feed phase and during the induction of the fed-batch process has been reported (Hang, Ye *et al.*, 2009; Arnau, Ramon *et al.*, 2010). The behavior of cell growth was quite similar to with and without PTM supplementation (figure 1A), showing no influence in biomass formation, so none alteration was observed in biomass production and specific growth rate (Table 1). Although the PTM addition had been related to overcome the basal salts limitation by yeast cell and it reverberate in biomass production, none alteration was visualized in this work. PTM supplementation function is correlated with nutritional supply during nutrients starvation by cells (Xiao, Zhou *et al.*, 2006). In fed-batch growth glycerol was a carbon source capable of providing the possibly nutritional requirement for cells growth. Another author also have been adopted PTM supplementation and did not detect any increase on biomass increase production by the supplementation with trace elements (Chauhan, Arora *et al.*, 1999).

Otherwise, the addition of PTM 12 $ml.L^{-1}$ during the methanol feed resulted in a cumulative increase in the levels of recombinant frutalin expression compared with the induction in the absence of supplementation (Table 1). PTM supplementation in *P. pastoris* cultivation was able to produced 13.4 $mg.L^{-1}$ of the recombinant frutalin, being 2.5 higher than in absence of PTM. In shaker-flasks the production of 3.3 $mg.L^{-1}$ of frutalin represents an increase of 1.5-fold in presence of PTM. *P. pastoris* were previous related to produce higher recombinant protein after the trace elements supplementation using fed-batch process: HBsAg ($500mg.l^{-1}$), phytase ($350mg.l^{-1}$), angiostatin

(360mg.l^{1}) and alpha-amylase (108mg.l^{-1}) (Chauhan, Arora *et al.*, 1999; Lee, 2003; Chen, 2004; Xie, Zhou *et al.*, 2005).

The cultivation of *P. pastoris* on defined media is associated with a high rate of protease expression, especially in high cell density cultures. In defined media, nitrogen depletion results in an increase in protease activity, which can be avoided by increasing the initial nitrogen concentration or by supplementation. Nutrient starvation leads to autophagic cell degradation and lyses, leading to the release of vacuolar proteases (Todde, Veenhuis *et al.*, 2009). The use of PTM should increase protein production by increase in cell viability as well as improved the final protein concentration due to strong decrease in proteolytic degradation of recombinant proteins (Chauhan, Arora *et al.*, 1999; Boze, Céline *et al.*, 2001; Xiao, Zhou *et al.*, 2006; Ghosalkar, Sahai *et al.*, 2008; Roepckea, Vandenberghea *et al.*, 2011). Also, recombinant protein stability is further enhanced by addition of PTM to the culture medium, possibly by acting as alternative and competing substrates for one or more proteases, and these supplements can also repress protease induction caused by nutrients limitation (Macauley-Patrick, Fazenda *et al.*, 2005).

SDS–PAGE showed the frutalin production by *P. pastoris* KM71H after 24 hours of methanol induction (Figure 1B). This protein migrated in SDS–PAGE as a double band where the upper band corresponds to the chain of the glycosylated isoforms (highly glycosylated) and the lower band represents the non-glycosylated isoforms (or slightly glycosylated). The β-chain is not visible due to its low molecular weight (2.1 KDa). Recombinant frutalin showed identical SDS–PAGE migrations pattern to native frutalin, fact also observed by Oliveira and collaborators (2008). Size-exclusion chromatogram illustrates recombinant frutalin profile elution during purification process (Figure 3). The SDS-PAGE analysis followed by silver nitrate staining confirmed the recombinant frutalin purity.

