POLLYANNA AMARAL VIANA

CARACTERIZAÇÃO BIOQUÍMICA E ESTUDOS ESTRUTURAL E TERMODINÂMICO DE α-GALACTOSIDASES DE *Debaryomyces hansenii* UFV-1

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

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Aprovada em: 19 de fevereiro de 2009.

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(Co-orientador)

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Dedico este trabalho

Aos meus pais, Adalberto e Eliane, pela vida e pela oportunidade de estar vivendo mais um momento especial como este; aos meus irmãos, Giulliano e Dagoberto, pela amizade e apoio; e ao meu marido Germano, por todo amor, carinho e companheirismo.

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"As pessoas que vencem neste mundo, são as que procuram as circunstâncias de que precisam, e quando não as encontram, as criam."(George *Bernard Shaw*).

BIOGRAFIA

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ABREVIATURAS E SÍMBOLOS

A	Absorbância
BCA	Ácido bicinconínico
BSA	Albumina sérica bovina
C	Carbono
CD	Dicroísmo Circular
DEAE	Dietil aminoetil
DSC	Calorimetria de Varredura Diferencial
EDTA	Ácido etilenodiaminotetracético
g	Grama
GO	Galacto-oligossacarídeos
h	Hora
K	Kelvin
K_{cat}	Constante catalítica
kDa	Kilodalton
K_i	Constante de inibição
K_m	Constante de Michaelis-Menten
log	Logaritmo
M	Molaridade (mol/L)
MALDI-TOF	Desorção/ionização por laser assistida por matriz e detecção por tempo de vôo
min	Minuto
mg	Miligrama
mM	Milimolar
Mr	Massa molecular
nm	Nanômetro
o-NP-β-Gal	*o*-nitrofenil-β-D-galactopiranosídeo
o-NP-β-Glc	*o*-nitrofenil-β-D-glicopiranosídeo
PAGE	Eletroforese em gel de poliacrilamida
pH	Potencial hidrogeniônico

pI	Ponto isoelétrico
p-NP-α-Gal	*p*-nitrofenil-α-D-galactopiranosídeo
p-NP-β-Gal	*p*-nitrofenil-β-D-galactopiranosídeo
p-NP-α-Glc	*p*-nitrofenil-α-D-glicopiranosídeo
p-NP-β-Xil	*p*-nitrofenil-β-D-xilopiranosídeo
p-NP-α-Man	*p*-nitrofenil-α-D-manopiranosídeo
p-NP-α-Ara	*p*-nitrofenil-α-D-arabinopiranosídeo
p-NP	*p*-nitrofenol
p/p	Peso/peso
p/v	Peso/volume
R	Constante universal dos gases (8,31 J/mol.K)
RCI	Índice relativo de cooperatividade
RNM	Ressonância Magnética Nuclear
RO	Oligossacarídeos de rafinose
RP-HPLC	Cromatografia líquida de alta eficiência-fase reversa
rpm	Rotação por minuto
S	Substrato
s	Segundo
SD	Desvio padrão
SDS	Dodecil sulfato de sódio
T	Temperatura
$t_{1/2}$	Meia-vida
T_m	Temperatura de desnaturação (50 %)
Tris	Tris (hidroximetil) aminometano
U	Unidade de atividade enzimática
UV	Ultravioleta
v/v	Volume/volume
V_{max}	Velocidade máxima
V	Volts
w/v	Peso/volume
$\Delta H_{(D)}$	Variação de entalpia
$\Delta S_{(D)}$	Variação de entropia
$\Delta G_{(D)}$	Variação de energia livre
ΔCp^{D}_{N}	Variação de capacidade calorífica
$\Delta T_{1/2}$	Índice relativo de cooperatividade
λ	Comprimento de onda
μg	Micrograma
μL	Microlitro

RESUMO

VIANA, Pollyanna Amaral, D.Sc., Universidade Federal de Viçosa, Fevereiro de 2009. **Caracterização bioquímica e estudos estrutural e termodinâmico de α-galactosidases de *Debaryomyces hansenii* UFV-1.** Orientadora: Valéria Monteze Guimarães. Co-orientadores: Sebastião Tavares de Rezende, Marcelo Matos Santoro e Ronaldo Alves Pinto Nagem.

O consumo humano de produtos derivados da soja foi limitado pela presença de oligossacarídeos não digeríveis, tais como os α-galacto-oligossacarídeos rafinose e estaquiose (GO). A maioria dos mamíferos, incluindo o homem, não possui α-galactosidase pancreática, necessária para a hidrólise destes açúcares. Entretanto, os GO podem ser fermentados por microrganismos presentes no intestino grosso, produzindo gases, que podem induzir flatulência e outras desordens gastrintestinais. A utilização de microrganismos que expressam α-galactosidase é uma solução promissora à eliminação dos GO, antes que estes alcancem o intestino grosso. No estudo atual, nós relatamos a produção e a purificação das α-galactosidases extracelular e intracelular de *Debaryomyces hansenii* UFV-1, para estudos de caracterizações moleculares (dicroísmo circular) e cinético-bioquímicas destas enzimas. Nós também realizamos análises termodinâmicas usando a microcalorimetria (DSC), visando o melhor conhecimento das características individuais de cada α-galactosidase, com a finalidade

de utilizar estas enzimas na eliminação dos GO em produtos derivados da soja. Células de *Debaryomyces hansenii* UFV-1, cultivadas em galactose como fonte de carbono, produziram α-galactosidases extracelular e intracelular, com massas moleculares de 54,5 e 54,8 kDa (MALDI-TOF), 60 e 61 kDa (SDS-PAGE), e valores de *pI* de 5,15 e 4,15, respectivamente. α-Galactosidases extracelular e intracelular desglicosiladas apresentaram massas moleculares de 36 e 40 kDa, com conteúdo de carboidrato de 40 e 34 %, respectivamente. As seqüências do *N*-terminal das α-galactosidases foram idênticas. α-Galactosidases intracelular e extracelular de *D. hansenii* UFV-1 mostraram poucas variações em seus conteúdos de aminoácidos. α-Galactosidase intracelular mostrou menor termoestabilidade quando comparada com a enzima extracelular. α-Galactosidase extracelular de *D. hansenii* UFV-1 apresentou maior K_{cat} do que a enzima intracelular (7,16 vs 3,29 s^{-1}, respectivamente), para o substrato *p*NPαGal. O valores de K_{cat} da enzima intracelular para melibiose, estaquiose e rafinose foram 0,03, 6,05 e 4,12 s^{-1}, respectivamente. Incubação da isoforma intracelular com extrato hidrossolúvel da soja por 6 h a 55 ^{o}C reduziu os teores de estaquiose e rafinose em 100 e 73 %, respectivamente. Pode-se observar que, a α-galactosidase de *D. hansenii* UFV-1 foi eficiente na redução dos GO presentes em produtos derivados da soja, e é apropriada para uso industrial no processamento destes açúcares. Derivados de galactopiranosídeos foram sintetizados para explicar atividades inibitórias das α-galactosidases de *D. hansenii* UFV-1. α-D-Galactopiranosídeo de metila foi o inibidor mais potente comparado aos demais testados, com valores de *K*i de 0,82 e 1,12 mM, para as enzimas extracelular e intracelular, respectivamente. A presença do grupo hidroxila na posição C-6 foi importante para o reconhecimento pelas α-galactosidases de *D. hansenii* UFV-1. As glicoproteínas foram completamente hidrolisadas com 6 M de HCl a 80 °C, liberando os monossacarídeos após 2 h de incubação. A presença de galactose e manose foram observadas na α-galactosidase extracelular e xilose na enzima intracelular. Propriedades espectroscópicas, cinéticas e

