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ENZIMAS PECTINOLÍTICAS DE *KLUYVEROMYCES MARXIANUS*: SELEÇÃO E APLICAÇÃO EM PROCESSOS DA INDÚSTRIA DE ALIMENTOS

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Tese submetida ao Programa de Pós Graduação em Engenharia de Alimentos do Centro Tecnológico da Universidade Federal de Santa Catarina para obtenção do grau de Doutora em Engenharia de Alimentos

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RESUMO

Nesse trabalho, dezenove leveduras do gênero *Kluyveromyces* foram testadas quanto à sua capacidade de produzir pectinases. Dentre as linhagens testadas sobressaíram a NRRL-Y-7571, NRRL-Y-6373 e 17D, com diâmetros de halos de 4,6, 4,4 e 4,1cm, respectivamente. As maiores atividades pectinolíticas foram detectadas nas linhagens NRRL-Y-7571(62,79 $U.mL^{-1}$), seguida por NRRL-Y-6373 (30,88 $U.mL^{-1}$). Com base na secreção de pectinases totais, a levedura *K. marxianus* NRRL-Y-7571 foi selecionada para o tratamento enzimático de suco de uva e vinho. A preparação enzimática apresentou maiores atividades pectinolíticas em pH 4,8. Com relação à temperatura de reação da preparação pectinolítica melhores desempenhos foram obtidos nas faixas de 30-40°C. Quanto à estabilidade térmica das pectinases observou-se preservação de cerca de 80% das atividades após 150 minutos de exposição a temperaturas de até 40ºC. Foi avaliada a influência de diferentes concentrações de glicose e pectina sobre a produção de pectinase. Os resultados mostraram que concentrações de pectina de 1 e 2% (m/m) favorecem a produção de PG. Observa-se que os valores obtidos para a atividade de poligalacturonase na presença de 2% (m/m) de pectina (114 U/mL) sofreram uma redução de aproximadamente 50% (60 U/mL) com 1% (m/m) de pectina no meio. Na extração e clarificação de suco de uva, os resultados indicam que o extrato pectinolítico obtido a partir de *K. marxianus* NRLL-Y-7571 é promissor, contribuindo para características sensorias (cor) e propriedades funcionais. Os resultados mostraram que a adição de enzima no vinho tinto acelerou a extração de compostos fenólicos e antocianinas, apresentando características cromáticas que podem ser consideradas melhores que as do vinho controle. Também, o vinho tratado com a enzima apresentou menor turbidez. Os resultados deste estudo demonstram os benefícios do uso do tratamento enzimático para obter suco de uva e vinho com enzimas pécticas produzidas por leveduras.

ABSTRACT

A total of ninetten *Kluyveromyces* strains were evaluated in this study to select highly pectinolytic strains. Among the strains tested highlights the NRRL-Y-7571, NRRL Y-6373, and 17D, with halos diameters of 4.6, 4.4 and 4.1 cm respectively. The highest activities were found in pectinolytic strains NRRL-Y-7571 (62.79 U.mL $^{-1}$) followed by NRRL-Y-6373 (30.88 U.mL^{-1}). Based on the secretion of total pectinases, yeast *K. marxianus* NRRL-Y-7571 was selected for the enzymatic treatment of grape juice and wine. The enzymatic preparation showed higher pectinolytic activity in pH 4.8. As to reaction temperature the preparation pectinolytic best performances were obtained in the range of 30-40°C. The thermal stability of preservation pectinases observed around 80% activity after 150 minutes of exposure to temperatures of 40°C. To evaluate the effect of pectin and glucose concentrations on the production of polygalacturonase by the selected strain The results show that pectin concentrations of 1 and 2% (w/w) favor the production of polygalacturonase. It is observed that the values obtained for the activity of polygalacturonase in the presence of 2% (w/w) pectin (114 U/mL) decreased by approximately 50% activity (60 U/ml) with 1% (w/w) pectin in the middle. Results indicate that the pectinolytic extract obtained from *K. marxianus* NRLL-Y-7571, is promising for grape juice processing, contributing to sensory (color) characteristics and functional properties. The results show that the addition of enzyme in red wines presented chromatic characteristics which can be considered better than those of the control wine, and accelerates the extraction of phenolic compounds and anthocyanins. Also, the wine treated with the enzyme had a lower turbidity. The results of this study demonstrate the benefits of using enzyme treatment to obtain grape juice and wine with pectic enzymes produced by yeast.

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NOMENCLATURA

ATCC - *American Type Culture Collection*

DNS - ácido 3,5 dinitrosalicílico

Endo-PG - endo-poligalacturonase

Exo-PG - exo-poligalacturonase

FT - fenóis totais

GAE - equivalentes de ácido gálico

GR - grupos redutores

H/C - relação entre os diâmetros da colônia e do halo formado

IC - Intensidade de cor

LBVMA - Laboratório de Biotecnologia Vegetal e Microbiologia Aplicada

NRRL - *National Center for Agricultural Utilization Research*

U - unidade enzimática

PE - pectinesterase

PG - poligalacturonase

PGL - poligalacturonato liase ou pectato liase

PL - pectina liase

PME - pectina metilesterase

PMG - polimetilgalacturonase

PMGE - polimetilgalacturonato esterase ou pectina esterase

PMGL - polimetilgalacturonato liase

TPA - atividade total de pectinase

SUMÁRIO

Enzimas pectinolíticas ou pectinases constituem um grupo de enzimas que catalisam a degradação das substâncias pécticas presentes no material vegetal, hidrolisando ligações glicosídicas ao longo da cadeia carbônica, provocando a sua despolimerização e desesterificação, e são produzidas por plantas superiores, fungos filamentosos, leveduras e bactérias.

As pectinases são enzimas de grande importância ao se considerar sua aplicação industrial, e estão entre as mais comercializadas, sobretudo para a indústria alimentícia, sendo amplamente utilizadas no processamento de polpa e sucos de frutas, pois agem sobre as substâncias pécticas, aumentando o rendimento e otimizando o processo de clarificação dos sucos. O suco resultante deste tratamento apresenta uma quantidade menor de pectina e uma viscosidade mais baixa, o que é vantajoso e facilita os processos de filtração subsequentes, além de apresentar melhores características funcionais, visuais e de turbidez.

Na elaboração de vinhos, mais especificamente na vinificação em tinto, a maior importância e motivo de atenção estão na extração e difusão dos componentes nobres da casca das uvas, especialmente os compostos fenólicos e de aromas que contribuem com a cor e as características sensoriais dos vinhos. A extração destas substâncias depende de fatores bioquímicos, cuja caracterização se dá através do emprego de preparados enzimáticos, principalmente enzimas pectinolíticas, que quando não produzidas pela cepa de levedura utilizada na fermentação, são adicionados ao mosto.

Apesar dos vários estudos que mostram a potencialidade de diferentes tipos de organismos como produtores de enzimas degradantes de substâncias pécticas, em escala industrial, as preparações enzimáticas usadas na indústria de alimentos são produzidas principalmente por fungos filamentosos, com a composição dos complexos enzimáticos pectinolíticos variando com o gênero, a espécie e com a linhagem de micro-organismo utilizada. *Aspergillus niger*, que produz poligalacturonase (PG), pectina liase (PL) e pectina metilesterase (PME) é a principal fonte de enzima pectolítica utilizada na indústria de alimentos. A hidrólise da pectina por PME leva a produção de metanol, composto tóxico e indesejável em alguns produtos alimentícios, como bebidas fermentadas.

Sendo consideradas como uma fonte alternativa na produção destas enzimas, as leveduras apresentam um potencial para a produção de enzimas microbianas para a indústria de alimentos. Leveduras produzem diferentes enzimas pectolíticas de acordo com o ambiente e a origem genética, podendo produzir PG, PL, PME ou pectato liase (PAL), dependendo das condições de temperatura, pH e da disponibilidade do substrato. Contudo, *Kluyveromyces*, *Saccharomyces* e *Candida* produzem somente PG (principalmente endopoligalacturonase). Consequentemente, suas pectinases podem ser usadas para clarificar suco de fruta e vinho sem liberação de metanol, apresentando vantagens com relação aos preparados enzimáticos comerciais. Na busca por novos produtos, as pesquisas que envolvem o estudo e a produção de enzimas com potencial industrial a partir de novas fontes microbianas é de interesse estratégico, pois garantem o suprimento de enzimas aos mais variados processos industriais.

Diante deste cenário, este trabalho está apresentado em capítulos, com objetivos, revisão bibliográfica geral, artigos, depósito de patente, discussão geral e bibliografia complementar. No CAPÍTULO I estão descritos os objetivos geral e específicos do presente estudo. O CAPÍTULO II refere-se a uma revisão bibliográfica geral. Os artigos estão descritos nos CAPÍTULOS III, IV e V, e o depósito de patente é apresentado no CAPÍTULO VI, conforme segue.

CAPÍTULO III - Artigo 1: "Screening and Breeding of *Kluyveromyces* Strains for Pectinolytic Activity: Polygalacturonas e Production by *Kluyveromyces marxianus* NRRL-Y-7571". Submetido para publicação ao periódico *Process Biochemistry* em abril de 2012. Artigo redigido conforme as instruções e normas do periódico.

Este estudo teve como objetivo selecionar uma levedura do gênero *Kluyveromyces* com capacidade pectinolítica e avaliar diferentes concentrações de pectina, como indutor, e de glicose, como fonte de carbono, no meio de produção de poligalacturonases por *K. marxianus.*

CAPÍTULO IV - Artigo 2: "Grape Juice Extraction and Clarification using an Enzymatic Preparation of the highly Pectinolytic Yeast *Kluyveromyces marxianus* NRRL-Y-7571." Submetido para publicação ao periódico *LWT - Food Science and Technology* em maio de 2012. Artigo redigido conforme as instruções e normas do periódico.

Este trabalho teve como objetivo estudar o uso de extrato enzimático bruto produzido por *Kluyveromyces marxianus* NRRL Y-7571 na maceração e clarificação de suco de uva da cultivar Bordô (*Vitis labrusca*) em comparação com preparado enzimático comercial.

CAPÍTULO V - Artigo 3: "Pectinolytic Extract of *Kluyveromyces marxianus* in Red Wine Making". Submetido para publicação ao periódico *Journal of Agricultural and Food Chemistry* em junho de 2012. Artigo redigido conforme as instruções e normas do periódico.

O presente estudo teve como objetivo avaliar o efeito da adição de extrato enzimático bruto de *Kluyveromyces marxianus* NRRL-Y-7571 na vinificação de uva da variedade *Cabernet Sauvignon* sobre as características visuais do vinho (cor, compostos fenólicos, antocianinas e turbidez).

CAPÍTULO VI - Depósito de Patente: "Processo de Produção de Extrato Enzimático, Extrato Enzimático, Uso de Extrato, Composição compreendendo Extrato e Método de Clarificação de Bebidas Utilizando Extrato obtido a partir de *Kluyveromyces*". Patente depositada em 24 de novembro de 2011. Patente redigida conforme as instruções e normas do depósito.

A presente invenção descreve produtos e processos compreendendo um excelente extrato enzimático, com elevada atividade de poligalacturonase, produzido por cepas selecionadas de *Kluyveromyces* sp., especialmente para a extração e clarificação de bebidas, em especial, bebidas fermentadas como suco de frutas ou vinho, por exemplo.

No CAPÍTULO VII é realizada uma discussão geral em que se procura destacar os aspectos principais do presente trabalho. No CAPÍTULO VIII são apresentadas as conclusões e no CAPÍTULO IX a bibliografia complementar utilizada no presente estudo.

CAPÍTULO I - OBJETIVOS

1.1 OBJETIVO GERAL

Avaliar a capacidade de produção de enzimas pectinolíticas por leveduras do gênero *Kluyveromyces* como fonte alternativa as produzidas comercialmente, para aplicação na indústria de sucos e nos processos de elaboração de vinhos.

1.2 OBJETIVOS ESPECÍFICOS

- Selecionar linhagens de leveduras do gênero *Kluyveromyces* com alta atividade pectinolítica;
- Avaliar o efeito de diferentes concentrações de pectina e glicose na produção de pectinase total pela levedura selecionada;
- Estudar o efeito do uso do extrato enzimático bruto produzido por leveduras do gênero *Kluyveromyces* na extração e clarificação de suco de uva da variedade Bordô;
- Avaliar a aplicação do extrato enzimático bruto produzido por leveduras do gênero *Kluyveromyces* no processo de vinificação em tinto com variedade *Cabernet Sauvignon.*

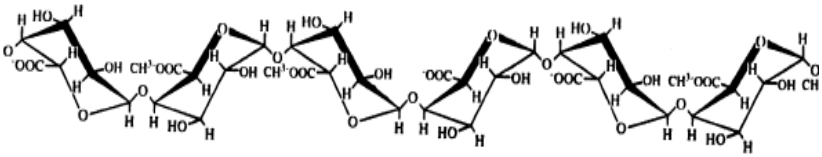
2.1 SUBSTÂNCIAS PÉCTICAS

As substâncias pécticas são polissacarídeos de alta massa molecular, presentes em todos os tecidos vegetais superiores, responsáveis pela manutenção da integridade dos tecidos e que participam, junto com outros componentes (celulose e hemicelulose), da organização da parede celular (ALKORTA et al., 1998; VRIES; VISSER, 2001; MOHNEN, 2008).

Quimicamente, as substâncias pécticas são um complexo coloidal de polissacarídeos ácidos, composto de resíduos de ácido galacturônico unidos por ligações α- 1,4, parcialmente esterificados por grupos metila (KASHYAP et al., 2001) e parcial ou completamente neutralizadas por uma ou mais bases (íons sódio, potássio ou amônio) (SAKAI et al., 1993).

Estruturalmente, as moléculas de pectina são constituídas de uma cadeia principal linear de unidades repetidas de ácido α-(1→4)-D galacturônico, sendo que parte destas unidades apresenta-se esterificada, como éster metílico (Figura 1). As cadeias de resíduos galacturonato são, porém, interrompidas por unidades de α□-(1→2)-L-ramnose, às quais estão ligadas cadeias laterais, formadas por açúcares neutros. Essas cadeias laterais são responsáveis pela união das moléculas de pectina à matriz polissacarídica da parede celular vegetal (ALKORTA et al., 1998; LANG; DÖRNENBURG, 2000; VRIES e VISSER, 2001; CAFFALL e MOHNEN, 2009).