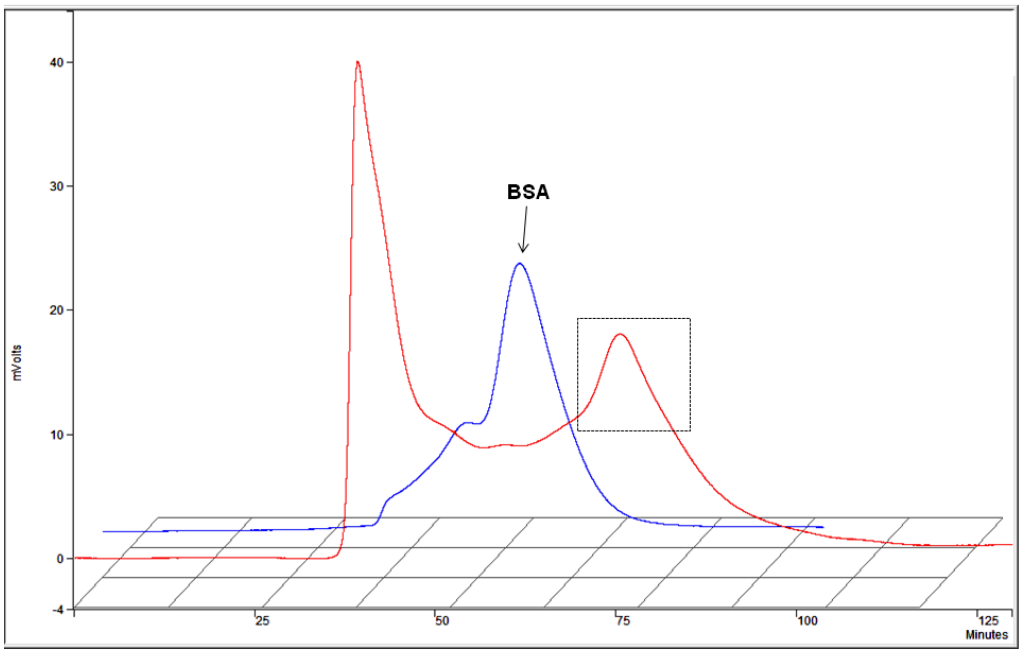

Figure 2. Elution profile on Sephacryl ® S-100HR column of the fed-batch process supernatant. BSA chromatogram eluted in the same conditions of the recombinant frutalin is marked in the figure.

Conclusions

Our results showed that recombinant frutalin production can be significantly increased with PTM supplementation using fed-batch process. Optimization of the strategies that allows higher recombinant frutalin production may broaden its application in cancer diagnostic and therapy.

Acknowledgments

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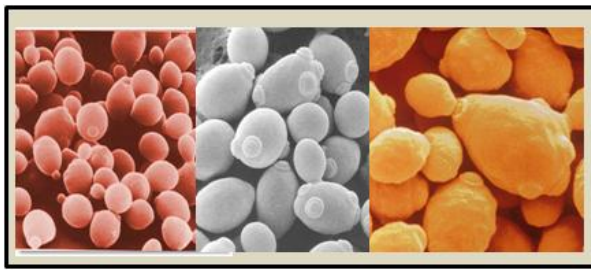

Capítulo III

"A curiosidade é mais importante que o conhecimento."
(Albert Einstein)

Capítulo III

Submetido para Enzyme and Microbial Technology

Influence of the carbon source and ionic stress in β-galactosidase activity from Kluyveromyces lactis DSM3795

Influence of the carbon source and ionic stress in β-galactosidase activity from *Kluyveromyces lactis* DSM3795

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Abstract

Kluyveromyces lactis is able to grow on cheap substrates such as lactose and residual whey from dairy industries due to the presence of β-galactosidase enzyme. However, carbon source and major minerals have been described to affect its activity. This study evaluated the influence of different carbon sources from commercial reagents and natural extract. Results showed the highest specific β-galactosidase activity was found in lactose media (223.78 $U.mg^{1}$), despite low activity was found in skimmed and integral milk. Ions Na^{+}, K^{+} and Mg^{2+} were also studied showing to be able to increase β-galactosidase activity. Ca^{2+} cation seems to be an inhibitor for enzyme activity.

Key words: *Kluyveromyces lactis*, β-galactosidase, Carbon sources and ionic stress

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1. Introduction

β-Galactosidase (EC 3.2.1.23), enzyme responsible for lactose hydrolysis into glucose and galactose, has important applications in the fields of medicine (lactose intolerance), food (to prevent lactose crystallization and increase sweetening power in dairy food) and the environmental (cheese whey utilization). It is widely distributed in nature, but microorganisms are the best sources for industrial production. Microbial β-galactosidases exhibit different properties depending on their source, being obtained from bacteria, fungi and yeasts including *Escherichia coli* (Matthews, S. B., 2005), *Bifidobacterium* (Hsu, Yu *et al.*, 2005), *Kluyveromyces lactis* (Ornelas, Silveira *et al.*, 2008) and *Kluyveromyces marxianus* (Martins, De Souza Jr *et al.*, 2002).