termodinâmicas foram determinadas para α-galactosidases de *D. hansenii* UFV-1. Efeitos de pH e temperatura na estrutura destas enzimas foram investigadas usando Dicroísmo Circular (CD). α-Galactosidases de *D. hansenii* UFV-1 apresentaram composições de estruturas secundárias (α-hélice, folhas-β paralelas e voltas-β) semelhantes. Calorimetria de Varredura Diferencial (DSC) foi utilizada para a determinação de alguns parâmetros termodinâmicos durante desnaturação térmica protéica. Imediatamente, não foi observada reversibilidade da desnaturação térmica para α-galactosidases de *D. hansenii* UFV-1, entretanto, ocorreu como um processo termodinamicamente controlado. α-Galactosidase extracelular, em pH 5,5, mostrou um valor de T_m menor (86,1 °C) quando comparado com a enzima intracelular (87,3 °C). O índice relativo de cooperatividade para a α-galactosidase intracelular ($\Delta T_{1/2}$ = 5,0 ºC) foi menor do que para a enzima extracelular ($\Delta T_{1/2}$ = 5,6 ºC). Assim, os dados de CD e DSC sugerem que, as duas enzimas tenham comportamentos diferentes e discretos, embora possuam estrutura secundária similar. A energia de ativação de Arrhenius reportada para as α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1 (88 e 95 KJ/mol, respectivamente) foi correspondentemente elevada e indica uma mudança química transiente considerável durante o processo de ligação. As condições testadas para a cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1 foram os “Kits” 1 e 2 (*Crystal Screen),* utilizando a técnica da difusão de vapor em gota suspensa. Em determinadas condições houve aparecimento de cristais, entretanto, análises de difração em raios-X indicaram a formação dos sais usados na cristalização.

ABSTRACT

VIANA, Pollyanna Amaral, D.Sc., Universidade Federal de Viçosa, February, 2009. **Biochemical characterization and structural and thermodynamic studies of *Debaryomyces hansenii* UFV-1 α-galactosidases.** Advisor: Valéria Monteze Guimarães. Co-Advisors: Sebastião Tavares de Rezende, Marcelo Matos Santoro and Ronaldo Alves Pinto Nagem.

Human consumption of soy-derived products has been limited by the presence of non-digestible oligosaccharide, such as the α-galacto-oligosaccharides raffinose and stachyose (GO). Most mammals, including man, lack pancreatic α-galactosidase, which is necessary for the hydrolysis of these sugars. However, GO can be fermented by gas-producing microorganisms present in the large intestine, which in turn can induce flatulence and other gastrointestinal disorders. The use of microorganisms expressing α-galactosidase is promising solution to the elimination of GO before they reach the large intestine. In the present study, we reported the production and purification of *Debaryomyces hansenii* UFV-1 extracellular and intracellular α-galactosidases, for studies of molecular (circular dichroism) and kinetic-biochemical characterizations of these enzymes. We also performed the thermodynamic analysis using microcalorimetry (DSC), aiming for better knowing the individual

characteristics of each α-galactosidase, with the purpose for using these enzymes in the elimination of the GO in soy-derived products. *Debaryomyces hansenii* UFV-1 cells, cultivated on galactose as carbon source, produced extracellular and intracellular α-galactosidases, with 54.5 and 54.8 kDa molecular mass (MALDI-TOF), 60 and 61 kDa (SDS-PAGE), and 5.15 and 4.15 *pI* values, respectively. Extracellular and intracellular α-galactosidases deglycosylated presented 36 and 40 kDa molecular mass, with 40 and 34 % carbohydrate content, respectively. The *N*-terminal sequences of the α-galactosidases were identical. Amino acid analysis from *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases showed few variations in their contents. Intracellular α-galactosidase showed smaller thermostability when compared to the extracellular enzyme. *D. hansenii* UFV-1 extracellular α-galactosidase presented higher K_{cat} than the intracellular enzyme (7.16 vs 3.29 s^{-1}, respectively), for the *p*NPαGal substrate. The intracellular enzyme K_{cat} values for melibiose, stachyose, and raffinose were 0.03, 6.05 and 4.12 s^{-1}, respectively. Incubation of intracellular isoform with soymilk for 6 h at 55 ^{o}C reduced stachyose and raffinose amounts by 100 and 73 %, respectively. It can be observed that *D. hansenii* UFV-1 α-galactosidase was efficient in reducing the GO presents in soy-derived products, and it is appropriate for industrial use in the processing of these sugars. α-D-Galactopyranosides derivatives were synthesized for elucidate inhibitory activities of *D. hansenii* UFV-1 α-galactosidases. Methyl α-D-galactopyranoside was the most potent inhibitor compared to the others tested, with *K*i values of 0.82 and 1.12 mM, for extracellular and intracellular enzymes, respectively. The presence of hydroxyl group in the C-6 position was important for the recognition by *D. hansenii* UFV-1 α-galactosidases. The glycoproteins were completely hydrolyzed with 6 M HCl at 80 °C, releasing the monosaccharides after 2 h of incubation. The presence of galactose and mannose was observed in the extracellular α-galactosidase and xylose in the intracellular enzyme. Spectroscopic, kinetic and thermodynamic properties were determined

for *D. hansenii* UFV-1 α-galactosidases. Effects of pH and temperature on the structure of these enzymes were investigated using Circular Dichroism (CD). *D. hansenii* UFV-1 α-galactosidases showed very similar secondary structure compositions (α-helix, β-sheet paralel and β-turn). Differential Scanning Calorimetry (DSC) was employed for the determination of some thermodynamic parameters during protein denaturation. Thermal denaturation reversibility was not immediately observed for *D. hansenii* UFV-1 α-galactosidases; however it occurred as a thermodynamically controlled process. Extracellular α-galactosidase, at pH 5.5, showed a T_m value smaller (86.1 °C) when compared to the intracellular enzyme (87.3 °C). The cooperativity relative index for intracellular α-galactosidase ($\Delta T_{1/2}$ = 5.0 ºC) was smaller than that of the extracellular enzyme ($\Delta T_{1/2}$ = 5.6 ºC). Thus, the CD and DSC data suggest that the two enzymes have different and discrete behaviors although they possess similar secondary structure. The related Arrhenius activation energy for extracellular and intracellular α-galactosidase from *D. hansenii* UFV-1 (88 and 95 KJ/mol, respectively) was correspondingly high and indicates a considerable transient chemical change during the binding process. The conditions tested for *D. hansenii* UFV-1 extracellular α-galactosidase crystallization were the Kits 1 and 2 (Crystal Screen*)* using the vapor diffusion technique in hanging drop. It had crystal appearance in determined conditions; however, the X-ray diffraction analysis had indicated salts formation used in the crystallization.