Figura 1 - Estrutura de uma molécula de pectina



Fonte: ALKORTA et al., 1998.

Segundo a American Chemical Society as substâncias pécticas são classificadas em protopectinas, ácidos pectínicos, pectinas e ácido péctico (ALKORTA et al., 1998).

As protopectinas, compostas por unidades de ácidos galacturônicos ligadas ao cálcio por ligações iônicas, são insolúveis em água e estão presentes nos tecidos vegetais intactos (JAYANI et al., 2005).

Os ácidos pécticos são substâncias pécticas compostas de ácidos poligalacturônicos coloidais (ALKORTA et al., 1998; KASHYAP et al., 2001), onde os grupos carboxilas estão essencialmente não esterificados (SAKAI et al., 1993; KASHYAP et al., 2001) e cujos sais são denominados de pectatos (WHITAKER, 1990; ALKORTA et al., 1998).

O termo geral pectina designa ácidos pectínicos solúveis em água, com quantidade variável de grupos metil éster e um grau de neutralização capaz de formar gel com açúcares e ácidos em condições determinadas (SAKAI et al., 1993). As pectinas são polímeros nos quais pelo menos 75% dos grupos de galacturonatos estão esterificadas com metanol (JAYANI et al., 2005).

2.2 ENZIMAS PECTINOLÍTICAS

As enzimas pectinolíticas, de grande utilização industrial, são amplamente encontradas na natureza, originadas de muitas plantas e de fontes microbianas (fungos, leveduras e bactérias) (JAYANI et al., 2005; YADAV et al., 2009; ALIMARDANI-THEUIL et al., 2011). As pectinases correspondem a um grupo de enzimas que atuam sobre substâncias pécticas através de reações de hidrólise e de trans-eliminação, provocando a despolimerização das moléculas, e através de reações de desesterificação, hidrolisando a ligação éster entre os grupos carboxila e metil das pectinas (KASHYAP et al., 2001; DARTORA et al., 2002).

Visto a grande diversidade de substâncias pécticas presentes em diferentes tecidos de plantas, também ocorre uma variação nas pectinases, que pelo mecanismo de ação encontram-se divididas em três grupos: (1) as protopectinases, que hidrolisam a protopectina insolúvel originando pectina solúvel; (2) as desmetoxilases, que catalisam a desesterificação da pectina a ácido péctico por remoção dos resíduos metoxila; e (3) as despolimerases, que catalisam a clivagem hidrolítica das ligações α-(1→4) das cadeias glicosídicas no ácido D – galacturônico (ALKORTA et al., 1998; KASHYAP et al., 2001; JAYANI et al., 2005).

2.2.1 Protopectinases

Estas enzimas também conhecidas por pectinosinases catalisam a hidrólise da protopectina (JAYANI et al., 2005), liberando pectina altamente polimerizada e solúvel (ALKORTA et al., 1998; KASHYAP et al., 2001). São classificadas em dois tipos: o tipo A, que reage com a região de ácido poligalacturônico da protopectina e as do tipo B, que reage com as cadeias de polissacarídeos que podem se conectar com as cadeias de ácido poligalacturônico e os constituintes das paredes celulares (ALKORTA et al., 1998).

2.2.2 Enzima desmetoxilante

A classificação enzima pectinolítica desmetoxilante inclui apenas uma representante, a pectinesterase (PE) (E.C.3.1.1.11), que catalisa a desesterificação dos grupos metoxila da pectina, transformando-a em ácido péctico e liberando metanol. Durante a reação ocorre a quebra da molécula da água e a adição de um hidrogênio a um carbono da ligação e adição do grupo hidroxila a outro carbono, produzindo metanol e pectina com baixo grau de metoxilação (ALKORTA et al., 1998).

2.2.3 Enzimas despolimerizantes

De acordo com Kashyap et al. (2001), as despolimerases são classificadas segundo os critérios: (1) modo pelo qual a ligação é rompida (hidrólise ou trans-eliminação); (2) posição de ataque no substrato (endopectinase ou exopectinase); e (3) tipo de substrato hidrolisado (ácido péctico ou pectina). Envolvem as hidrolases (catalisam a hidrólise de ligações α-1,4) e as liases (catalisam β-eliminação).

As enzimas pertencentes ao grupo das pectinases despolimerizantes são apresentadas na sequência (KASHYAP et al., 2001; JAYANI et al., 2005).

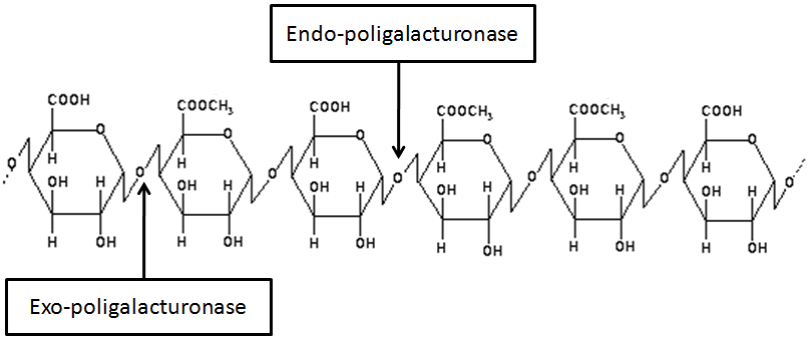
Poligalacturonases (PG)

- Endo-PG (E.C.3.2.1.15): hidrolisa aleatoriamente as ligações α-1,4 entre unidades de ácido poligalacturônico.

- Exo-PG 1 (E.C.3.2.1.67): hidrolisa as ligações sucessivas do ácido poligalacturônico, a partir da extremidade não-redutora, liberando moléculas de ácido galacturônico.
- Exo-PG 2 (E.C.3.2.1.82): hidrolisa as ligações alternadas do ácido poligalacturônico, a partir do terminal não-redutor, liberando moléculas de ácido digalacturônico.

O modo de ação das enzimas endo e exo-poligalacturonase, é apresentado na Figura 2 .

Figura 2 - Modo de ação da poligalacturonase (Endo-PG e Exo-PG)



Fonte: adaptado de BOBBIO; BOBBIO, 1989.

Polimetilgalacturonases (PMG)

- Endo-PMG (EC 3.2.1.15): hidrolisa a ligação glicosídica α-1,4 da pectina, preferencialmente, na pectina altamente esterificada;
- Exo-PMG (EC 3.2.1.15): hidrolisa a ligação glicosídica α-1,4 da pectina na extremidade não redutora da cadeia da pectina.

Polimetilgalacturonato liases (PMGL)

- Endo-PMGL ou endo-pectina liase (E.C.4.2.2.10): causa a quebra das ligações glicosídicas entre resíduos de ácidos galacturônicos por um mecanismo de trans-eliminação.
- Exo-PMGL ou exo pectina liase: causa a quebra da pectina, sequencialmente, por trans-eliminação.

Poligalacturonato liases (PGL)

- Endo-PGL ou endo-pectato liase (E.C.4.2.2.2): rompe as ligações glicosídicas internas entre resíduos de ácido péctico por trans-eliminação.
- Exo-PGL ou exo-pectato liase (E.C.4.2.2.9): rompe as ligações glicosídicas entre resíduos de ácido galacturônico por trans-eliminação, a partir de terminais não-redutores.

2.3 PECTINASES MICROBIANAS

As substâncias pécticas podem ser degradadas por enzimas pectinolíticas, produzidas em diferentes combinações pelas plantas e por micro-organismos como fungos, leveduras e bactérias. Diversos micro-organismos como *Bacillus, Erwinia, Kluyveromyces, Aspergillus, Rhizopus, Trichoderma, Pseudomonas, Penicillium* e *Fusarium* são conhecidos como bons produtores de pectinases (KASHYAP et al., 2001; DE GREGORIO et al., 2002; GUMMADI; PANDA, 2003).

Apesar dos vários estudos que mostram a potencialidade de diferentes tipos de organismos como produtores de enzimas pectinolíticas, atualmente, a maioria das pectinases que são utilizadas na indútria de alimentos ainda é obtida a partir de fungos filamentosos (KASHYAP et al., 2001; BLANDINO et al., 2002; HOONDAL et al., 2002; MARTIN et al., 2004; JAYANI et al., 2005; LINDE et al., 2007). As pectinases usadas na indústria de alimentos são produzidas comercialmente por *Aspergilus niger* (ACUÑA-ARGÜELLES et al., 1995; JIA; WHEALS, 2000; GUMMADI; PANDA, 2003). Esta espécie fúngica produz várias pectinases, incluindo a pectina metilesterase (PME), poligalacturonase (PG) e pectina liase (PL). Entretanto, há casos onde as pectinases são usadas para finalidades específicas. Muitas pectinases comerciais obtidas do *Aspergilus niger* apresentam baixa atividade de PG e elevada atividade de PL e de PME. A hidrólise da pectina por PME leva a produção de metanol (DA SILVA et al., 2005), composto tóxico e indesejável em alguns produtos alimentícios, como bebidas fermentadas.

Sendo consideradas como uma fonte alternativa na produção destas enzimas, as leveduras apresentam potencial para a produção de enzimas microbianas para a indústria de alimentos (DA SILVA et al., 2005; ALIMARDANI-THEUIL et al., 2011), com algumas vantagens sobre fungos filamentosos (BLANCO et al., 1999). Ambas as espécies

de leveduras, *Saccharomyces* e *Kluyveromyces*, são classificadas como organismos *Generally Recognized As Safe* (GRAS) pela *Food and Drug Administration* (FDA), órgão do governo dos EUA responsável pelo controle de alimentos e medicamentos, cujas normas são internacionalmente aceitas, sendo esse um importante critério técnico para aplicações alimentícias (VAN DER BERG et al., 1990; BONEKAMP; OOSTEROM, 1994).

Leveduras pectolíticas produzem diferentes enzimas de acordo com o ambiente e sua origem genética. Elas podem produzir PG, PL e PME, dependendo das condições de temperatura, pH e da disponibilidade do substrato. Por exemplo, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus e K. lactis*, são capazes de produzir somente PG e não apresentam atividade PME (BLANCO et al., 1999). Consequentemente, suas pectinases podem ser usadas para clarificar suco de fruta e vinho sem liberar metanol (FERNÁNDEZ-GONZALÉZ et al., 2004), apresentando certa vantagem com relação aos preparados enzimáticos comerciais. O estudo de micro-organismos produtores de pectinases é fundamental para a obtenção de enzimas com características adequadas a processos industriais específicos.

As leveduras do gênero *Kluyveromyces* são utilizadas em bioprocessos para produção de diversas enzimas, como lactase, inulinase, pectinase e lipase (FONSECA et al., 2007). *K. lactis* é atualmente a espécie mais estudada dentro do gênero *Kluyveromyces*. Porém, sua congênere *K. marxianus* também apresenta potenciais aplicações em diversos segmentos da biotecnologia (FONSECA et al., 2008). Estudos e análises dos genes da pectinase de leveduras tem sido realizados (GAINVORS et al., 1994a; HIROSE et al., 1998; GOGNIES et al., 1999; JIA; WHEALS, 2000), mas, desde 2009, a caracterização destes genes foi acelerada devido à possibilidade de utilizações biotecnológicas (SIEIRO et al., 2009; VAN WYK; DIVOL, 2009). Dentre as leveduras promissoras para a produção de enzimas pectinolíticas destacam-se, portanto, as do gênero *Kluyveromyces*. Em especial, *K. marxianus* que possui uma alta capacidade de produção de enzimas pectinolíticas (SCHWAN et al., 1997).

2.4 APLICAÇÕES DAS PECTINASES NO PROCESSAMENTO DE SUCOS

As enzimas pectinolíticas são extensivamente usadas na indústria para clarificação de sucos de frutas, devido à sua ação na degradação de

substâncias pécticas que são constituintes das frutas (ALKORTA et al., 1998; UENOJO; PASTORE, 2007). O tratamento de frutas com enzimas pectinolíticas auxilia o processo de extração e ainda diminui a viscosidade e a turbidez dos sucos (KAUR et al., 2004; ABDULLAH et al., 2007; RIBEIRO et al., 2010). Alguns tipos de sucos apresentam acentuada turbidez que nem sempre é eliminada com a centrifugação, visto que, mesmo após esse processo, pequenas partículas contendo pectina podem manter-se em suspensão. A despectinização de sucos por tratamento enzimático com pectinases é uma alternativa eficiente para esse setor da indústria (KASHYAP et al., 2001; LANDBO; MEYER, 2007), pois ocorre a degradação das pectinas solúveis que geram a viscosidade e que causam agregação de partículas responsáveis pela turbidez (formando complexo pectina-proteína) (SIEBERT, 2006).

Na indústria de sucos de frutas, as enzimas pécticas são usadas para promover a degradação da pectina, pois esta se apresenta na forma de suspensão coloidal, mantendo consigo outras substâncias também suspensas (PINELO et al., 2010). A extração do suco é facilitada pela desintegração dos tecidos, ocorrendo a quebra das cadeias poliméricas de carboidratos como pectinas, hemiceluloses e amidos (BLANCO et al., 1999; BASTOS et al., 2002; HOONDAL et al., 2002).

No processamento de frutas, as pectinases são utilizadas após o corte da matéria-prima, para macerar a polpa até a liquefação parcial ou total da fruta, diminuindo o tempo de processamento e melhorando a extração dos componentes da fruta (CARDOSO et al., 1999; KAUR et al., 2004). Quando o processo de maceração é realizado sem a adição de enzimas pectinolíticas, um gel altamente viscoso de pectina é formado, o que dificulta a prensagem e o rendimento (MOJSOV et al., 2011). Após a extração, as pectinases são adicionadas para clarificação e diminuição de viscosidade, agem desestabilizando as substâncias floculantes, provocando a coagulação e precipitação com consequente clarificação. Durante o tratamento enzimático, ocorre o aumento do tamanho das partículas insolúveis devido à redução da repulsão eletrostática entre as partículas coloidais, agrupando-as para remoção durante a filtração (PILNIK; VORAGEN, 1993, BARROS et al., 2004).