Research on β-galactosidases from *Kluyveromyces* has focused mainly on immobilization, production and purification of the enzyme to be used for lactose hydrolysis in milk and whey. The yeast *Kluyveromyces lactis* is a unicellular eukaryote which is widely used in biotechnology applications including as host for heterologous protein production (Van Ooyen, Dekker *et al.*, 2006). It is able to grow on cheap substrates such as residual whey from dairy industries and alternative non-fermentable carbon sources, such as ethanol or glycerol (Gonzalez-Siso, Ramil *et al.*, 1996; Merico, 2004). This yeast has competitive secretory properties, shows excellent large-scale fermentation characteristics and has food grade status (Merico, 2004). The effect of carbon source on proteins production by *K. lactis* has been investigated for different enzymes like inulinase, glucoamylase and thermostable bacterial xylanase activity (Wei, Zheng *et al.*, 1998; Merico, 2004; Yin, Miao *et al.*, 2010). *K. lactis* secretome has also been studied according to the carbon sources (Madinger, Sharma *et al.*, 2009).

However, minerals such as natrium, magnesium, calcium and potassium have been described to affect the growth, yeast metabolism and protein production (Ryad, Lakhdar *et al.*, 2010). Ions homeostasis is maintained by transporters both on the plasma membrane and on endomembranes. In

K. lactis, potassium and natrium ions uptake through plasma membrane occurred by KlTrK1p and Nha1p, respectively. Calcium homeostasis is regulated by PMR1 and Mg-ATPase is responsible for intracellular Mg^{2+} regulation (Okubo, Morishita *et al.*, 2005; Bollo, Bonansea *et al.*, 2006). The influence of the ionic stress in protein production and processing, cell wall and mitochondrial metabolism in *K. lactis* was previously studied (Farina, 2004). Once these factors could also influence in β-galactosidase production, this study reports the influence of the carbon source and ionic stress over this enzyme activity from *K. lactis* DSM3795.

2. Materials and Method

2.1. Microorganism and Reagents

All assays were conducted using *Kluyveromyces lactis* strain DSM 3795 (kindly provided by Dr. Rainer Jonas, Germany). Stock cultures were maintained at -80°C in vials containing 50% (v/v) glycerol until used. *K. lactis* cells from stock cultures were transferred to sterile pre-culture 18 hours before the assays. All reagents were analytical grade (Merck Co.).

2.2. Growth conditions

K. lactis cells from stock cultures were transferred to 50 ml flasks containing 10 ml sterile medium and incubated at 30°C for 8 h for activating the cells. A volume of 2 ml of this culture was transferred to 20 ml of fresh medium in 250 ml flasks and incubated for 12 h to obtain enough cell to perform the growth tests. The grown cells were harvested by centrifugation (3000 *x*g, 20 min, 4^oC) and used as inoculum.

Fermentations were carried out at 30°C in aerated shaker-flasks containing complex medium YP (2% peptone and 1% yeast extract) supplemented with 2% (w/v) of different carbon sources (dextrose, lactose, galactose and sucrose). Integral milk, skimmed milk, glycerol, methanol, cashew

fruit and palm extract were also used at final concentration of 2% (v/v). Experiments were carried out in 500ml Erlenmeyer flask containing 100 ml medium. Flasks were incubated in rotatory shaker at 200 rpm and 30°C during 48 hours. 5 mL were withdrawn at different time and centrifuged at 3000 *x*g for 10 min. Scale-up study was performed in the same conditions using 2L Erlenmeyer flask containing 500 ml of complex medium. Ionic stress study was carried out in aerated shake-flasks (2L) containing 500 ml YP medium (2% peptone and 1% yeast extract) supplemented with 2% (w/v) lactose and NaCl (100mM), KCl (100mM), $CaCL_2$ (10mM) and $MgSO_4$ (5mM). The supernatants were filtered through 0.22 µm cellulose acetate filter to sugar and ions measurement and pH values determination. Cells were disrupted for β-galactosidase activity and protein measurement. All the experiments were carried out in triplicate.

2.3- *Analytical methods*

Cell concentration (dry weight per volume) was determined by measuring the optical densities (OD) of the yeast culture samples at 660 nm, which was correlated to a calibration curve (dry weight × OD). Sugar concentration was measured by DNS method (Miller, 1972) and pH values were determinate thought pH indicator strips (Merck Co.). Total intracellular protein concentration was determined by the Bradford protein commercial (Sigma) assay using bovine serum albumin as the concentration standard.