1. INTRODUÇÃO

Debaryomyces hansenii, uma levedura metabolicamente versátil, não-patogênica e osmotolerante, é considerada um alvo atrativo para pesquisas básicas e biotecnológicas. *D. hansenii* é a espécie de levedura mais freqüente encontrada em produtos fermentados ricos em proteínas, como salsichas e queijos (ENCINAS *et al*., 2000; PETERSEN; WESTALL; JESPERSEN, 2002). Possui alta tolerância ao sal e capacidade de crescimento em baixas temperaturas, quando comparada à levedura *Saccharomyces cerevisiae* (HARO *et al*., 1993; PRISTA *et al*., 1997). Essas duas espécies são capazes de metabolizar ácidos orgânicos e aminoácidos, regulando a acidez de produtos fermentados e apresentam atividades lipolíticas e proteolíticas, contribuindo assim para o desenvolvimento do aroma destes produtos (COOK, 1995; SORENSEN; SAMUELSEN, 1996; OLENSEN; STAHNKE, 2000; DURÁ; FLORES; TOLDRÁ, 2002).

A levedura *Debaryomyces hansenii* tem sido utilizada, em pesquisas, para produção de xilitol/etanol a partir de xilose (ROSEIRO; PEITO; AMARAL-COLLAÇO, 1991; PARAJÓ; DOMINGUEZ, H.; DOMINGUEZ, J., 1995; SAMPAIO *et al*., 2006), produção de proteases

(BOLUMAR *et al.*, 2002) e de α-galactosidases extracelular e intracelular (VIANA *et al.*, 2006, 2007).

α-Galactosidases (α-D-galactosídeo galactohidrolase; E.C 3.2.1.22) catalisam a hidrólise de ligações α-1,6 de resíduos de α-galactosídeos em oligossacarídeos simples, como estaquiose, rafinose e melibiose, em polissacarídeos, como galactomananas e em substratos sintéticos, como *para*-nitrofenil-α-D-galactopiranosídeo, *p*NPαGal (MEYER; REID, 1982). As α-galactosidases estão amplamente distribuídas em animais, plantas e microrganismos. Em humanos, a α-galactosidase é uma hexoglicosidase lisossomal que cliva ligações α-1,3 de resíduos de galactose em glicolipídeos e glicoproteínas, e mutações no gene dessa enzima causam degradação incompleta dos carboidratos, resultando na Doença de Fabry (IOANNOU *et al.*, 2001). Durante a germinação de sementes, α-galactosidase é uma das principais enzimas envolvidas na hidrólise das galactomananas, um dos maiores polissacarídeos da parede celular vegetal (REID, 1985; REID *et al.*, 1992). Rafinose e estaquiose (RO: raffinose oligosaccharides) presentes em leguminosas, causam flatulência em animais monogástricos e humanos. As α-galactosidases possuem potencial para aliviar estes sintomas indesejáveis, por hidrolisar esses oligossacarídeos (DEY; PATEL; BROWNLEADER, 1993). α-Galactosidases de plantas e microrganismos foram adicionadas em extrato hidrossolúvel e melaço de soja para converter os RO em açúcares digeríveis, moderando as propriedades causadoras de flatulência destes produtos (GUIMARÃES *et al.*, 2001; VIANA *et al.*, 2005; VIANA *et al.*, 2007).

O estudo termodinâmico de proteínas tem atraído atenção de várias linhas de pesquisa (PRIVALOV, 1989). A estabilidade termodinâmica de uma proteína é a medida de quanto mais estável é sua conformação globular ou nativa, em comparação com seu estado desnaturado (PACE; SHIRLEY; THOMSON, 1990; AHMAD; YADAV; TANEJAS, 1992). A forma nativa de uma enzima, ou seja, sua estrutura terciária ou quaternária está em um

mínimo de energia e demonstra vários graus de flexibilidade, principalmente na sua superfície, onde as cadeias laterais e as alças possuem conformações alternativas. Para que ocorra um processo, onde a proteína passe do estado nativo para o desnaturado, à temperatura ambiente e na ausência de desestabilizantes, é necessário um aumento significativo da energia livre (PACE; SHIRLEY; THOMSON, 1990). Entretanto, a conformação nativa das proteínas pode ser facilmente desfeita e a cadeia polipeptídica pode se desenovelar, por meio de processos físicos, como temperatura e pressão, ou químicos, como variação do pH e adição de substâncias desnaturantes (PRIVALOV, 1997). O entendimento dos mecanismos relacionados à estabilidade das proteínas é importante, uma vez que a estrutura nativa da proteína deve ser mantida. Entretanto, a estabilidade não pode ser tão rígida a ponto de impedir mudanças conformacionais ou ajustes necessários às funções das proteínas (BECKTEL; SCHELLMAN, 1987).

O processo reversível dos dois estados, enovelamento e desenovelamento das proteínas, podem ser estudados por métodos sensíveis, capazes de detectar mudanças na estrutura da molécula, como Espectrofotometria e Dicroísmo Circular (CD) (MARTINS, 1999; MARTINS; SANTORO, 1999; BRUMANO; ROGANA; SWAUSGOOD, 2000; MARTINS *et al.*, 2003;), Ressonância Magnética Nuclear (RNM) (KNUBOVETS; OSTERHOUT; KLIBANOV, 1999), Fluorescência (HILSER *et al.*, 2001), etc. Apesar de estes métodos espectroscópicos serem extremamente sensíveis e capazes de fornecer informações estruturais de alta resolução e parâmetros termodinâmicos, as informações são indiretas, ou seja, é uma determinação modelo dependente da termodinâmica do sistema. Portanto, a análise calorimétrica complementa os estudos espectroscópicos, fornecendo uma descrição mais completa do sistema de interesse (COOPER; JOHNSON, 1994; HAYNIE, 2001).

Neste trabalho, foram realizados estudos sobre a indução da síntese das α-galactosidases extracelular e intracelular em culturas de *Debaryomyces hansenii* UFV-1, e estudos bioquímicos, cinéticos, termodinâmicos e moleculares destas α-galactosidases.

O trabalho foi dividido nos seguintes Capítulos:

⇒ **Capítulo 1:** α-Galactosidases Production by *Debaryomyves hansenii* UFV-1.

⇒ **Capítulo 2:** *Debaryomyces hansenii* UFV-1 Intracellular α-Galactosidase Characterization and Comparative Studies with the Extracellular Enzyme.

⇒ **Capítulo 3:** Evaluation of *Debaryomyces hansenii* UFV-1 α-Galactosidases Inhibitory Activity of some α-D-Galactopyranoside Derivatives.

⇒ **Capítulo 4:** Comparative Spectroscopic, Thermodynamic and Kinetic Properties of *Debaryomyces hansenii* UFV-1 α-Galactosidases.

⇒ **Capítulo 5:** Testes de Cristalização por Difusão de Vapor da α-Galactosidase Extracelular de *Debaryomyces hansenii* UFV-1.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral deste trabalho foi realizar estudos básicos e aplicados sobre as α-galactosidases de *Debaryomyces hansenii* UFV-1, envolvendo caracterizações bioquímica, cinética, termodinâmica e estrutural dessas enzimas, para aplicação na eliminação dos oligossacarídeos de rafinose presentes em derivados da soja.

2.2. Objetivos Específicos

⇒ Produzir e purificar as α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1.

⇒ Determinar a seqüência do terminal amínico da α-galactosidase intracelular de *D. hansenii* UFV-1.

⇒ Avaliar potencialidades para aplicações biotecnológicas da α-galactosidase intracelular de *D. hansenii* UFV-1, no processamento dos oligossacarídeos de rafinose presentes em produtos de soja.

⇒ Determinar a massa molecular das α-galactosidases nativas de *D. hansenii* UFV-1 por Espectrometria de Massa e das enzimas desglicosiladas por SDS-PAGE.