As enzimas pectinolíticas agem de formas diferentes nos sucos de frutas. Nos sucos límpidos e brilhantes (maçã, pêra e uva), as enzimas aumentam a produção de suco durante a prensagem e a filtração do mesmo, promovendo a remoção do material em suspensão, já nos sucos com turbidez (laranja e ameixa), a poligalacturonase degrada rapidamente o ácido péctico formado, evitando, dessa forma, precipitação e originando uma opacidade estável (WHITAKER, 1984).

Em sucos não clarificados como o de laranja, as pectinases podem ser usadas em diferentes estágios, permitindo uma melhor extração de açúcares e sólidos solúveis, resultando em maior rendimento e, portanto, em viscosidade mais baixa (PILNIK; VORAGEN, 1993).

A utilização de pectinases em combinação com hidrolases na indústria de alimentos é reforçada em diversos estudos. Chamadas coletivamente de enzimas de maceração, a combinação de pectinases, celulases e hemicelulases, são usadas na extração e na clarificação de sucos de frutas e vegetais (GAILING et al., 2000; KAUR et al., 2004). O efeito sinérgico da combinação de pectinases e celulases é importante no tratamento enzimático de muitas polpas de frutas (DEMIR et al., 2001). Barros et al. (2004) em estudo realizado na obtenção de sucos de frutas, comprovaram a redução da viscosidade dos sucos com a utilização de uma mistura de pectinases, celulases e hemicelulases.

Diversos estudos vêm sendo conduzidos com a adição de enzimas pectinolíticas na extração e clarificação dos sucos de frutas. Por exemplo, maiores níveis na extração da polpa de cupuaçu foram obtidos quando enzimas pectinolíticas foram utilizadas durante o processamento (BASTOS et al., 2002). De forma semelhante, Paranjpe et al. (2012) observaram um aumento no rendimento (32-48%) do suco de uva da variedade Concord, e Threlfall et al. (2005) um aumento entre 24 e 33% no rendimento de suco de uva após tratamento enzimático. Granada et al. (2001) observaram um aumento no rendimento de sucos de amora-preta e Mutlu et al. (1999), na produção de suco de cenoura. De acordo com Granada et al. (2001), em frutos como amora, morango, cereja e ameixa, o suco está retido dentro da estrutura celular e as preparações enzimáticas promovem a quebra desta estrutura, dissolvendo os compostos pectinolíticos, e consequentemente liberando mais facilmente o suco.

A utilização de leveduras pectolíticas ou suas enzimas pode ser altamente vantajosa no desenvolvimento de sucos de frutos. Gainvors et al. (1994b) demonstraram que o extrato enzimático bruto obtido a partir da linhagem SCPP de *S. cerevisiae*, apresentou o mesmo efeito sobre a turbidez na clarificação de sucos quando comparado com o preparado enzimático comercial (Endozyme). Gomez-Ruiz et al. (1988) também não detectaram diferença significativa entre a clarificação de suco de fruta utilizando a endopoligalacturonase de *Kluyveromyces fragilis* ou um preparado enzimático comercial.

2.5 APLICAÇÕES DAS PECTINASES NA ELABORAÇÃO DE VINHOS

A obtenção de vinhos de qualidade envolve a utilização de uma série de técnicas de processamento que garantam obtenção de um produto final, cujas características incluem, entre outros, uma boa aparência visual, transparência, cor, que também deve ser estável ao longo da vida do vinho (RIBÉREAU-GAYON et al., 2003). Segundo Peynaud (1982) o vinho é um produto obtido exclusivamente pela fermentação alcoólica, total ou parcial, de uvas frescas, esmagadas, ou não, ou do mosto de uvas.

Na elaboração do vinho tinto pelo processo clássico, a fermentação alcoólica e a maceração ocorrem simultaneamente. Nessa fase, os constituintes da parte sólida da uva, principalmente da película, passam para o mosto através dos fenômenos de dissolução e de difusão (UBIGLI, 1988). A extração das substâncias responsáveis pela cor do vinho depende de fatores químicos, físicos e bioquímicos. Os fatores bioquímicos mais importantes correspondem ao emprego de preparados enzimáticos, principalmente enzimas pectinolíticas.

Enzimas são muito utilizadas na indústria de alimentos e em particular nos processos enológicos (ARMADA et al., 2010). Enzimas pécticas são sintetizadas naturalmente pelas plantas e estão presentes na uva. No entanto, elas têm baixa atividade durante o processo de produção de vinho (DUCASSE et al., 2011). Fundamentalmante, enzimas pectinolíticas podem ser adicionadas durante o processo de vinificação para: (1) degradar polissacarídeos estruturais do mosto que interferem com o processo de extração (ROLDÁN et al., 2006); (2) aumentar o volume do suco e reduzir o tempo de prensagem, durante o esmagamento da uva (MOJSOV et al., 2011); (3) antes ou após a fermentação do mosto, para diminuir as partículas em suspensão; e (4) para assegurar a filtração e clarificação do vinho (UGLIANO, 2009).

A composição química do vinho é complexa em variedade e quantidade de substâncias provenientes da própria uva ou formadas durante o processo de elaboração. Todas as substâncias afetam de alguma forma a qualidade do vinho e especialmente as características sensoriais. Entre as substâncias mais importantes para a qualidade do vinho estão os compostos fenólicos e aromáticos. A composição destas substâncias, quando oriundas da própria uva, encontram-se em grande parte na casca sendo extraídas durante o processo de maceração (FLANZY, 2000).

A maceração é responsável por grande parte das características específicas, visuais, olfativas e gustativas que diferenciam os vinhos tintos dos vinhos brancos, contribuindo essencialmente com os compostos fenólicos (antocianinas e taninos) que participam da cor e estrutura geral do vinho, bem como dos compostos aromáticos, substâncias nitrogenadas, polissacarídios (especialmente pectinas), substâncias minerais, entre outras. A vinificação em tinto mais comum é caracterizada pelo contato das partes sólidas das uvas (película, sementes, e eventualmente, partes do engaço) com a fração líquida do mosto, sendo o período de contato dependente da qualidade e características desejadas (RIBÉREAU-GAYON et al., 2003).

Dentre os compostos orgânicos que contribuem para a composição química e a qualidade do vinho tinto, os polifenóis ocupam lugar de destaque. No caso de mostos obtidos a partir de uvas vermelhas, a degradação da parede celular da casca das uvas através do tratamento com enzimas pectolíticas resulta em um aumento da liberação de compostos fenólicos responsáveis pela cor (PINELO et al., 2006; BUSSE-VALVERDE et al., 2011). As antocianinas (pigmentos) provêm das cascas e são extraídas principalmente no início da maceração (REVILLA; GANZÁLEZ-SAN JOSÉ, 2003; KELEBEK et al., 2007). Os taninos (principalmente flavanóides) são extraídos das cascas e sementes (CHEYNIER et al., 2006), e podem contribuir para a estabilidade da cor dos vinhos tintos, reagindo com as antocianinas e formando pigmentos derivados (SALAS et al., 2003).

A extração de compostos fenólicos geralmente depende da variedade e qualidade das uvas, além de parâmetros tecnológicos de esmagamento, tempo de maceração, temperatura, recirculação, entre outros. Embora a extração da cor seja difícil de controlar, bons resultados são descritos com a utilização de tratamentos enzimáticos (FLANZY, 2000; GERBAUX et al., 2002). As enzimas pécticas de grande interesse na indústria de vinhos são as poligalacturonases. Quando não produzidas pela levedura, estas enzimas são geralmente adicionadas ao mosto na forma de preparados enzimáticos contendo atividades de celulases, hemicelulases e pectinases, conhecidas como enzimas de maceração (GONZÁLEZ-SANJOSÉ, 2005). O uso combinado destas enzimas proporciona uma maior eficiência na maceração da casca com o aumento da extração de pigmentos, facilitando a clarificação e a filtração do mosto e aumentando a qualidade e a estabilidade do vinho (ALKORTA et al., 1998; GAILING et al., 2000; TAKAYANAGI et al., 2001; KAUR et al., 2004).

Da mesma forma que em sucos de frutas, a utilização de leveduras pectolíticas ou suas enzimas pode ser altamente vantajosa na elaboração de vinhos. Blanco et al. (1997) demosntraram que na fermentação do vinho onde foram usadas linhagens pectinolíticas de *S. cerevisiae*, o processo de clarificação foi facilitado, com redução em alguns casos de até 50% no tempo de filtração. Da mesma forma, Vilanova et al. (2000) construíram uma linhagem de levedura com elevada expressão *pgu1*. Esta linhagem utilizada na fermentação enológica reduziu o tempo de filtração e os níveis de metanol no vinho. Pretorius (2000) previu que leveduras pectolíticas podem melhorar a liquefação, a clarificação e a filtração do mosto de uvas, com maior liberação de compostos de cor e sabor aprisionados nas cascas da uva e, assim, contribuir positivamente para o *bouquet* do vinho.

CAPÍTULO III - ARTIGO 1:

SCREENING AND BREEDING OF *Kluyveromyces* STRAINS FOR PECTINOLYTIC ACTIVITY: POLYGALACTURONASE PRODUCTION BY *Kluyveromyces marxianus* NRRL-Y-7571

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Screening and Breeding of *Kluyveromyces* Strains for Pectinolytic Activity: Polygalacturonase Production by *Kluyveromyces marxianus* NRRL-Y-7571

ABSTRACT

Kluyveromyces strains were screened for pectinase production. All the strains grew on citric pectin as sole carbon source, and most of them exhibited pectinolytic and viscosity reduction activities. Breeding and selection of pectinolytic segregants resulted in important increase on extracellular polygalacturonase activities (PG). The evaluation of the best strain (NRRL-Y-7571) under different condition showed that oxygen depletes PG activity, pectin acts as an inducer, and glucose stimulates grown but did not affect enzyme production. The cultivation of *K. marxianus* NRRL-Y-7571 in 2% pectin medium under a restricted oxygen condition resulted in the highest extracellular polygalacturonase activity (114 U. mL^{-1}).

Keywords: *Kluyveromyces marxianus*, yeast breeding, polygalacturonase, pectinolytic activity.

1. INTRODUCTION

Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that promote the degradation of pectic substances [1]. Pectins are complex polymers constituted by a backbone of (1→4)-α-D-galacturonic acid residues, partially methyl-esterified, that make part of the jelly-like matrix that cement plant cells together, and in which cell wall components are imbibed. Based in their mode of action, pectinases are classified in three broad groups:(1) protopectinases, that degrade the insoluble protopectins and give rise to highly polymerized soluble pectins, (2) depolymerases, that break the 1→4 glycosidic bonds of the principal pectin chain (polygalacturonase, pectin lyase, and pectate lyase), and (3) de-methoxylating enzymes such as pectin methylesterase, which remove methoxy esters [1,2].

Several organisms are able to produce one or more pectinases, including plants, filamentous fungi, bacteria, and yeasts [1-4]. Pectinases are commonly used in industrial processes involving the degradation of plant materials, because their action on pectins enhance

and speed up the extraction of fruit juices [5, 6], and contribute for the clarification of the final product, reducing its viscosity and increasing its stability [7].

Currently, the main source of pectic enzymes for industrial use is the mold *Aspergillus niger* [8]. This fungal species produces a pectinase complex with high pectin methylesterase and pectin lyase activities, and low polygalacturonase activity [9, 10]. The hydrolysis of pectin by pectin methylesterases represents a concern in food industries, as their action on pectins produces methanol, a toxic alcohol [11]. In this context, yeasts have been proposed as an alternative for the large scale production of pectinases [1, 2, 12, 13]. Among yeast species, only a few produce pectinolytic enzymes [2]. Traditionally, pectinolytic yeasts have been isolated from fruits or related products, like tropical fruits (11), citrus (14), cocoa pulp (4), and olives (15). The fermentative yeasts *Saccharomyces* and *Kluyveromyces* secrete just polygalacturonases [2, 8, 16], and did not increase the methanol content of fruit juices and wines during clarification processes [17, 18].

The production of pectinolytic enzymes depends on microbial strains and production conditions. Wild type strains selection and heterologous expression of bacterial, fungal and yeast pectinase genes have been used to improve pectinolytic yeasts. However, although currently applied in filamentous fungi, mutagenesis or breeding have been poorly used for pectinolytic yeasts improvement [2]. Moreover, the production of pectinases by molds and yeasts is affected by several physical (e.g. temperature, pH, oxygen supply) and chemical (culture medium composition) parameters, which should be optimized in order to obtain high enzymatic yields [19].

The aim of this study was to select highly pectinolytic strains of *Kluyveromyces sp.*, and to evaluate the effect of the agitation and pectin and glucose concentrations on the production of polygalacturonase by the selected strain.

2. MATERIAL AND METHODS

2.1. Yeast strains and breeding

A total of 19 *Kluyveromyces* strains (Table 1) were evaluated in this study. These strains include 11 isolates (six obtained from the National Center for Agricultural Utilization Research (NRRL), one from the American Type Culture Collection (ATCC), two from the Instituto Zimotécnico (ESALQ, USP, Brazil), and two from the Fundação de

Tecnologia Industrial (FTI, SP, Brazil)), four auxotrophic mutants obtained from ESALQ, a hybrid, and three meiotic segregants selected based on their growth on minimal medium with pectin as sole carbon source, obtained from Laboratório de Biotecnologia Vegetal e Microbiologia Aplicada (LBVMA).

K. marxianus hybrid was obtained by mass mating and selection by auxotrophic marks complementation on minimal medium (2% glucose, 0.67% YNB without aminoacids, 2% agar). Sporulation was induced on raffinose-acetate medium, vegetative cells eliminated by diethyl ether treatment [20], and spores plated on pectin-YP medium (1% yeast extract, 2% peptone, 1% pectin, 2% agar).

Table 1. Yeast strains evaluated in this study.