Natrium, potassium and calcium ions concentrations were measured by flame photometer DM-61 (DigMed) previously calibrated by calcium, potassium, natrium and litium standard (Merck®). Magnesium concentrations by a high-performance liquid chromatography (SCL-6B, Shimadzu) accomplished with ion exclusion column HPX Aminex 87C (Bio-Rad). Ultrapure water was used as the mobile phase. The measurement was performed at 65 ºC at a flow rate of 0.6

mL/min by refractive index detector (Differential refractometer, Waters 410). Mg^{2+} concentration was calculated through a standard curve relating the area to concentration (mM).

2.4. Enzymes activity

β-Galactosidase activity was measured using *O*-nitrophenil-β-D-galactopyranoside (*O*NPG; Sigma, St. Louis, USA) as the substrate. The hydrolysis of substrate was assayed at 30°C, using *O*NPG at 8mg/mL in buffer solution (100 mM potassium phosphate buffer, pH 7.0). The reaction was stopped by adding 0.5 mL of 1M $NaCO_3$ and the time of reaction registered. The yellow colour formation was measured spectrophotometrically at 420 nm. One enzyme unit is defined as the amount of enzyme releasing 1 mol of *O*NPG per mL per minute at 30°C, pH 7.0.

3. Results and Discussion

3.1- Effect of carbon source in β -galactosidase activity

The substrate influence over β-galactosidase activity has been related with divergent values, probably because the sensitivity of the enzyme varies with the substrates present in the media (Merico, 2004). Aiming to observe the different enzyme activity, commercial (dextrose, lactose, galactose, sucrose, glycerol, methanol) and natural substrates (skimmed and integral milk and cashew fruit and palm extracts) were used as carbon source.

Our results showed that *K. lactis* DSM 3795, cultivated in 500mL Erlenmeyer-flask containing 100mL of the media, produced 28.23 $g.L^{-1}$ final biomass in dextrose, followed by lactose, galactose and sucrose based media in similar concentration (~17 $g.L^{-1}$) (*Table 1*). Palm extract leaded to the lowest biomass formation (6.12 $g.L^{-1}$) although specific β-galactosidase activity showed to be likely in integral milk (44.5 $U.mg^{-1}$). The highest specific β-galactosidase

activity was found in lactose media (223.78 $U.mg^{1}$) followed by galactose (175.11 $U.mg^{-1}$) (Table 1), as expected once they are assumed as inducers of β-galactosidase activity in *K. lactis* (Ornelas, Silveira *et al.*, 2008). Low enzyme activity on skimmed and integral milk was observed, probably related to low concentration of lactose in the media, 4.22 g/l and 2.28 g/l, respectively. Similar β-galactosidase activity was found in palm extract, a substrate rich in pectin that undergo hydrolysis release saccharides such as arabinose, fucose, rhamninose and galactose (Willats, Knox *et al.*, 2006). It indicates that galactose concentration in medium can induce LAC system without the presence of a strong repressor. Otherwise, glycerol, methanol and sucrose media showed great effect in repressing the LAC system, leading to β-galactosidase activity values lower than in dextrose. The period for highest enzyme activity was found in the beginning of stationary phase, with decrease to low levels after the peak found at 10 and 12h on galactose and lactose media, respectively.

Cells started stationary phase after 12h of growth with simultaneous pH decreased, reaching to final values of 4.0–5.0 in 48h. Reducing sugar measured at this time showed an incomplete utilization some carbon sources, probably caused by the depletion of nutrients in the medium or due to pH and oxygen concentration, critical parameters in shake-flask cultures (Rubio-Texeira, 2006).

Maximum yield found in lactose (40.51 $U.g^{-1}.L^{1}$) was 4-fold higher than in galactose medium, showing to be more efficient to induce the LAC system, despite glucose presence after lactose hydrolysis. Skimmed and integral milk showed the lowest yield once they were not able to induce efficiently the β-galactosidase activity by *K. lactis*. LAC promoter has been used efficiently to heterologous protein production using lactose induction. Yields obtained to Hydroquinone galactoside (2.01 $U.g^{-1}.L^{-1}$) (Kim, Lee *et al.*, 2010) and recombinant β-galactosidase (12.3 $U.mg^{-1}.L^{-1}$) (Becerra, 2004) by *K.lactis* showed the efficiency of LAC induction system in lactose medium (Gonzalez-Siso, Ramil *et al.*, 1996; Becerra, 2004).