⇒ Determinar a composição de aminoácidos das α-galactosidases de *D. hansenii* UFV-1.

⇒ Hidrolisar, identificar e quantificar os monossacarídeos presentes nas α-galactosidases de *D. hansenii* UFV-1 por CLAE.

⇒ Produzir anticorpos anti-α-galactosidase extracelular de *D. hansenii* UFV-1 pela imunização de camundongos.

⇒ Realizar estudos de cinética enzimática: determinação da constante de Michaelis-Menten (K_m) e da constante catalítica (K_{cat}), energia de ativação (E_a), e constantes de inibição (K_i) das α-galactosidases de *D. hansenii* UFV-1 com compostos derivados de α-D-galactopiranosídeos.

⇒ Determinar o ponto isoelétrico (*pI*) das α-galactosidases de *D. hansenii* UFV-1 por cromatofocalização.

⇒ Fazer a análise conformacional das α-galactosidases de *D. hansenii* UFV-1 por Dicroísmo Circular (CD) para determinação da porcentagem de estruturas secundárias em diferentes temperaturas e pH.

⇒ Fazer a análise termodinâmica das α-galactosidases de *D. hansenii* UFV-1 por Calorimetria de Varredura Diferencial (DSC), para determinação da T_m, desnaturação térmica, teste de reversibilidade, e índice relativo de cooperatividade.

⇒ Fazer a análise estrutural da α-galactosidase extracelular de *D. hansenii* UFV-1 por meio de testes de cristalização pela técnica da difusão de vapor.

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4. CAPÍTULOS

Capítulo 1

α-Galactosidases Production by *Debaryomyces hansenii* UFV-1

α-Galactosidases Production by *Debaryomyces hansenii* UFV-1

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4.1.1. Abstract

Extracellular and intracellular α-galactosidases were produced by *Debaryomyces hansenii* UFV-1 grown on different media with several carbon sources. *D. hansenii* UFV-1 grown in YP medium presented maximum cell mass (8.45 mg/mL) after 36 h of cultivation, with lactose as a carbon source, followed by sucrose, glucose, raffinose and galactose. Higher extracellular and intracellular α-galactosidases activities were observed at 48 h of *D. hansenii* cultivation in YP medium containing galactose (0.97 and 5.27 U/mL), lactose (1.28 and 4.88 U/mL), raffinose (0.23 and 4.63 U/mL), sucrose (0.52 and 4.28 U/mL) and glucose (0.83 and 3.28 U/mL). Galactose and lactose were the carbon sources that induced greatest cells mass and α-galactosidase activities from *D. hansenii* UFV-1, supporting the evidence for the model of induction for the yeast *GAL/MEL* regulon, such as described in *Sacharomyces cereviseae.*

4.1.2. Keywords

α-Galactosidases*; Debaryomyces hansenii* UFV-1; Culture media; Carbon sources; Incubation times; Cell mass; Enzymes production.

4.1.3. Introduction

Debaryomyces hansenii, a metabolically versatile, non-pathogenic, osmotolerant and oleaginous microorganism, represents an attractive species for fundamental and applied biotechnological research. *D. hansenii* is yeast species most frequently found in protein-rich fermented products, such as sausages and cheeses (***1***,***2***). The better adaptation of this species to certain ecosystems, compared to *Saccharomyces*, is due to its high salinity tolerance and ability to grow at low temperatures (***3***). Therefore, interest in the physiology and biochemistry of *D. hansenii* is increasing. This species metabolizes organic acids and amino acids, regulating the acidity of the fermented product, and also provides lipolytic and proteolytic

activities contributing to flavor development (***4***,***5***,***6***). *D. hansenii* is a xylose-utilizing yeast that exhibits an interesting xylitol/ethanol production ratio for industrial use (*7*,***8***).

It was recently reported that *D. hansenii* UFV-1 presents the ability to produce α-galactosidases, which hydrolyzed indigestible flatus-inducing oligosaccharides in soybean derivatives, enhancing the nutritional value of these products (***9***). α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is an exo-acting enzyme that mainly cleaves terminal α-1,6-linked D-galactosyl residues from a wide range of substrates including oligosaccharides, such as raffinose, stachyose, verbascose, and polysaccharides of galactomannans like locust bean gum and guar gum (***10***). α-Galactosidase has particular interest for its potential biotechnological applications, mainly in the sugar industry, where they improve crystallization of sucrose by hydrolysis of raffinose (***11***). Moreover, they can enhance the bleaching effect of endo-1,4-mananases in softwood kraft pulp (***12***,***13***). α-Galactosidases are also of interest in biomedical applications, *e.*g., the treatment of Fabry's disease by enzyme replacement therapy (***14***) or blood type conversion (***15***).

In this study, the effect of different sugars used as carbon sources for the extracellular and intracellular α-galactosidases production by *Debaryomyces hansenii* UFV-1 was reported.

4.1.4. Material and Methods

4.1.4.1. Microorganism and culture conditions

D. hansenii UFV-1 stored at -80 ^{o}C in glycerol and YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) was streaked on an YPD agar surface (1.5 % agar) and kept in an incubation chamber for 36 h at 30 ^{o}C. The yeast was then activated in an YPD liquid medium and incubated for 12-15 h at 200 rpm and 30 ^{o}C. An aliquot of the cells obtained after centrifugation (4000*g* for 5 min at 4 °C) was transferred to one of three different media (100 mL): YP-medium (1 % yeast extract and 2 % peptone); MM-mineral medium (0.62 g/L

KH_2PO_4, 2.0 g/L K_2HPO_4, 1.0 g/L $(NH_4)_2SO_4$ and 0.1 g/L $MgSO_4.7H_2O$), and MME-mineral medium (0.62 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$ and 5.0 g/L yeast extract). The sugars glucose, sucrose, lactose, galactose, melibiose, and raffinose 1 % (w/v) were used as carbon sources, and the incubation times tested were 12, 27, 36 and 48 h. After each incubation period at 30 ^{o}C and 200 rpm, samples of 3 mL were colleted and frozen for analyses of cell mass production, enzymatic activity and protein concentration. The growth of the different cultures was determined spectrophotometricaly, in which one unit of $O.D_{600}$ corresponded at 0.53 ± 0.12 mg of dry weight/mL. After cultivation, the supernatant was separated by centrifugation (4000*g* for 5 min at 4 ^{o}C) and used as extracellular α-galactosidase source and the biomass was used as intracellular enzyme source. The average standard deviation for triplicate analysis was less than 8 %.

4.1.4.2. Enzyme assay

A reaction mixture containing 650 µL of 0.1 M sodium acetate buffer, pH 5, 100 µL of extracellular or intracellular enzyme solution, and 250 µL of 2 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPαGal) was preincubated for 15 min at 40 °C. The reaction was terminated by adding 1 mL of 0.5 M sodium carbonate and the released *p*-nitrophenol (*p*-NP) was determined at 410 nm, according to the standard curve (0-0.16 µmol *p*-NP). This procedure was defined as the standard assay. Samples of 1 mL yeast cultures were permeabilized (***9***) and 100 µL fractions of the intracellular enzyme solution were used for α-galactosidase activity determination. One α-galactosidase unit (U) was defined as the amount of enzyme that liberated 1 µmol of *p*-NP per minute under the assay conditions.

4.1.4.3. Determination of protein concentration

Protein concentration in the enzymatic preparations of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases was determined by the Bradford method (***16***) and by the BCA (bicinchoninic acid) method (***17***), respectively, with BSA (albumin serum bovine) as the standard.