Code	Species	Source	Observations
NRRL-Y-8278	*K. lactis var. drosophilarum*	NRRL	Isolated from *Drosophila azteca*, USA
NRRL-Y-7571	*K. marxianus*	NRRL	Isolated from pozol, México
NRRL-Y-6373	*K. marxianus*	NRRL	Isolated from Orange peel
NRRL-Y-1190	*K. marxianus*	NRRL	Isolated from yogurt
NRRL-Y-1195	*K. marxianus*	NRRL	----------
NRRL-Y-1207	*K. marxianus*	NRRL	----------
FTI 20014	*K. marxianus*	FTI	----------
FTI 20015	*K. marxianus*	FTI	----------
IZ 1339	*K. marxianus*	ESALQ	Isolated from *Drosophila*, Brazil
IZ 610	*K. fragilis*	ESALQ	----------
ATCC 10606	*K. marxianus*	ATCC	Isolated from air, Nederland
KM9	*K. marxianus*	ESALQ	met^- mutant of IZ1339
KM21	*K. marxianus*	ESALQ	arg^- mutant of IZ 619
KM14	*K. marxianus*	ESALQ	ura^- mutant of IZ1821
KF4	*K. fragilis*	ESALQ	ser^- mutant of IZ610
9x21A	*K. marxianus*	LBVMA	Km9 x Km21 hybrid
9D	*K. marxianus*	LBVMA	Segregant of 9x21A
17D	*K. marxianus*	LBVMA	Segregant of 9x21A
45E	*K. marxianus*	LBVMA	Segregant of 9x21A

2.2 Media and Experimental conditions for the selection and production of enzymes

Pectinolytic strains were selected by the method proposed by McKay [21]. Briefly, the microorganisms were inoculated on YNB-pectin (1% citric pectin Genu® 105, 0.5% yeast extract, 0.67% Yeast Nitrogen Base (YNB), and 2% agar, pH 5.0), and grown at 30°C for 96 hrs. The plates were overlaid with an aqueous solution of ruthenium-red (0.05%), incubated for 5 minutes, and washed with distilled water. The clear halos of pectin degradation were measured, and the relative pectinolytic activity (RPA) calculated as a relation between the diameter of the degradation halo and the colony diameter.

To evaluate the extracellular pectinolytic activity, yeast strains were grown for 48h at 30ºC and 150 rpm in YEPD (2% glucose, 2% peptone, 1% yeast extract, pH 6.8), and used to inoculate (2×10^7 cells/mL) 50 mL of citric-pectin broth (0.5% yeast extract, 1% citric pectin, 0.67% yeast nitrogen base, pH 5.0). Cultures were maintained at 30ºC for 48h with no shacking, centrifuged at 14000 rpm for 10 minutes, and the supernatant used as crude extract for enzymatic analyses.

To evaluate the influence of culture medium composition on yeast growth and polygalacturonase production, four media with different concentrations of glucose and citric pectin (ERKISA S.A., Brazil) were formulated: (1) 1% pectin, (2) 2% pectin; (3) 1% pectin and 1% glucose, and (4) 1% glucose. These carbon sources were incorporated on the basal medium (0.67% Yeast Nitrogen Base, 0.5% Yeast Extract, pH 5.0) proposed by Kashyap et al. [22]. The culture media (50 mL) were inoculated (2×10^6 cells/mL final concentration) from a two day old culture in YEPD at 28°C and 150 rpm. The experiments were conducted at 28°C for 96 h. Samples were collected every 8 h, centrifuged (10 min, 10000 rpm), and the supernatant (crude extracts) stored at 4°C. The experiments were conducted twice with three replications of each treatment.

2.3. Analytical methods

The polygalacturonase activity was determined by measuring the release of reducing groups using the dinitrosalicilic acid (DNS) method proposed by Miller [23] adapted to small volumes. Briefly, 50 µL of crude extract were mixed with 50 µL of polygalacturonic acid solution (0.1% polygalacturonic acid in 25 $mmol.L^{-1}$ acetate buffer, pH 5.0) in

200 µL PCR plates for 96 samples. The plates were incubated at 40°C for 1 h in a MJ Research Thermal Cycler PTC100 (Perkin-Elmer). The reactions were stopped by the addition of 60 µL of DNS reagent. The plates were replaced in the thermo cycler, and incubated for 15 min at 95°C. The samples were transferred to a microplate (96 holes), completed to 200 µL, and the absorbance (595 nm) determined in a microplate reader (Biochrom Libra S12). Three replications were used for each sample. One unit of polygalacturonase activity was defined as the amount of extract required to release 1 µmol (galacturonic acid equivalent) of reducing groups per minute. The quantification of the reducing groups was performed by comparison with a standard curve of galacturonic acid (0 to 80 µg) under the same conditions.

PG activity was also determined by measuring the reduction of viscosity of a standard pectin solution. For this estimation 3.2 mL of diluted sample were mixed with 14.8 mL of a 1% (w/v) solution of citric pectin (Delaware, Porto Alegre, Brazil) in 0.05 $mol.L^{-1}$ acetic acid-acetate buffer (pH 4) and the reaction mixture incubated at 30 C for 30 min, after which the viscosity was measured in a DV-II+ viscometer (Brookfield model DV-II+, USA). One relative viscosity unit (RVU) was defined as the enzyme quantity required decreasing the initial viscosity by 50% in 30 min under the experimental conditions [24].

The concentration of reducing groups in the culture supernatant was determined by the DNS method described above, but using water instead of polygalacturonic acid solution, and incubating the samples directly at 95°C for 15 min. A standard curve of glucose (0 to 10 g/L) was used. Yeast growth was estimated by optical density at a wavelength of 600 nm.

2.4. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA), and means comparison by the Tuckey´s test with a probability level below 5% ($p < 0.05$). The analyses were conducted on the PASW 18.0.0 statistics.

3. RESULTS AND DISCUSSION

3.1. Selection of pectinolytic strains

Among the 19 *Kluyveromyces* strains evaluated, 16 (84%) showed pectinolytic activity on agar plates (Table 2). The three strains

(NRRL-Y-8278, NRRL-Y-1195, NRRL-Y-1207) that did not exhibited halo, grew on YNB-pectin medium, indicating that there are able to use pectin as sole carbon source. These strains showed just a basal extracellular polygalacturonase activity (1.07 to 1.69 $U.mL^{-1}$) when compared with the other strains.

Table 2. Pectinolytic activity of *Kluyveromyces* strains evaluated in this study

Strains	Relative activity (RA)	Pectinolytic activity ($U.mL^{-1}$)	Viscosity Reduction (RVU)
NRRL-Y-8278	0.00 ± 0.00^{i}	1.07 ± 0.17^{g}	1.39 ± 0.00^{h}
NRRL-Y-7571	4.60 ± 0.10^{b}	62.79 ± 1.03^{a}	9.95 ± 0.26^{a}
NRRL-Y-6373	3.67 ± 0.11^{c}	30.88 ± 1.03^{b}	9.58 ± 0.13^{a}
NRRL-Y-1190	3.09 ± 0.09^{de}	10.71 ± 0.16^{d}	3.21 ± 0.33^{f}
NRRL-Y-1195	0.00 ± 0.00^{i}	1.07 ± 0.14^{g}	1.12 ± 0.26^{h}
NRRL-Y-1207	0.00 ± 0.00^{i}	1.69 ± 0.18^{g}	1.44 ± 0.06^{h}
FTI 20014	3.67 ± 0.11^{c}	16.96 ± 1.84^{c}	2.46 ± 0.06^{g}
FTI 20015	3.34 ± 0.05^{cd}	12.61 ± 0.61^{cd}	4.27 ± 0.13^{de}
IZ 1339	2.09 ± 0.09^{h}	9.92 ± 0.41^{de}	1.07 ± 0.19^{h}
IZ 610	2.50 ± 0.06^{fg}	10.44 ± 1.05^{d}	1.12 ± 0.26^{h}
ATCC 10606	3.40 ± 0.10^{cd}	17.22 ± 0.61^{c}	4.84 ± 0.13^{cd}
KM9	2.75 ± 0.12^{ef}	13.79 ± 0.01^{cd}	1.39 ± 0.00^{h}
KM21	3.67 ± 0.08^{c}	16.45 ± 0.11^{c}	3.07 ± 0.06^{fg}
KM14	2.30 ± 0.10^{gh}	4.02 ± 0.81^{f}	1.49 ± 0.00^{h}
KF4	2.44 ± 0.04^{fgh}	8.49 ± 0.00^{e}	0.97 ± 0.06^{h}
9x21A	3.44 ± 0.11^{cd}	12.50 ± 0.84^{cd}	3.05 ± 0.06^{fg}
9D	4.33 ± 0.11^{b}	27.23 ± 1.32^{b}	4.00 ± 0.26^{e}
17D	5.13 ± 0.12^{a}	29.85 ± 0.00^{b}	6.19 ± 0.06^{b}
45E	4.50 ± 0.20^{b}	30.15 ± 1.03^{b}	5.37 ± 0.13^{c}

The values are the average of three replications, and treatments with the same letter in a column are no statistically different (Tukey test, $p<0.05$). One unit (U) is equal to amount of extract required to release 1 µmol (galacturonic acid equivalent) of reducing groups per minute.

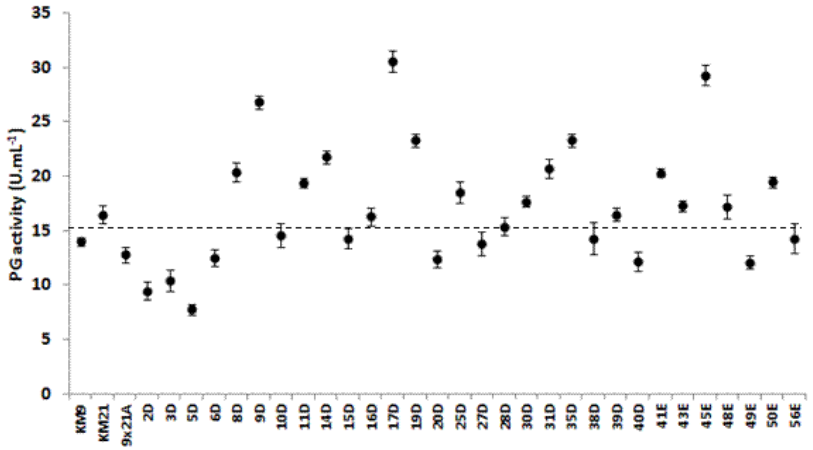
A large and significant variation was observed on the polygalacturonase activity among the *Kluyveromyces* strains evaluated (Table 2), and a significant correlation (R = 0.755) was detected

between relative activity (halo) and PG activity, indicating that relative activity can be used for strains selection. The highest extracellular PG activity was detected on NRRL-Y-7571 (62.79 $U.mL^{-1}$), followed by NRRL-Y-6373 (30.88 $U.mL^{-1}$). The PG activities detected in these strains were higher than those previously reported in different wild yeast species, including *Kluyveromyces* [11, 25, 26], and a recombinant *S. cerevisiae* strain [13]. *K. marxianus* NRRL-Y-7571 has high inulinolytic activity [27], and may be used for the production of both enzymes.

As expected, high correlation (R = 0.878) was observed between the polygalacturonase activity and the reduction of viscosity (Table 2). Crude extracts of NRRL-Y7571 and NRRL-Y6373 were able to drastically (>50%) reduce the viscosity of a pectin solution, confirming the potential of *Kluyveromyces* pectinases for industrial clarification and stabilization processes [16].

The auxotrophic mutants KM9 (met^-) and KM21 (arg^-) were crossed producing the 9x21A hybrid. This hybrid was sporulated and 30 segregants were initially selected by their pectinolytic halos on YNB-pectin medium. The segregants exhibited a large variation on extracellular pectinolytic activity and 40% were significantly higher than the parental strains (Figure 1).

Figure 1. Extracellular polygalacturonase activity of parental strains, hybrid and selected segregants. Dotted line indicates the mean PG activity of the parental strains KM9 and KM21.

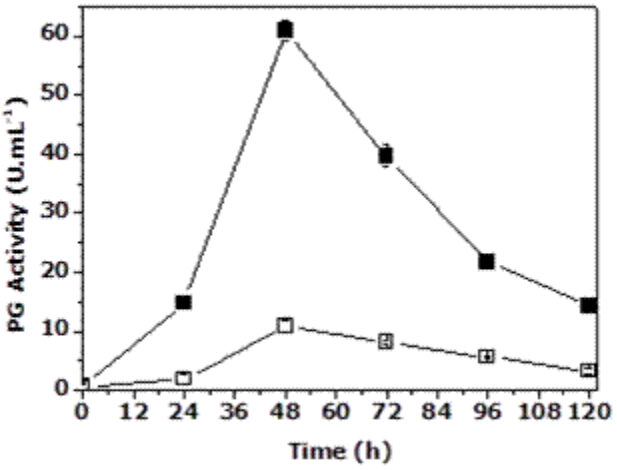


The pectinolytic activity of the selected segregants 9D, 17D and 45E was 80.5, 94.4 and 99.4% superior than the average of KM9 and KM21, indicating that conventional breeding methods can be efficiently used to increase extracellular polygalacturonase activity in *Kluyveromyces*.

3.2. Effect of medium composition and aeration agitation on the polygalacturonase production by K. marxianus NRRL-Y-7571

Based on results, NRRL-Y-7571 was selected for further analysis. A first experiment in pectin1%-YNB medium, conducted under constant shaking (150 rpm) and static condition, confirmed the negative effect of dissolved oxygen on polygalacturonase production by *Kluyveromyces marxianus* [28], with maximum activity of 10.9 U/mL in ~~aerated~~ agitated and 60.8 U/mL in non-~~aerated~~ agitated cultures, respectively (Figure 2).

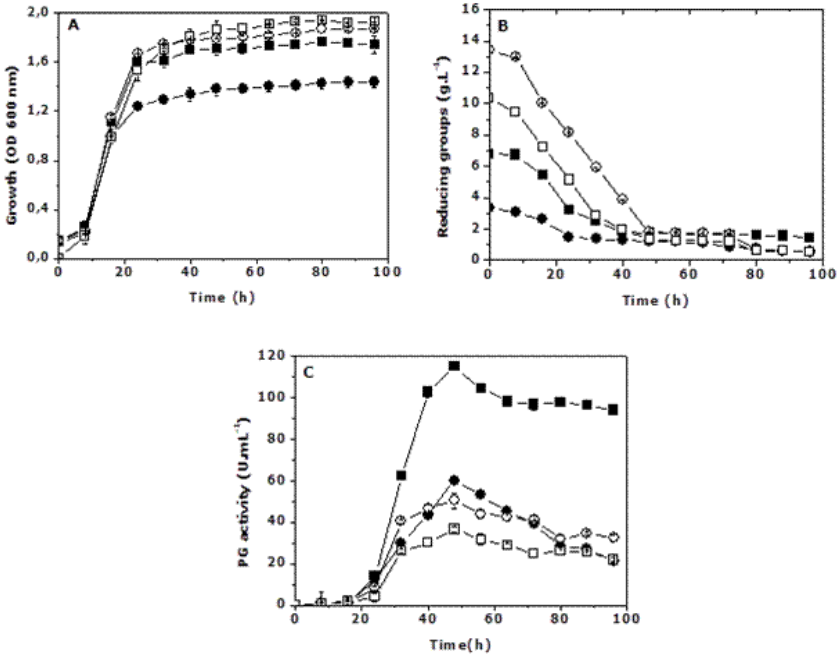
Figure 2. Extracellular polygalacturonase activity of *K. marxianus* NRRL-Y-7571 in static (■) and shacked (□) culture.