Table 1. Influence of the carbon source in dry weight production and β-galactosidase activity by *K. lactis* DSM 3795 cultivated in shaker-flask at 30°C during 48 hours.

Carbon source	ΔX ($g.L^{-1}$)	Enzyme activity* ($U.mg^{-1}$)	Final pH	Sugar final concentration ($g.L^{-1}$)	ΔS ($g.L^{-1}$)	Y* ($U.g^{-1}.L^{-1}$)
Dextrose	28.26	57.18	5.0	1.2	21.05	1.03
Skimmed milk	17.60	63.41	4.5	1.49	2.42	0.27
Integral milk	13.87	44.63	4.5	1.96	0.27	0.33
Glycerol	9.06	22.16	4.0	ND	ND	2.6
Galactose	17.46	175.11	5.0	1.12	21.61	10.92
Sucrose	17.41	9.02	5.0	0.74	2.56	0.1
Lactose	11.29	223.78	4.5	17.2	1.57	40.51
Methanol	7.62	23.07	4.5	ND	ND	0.86
Cashew fruit	8.89	12.42	5.0	1.01	3.44	2.66
Palm extract	6.12	44.51	5.0	0.91	3.14	7.05

N.D. – not determined, (*) maximum values obtained during growth cellular.

Data are means of triplicates and P-values were less than 0.05.

The scale-up test for *K. lactis* DSM 3795 was performed in 2L Erlenmeyer-flask containing 500mL of lactose and galactose 2% at 30°C. The biomass formation was similar in both substrates (Figure 1), but showed 26% increment in lactose media. This was the opposite behavior in previous test containing 100mL media, which galactose produced 35% more biomass than lactose.

β-galactosidase activity profile showed no difference in 2L Erlenmeyer-flask for both media, but a slight difference was observed in comparison to galactose in 500mL Erlenmeyer-flask containing 100 mL (data not shown). The highest activity was therefore reached in lactose medium (365.21 $U.mg^{-1}$) when cells had already stopped the growth, being followed by slow decline in enzyme activity. Similar β-galactosidase activity profile was observed in galactose medium, with lower enzyme activity and faster decrease.

The β-galactosidase *K. lactis* DSM3795 activity showed a steep increase during exponential phase of growth for both media and the highest activity was detected in early stationary growth phase. In follow, a continuous decrease in enzyme activity was visualized during stationary phase when occur nutritional starvation and cellular lyses with simultaneous pH decrease (4.5 was final values). This profile was similar for glucoamylase activity produced in *K. lactis* grown in lactose and galactose medium (Merico, 2004), enforcing the mechanism of induction by these carbon sources over LAC promoter (Gonzalez-Siso, Ramil *et al.*, 1996).

It was established that induction of Lac⁄Gal regulon depends on the intracellular concentration of the inductor (Cardinali, Vollenbroich *et al.*, 1997). Then, lactose hydrolyses by β-galactosidase release galactose and glucose residues, which could contribute to the maintenance of the inductor in the media for longer time compared to galactose medium. So, lactose proved to be the optimal carbon source to allow the highest β-galactosidase activity. Thus, it was used as carbon source to evaluate the influence of ionic stress on β-galactosidase activity.