4.1.4.4. Antibodies anti-extracellular α-galactosidase production

The *Debaryomyces hansenii* UFV-1 extracellular α-galactosidase was purified (***18***) and used for the production of anti-α-galactosidase antibodies. Groups of three, males, 4-6 weeks old, BALB/c mice (Institute of Biological Sciences, ICB, UFMG, Belo Horizonte, Brazil) were immunized subcutaneously in their footpads. For the first immunization, 40 μg of α-galactosidase adsorbed to alum (1.0 μg alum: 1.5 μg antigen; Rehydragel Low Viscosity Gel, Reheis, Inc., Berkeley Heights, NJ, USA), as adjuvant was used. For a period of three weeks, the mice were weekly immunized using 20 μg of antigen. Two weeks after the last dose, the mice were sacrificed and serum samples were collected for immunological analysis.

4.1.4.5. Immuno Blot analysis

D. hansenii UFV-1 was cultivated in MME-mineral medium with galactose as a carbon source (1 %), and samples were colleted at 12, 27, 36 and 48 h. The proteins extracts (500 μg) were separated on a SDS-PAGE (***19***), where 5 % stacking and 12.5 % resolving polyacrylamide gels were used, and transferred to nitrocellulose membranes (0.2 μm pore size, Sigma, St. Louis, MO, USA) using standard Western blot protocols (***20***). Membranes were blocked with PBS/BSA2% and incubated for 16 h at 37 ^{o}C before the first incubation with a pool of sera collected from the immunized mice, diluted to 1:20 in PBS. The membrane was incubated with goat anti-mouse IgG peroxidase conjugate (1:500, Sigma),

used as a second antibody, in PBS solution containing 0.025 % 4-chlro-1-naphtol diluted in 1 mL of methanol, 0.05 % diaminobenzidine (DAB) in the presence of 0.04 % (v/v) H_2O_2.

4.1.5. Results and Discussion

Debaryomyces hansenii UFV-1 was cultivated in shaker flasks on three culture media containing hexoses (glucose and galactose), disaccharides (sucrose, lactose and melibiose) and trisaccharides (raffinose) as carbon sources (1 % w/v) in order to determine their effect on cell mass and extracellular and intracellular α-galactosidases production.

Cultivation in YP medium resulted in higher cell mass, followed by MME-mineral and MM-mineral media (**Figure 1**). *D. hansenii* UFV-1 grown in YP medium presented maximum cell mass (8.45 mg/mL) after 36 h of cultivation, with lactose as a carbon source, followed by sucrose, glucose, raffinose and galactose. Cell mass production was observed to be lower with melibiose as a carbon source. On the other hand, higher extracellular and intracellular α-galactosidases activities were observed at 48 h of *D. hansenii* UFV-1 cultivation in YP medium (**Table 1**) containing galactose (0.97 and 5.27 U/mL), lactose (1.28 and 4.88 U/mL), raffinose (0.23 and 4.63 U/mL), sucrose (0.52 and 4.28 U/mL) and glucose (0.83 and 3.28 U/mL). Melibiose was the poorest inducer of extracellular and intracellular α-galactosidases registering activities of 0.16 and 1.26 U/mL, respectively (**Table 1**). In *Saccharomyces* spp. the ability to use melibiose depends on the presence of *MEL* genes encoding α-galactosidases (***21***). The *N*-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase was determined as YENGLNLVPQMGWN similar to other microbial α-galactosidases, which belong to the glycoside hydrolase family 27. The alignment of the *N*-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase (14 amino acid residues) with the sequence of *D. hansenii* CBS767 α-galactosidase showed two nonconservative changes (***18***).

Galactose and lactose were the carbon sources that induced greatest cells mass and α-galactosidase activities from *D. hansenii* UFV-1 (**Table 1 and Figure 2**). There is evidence for a model of induction for the yeast *GAL/MEL* regulon. An example of a *GAL* gene with critical basal expression is the α-galactosidase-encoding *MEL1* gene. Mel1p is a secreted enzyme necessary for the extracellular breaking down of melibiose into galactose and glucose. Galactose enters the cell and triggers induction of the *GAL* genes, including *MEL1* via the Gal3p/Gal80p/Gal4p pathway (***22***).

D. hansenii UFV-1 cultivated in medium containing different sugars, present intracellular β-galactosidase activity, with 36 h of cultivation in the presence of lactose (5.27 U/mL), galactose (2.78 U/mL), and melibiose (0.73 U/mL). However, in the same conditions, did not present extracellular β-galactosidase activity. These sugars induced more *D. hansenii* UFV-1 intracellular α-galactosidase activity when compared to the extracellular enzyme activity (**Table 1**). Cellular uptake of sugars requires the action of "transporters", since these compounds do not freely permeate biological membranes. In *Saccharomyces cerevisiae* two different sugar transport mechanisms were already described: carrier mediated facilitated diffusion, responsible for the uptake of hexoses (glucose, fructose, mannose and galactose), and active H^+-symporters responsible for the uptake α-glucosides (***23***). In the case of hexoses, at least seven different permeases (*HXT1-7*) are involved in the transport mechanism (***24***). For α-glucosides two different permeases were characterized at the molecular level: a maltose-H^+ symporter encoded by *MAL* gene and a general α-glucoside-H^+ symporter encoded by *AGT1* (***25,26,27***). In the case of *D. hansenii* UFV-1, lactose was probably transported by a permease and it was hydrolyzed intracellularly by β-galactosidase, releasing galactose which induced higher intracellular α-galactosidase activity. The expression of the α-galactosidase in *Zygosaccharomyces cidri* also was induced with galactose (***28***).

The *Torulaspora delbrueckii* IFO 1255 yeast cultivated in YP medium (***29***) produced extracellular α-galactosidase, reaching maximum activity after 16 h, with 1 % melibiose (0.39 U/mL) and 1 % galactose (0.25 U/mL). Similar results were obtained when *D. hansenii* UFV-1 was cultivated for 27 h with 1 % galactose (0.26 U/mL) (**Table 1**). The yeast strains, such as *Debaryomyces castelli* IFO 1359 and *Debaryomyces nepalensis* IFO 1428 (***30***) produced α-galactosidases in YP medium with 0.05 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 0.01 % KCl and 1 % galactose, after 48 h of cultivation (**Table 2**). *D. hansenii* UFV-1 cultivated in YP medium with 1 % galactose, showed higher α-galactosidases activity (6.2 U/mL) when compared to the IFO 1359 and IFO 1428 strains (2.8 and 1.1 U/mL, respectively), but lower than *Streptomyces erythrus* cultivated in Czapek-Doxs medium with 2 % galactose (**Table 2**).

The expression of *D. hansenii* UFV-1 extracellular α-galactosidase increased during the cultivation period in the MME-mineral medium, with 1 % galactose (**Figure 3**). In this medium, the effect of different carbon sources on cell mass production is depicted in **Figure 1B**. Greater quantities of cell mass were produced with lactose (6.11 mg/mL) and raffinose (5.50 mg/mL), during 27 h of cultivation. Lactose yielded higher extracellular and intracellular α-galactosidases activities after 48 h of cultivation (3.87 U/mL), followed by galactose (1.55 U/mL) and melibiose (1.46 U/mL) (**Table 1**). α-Galactosidases from *Saccharomyces carlsbergensis* were inducible enzymes secreted into the culture medium and galactose proved to be a good inducer (***31***). Higher specific activity values of extracellular α-galactosidase from *D. hansenii* UFV-1, 8.3 and 6.5 U/mg of protein were determined after cultivation for 36 and 48 h, respectively (data not shown). The α-galactosides (except *p*-nitrophenyl-α-D-galactoside) and galactose were inducers of α-galactosidase (***32***).