Yeast growth, reduction products variation, and polygalacturonase activity during static cultures of *K. marxianus* NRRL-Y-7571 on four media with different concentrations of glucose and pectin are shown in Figure 3. As can be observed, independent of the

culture media, NRRL-Y-7571 showed an initial lag phase of 8 h followed by a logarithmic growth phase of 8 h in 1% pectin to 16 h on the other media (Figure 3A). Moreover, no differences were observed on yeast growth on the pectin, glucose or pectin/glucose media (Figure 3A), indicating that polygalacturonic acid is efficiently metabolized by NRRL-Y-7571.

Figure 3. Effect of glucose and pectin on yeast growth and extracellular polygalacturonase activity. Glucose 1% (□), glucose 1% with pectin 1% (○), pectin 1% (●), and pectin 2% (■).



Kluyveromyces growth was accompanied by a decrease on reducing groups in culture media (Figure 3B). The absence of reducing products accumulation in pectin and pectin/glucose media, indicates that polygalacturonic acid released by polygalacturonase activity is rapidly incorporated and used by yeast cells. Filamentous fungi D-galacturonic acid metabolism operates by a specific pathway that involves a sequential conversion of D-galacturonic acid to L-galactonate, 2-keto-3-deoxy-L-galactonate, and glycerol and pyruvate [29]. Although

conserved in fungi, we were not able to find homologous (pBLAST) to fungal D-galacturonic acid reductase and L-galacturonate dehydrogenase, the two initial enzymes in D-galacturonic acid pathway, in *Saccharomyces cerevisiae* and *Kluyveromyces lactis* genomes, posing an interesting question on the metabolism of D-galacturonic acid residues by pectinolytic yeasts.

Independent of the culture media, polygalacturonase activity reached its maximum after 48 h of culture, when the yeast attained the stationary phase, followed by a low but significant decay (Figure 3C). The association between *K. marxianus* growth and polygalacturonase activity was previously evidenced [25].

The highest polygalacturonase activity of *K. marxianus* NRRL-Y-7571 was obtained in 2% pectin medium (114 U/mL) (Figure 3C). This value is higher than that reported in other studies using other strains [11, 25, 26, 29].

The polygalacturonase activity in 1% glucose medium (36 U/mL) confirmed the constitutive production of this enzyme by *Kluyveromyces* [21]. However, in 1% glucose plus 1% pectin medium, the activity was significantly higher (50 U/mL), indicating that although constitutive this enzyme is induced by pectin or their products. Polygalacturonase induction by pectin was observed in different yeast species, including *S. cerevisiae* [30, 31] and *Kluyveromyces marxianus* [25, 32]. Conversely, Schwan and Rose [33] did not evidence polygalacturonase induction by pectin in the *Kluyveromyces marxianus* CCT 3172 isolate, indicating that pectin induction may be strain specific.

In summary, the present results showed that the selection and breeding of *K. marxianus* can significantly increase the polygalacturonase production, and confirmed that the establishment of adequate culture conditions for specific strains is essential to obtain high pectinolytic extract for industrial purposes.

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CAPÍTULO IV - ARTIGO 2

GRAPE JUICE EXTRACTION AND CLARIFICATION USING AN ENZYMATIC PREPARATION OF THE HIGHLY PECTINOLYTIC YEAST *Kluyveromyces marxianus* NRRL-Y-7571

Artigo submetido para publicação no periódico *LWT - Food Science and Technology* em maio de 2012

Grape Juice Extraction and Clarification using an Enzymatic Preparation of the highly Pectinolytic Yeast *Kluyveromyces marxianus* NRRL-Y-7571

ABSTRACT

This study analyses the efficiency of yeast pectinolytic preparations produced in laboratory compared with commercial products used in the maceration and clarification process of grape juices. A crude enzymatic extract, produced by *Kluyveromyces marxianus* NRRL-Y-7571, was tested in comparison to the commercial preparations Novozym®33095 and Pectinex®Ultra Color, used for the maceration and clarification of juice, respectively. With pectinases total activity at 1 U/mL of fruit juice, reactions were conducted at 40ºC for 60 min. After treatment with the crude enzymatic extracts, increases in extraction yield (28.02%) and a decrease in viscosity (50.70%) during the maceration step for obtaining the pulp, and decrease in turbidity (11.91%) during the juice production step were observed. With commercial enzyme preparation, higher values for these parameters: 42.36, 63.20, and 26.81% respectively, were achieved. By using commercial enzyme preparation, a decrease in polyphenol content compared to the control in the juice (11%), and an increase (15%) when the crude enzyme extract was applied, was observed. The anthocyanins content in the grape juice was decreased after treatment with the commercial preparation (18%), and increased with the use of crude enzymatic extracts (20%). Considering all comparison criteria, the crude enzymatic extract showed promising results for red grape juice processing.

Key-words: pectinase, maceration, clarification, grape juice

1. INTRODUCTION

Due to the presence of polysaccharides (pectin, cellulose, hemicelluloses and starch), proteins, tannins and metals, the fruit juices are naturally cloudy (Vaillant, Millan, Dornier, Decloux, & Reynes, 2001). Pectins are responsible for the turbidity and consistency of the juice causing an increase in their viscosity which hinders its clarification, filtration and concentration (Abdullah, Sulaiman, Aroua, & Noor, 2007).

The main industrial application of pectinases is the extraction and clarification of fruit juices (Gummadi & Panda, 2003). Most of the microbial pectinases produced by the industry are dedicated to this purpose. The degradation of pectic substances in fruit juices is achieved through the addition of pectinolytic enzymes resulting in an increase in juice yield and its clarification, as well as a decrease in viscosity. Moreover, treatment with pectinases facilitates juice filtering (Sandri, Fontana, Barfknecht, & Silveira, 2011).

Grape juice is among the fruit processing industry which includes extraction and enzymatic clarification. More than 98% of the grape production in Brazil is processed to produce wine, juices and other products (IBRAVIN, 2011). To obtain the grape juice, the addition of pectinase is required at different stages, improving the visual characteristics (color and turbidity), assisting in the release of anthocyanins of red grapes, and increasing the extraction yield (Gómez-Plaza, Romero-Cascales, & Bautista-Ortín, 2010).

Considering the variety of enzymes, traditionally the majority of studies refer to the pectinases of the bacteria and various fungi (Jayani, Saxena, & Gupta, 2005; Yadav, Yadav, Yadav, & Yadav, 2009) although there has been a major advance in the description and characterization of pectic enzymes produced by yeast (Alimardani-Theuil, Gainvors-Claisse, & Duchiron, 2011; Blanco, Sieiro, & Villa, 1999).

The *Kluyveromyces* yeasts are used in bioprocesses for the production of various enzymes such as lactase, inulinase, lipase and pectinase (Fonseca, Gombert, Heinzle, & Wittmann, 2007). *K. lactis* is the most studied species in the genus *Kluyveromyces*, but his counterpart *K. marxianus* also has potential applications in various segments of biotechnology (Fonseca, Heinzle, Wittmann, & Gombert, 2008) and has a high capacity for production of pectinolytic enzymes (Schwan, Cooper, & Wheals, 1997). In this context, this study aimed to use the crude enzymatic extract produced by *Kluyveromyces marxianus* NRRL Y-7571 in the maceration and clarification of grape juice from Bordô cultivar (*Vitis labrusca*), in comparison with the commercial enzyme preparation.

2. MATERIALS AND METHODS

2.1 Production of experimental enzymatic extract

K. marxianus NRRL-Y-7571 was used for the production of

enzymatic extract in liquid medium. The liquid medium YNB-pectin (50 mL) used in the process was defined by Kashyap, Chandra, Kaul, & Tewari (2000) and had the following composition: 0,5% yeast extract, 1% citric pectin (ESKISA S.A., Brazil), 0,67% Yeast Nitrogen Base, pH 5,0. Duran flasks were inoculated with *K. marxianus* NRRL-Y-7571 (2×10^7 cells/mL), and incubated at 28°C for 48 hours, under static conditions. The obtained solution was then centrifuged for 10 minutes at 5000 g. The supernatant (enzymatic extract) was kept at 4°C.

2.2 pH and temperature effect on the pectinolytic activity of K. marxianus extracts

The optimal pH of the reaction crude enzyme extract of *K. marxianus* NRRL-Y-7571 was evaluated using acetate buffer solution (pH 3.2, 3.6, 4.0, 4.4, 4.8, 5.2, 5.6 and 6.0) at 40ºC for 60 min. The stability of pectinase produced by *K. marxianus* was evaluated at different incubation temperatures of crude extracts (20, 30, 40, 50, 60 and 70°C) at different times (0, 15, 30, 45, 60, 90, 120 and 150 minutes) at pH 5.0.

2.3 Production of grape pulp and juice

Bordô grape (*Vitis labrusca*) used in this work was cultivated in Caxias do Sul/Brazil and harvested in March 2011. To obtain the pulp, 200g samples of selected and cleaned fruit were manually crushed, and either treated with the commercial enzyme preparation Novozym®33095 (Novozymes Latin America LTDA, Brazil) obtained from *Aspergillus aculeatus* e *A. niger*, or enzymatic crude extract produced by *K. marxianus* NRRL-Y-7571, diluted in 10 mL of distilled water, both with total pectinase activity (TPA) of 1U per gram of fruit. For each assay, control samples free of pectinolytic enzymes were also evaluated. Controls and treatments were incubated in a thermostatic bath (B. Braun Biotech model Certomat WR, Germany) at 40°C for 60 min, with the enzymatic activity being stopped by cooling to 4 °C in an ice bath. Pulp preparation was finished by grinding and mixing the paste in an industrial blender (Metvisa, Brazil). The pulp yield was determined gravimetrically, by taking into account the masses of the control pulps and of the enzymatically treated masses.

For juice production, 10 mL of solution from each enzyme preparation, commercial enzyme preparation Pectinex®Ultra Color (Novozymes Latin America LTDA, Brazil) obtained from *Aspergillus*

aculeatus e *A. niger*, or enzymatic crude extract produced by *K. marxianus* NRRL-Y-7571, were added to 50 mL of pulp contained in 100 mL beakers to obtain 1U of TPA per mL of pulp. The conditions were the same as those used to obtain the pulp. After the treatment, the materials were filtered (Whatman No. 1) to obtain the juice.

2.4 Analytical Methods

Total pectinase activity (TPA) was determined by measuring the release of reducing groups by using the method dinitrosalicílico acid (DNS) proposed by Miller (1959), adapted for small volumes. Reading was performed in an ELISA reader (Biochrom Pound S12) with a wavelength of 595ηm. For each experiment there were three replicates. One unit of activity was defined as the amount of enzyme required to release 1 μmol (equivalent of galacturonic acid) reducing groups per minute. The quantification of the reducing groups was performed by comparison to a standard curve of galacturonic acid (0-80 μg) under the same conditions.

The pH value of grape was measured using a digital pH meter (Horiba Fseries, Model F21). Buffer solutions at pH 4.0 and 7.0 were used to standardize the equipment.

Protein content in grape was determined by Lowry procedure (Lowry, Rosebrough, Farr, & Randall, 1951) adapted by Pinelo, Zeuner, & Meyer (2010). Briefly, 0.5 mL of $CuSO_4 \cdot 5H_2O$ (10 $g.L^{-1}$) was mixed with 50 mL of 10 $g.L^{-1}$ of Na_2CO_3-KNa-tartrate, and 1.0 mL of the resulting solution was mixed with 0.1 mL of a suspension prepared with 1 g of crushed fruit to which 49 mL of water were added. After 10 min, 0.1 mL of Folin-Ciocalteu reagent was added to the reaction. The absorbance was measured at 660 nm after 30 minutes of incubation. The protein content was expressed as bovine serum albumin (BSA) equivalents in g/100 g of fresh fruit.

For determining pectin content in grape, polysaccharides were extracted from samples prepared with 5 g of crushed fruit suspended in 245 mL of water acidified with citric acid to pH 2.5, under reflux in a condensation system operated at 97 °C for 30 minutes. Pectin was precipitated by alcohol-juice treatment 2:1. The mixture of solvent and precipitate was stirred for 10 minutes and then left to rest for one hour to allow pectin flotation. After this procedure, the pectic substances float on the surface of the alcohol/water mixture and thus they are easier to be removed in a quantitative way. The floating pectin was filtered through cheesecloth, rinsed with 95 °GL alcohol, and pressed. The pressed

pectin was dried to constant weight at 55 °C, cooled in a desiccator, and the yield was calculated on a dry weight basis (initial weight of sample) (Canteri- Schemin, Fertonani, Waszczynskyj, & Wosiacki, 2005).

Viscosity of the grape pulp was measured in viscometer (Brookfield model DV-II +, USA) in 20 mL samples at 30ºC (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2010). The yield of grape pulp enzymatically treated was determined gravimetrically by considering the masses of the untreated control pulps. The degrees of viscosity and yield were expressed as percentage of viscosity or yield, calculated with the results obtained from the control samples of each assay.

The turbidity in grape juice following each treatment was evaluated in spectrophotometer (Genesys 10UV Thermo, USA) at 540 nm (Chatterjee, Chatterjee, Chatterjee, & Guha, 2004). The degrees of turbidity were expressed as percentage of turbidity, calculated with the results obtained from the control samples of each assay.

Colour intensity (CI) was calculated as the sum of absorbance at 620 nm, 520 nm and 420 nm, and expressed as the ratio between absorbance at 420 nm and the absorbance at 520 nm (Amerine & Ough, 1988).