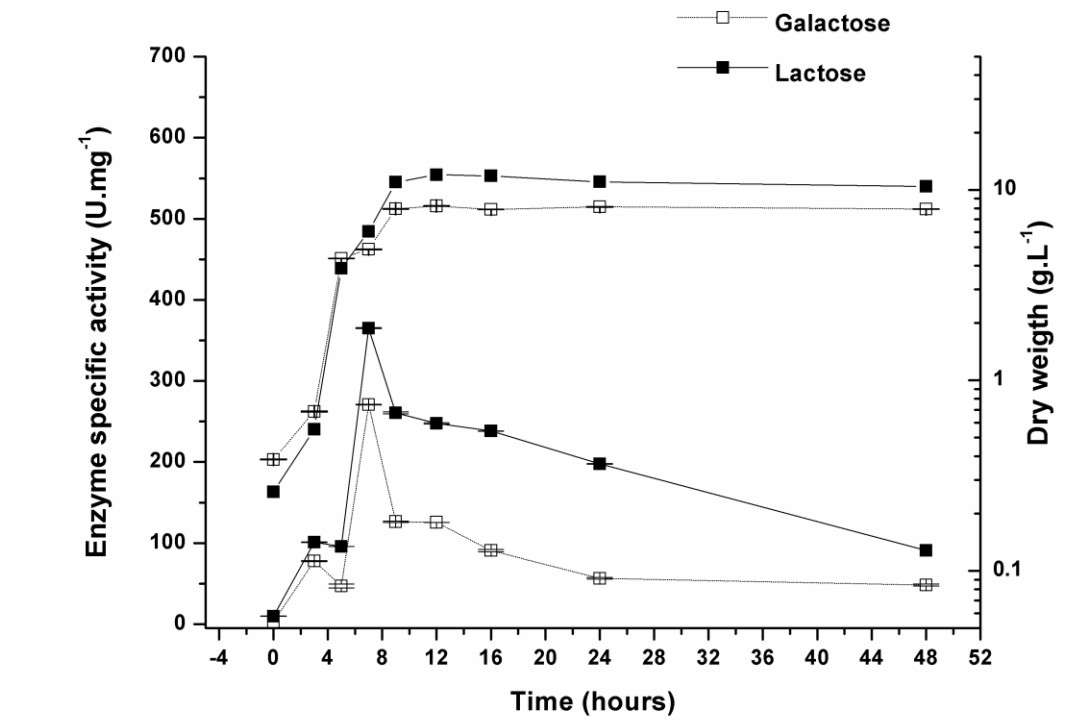

Figure 1. Biomass production and specific *β*-galactosidase activity per total protein by *K. lactis* during shaker-flasker cultivation (30°C, 150 rpm).

3.2- Ionic stress X β-galactosidase activity

Protein production depends on numerous factors but nutritional status leads to major effects. Major minerals such as nitrates, calcium and potassium among the most essential ingredients of the nutrient medium are known to affect yeast metabolism(Amdoun, Khelifi *et al.*, 2009). The activation or inhibition of β-galactosidase from different sources by mono- and divalent cations has been well documented in *Escherichia coli*, *Aspergillus oryzae* and *Saccharomyces fragilis* (Sutendra, Wong *et al.*, 2007; Neri, Balcão *et al.*, 2009). Our results show that β-galactosidase activity from *K. lactis* DSM 3795 increased in the presence of Na^{+}, K^{+}, Mg^{2+} compared to the values in absence of these ions and decrease in the Ca^{2+} presence (figure 2). Ions levels decrease

until 8 hour and remained constant during growth in all media tested, suggesting ions uptake by yeast.

The addition of 5mM $MgSO_4$ leaded to the highest β-galactosidase activity (734.71 $U.mg^{-1}$), being 2.9-fold higher than control medium. Magnesium sulphate was also found to enhance β-galactosidase activity in *E. coli*(Sutendra, Wong *et al.*, 2007), *Arthrobacter* B7 and *Bifidobacterium bifidum* (Lee, 2002), despite each enzyme has it particular structural characteristic, like size and molecular structure (Lee, 2002; Becerra, Gonzalezsiso *et al.*, 2006; Katrolia, Yan *et al.*, 2011). Divalent cation Mg^{2+} has been required for maximal activity the β-galactosidases in *E. coli* (162.3 $U.mg^{-1}$) (Garman, Coolbear *et al.*, 1996) and *Lactobacillus reuteri* (18.8 $U.mg^{-1}$) (Schwab e Gänzle, 2011) Crystallization and diffraction analysis of β-galactosidase from several species were able to show binding sites for Mg^{2+} near to enzyme activate site (Matthews, B. W., 2005), explaining the enhancement of enzyme activity. Mg^{2+}-ATPase transporter present in cellular membrane allows Mg^{2+} exchange and its participation in enzyme activity (Juers, Rob *et al.*, 2009).

The addition of either 100 mM KCl or 100 mM NaCl led to increase in enzyme activity, 1.79 and 1.67-fold, respectively. Additionally, Na^+ and K^+ inducing prolife is similar for β-galactosidase produced by different microorganisms (Song et al, 2010). However, the effects of these ions over enzyme activity varied greatly between species (Kim et al, 1997). Association of these monovalent cations with β-galactosidase could induce conformational changes in the enzyme structure (Miranda, Saldaa *et al.*, 2002). In fact, X-ray data for β-galactosidase crystals also identified sodium-binding sites in activation site (Juers, Rob *et al.*, 2009) justifying their influence on enzyme activity. However, natrium ion also may provide higher enzyme activity by ion homeostasis regulation, like occurred for K^+ ion. Homeostasis maintenance is controlled through membrane transporters KlTrK1p and Nha1p for K^+ and Na^+, respectively (Ohgaki, Nakamura *et al.*, 2005; Bollo, Bonansea *et al.*, 2006). The K^+ and Na^+ transporters play a significant role in the

regulation on ions homeostasis resulting cytoplasmic pH values maintained and undergone all condition required for protein processing by yeast cell (Naseem, Holland *et al.*, 2008).