D. hansenii UFV-1 cultivated in MM-mineral medium presented greater cell mass after 27 h, with melibiose (4-fold), raffinose (3-fold), and lactose (2-fold), compared with the glucose sugar (**Figure 1C**). Cell mass production and α-galactosidase activity were not

detected in the culture medium without sugar (control). The maxima α-galactosidases activities were observed after 48 h of cultivation with raffinose (2.90 U/mL), followed by lactose (1.35 U/mL) and sucrose (1.19 U/mL) (**Table 1**).

The YP medium promoted greater cell mass production and α-galactosidase activity, likely due its highly nutrient rich composition, compared to the others media used. The results suggest that in *Debaryomyces hansenii*, the α-galactosidase induction mechanism by different sugars is similar to that of other yeasts, such as *Sacharomyces cerevisiae*.

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4.1.6. References

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Figures

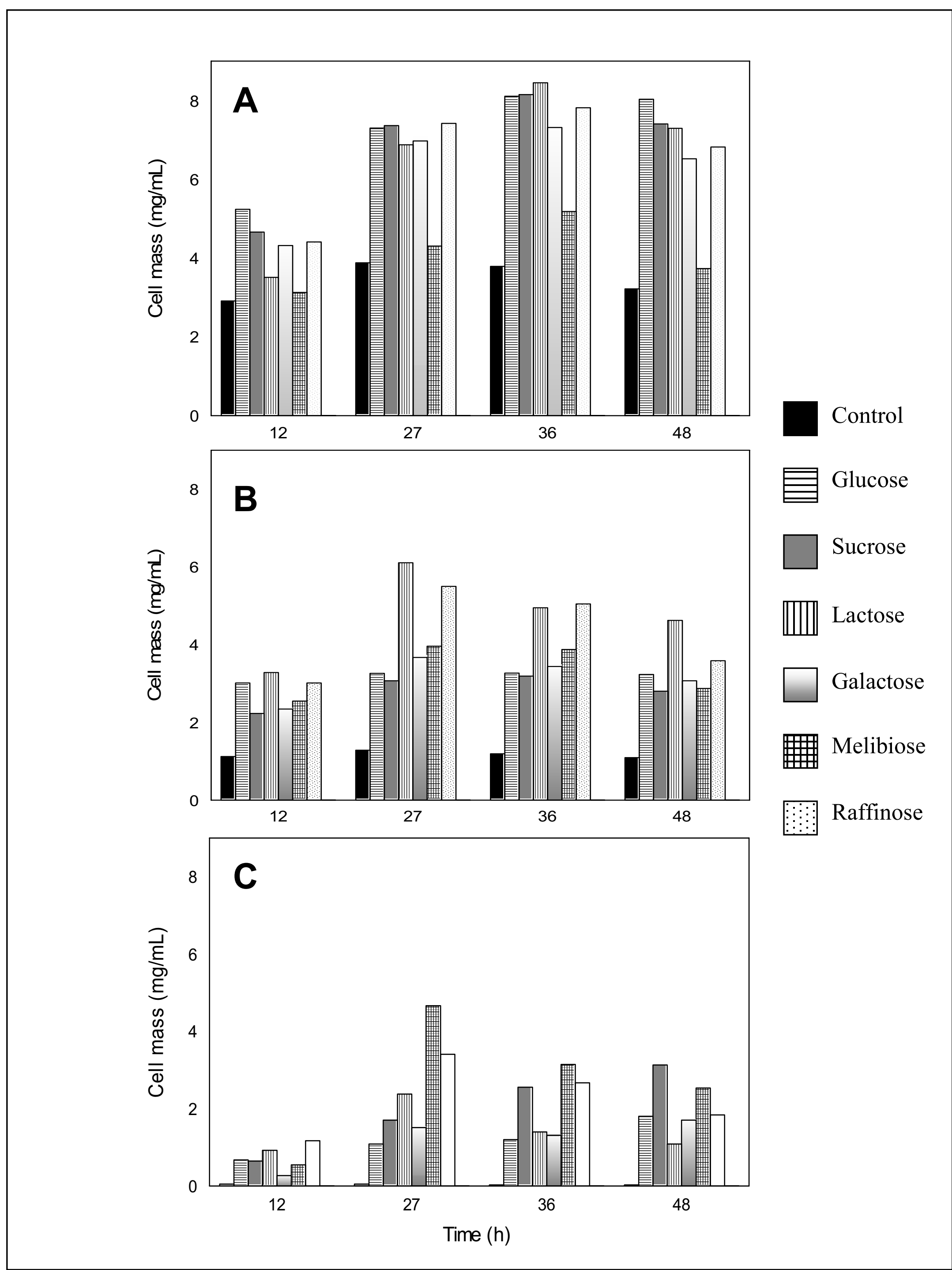

Figure 1 – Cell mass (mg/mL) of the *D. hansenii* UFV-1 culture in YP (**A**); MME-mineral (**B**); and MM-mineral (**C**) media with different carbon sources and incubation times at 30 ^{o}C.

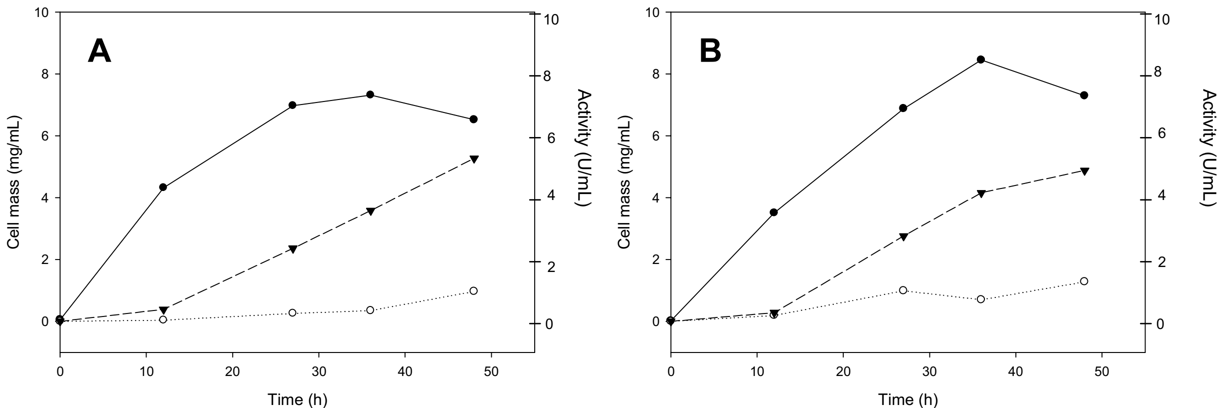

Figure 2 – Effect of sugars on cell mass (●) and *D. hansenii* UFV-1 extracellular (o) and intracellular (▲) α-galactosidases production in the YP medium with galactose (**A**) and lactose (**B**) as carbon source (1 %).