Total phenols in grape pulp and juice were determined by the Folin-Ciocalteu procedure (Singleton & Rossi, 1965), as follows: 1 mL of 10-fold diluted Folin-Ciocalteu reagent was added to 0.2 mL of 1:10 diluted sample, incubated at room temperature (approximately 25°C). After 1 min, 0.8 mL of Na_2CO_3 solution (75 g/L) was added and the mixture was shaken. After two hours, the absorbance was measured at 765 nm. The phenolic content was expressed as gallic acid equivalents in µg/mL.

The total anthocyanin content was determined using the pH differential method described by Giusti & Wrolstad (2001), which relies on the structural transformation of the anthocyanin chromophores as a function of pH. The anthocyanin content was calculated as cyanidin-3-glycosid and the results were expressed as µg cyanidin-3-glycoside/mL.

2.5 Statistical analysis

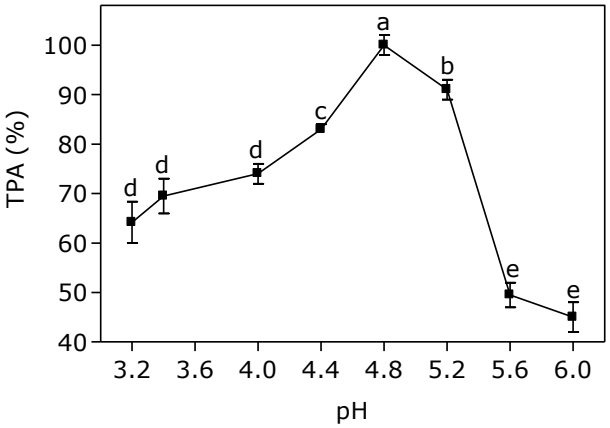
The data were submitted to variance analysis (*one-way* ANOVA) and means were compared by the Tuckey's test, with probability level below 5 % ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Characterization of yeast pectic enzymes

The pH effect on total pectinase activity in *K. marxianus* enzymatic extracts is presented in the Figure 1. Maximal activities were obtained at pH value of 4.8, and over 80% of maximum activity was maintained between pH 4.4 to 5.2. These values are within the range observed in PG enzymes from *Saccharomyces paradoxus* (Eschstruth & Divol, 2011), *Kluyveromyces marxianus* (Serrat, Bermúdez, & Villa, 2002), *K. marxianus* CECT 1043 (Siekstele, Bartkeviciute, & Sasnauskas, 1999), *S. cerevisiae* (Blanco, Sieiro, Díaz, & Villa, 1994), and *K. wickerhamii* IFO 1675 (Yoshitake, Numata, Katsuragi, Hours, & Sakai, 1999).

Figure 1. pH effect on total pectinase activity (TPA) in experimental enzymatic extracts under standard conditions. The TPA (%) values correspond to the average of three tests. Diferent letters (a – e) indicate significant differences ($p < 0.05$). Total pectinase activity (TPA) as a function of pH (A) at 40°C.

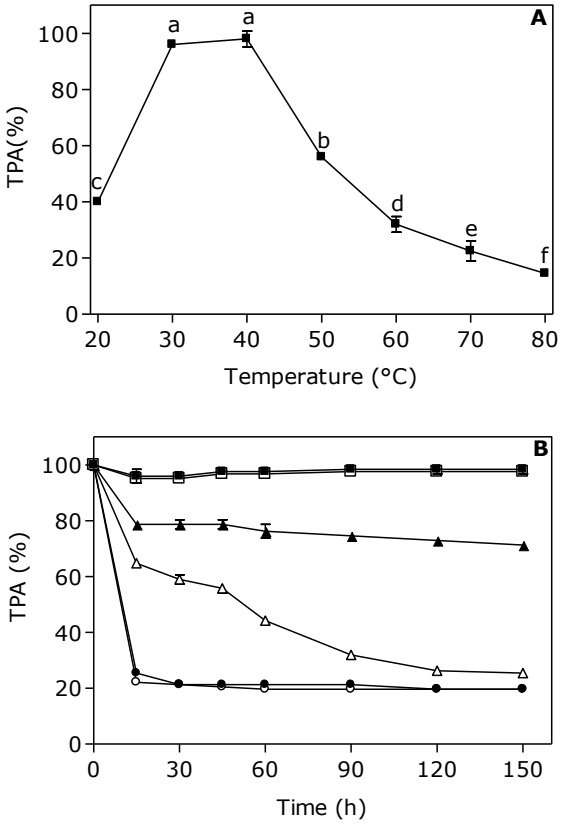


For biotechnological applications, another important factor is the optimum enzymatic temperature. The results obtained with *K. marxianus* crude extracts are shown in Figure 2A in which can be observed that the highest activities were attained between 30 and 40ºC,

with a maximum at 40ºC. Similar results were found for the pectinase produced by *Saccharomyces cerevisiae* (Blanco, Sieiro, & Villa, 1999) e *S. paradoxus* (Eschstruth & Divol, 2011).

The profiles of PG stability at different temperature over time (Figure 2B) showed that the pectinase activity is stable at 20, 30 and 40ºC and progressively decrease at 50°C, and rapidly decrease above 60ºC and 70°C.

Figure 2. Temperature effect (A) and thermostability (B) of the total pectinase activity (TPA) of enzymatic extract of *K. marxianus*. 20°C (solid square), 30ºC (open square), 40ºC (solid triangle), 50ºC (open triangle), 60ºC (solid circle), 70ºC (open circle). The TPA (%) values correspond to the average of three tests. Different letters (a – f) indicate significant differences ($p < 0.05$).



3.2 Enzymatic treatments in fruit pulp and juices

Initially a physico-chemical characterization of the fresh grape was carried out. Proteins present in the fruit can affect the activity of these enzymes as they interact with phenolic compounds (Siebert, 2006), forming complexes that are difficult to remove (Pinelo, Zeuner, & Meyer, 2010). However, the analyses in the grape fruits tested showed that protein concentration was low, around 0.61 g/100 g of fresh fruit, not affecting juice turbidity. However, it was observed that Bordô grape contains a high amount of pectin (4.56 g/100 g of fresh fruit), hindering the maceration step, since the main components that influence the structure and viscosity of the juice are pectic polysaccharides (Grassin & Fauquembergue, 1996), and justifing enzymatic treatment.

The effect of exogenous pectinases addition during Bordô grape pulp maceration on juice yield and viscosity, as well as in the turbidity during juice clarification is present in Table 1. The enzymatic extract of the *K. marxianus* increased juice yield and reduced pulp viscosity. However, the results were lower than those obtained with Novozym®33095. This difference can be attributed to the presence of other hydrolytic enzymes, such as cellulases, amylases, and xylanases in the commercial preparation, which increase cell wall degradation. The combination of pectinases and cellulases in the treatment of fruit pulp provides an increase in yield after processing, when compared to using only pectinases (Câponouvá & Drdák, 2002). The reduction of juice viscosity is a result of enzymatic degradation in the cell wall matrix and the release of water and lower molecular weight polysaccharides. Although the yield and viscosity parameters are related, the former is mainly the result of joint action of several hydrolytic enzymes, while the viscosity reduction is mainly due to pectinolytic enzymes. Moreover, the pH of fresh grape was 3.18, a pH in which the pectinolytic activity of *K. marxianus* NRRL-Y-7571 extract is approximately 60% of its maximum (Figure 1), but is within the optimal pH range (2.8 – 3.8) recommended for Novozymes.

Enzymatic processing improved the juice yield by 28-42%. The increase in yield obtained by enzymatic processing was higher than the range observed by Threlfall, Morris, Howard, Brownmiller, & Walker (2005) on Black beauty and Sunbelt grape juices. They observed an increase of 24-33% on similar comparison in Sunbelt and Black beauty grapes. Similar values for juice yield (32-48%) were observed by Paranjpe, Ferruzzi, & Morgan (2012) in Thompson seedless grapes.

These differences may be solely due to the variety of grapes or the time for the heating process.

A major problem found in the preparation of fruit juices is the turbidity, due to the presence of insoluble materials, mainly protopectins responsible for the firmness and hardness of unripe fruits, and which move to soluble form during ripening. When the preparation of fruit juice is performed without the addiction of pectinolytic enzymes, high turbidity and viscosity hinders juice pressing and filtration. Both *K. marxianus* pectinolytic extract and Pectinex®Ultra Color reduced juice turbidity, with better results for the commercial enzyme (Table 1).

Table 1. Effect of enzymatic treatment on the production of pulp and juice of grape in relation to control sample.

Parameters	Commercial enzyme preparation (PEC)	Crude enzymatic extract of the *K. marxianus* (EEB)
Pulp yield increment (%)[1]	42.36 ± 1.40[a]	28.02 ± 1.80[b]
Pulp viscosity reduction (%)[1]	63.20 ± 1.77[a]	50.70 ± 2.30[b]
Juice turbidity reduction (%)[2]	26.81 ± 0.42[a]	11.91 ± 0.69[b]

Same letters for each row do not differ statistically at 5% ($p<0.05$). [1] Parameter determined in the macerated pulp. [2] Parameter determined in the clarified juice.

In general, the action of commercial enzyme preparations was more efficient than the crude enzyme extract of *K. marxianus*. However, the results found in this study are promising since the pectinolytic extract of *K. marxianus* was just a crude extract, and that the pH of Bordô grape juice used (pH 3.18) was lower than the optimum for the pectinolytic activity of the extract (Figure 1).

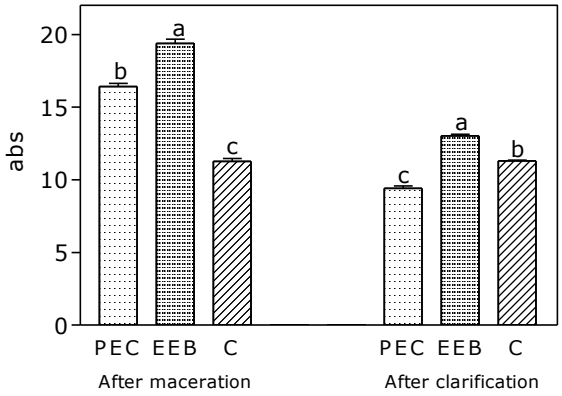
According to Busse-Valverde, Gómez-Plaza, López-Roca, Gil-Muñoz, & Bautista-Ortín (2011) in red grapes juice the degradation of cell walls in the skin of the grapes through pectinolytic enzyme treatment results in an increased release of phenolic compounds responsible for color. Colour is the most important attribute used, along with other variables, as an indicator of the quality of grape juice. This characteristic is directly dependent on the phenolic composition of the juice and the anthocyanins present in the grape skin. The anthocyanins participate in many reactions that promote changes in the colour of

grape products, mainly through co-pigmentation and formation of polymeric pigments (Wrolstad, Durst, & Lee, 2005).

The color intensity values ranged from 11.27 to 19.38 after maceration of pulp and 9.42 to 15.11 after clarification of juice (Figure 3). All juices showed a higher percentage of red colour (85%), followed by yellow (10%) and blue (5%).

The juice samples treated with commercial enzyme and crude enzymatic extract of *K. marxianus* NRRL-Y-7571 showed a significant difference ($p<0.05$) regarding color intensity. Some commercial enzyme preparations have in their composition secondary enzymatic activities that can change the profile of phenolic compounds (Pinelo, Arnous, & Meyer, 2006). A example is the degradation of anthocyanins by beta-glycosidase or phenol esterase, as reported by Bautista-Ortín, Martínez-Cutillas, Ros-García, López-Roca & Gómez-Plaza, (2005).

Figure 3. Color intensity of the grape juice after maceration and clarification. PEC: Commercial enzyme; EEB: Crude enzymatic extract of *K. marxianus* NRRL-Y-7571. C: control. Diferent letters (a – c) indicate significant differences ($p < 0.05$) for each process.



Polyphenol and anthocyanins content in grape juices are important variables due to its contribution to juice color and antioxidant activity. According to Scola et al. (2010), *V. labrusca* species, mainly Bordô variety can be used to obtain extracts with important antioxidant activity. The main phenolic compounds in *V. labrusca*, mainly Bordô variety extracts were catechin and epicatechin, followed by procyanidin

B3, procyanidin B1, procyanidin B2, gallic acid, epigallocatechin, and procyanidin B4.

As can be observed in Table 2, both *K. marxianus* extract and commercial enzyme treatments increased the concentration of polyphenols and anthocyanins in the pulp. Polyphenols and anthocyanins are located in the skin cell vacuoles and transferred from grape skins (Paranjpe, Ferruzzi, & Morgan, 2012), to juice during the maceration stage. The degradation of the cell walls of the grape cells by pectinase and cellulase allows a higher diffusion of the components located inside the vacuoles, facilitating a better extraction of the juice during pressing (Gómez-Plaza, Romero-Cascales & Bautista-Ortín, 2010).

Table 2. Polyphenols and anthocyanins content in grape pulp and juice after enzymatic treatments.

Treatment	Polyphenols (µg/mL)		Anthocyanins (µg cyanidin-3-glucoside/mL)	
	Pulp	Juice	Pulp	Juice
Control	1733.99 ± 98.27^{b}	1699.52 ± 150.55^{b}	21.07 ± 1.47^{b}	18.45 ± 0.50^{b}
PEC	1952.35 ± 203.68^{a}	1509.23 ± 65.76^{b}	24.30 ± 0.83^{a}	15.04 ± 0.69^{c}
EEB	2168.98 ± 195.57^{a}	1924.04 ± 210.50^{a}	27.62 ± 2.12^{a}	22.25 ± 0.54^{b}

Values correspond to the average of three tests. Different letters (a-b) indicate significant differences ($p < 0.05$) for each analysis. PEC: Commercial enzyme; EEB: Crude enzymatic extract of *K. marxianus* NRRL-Y-7571.

After clarification, the polyphenol and anthocyanin content of control juice decreased 2 and 12.4%, respectively. However, juice treatment with commercial enzyme caused a reduction (pulp vrs. juice) of 23% and 38% in polyphenol and anthocyanin content, respectively, when these reductions were of 11.3 and 20% in juices treated with *K. marxianus* extracts. This occurs because, the clarification of the juice to precipitate pectin drags with other compounds which are removed by centrifugation (Landbo, Pinelo, Vikbjerg, Let, & Meyer, 2006).