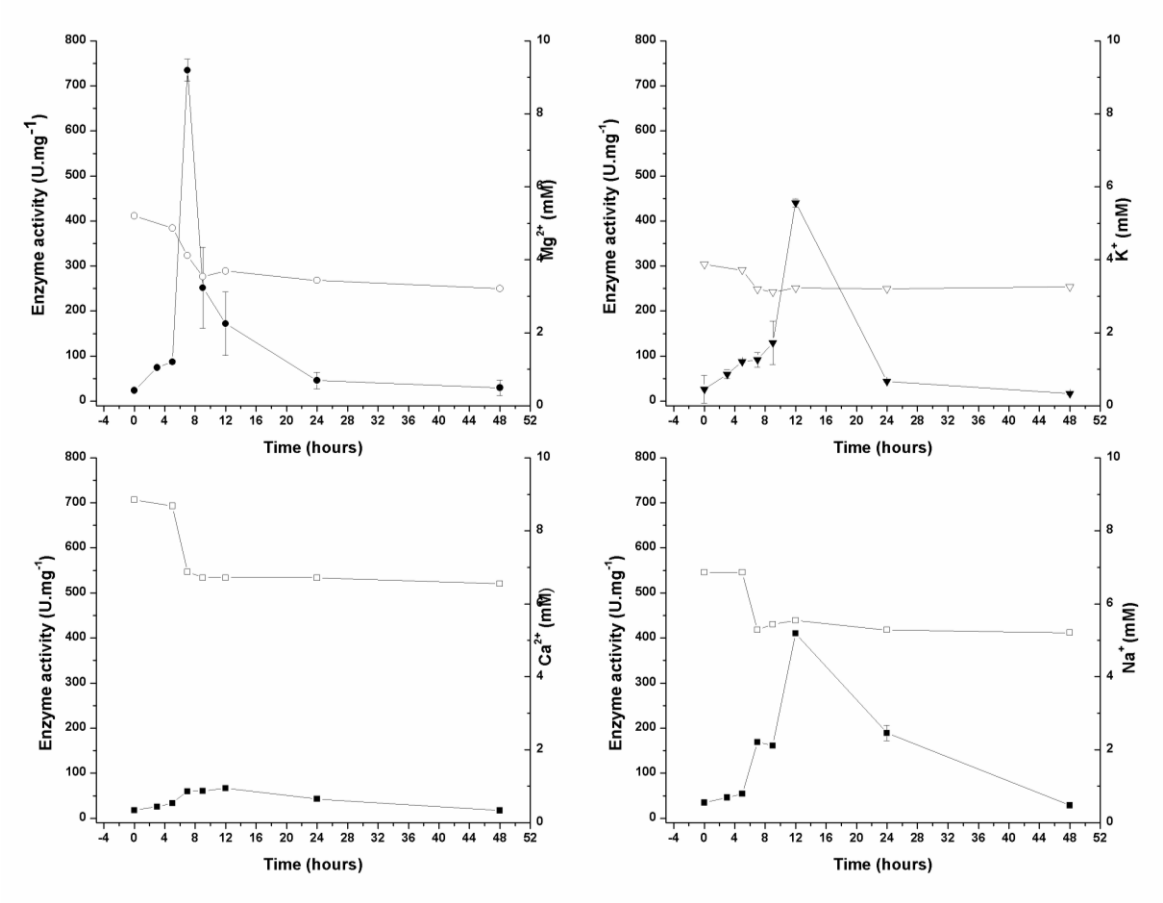

Figure 2. Influence of the ions Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} in β-galactosidase activity produced by *K. Lactis* DSM3795 in shaker-flasker (30°C, 150rpm).

Symbols full represent ions concentration (mM) and empty indicate β-galactosidase activity ($U.mg^{-1}$). Lactose 2% was used like carbon source in all samples.