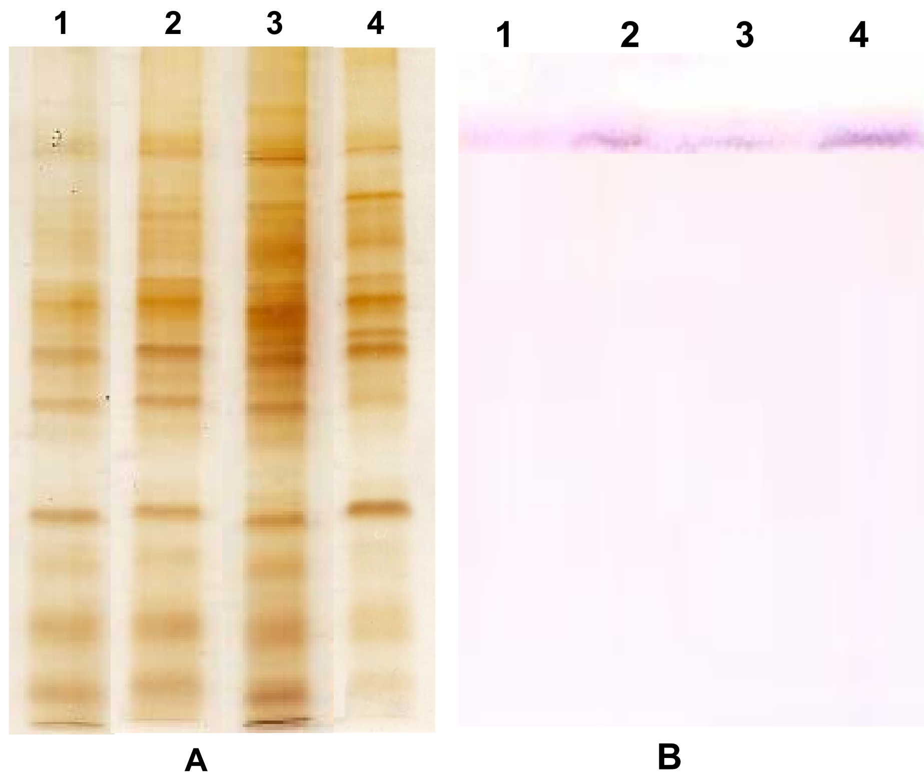

Figure 3 – SDS-PAGE (12.5 %) analysis (**A**) and Western Blot of sera with anti-extracellular-α-galactosidase antibodies (**B**) from *D. hansenii* UFV-1 culture in MME-mineral medium with galactose (1 %), after 12 h (1); 27 h (2); 36 h (3) and 48 h (4) of cultivation at 30 °C, 200 rpm.

Tables

Table 1 – α-Galactosidase from *D. hansenii* UFV-1 activity (U/mL) in different culture media, carbon sources and incubation times at 30 °C.

	α-Galactosidase Activity (*U/mL)											
	Extracellular				**Intracellular**				**Total**			
Medium	12 h	27 h	36 h	48 h	12 h	27 h	36 h	48 h	12 h	27 h	36 h	48 h
YP												
+Control	0,02	0,03	0,04	0,04	0.0	0.0	0.0	0.0	0,02	0,03	0,04	0,04
Glucose	0.05	0.25	0.25	0.83	0.27	1.60	2.97	3.28	0.32	1.85	3.22	4.11
Sucrose	0.14	0.26	0.34	0.52	0.38	2.43	3.41	4.28	0.52	2.69	3.75	4.80
Lactose	0.19	0.99	0.70	1.28	0.28	2.76	4.16	4.88	0.47	3.75	4.86	6.16
Galactose	0.04	0.26	0.35	0.97	0.39	2.36	3.58	5.27	0.43	2.62	3.93	6.24
Melibiose	0.02	0.09	0.08	0.16	0.36	1.76	1.85	1.26	0.38	1.85	1.93	1.42
Raffinose	0.16	0.77	0.92	0.23	0.54	3.51	2.94	4.63	0.70	4.28	3.86	4.86
MME												
+Control	0,01	0,02	0,02	0,02	0.0	0.0	0.0	0.0	0,01	0,02	0,02	0,02
Glucose	0.02	0.06	0.07	0.07	0.16	0.88	1.15	0.70	0.18	0.94	1.22	0.77
Sucrose	0.07	0.06	0.11	0.10	0.38	0.69	0.74	0.67	0.45	0.75	0.85	0.77
Lactose	0.16	0.52	0.44	0.76	0.47	2.71	2.06	3.11	0.63	3.23	2.50	3.87
Galactose	0.02	0.14	0.15	0.14	0.66	2.05	1.51	1.41	0.68	2.19	1.66	1.55
Melibiose	0.04	0.13	0.21	0.26	0.17	0.81	0.60	1.20	0.21	0.94	0.81	1.46
Raffinose	0.13	0.25	0.26	0.20	0.42	1.38	1.26	1.06	0.55	1.63	1.52	1.26
MM												
+Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glucose	0.03	0.08	0.10	0.05	0.19	0.30	0.53	0.64	0.22	0.38	0.63	0.69
Sucrose	0.10	0.16	0.02	0.0	0.18	0.70	0.51	1.19	0.28	0.86	0.53	1.19
Lactose	0.18	0.02	0.09	0.45	0.10	1.08	1.58	0.90	0.28	1.10	1.67	1.35
Galactose	0.02	0.03	0.0	0.0	0.11	0.33	0.41	0.30	0.13	0.36	0.41	0.30
Melibiose	0.06	0.05	0.0	0.0	0.12	0.35	0.43	0.81	0.18	0.40	0.43	0.81
Raffinose	0.16	0.02	0.05	0.37	0.24	1.38	1.82	2.53	0.40	1.40	1.87	2.90

* One α-galactosidase unit (U) was defined as the amount of enzyme that liberates 1 μmol of *p*-NP per minute under the assay conditions. + Without sugar.

Table 2 – α-Galactosidase activity from different yeasts

Microorganism	Culture medium	Substrate	*Activity (U)	Reference
Debaryomyces hansenii UFV-1	YP	Lactose (1 %)	6.1	This work
		Galactose (1 %)	6.2	
		Raffinose (1 %)	4.9	
	MME	Lactose (1 %)	3.9	
		Galactose (1 %)	2.2	
Streptomyces erythrus	Czapek-Doxs	Galactose (2 %)	12.9	(*33*)
		Raffinose (2 %)	11.6	
		Melibiose (2 %)	10.7	
Debaryomyces castellii IFO 1359	YP with 0.05 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 0.01 % KCl	Galactose(1 %); or Melibiose (0.5 %)	2.8	(*30*)
Debaryomyces nepalensis IFO 1428			1.1	
Saccharomyces cerevisiae IFO 1997			0.1	
Pichia guilliermondii IFO 10106			0.6	
Schvanniomyces occidentalis IFO 1839			0.8	
Torulaspora delbruekii IFO 1255	YP	Galactose (2 %)	0.5	(*29*)
Saccharomyces carlsbergensis 1317	Yeast extract 0.3 %	Galactose (1 %)	4.5	(*31*)

*Each of the α-galactosidase activities in this table was enzyme activity obtained from 1 mL of culture broth.