4. CONCLUSIONS

The results of this study demonstrate the benefits of using enzyme treatment to obtain grape pulp and juice. The enzyme treatment with either *K. marxianus* extract or commercial enzymes positively influenced the process increasing yield and clarity, and decreasing juice turbidity. However, crude pectinolytic extract of *K. marxianus* resulted in higher polyphenol and anthocyanin extraction and final concentration compared with commercial enzymes. These results indicates that the pectinolytic extract obtained from *K. marxianus* NRLL-Y-7571, are promising for grape juice processing, contributing to sensory (visual) and nutritional qualities.

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CAPÍTULO V - ARTIGO 3

PECTINOLYTIC EXTRACT OF *Kluyveromyces marxianus* IN RED WINE MAKING

Artigo submetido para publicação no periódico *Journal of Agricultural and Food Chemistry* em junho de 2012

Pectinolytic Extract of *Kluyveromyces marxianus* in Red Wine Making

ABSTRACT

The effect of the addition of enzymatic extract of *Kluyveromyces marxianus* NRRL-Y-7571 during the maceration and fermentation steps of Cabernet Sauvignon wine production was evaluated. The results obtained in the analytical determinations of the wines showed levels within the limits established by Brazilian legislation and similar to values found in other studies conducted. The results show that the addition of enzyme in red wines presented chromatic characteristics which can be considered better than those of the control wine, and accelerates the extraction of phenolic compounds and anthocyanins. Moreover, wines treated with the *K. marxianus* enzymatic extract exhibited lower turbidity that greatly facilitated wine clarification.

Keywords. *Kluyveromyces marxianus*. Enzymatic extract. Wine. Color.

INTRODUCTION

A common red wine making procedure is characterized by a maceration step in which the solid parts of grapes grains (skins and seeds) remain in contact with the must during fermentation. During this period, the aromatic and colored compounds liberated determine important varietal organoleptic characteristics of the final product [1]. Phenolic compounds and anthocyanins, present in grape skin cell vacuoles and extracted during maceration, are responsible for color intensity and tonality of red wines [2].

The extraction and stability of colored compounds is influenced by the maceration time, enzymes, pH, ethanol, temperature, among other factors. Endogenous pectinases present in grapes play an important role in fruit ripening, but they are poorly active under the pH conditions and SO_2 levels of wine fermentation [3]. Commercial pectinases, currently obtained from *Aspergillus* sp., are used in winemaking to enhance the extraction of grape compounds by degrading structural polysaccharides [4]. Fundamentally, these enzymes are added to the must during the pellicle maceration stage (maceration enzymes) or during the debourbage of the must or wine (clarification enzymes) [5].

The use of pectinolytic yeasts or their enzymes could be highly advantageous in the development of wine. Unlike filamentous fungi, yeasts do not usually secrete pectin esterase. Therefore, their pectinases can be used to wines while maintaining low methanol levels [6]. Pectinolytic wine yeasts may improve liquefaction, clarification and filterability of grape must, releasing more color and flavor compounds entrapped in the grape skins and thereby make a positive contribution to the wine bouquet [7].

Yeast pectinases have attracted a great deal of attention from various research groups worldwide as an alternative to fungal pectinases [8]. When added to grape must, a crude pectolytic extract from a *S. cerevisiae* strain had the same effect on the turbidity as the same quantity of a commercial pectinase preparation [9]. The clarification process is greatly facilitated, with the filtration time being reduced by up to 50% in some cases, when wine fermentations are carried out using pectolytic strains of *S. cerevisiae* [10]. In this context, pectinolytic enzymes derived from *Kluyveromyces* would provide a useful alternative to mould derived pectinases. The aim of this study was to evaluate the effect of the enzymatic extract of *K. marxianus* NRRL-Y-7571 in winemaking grape of Cabernet Sauvignon. For this, the fermentation parameters and the effect of enzymes on the polyphenol concentration and colour of the wines were determined.

MATERIAL AND METHODS

Production of experimental enzymatic extract

K. marxianus NRRL-Y-7571 was used for the production of enzymatic extract (EB) in liquid medium. The liquid medium YNB-pectin used in the process was defined by Kashyap et al. [11] and had the following composition: 0.5% yeast extract, 1% citric pectin, 0.67% Yeast Nitrogen Base, pH 5.0. Duran flasks (100 mL) with liquid medium were used to obtain the enzymatic extract. The flasks were inoculated with *K. marxianus* NRRL-Y-7571, and incubated at 28°C for 48 hours, under anaerobic conditions. Cultures were centrifuged for 10 minutes at 5000 g, and the supernatant (enzymatic extract - EB) was kept at 4°C.

Total pectinolytic activity assay

The total pectinolytic activity (PG) was determined by measuring

the release of reducing groups by using the method dinitrosalicílico acid (DNS), proposed by Miller [12] adapted to small volumes. Briefly, the crude extract (50 µL) was mixed with 50 µL of polygalacturonic acid solution (0.1% in 25mM acetate buffer pH 5.0) in PCR plates with 96 holes (200 µL). The plates were incubated at 40ºC for 1 hour in Research thermal cycler model PTC 100, and the reaction was stopped by adding 60 µL of DNS. The plates were again placed in the thermocycler and maintained at 95ºC for 15 minutes, and after completion of the volume to 200 µL with distilled water. Samples (150 µL) were transferred to microplate and the absorbance (595nm) evaluated in a Biochrom Libra S12 microplate reader. One unit of activity was defined as the amount of enzyme required to release 1 µmol (equivalent of galacturonic acid) reducing groups per minute. The quantification of the reducing groups was performed by comparison with a standard curve of galacturonic acid (0-80 µg) under the same conditions. All the tests were made in triplicate.

Grape samples and winemaking

Grapes from *Vitis vinifera* L. var. Cabernet Sauvignon, cultivated at Caxias do Sul/Brazil, were harvested into 10 kg plastic bags and transported to the laboratory. The grapes were weighed, divided into batches of 400 g, destemmed, crushed manually, sulfited (30 mg. L^{-1} sodium metabisulfite) and transferred to 500 mL flasks covered with Müller valves. Two wines were elaborated, each in triplicate, one with 0.8 $U.mL^{-1}$ enzymatic extract of *K. marxianus* NRRL-Y-7571 (Sc+EB) and a control (Sc). Both treatments were inoculated with 5 x 10^6 $UFC.mL^{-1}$ of *Sacharomyces cerevisiae* EC1118 (Lallemand Inc.). The alcoholic fermentation was followed by the release of CO_2 every 24 hours. On the sixth day of fermentation, husks and sediments were removed. After completion of fermentation, the wines were transferred to sterilized containers and maintained at 10ºC for further analysis.

Must and Wine analyses

Ethanol (% v/v), reducing sugars ($g.L^{-1}$), pH, total acidity (expressed as $g.L^{-1}$ of tartaric acid), volatile acidity (expressed as $g.L^{-1}$ of acetic acid), total soluble solids (ºBrix) were determined according to the official methods [13]. Glycerol ($mg.L^{-1}$) was analysed by enzymatic methods.

Turbidity of must and wine were measured using a benchtop turbidity meter (Cyberscan Turbidimeter TB1000) and expressed in nephelometric turbidity unit (NTU).

Color intensity (CI) was calculated as the sum of absorbance at 620 nm, 520 nm and 420 nm, and expressed as the ratio between absorbance at 420 nm and absorbance at 520 nm [14].

Total phenols in wines were determined by the Folin-Ciocalteu procedure [15], as follows: 1 mL of 10-fold diluted Folin-Ciocalteu reagent was added to 0.2 mL of 1:10 diluted sample, incubated at room temperature (approximately 25ºC). After 1 min, 0.8 mL of Na_2CO_3 solution (75 $g.L^{-1}$) was added and the mixture was shaken. After two hours, the absorbance was measured at 765 nm. The phenolic content were expressed as mg of gallic acid equivalents (GAE).L^{-1}, averaged from three measurements.

The total anthocyanin content was determined using the pH differential method described by Giusti and Wrolstad [16], which relies on the structural transformation of the anthocyanin chromophores as a function of pH. The anthocyanin content was calculated as cyanidin-3-glycosid and the results were expressed as mg cy-3-glycoside.L^{-1}.

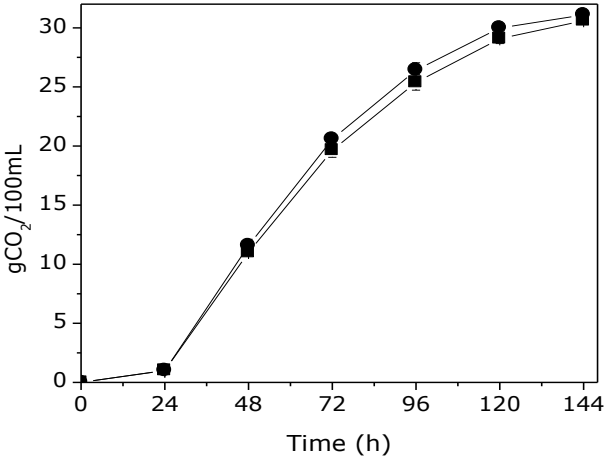
Statistical analysis

The data were submitted to variance analysis (one-way ANOVA) and means were compared by the Tuckey's test, with probability level below 5 % ($p<0.05$).

RESULTS AND DISCUSSION

Wine fermentations processed with and without enzymatic extract *K. marxianus* NRRL-Y-7571 showed similar behavior (Figure 1), indicating that the addition of the crude extract of *K. marxianus* did not affect the fermentation rate of EC1118. Although *K. marxianus* NRRL-Y-7571 exhibited a weak killer activity against EC1118 in co-cultures in solid medium (data none shown), the enzymatic extract did not affect *S. cerevisiae* growth. The function of *S. cerevisiae* EC1118 is to readily catalyze the conversion of the two main soluble sugars present in grape must, glucose and fructose, to ethanol and carbon dioxide, as well as other minor but important metabolites responsible for the organoleptic characteristics of the final product [17, 18].

Figure 1. Fermentation kinetics (gCO_2/100mL) without (solid square) and with enzymatic extract of *K. marxianus* NRRL-Y-7571 (solid circle), both inoculated with EC1118.



The average results of wine analyses (Table 1) showed that the fermentation parameters (alcohol, reducing sugars, pH, total and volatile acidity, soluble solids, and glycerol), did not significantly differ between the treatments with and without *K. marxianus* enzymatic extract, confirming that the addition of the enzymatic extract *K. marxianus* did not affect the fermentation performance. The alcohol content of wines (Table 1) were almost identical, leading to an average residual sugar concentration of 1.72 $g.L^{-1}$. Moreover, the pH values of wines produced in this study (3.66 to 3.68) were within the range observed by Cliff et al. [19] and Chira et al. [20] in Cabernet Sauvignon wine. Total acidity (6.25 and 6.35 $g.L^{-1}$) and volatile acidity were in accordance with Brazilian legislation and did not differ from that reported in Cabernet Sauvignon wines [19, 21, 22]. Glycerol concentration, the most abundant by-product of alcoholic fermentation, after ethanol and carbon dioxide [23], did not differed in wines produced with or without the enzymatic extract of *K. marxianus*, and were similar to that reported by Molina et al. [24]. However, wines produced with the addition of *K. marxianus* extract were significantly clearer than the controls, indicating that the pectinolytic enzymes of the extract efficiently reduce wine turbidity by degrading pectic compounds present in grape must. As pointed out by

Revilla and González-San José [25] and Pérez-Magariño and González-San José [26], wines that are not enzymatically treated show higher concentrations of pectins in suspension, which difficult the precipitation of impurities and increase turbidiy.

Table 1. Values obtained for the analysis parameters before and after alcoholic fermentation

	Initial analyses of must	Final analyses of wine	
		Sc	ScEB
Alcohol (% v/v)	0[b]	9.1 ± 0.14[a]	9.2 ± 0.17[a]
Reducing sugars ($g.L^{-1}$)	183.7± 0.22 [a]	1.69 ± 0.02 [b]	1.75 ± 0.08 [b]
pH	3.61 ± 0.03 [a]	3.68 ± 0.06 [a]	3.66 ± 0.02 [a]
Total acidity* ($g.L^{-1}$)	8.31 ± 0.06 [a]	6.35 ± 0.14 [b]	6.25 ± 0.04 [b]
Volatile acidity** ($g.L^{-1}$)	0 [b]	0.38 ± 0.05 [a]	0.36 ± 0.08 [a]
Total soluble solids (°Brix)	19.84 ± 0,29 [a]	0 ± 0.06[b]	0 ± 0.06[b]
Glycerol ($g.L^{-1}$)	0[b]	7.2 ± 0,22[a]	7.4 ± 0,09[a]
Turbidity (NTU)	76.13 ± 1.03[a]	75.05 ± 0.75[a]	61.85 ± 0.78[b]

Values are mean of triplicates. Same letters for each row does not differ statistically at 5% ($p<0.05$). *Expressed as g/L of tartaric acid. ** Expressed as g/L of acetic acid. Sc: *Sacharomyces cerevisiae*, ScEB: *Saccharomyces cerevisiae* added enzyme extract of *K. marxianus* NRRL-Y-7571.

Pectinases added during red wine maceration step are able to degrade pectic substances present in grape cell walls increasing the extraction efficiency of substances that contribute to the color and other organoleptic characteristics of wine [27]. Red wine color depends on the phenolic and anthocyanin compounds present in grape skin, their extraction during winemaking, and their reaction during wine fermentation and aging [28].

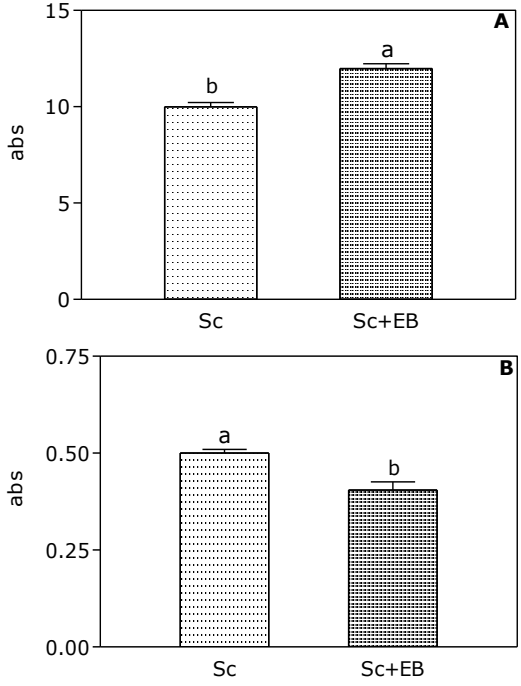
Wines obtained with the addition of enzymatic extract of *K. marxianus* NRRL-Y-7571 showed significant higher color intensity than those produced without enzymatic treatment, indicating that the pectinolytic enzymes present in the extract efficiently increase the extraction of colored compounds during wine maceration step (Figure 2A).