In other hand, metal stress with 10 mM $CaCl_2$ resulted in the lowest enzyme activity, being even lower than the control test. In these conditions, Ca^{2+} ion is related as complete or partial inhibitors for β-galactosidase activity. Additionally, in *K. lactis* metabolism this ion is involved in the transport of proteins from the endoplasmic reticulum. The *KlPMR1*gene encodes for Ca^{2+}-

ATPase localized in Golgi apparatus, responsible for the transport of calcium and manganese inside the lumen (Uccelletti, 2005). Addition of $CaCl_2$ to the medium could result in partially folded proteins, being retained in endoplasmic reticulum (ER) and eventually routed to ER-associated degradation (Zanni, Farina *et al.*, 2009). Similar enzyme inhibition by Ca^{2+} ion obtained in this study was observed in *Aspergillus oryzae* (Ansari e Husain, 2010) suggesting that this inhibition may be involved in protein processing.

4. Conclusions

Lactose proved to be the optimal carbon source among different carbohydrates as it allows the highest β-galactosidase activity and the magnesium, natrium and potassium improves substantially its activity. Study of the homologues protein production from *K. lactis* DSM 3795 is important to implement as a host for protein expression aimed to the purpose therapeutic protein production in the pharmaceutical industry.

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6. **References**

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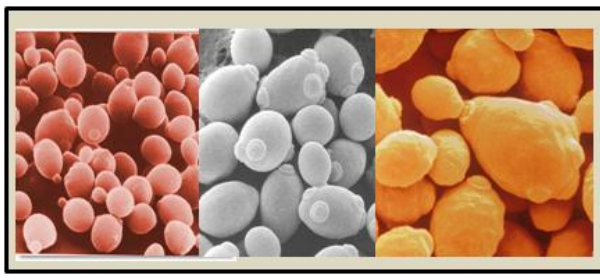

Conclusões

"O importante é não parar de questionar."

(Albert Einstein)

Conclusões

A tecnologia do DNA recombinante aliada ao desenvolvimento de estratégias produção de proteínas heterólogas em larga escala tem permitido o desenvolvimento de dispositivos com propósitos de diagnósticos e terapêuticos. Neste contexto, a *Pichia pastoris* mostrou ser um sistema eficiente para clonagem e expressão da proteína E7 do HPV16, bem como aumentar a produção da lectina frutalina recombinante pela suplementação com elementos traços usando o processo de batelada alimentada.

Adicionalmente, a caracterização das condições de cultivo e a avaliação do sistema promotor LAC sob diferentes fontes de carbono e estresse iônico na *Kluyveromyces* constitui um importante passo para a implementação dessa levedura como sistema de expressão de proteínas heterólogas. O aumento na produção da proteína β-galactosidase em meio enriquecido com lactose, bem como a potencialização da atividade enzimática na presença dos íons sódio, potássio e magnésio confirmam a capacidade da *K. lactis* de produzir proteína de importância biomédica a partir de meio de cultivo barato e de fácil acesso.

O cultivo da *P. pastoris* é ainda pode ser considerado caro e algumas modificações traducionais realizada por esta levedura podem comprometer a funcionalidade da proteína. Assim, a utilização da *K. lactis* como vetores de expressão para a produção de proteínas com propósitos terapêuticos, torna-se uma alternativa atrativa além representar um importante alvo no desenvolvimento de produtos para o diagnóstico, prevenção e tratamento do câncer.

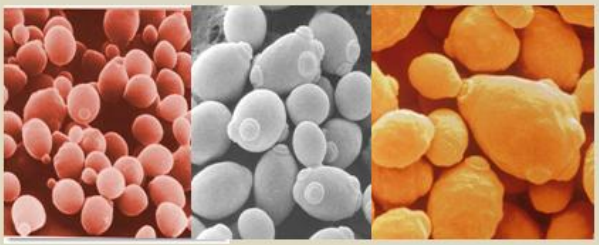

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TABLE OF CONTENTS

- Description p. 1
- Audience p. 1
- Impact Factor p. 1
- Abstracting and Indexing p. 2
- Editorial Board p. 2
- Guide for Authors p. 4

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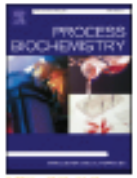
AUTHOR INFORMATION PACK

TABLE OF CONTENTS

- Description p.1
- Impact Factor p.1
- Abstracting and Indexing p.2
- Editorial Board p.2
- Guide for Authors p.4



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AUTHOR INFORMATION PACK

TABLE OF CONTENTS

- Description p.1
- Audience p.1
- Impact Factor p.1
- Abstracting and Indexing p.2
- Editorial Board p.2
- Guide for Authors p.4

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