The color intensity of the wine can be defined as the sum of optical densities, measured at a wavelength of 420nm, 520nm and

620nm. The 420nm measure reflects the yellow color of wine, which depends of tannins, tannin polymerization, and the combination between tannins and anthocyanins. The red intensity, measured at 520nm, is determined by anthocyanin concentration, and the purple-blue tint, measured at 620nm, reflects the degree of condensation between anthocyanins and catechins [29].

In the present study, the enzymatically treated wines exhibited better coloration (Figure 2B) characterized by a higher percentage of red color (65.3%) and lower yellow color (27.1%), compared with the control wines which showed 61.5% (red color) and 30.9% (yellow color). These results confirmed the positive contribution of enzymatic treatment in the color characteristics of red wines [21, 30].

Figure 2. Colour intensity (A) e coloration (B) obtained by microvinifications wine grape variety of Cabernet Sauvignon. Sc: *Sacharomyces cerevisiae*, Sc+EB: *Saccharomyces cerevisiae* added enzyme extract of *K. marxianus* NRRL-Y-7571. Different letters (a – b) indicate significant differences ($p < 0.05$).

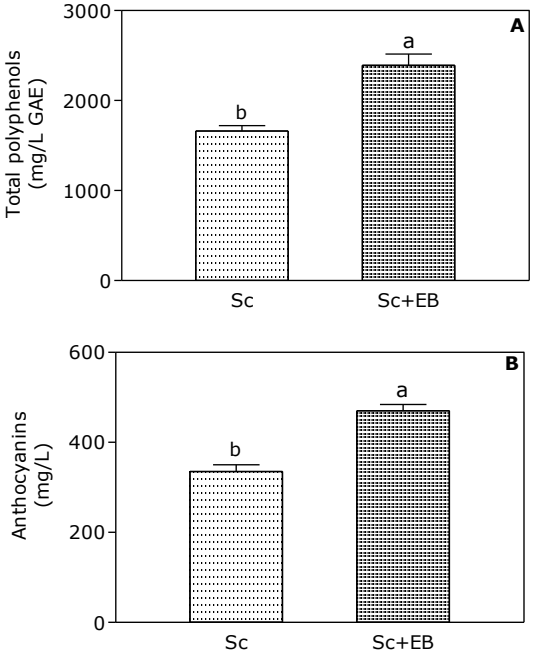


The phenolic compounds present in grape skins and seeds and liberated during the maceration process contribute to color [31], but also to wine flavor and astringency [32]. Among polyphenols, tannins (i.e. proanthocyanidins, which are oligomers and polymers of flavan-3-ols such as catechins) play a significant role in wine taste [33] and contribute to the color stability of red wines as they react with anthocyanins to form derived pigments such as tannin–anthocyanin and anthocyanin–tannin adducts [34].

The concentration of total polyphenols (Figure 3A) significantly differed between control wines and enzymatically treated wines, with values of 1670 mg L^{-1} and 2390 mg L^{-1}, respectively. This difference indicates that the addition of *K. marxianus* enzymatic extract efficiently contribute for polyphenols extraction, increasing the concentration of these compounds in approximately 45%. Although direct comparison with Cabernet Sauvignon wines produced in other regions and vintages is difficult, the total polyphenols concentration, in both control and enzymatically treated wines, was higher than that reported by Cliff et al. [19] and Rastija et al. [35], and within those determined by Chira et al. [20].

Anthocyanins are the main color compounds present in grape skin and their extraction during maceration/fermentation process influence the acceptability of red wines [30, 36]. Figure 3B shows the total anthocyanins content of control wines and enzymatically treated wines. As observed in the total phenolic compounds evaluation, the addition of *K. marxianus* enzyme extract significantly increased the concentration of total anthocyanins of Cabernet Sauvignon wine in 40%. The efficiency of pectinolytic enzymes on anthocyanin extraction, particularly proanthocyanins, in Merlot wines was evidenced by Ducasse et al. [37]. Although the concentration of phenolic compounds and anthocyanins vary considerably, depending on grape variety, environmental factors in the vineyard, processing techniques and storage conditions (maturation), the present results are consistent with those reported in the literature for wines made with Cabernet Sauvignon cultivar [38, 39].

Figure 3. Total phenolic content (A) and anthocyanins content (B) microvinifications of wine obtained from grapes of Cabernet Sauvignon. Sc: *Sacharomyces cerevisiae*, Sc+EB: *Saccharomyces cerevisiae* added enzyme extract of *K. marxianus* NRRL-Y-7571. Diferent letters (a – b) indicate significant differences ($p < 0.05$).



In summary, the results of this study clearly demonstrate that the addition of crude enzymatic extract of *K. marxianus* during the maceration process positively contribute for wine color intensity and tonality by increasing the extraction of polyphenols and anthocyanins present in grape skin, indicating that extracellular *K. marxianus* polygalacturonases are an alternative to filamentous fungi enzymatic preparations for wine making processes. Moreover, the addition of the yeast enzymatic crude extract reduced wine turbidity, but did not affect *S. cerevisiae* fermentation efficiency, final ethanol concentration, fixed and total acidity, among other important enological parameters.

ACKNOWLEDGMENTS

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CAPÍTULO VI - DEPÓSITO DE PATENTE

PROCESSO DE PRODUÇÃO DE EXTRATO ENZIMÁTICO, EXTRATO ENZIMÁTICO, USO DE EXTRATO, COMPOSIÇÃO COMPREENDENDO EXTRATO E MÉTODO DE CLARIFICAÇÃO DE BEBIDAS UTILIZANDO EXTRATO OBTIDO A PARTIR DE *KLUYVEROMYCES*

Depósito de Patende em 24 novembro de 2011

< Uso exclusivo do INPI >

INPI BRO 24/11/2011 020110120511 16:25 NPRJ 000022111227270

PI1105687-8

Espaço para etiqueta

DEPÓSI[illegible]E PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (71):

1.1 Nome: FUNDAÇÃO UNIVERSIDADE DE CAXIAS DO SUL - UCS

1.2 Qualificação: Instituto privado de pesquisa

1.3 CNPJ/CPF: 88648761000103

1.4 Endereço Completo: Rua Francisco Getúlio Vargas, 1130, Cidade Universitária, Caxias do Sul, RS, Brasil

1.5 CEP: 1.6 Telefone: 1.7 Fax:

1.8 E-mail:

☒ continua em folha anexa

2. Natureza: ◉ Invenção ○ Modelo de Utilidade ○ Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: INVENÇÃO

3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):
PROCESSO DE PRODUÇÃO DE EXTRATO ENZIMÁTICO, EXTRATO ENZIMÁTICO, USO DE EXTRATO, COMPOSIÇÃO COMPREENDENDO EXTRATO E MÉTODO DE CLARIFICAÇÃO DE BEBIDAS UTILIZANDO EXTRATO OBTIDO A PARTIR DE KLUYVEROMY

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

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Reivindicações

PROCESSO DE PRODUÇÃO DE EXTRATO ENZIMÁTICO, EXTRATO ENZIMÁTICO, USO DE EXTRATO, COMPOSIÇÃO COMPREENDENDO EXTRATO E MÉTODO DE CLARIFICAÇÃO DE BEBIDAS UTILIZANDO EXTRATO OBTIDO A PARTIR DE *KLUYVEROMYCES*

1. Processo de produção de extrato enzimático para extração e/ou clarificação de bebidas caracterizado por compreender a etapa de crescer *Kluyveromyces* sp. em meio para produção de poligalacturonase.
2. Processo de produção, de acordo com a reivindicação 1, caracterizado pelas leveduras utilizadas serem cepas selecionadas com alta atividade poligalacturonásica pertencentes ao gênero *Kluyveromyces*.
3. Processo de produção, de acordo com a reivindicação 1 ou 2, caracterizado pelo crescimento das cepas ocorrer em meios completos sintéticos ou naturais.
4. Processo, de acordo com a reivindicação 1, caracterizado pela produção de poligalacturonase nas cepas selecionadas de *Kluyveromyces* sp.ser realizada sob temperatura de 28ºC a 32ºC.
5 Processo, de acordo com a reivindicação 1, caracterizado pela produção de poligalacturonase nas cepas selecionadas de *Kluyveromyces* sp. ser realizada em meio sintético com pectina.
6. Processo, de acordo com a reivindicação 1 ou 5, caracterizado pela produção de poligalacturonase nas cepas selecionadas de *Kluyveromyces* sp. ser realizada utilizando pectina como única fonte de carbono e sem agitação.
7. Extrato enzimático caracterizado por ser obtido através do crescimento de *Kluyveromyces* sp. em meio para produção de poligalacturonase.
8. Uso de extrato enzimático caracterizado pelo extrato ser obtido a partir de *Kluyveromyces* sp. e por ser utilizado para extração e/ou clarificação de bebidas.
9. Uso, de acordo com a reivindicação 8, caracterizado por utilizar o extrato bruto ou concentrado com elevada atividade de poligalacturonase produzido por cepas selecionadas de *Kluyveromyces* sp. na extração e clarificação de suco de frutas.
9. Uso, de acordo com a reivindicação 8, caracterizado por utilizar o extrato bruto ou concentrado com elevada atividade de poligalacturonase produzido por cepas selecionadas de *Kluyveromyces* sp. na extração de cor em processo enológicos.

10. Composição para extração e/ou clarificação de bebidas caracterizado por compreender:
a) extrato enzimático obtido a partir de *Kluyveromyces* sp;
b) veículo aceitável.
11. Método de extração e/ou clarificação de bebidas caracterizado por compreender a etapa de contatar a bebida com extrato enzimático obtido a partir de *Kluyveromyces* sp.

Resumo

PROCESSO DE PRODUÇÃO DE EXTRATO ENZIMÁTICO, EXTRATO ENZIMÁTICO, USO DE EXTRATO, COMPOSIÇÃO COMPREENDENDO EXTRATO E MÉTODO DE CLARIFICAÇÃO DE BEBIDAS UTILIZANDO EXTRATO OBTIDO A PARTIR DE *KLUYVEROMYCES*

A presente invenção descreve produtos e processos compreendendo um excelente extrato enzimático, com elevada atividade de poligalacturonase, produzido por cepas selecionadas de *Kluyveromyces* sp., especialmente para a extração e clarificação de bebidas, em especial, bebidas fermentadas como suco de frutas ou vinho, por exemplo.

CAPÍTULO VII - DISCUSSÃO GERAL

As 19 linhagens de leveduras testadas no presente estudo quanto a atividade de poligalacturonases em sistemas líquidos obtiveram-se uma ampla faixa de valores, destacando-se a levedura NRRL-Y-7571 com a maior atividade, sendo esta superior a 60 $U.mL^{-1}$, e as menores atividades detectadas foram nas linhagens NRRL-Y-8278 (1,07 $U.mL^{-1}$) e NRRL-Y-1195 (1,07 $U.mL^{-1}$). Também foram apresentados dados que indicam que os métodos convencionais de melhoramento através de seleção, hibridização e segregação podem contribuir para a obtenção de leveduras com elevada atividade pectinolítica possibilitando a sua aplicação em diversos processos industriais.

A utilização de extrato pectinolítico da levedura pode ser altamente vantajosa na produção de sucos de frutos, como foi evidenciado neste estudo que o extrato enzimático bruto obtido a partir da linhagem NRRL-Y-7571 de *K. marxianus*, apresentou melhores efeito sobre as características visuais (coloração) e propriedades funcionais (concentração de compostos fenólicos e antocianinas) dos sucos quando comparado com o preparado enzimático comercial (Novozym®33095 e Pectinex®Ultra Color).

Da mesma forma, quando o extrato enzimático bruto obtido a partir da linhagem NRRL-Y-7571 de *K. marxianus* foi adicionado ao mosto de uva da variedade *Cabernet Sauvignon* em comparação ao vinho controle (sem adição de enzima), o produto final obtido após processo de vinificação com adição da enzima apresentou melhores características de coloração, maior concentração de compostos fenólicos e antocianinas, e menor turbidez, quando comparado ao vinho controle. De um modo geral, os resultados desse trabalho indicam que o extrato enzimático de *K. marxianus* tem potencial para ser utilizado na produção em maior escala dessas enzimas.

Assim como distintas linhagens de *Saccharomyces* variam na sua produção de metabólitos aromáticos, distintas espécies e linhagens de outras leveduras podem contribuir para as características organolépticas do produto final. Segundo Jolly *et al*. (2006), as leveduras do gênero *Kluyveromyces* contribuem com aromas florais e frutais em combinação com *Saccharomyces* na elaboração de vinhos. Portanto, as leveduras não-*Saccharomyces* podem ser utilizadas, principalmente em combinação com linhagens selecionadas de *Saccharomyces cerevisiae* para a obtenção de vinhos de alta qualidade.

CAPÍTULO VIII - CONCLUSÕES

A avaliação da linhagem com maior capacidade pectinolítica (NRRL-Y-7571), sob diferentes condições mostrou que a presença de oxigênio diminui a atividade da pectinase, que a pectina atua como indutor e a glicose estimula crescimento, mas não interfere na produção enzimática.

O tratamento enzimático com extrato enzimático de *K. marxianus* influenciou o aumento de rendimento e clarificação, bem como decréscimo da turbidez do suco de uva, além de resultar em um suco com altas concentrações de polifenóis, antocianinas e cor quando comparado com a enzima comercial.

A adição de extrato enzimático bruto de *K. marxianus* durante o processo de vinificação contribui positivamente para a intensidade de cor e coloração do vinho, aumentando a extração de polifenóis e antocianinas, diminuição da turbiez do vinho, e não interfere na eficiência fermentativa de *S. cerevisiae*.

CAPÍTULO IX - BIBLIOGRAFIA COMPLEMENTAR

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