Capítulo 2

Debaryomyces hansenii UFV-1 Intracellular α-Galactosidase Characterization and Comparative Studies with the Extracellular Enzyme

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Debaryomyces hansenii UFV-1 Intracellular α-Galactosidase Characterization and Comparative Studies with the Extracellular Enzyme

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Debaryomyces hansenii cells cultivated on galactose produced extracellular and intracellular α-galactosidases, which showed 54.5 and 54.8 kDa molecular mass (MALDI-TOF), 60 and 61 kDa (SDS–PAGE) and 5.15 and 4.15 p*I* values, respectively. The extracellular and intracellular deglycosylated forms presented 36 and 40 kDa molecular mass, with 40 and 34% carbohydrate content, respectively. The *N*-terminal sequences of the α-galactosidases were identical. Intracellular α-galactosidase showed smaller thermostability when compared to the extracellular enzyme. *D. hansenii* UFV-1 extracellular α-galactosidase presented higher k_{cat} than the intracellular enzyme (7.16 vs 3.29 s^{-1}, respectively) for the *p*-nitrophenyl-α-D-galactopyranoside substrate. The K_m for hydrolysis of *p*NPαGal, melibiose, stachyose, and raffinose were 0.32, 2.12, 10.8, and 32.8 mM, respectively. The intracellular enzyme was acompetitively inhibited by galactose (K_i = 0.70 mM), and it was inactivated by Cu(II) and Ag(I). Enzyme incubation with soy milk for 6 h at 55 °C reduced stachyose and raffinose amounts by 100 and 73%, respectively.

KEYWORDS: α-Galactosidases; *Debaryomyces hansenii* UFV-1; characterization; deglycosylation; galacto-oligosaccharides

INTRODUCTION

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) are known to occur widely in microorganisms, plants and animals, and some of them have been purified and characterized (*1, 2*). According to their source, mechanism and specificity, their properties differ markedly, and they are grouped into classes and clans (http://www.cazy.org/). Some of these enzymes catalyze the hydrolysis of α-1,6-linked galactosyl residues from galacto-oligosaccharides (GO) and polymeric galacto-(gluco)mannans. Several industrial applications of α-galactosidases are known, mainly in the sugar industry, where they improve the crystallization of sucrose by hydrolyzing raffinose in beet sugar syrups (*3*). Moreover, they can enhance the bleaching effect in the pulp and paper industry (*4*). They can also be used to improve the gelling properties of galactomannans used as food thickeners (*5*). α-Galactosidases are also of interest in biomedical applications, e.g., for the treatment of Fabry disease by enzyme replacement therapy (*6*) or for blood type conversion (*7*). The enzyme can be used for hydrolysis of raffinose, stachyose, and polysaccharides that induce gastrointestinal disturbances and flatulence in humans and which are present in soy milk and in other legume-derived foods (*8, 9*). Dietary supplementation with α-galactosidase can improve energy values and nutrient bioavailability, resulting in better growth performance and intestinal viscosity (*10*). Fungal α-galactosidases are the most suitable for technological applications mainly due to their acidic optima pH and broad stability profiles (*8*). However, the cost of production is one important factor which limits the use of this enzyme in industrial processes. It has been recently demonstrated that the yeast isolate *Debaryomyces hansenii* UFV-1 is very promising in the production of extracellular and intracellular α-galactosidases (*11*). *Debaryomyces hansenii* is a halophile yeast found in shallow seawaters

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Debaryomyces hansenii UFV-1 Intracellular α-Galactosidase Characterization and Comparative Studies with the Extracellular Enzyme

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4.2.1. Abstract

Debaryomyces hansenii UFV-1 cells cultivated on galactose produced extracellular and intracellular α-galactosidases, which showed 54.5 and 54.8 kDa molecular mass (MALDI-TOF), 60 and 61 kDa (SDS-PAGE) and 5.15 and 4.15 *pI* values, respectively. The extracellular and intracellular deglycosylated forms presented 36 and 40 kDa molecular mass, with 40 and 34 % carbohydrate content, respectively. The *N*-terminal sequences of the α-galactosidases were identical. Intracellular α-galactosidase showed smaller thermostability when compared to the extracellular enzyme. *D. hansenii* UFV-1 extracellular α-galactosidase presented higher K_{cat} than the intracellular enzyme (7.16 vs 3.29 s^{-1}, respectively) for the *p*-nitrophenyl α-D-galactopiranoside substrate. The K_m for hydrolysis of *p*-nitrophenyl α-D-galactopyranoside, melibiose, stachyose, and raffinose were 0.32, 2.12, 10.8 and 32.8 mM, respectively. The intracellular enzyme was acompetitively inhibited by galactose (K_i = 0.70 mM) and it was inactivated by Cu(II) and Ag(I). Enzyme incubation with soymilk for 6 h at 55 ^{o}C reduced stachyose and raffinose amounts by 100 and 73 %, respectively.

4.2.2. Keywords

α-Galactosidases; *Debaryomyces hansenii* UFV-1; characterization; deglycosylation; galacto-oligosaccharides.

4.2.3. Introduction

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) are known to occur widely in microorganisms, plants and animals, and some of them have been purified and characterized (***1,2***). According to their source, mechanism and specificity, their properties differ markedly, and they are grouped into classes and clans (http://www.cazy.org/). Some of these enzymes catalyze the hydrolysis of α-1,6-linked galactosyl residues from galacto-oligosaccharides (GO) and polymeric galacto-(gluco)mannans. Several industrial applications of α-galactosidases are known, mainly in sugar industry, where they improve the crystallization of sucrose by hydrolyzing raffinose in beet sugar syrups (***3***). Moreover, they can enhance the bleaching effect in pulp and paper industry (***4***). They can also be used to improve the gelling properties of galactomannans used as food thickeners (***5***). α-Galactosidases are also of interest in biomedical applications, e.g., for the treatment of Fabry disease by enzyme replacement therapy (***6***) or for blood type conversion (***7***). The enzyme can be used for hydrolysis of raffinose, stachyose, and polysaccharides that induce gastrointestinal disturbs and flatulence in human and which are present in soymilk and in other legume-derived foods (***8,9***). Dietary supplementation with α-galactosidase can improve energy values and nutrient bioavailability, resulting in better growth performance and intestinal viscosity (***10***). Fungal α-galactosidases are the most suitable for technological applications mainly due to their acidic optima pH and broad stability profiles (***8***). However, the cost of production is one important factor which limits the use of this enzyme in industrial process. It has been recently demonstrated that the yeast isolate *Debaryomyces hansenii* UFV-1 is very promising in the production of extracellular and intracellular α-galactosidases (***11***). *Debaryomyces hansenii* is halophile yeast found in shallow seawaters (12) but also in salty food products like meat and dairy products (***13***). *D. hansenii* UFV-1 extracellular α-galactosidase was characterized and its efficiency was demonstrated in the hydrolysis of GO present in soybean

products (***11,14***). In addition, *D. hansenii* UFV-1 permeabilized cells, containing the intracellular α-galactosidase was a good biocatalyser for GO hydrolysis in soybean products (***14***). Therefore, the study of *D. hansenii* UFV-1 α-galactosidases is interesting, since both enzymes could be used in biotechnological processes. In this paper we characterized the *D. hansenii* UFV-1 intracellular α-galactosidase and compared that with extracellular enzyme.

4.2.4. Materials and Methods

4.2.4.1. Yeast strain and culture conditions

The fungal strain used was the *Debaryomyces hansenii* UFV-1. It was isolated from a dairy plant and kept in the collection of the Departamento de Microbiologia, Universidade Federal de Viçosa (Viçosa, MG, Brasil). The taxonomic identification was carried out by the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands). *D. hansenii* UFV-1 stored at -80 ^{o}C in glycerol and YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) was streaked on YPD agar surface (1.5 % agar) and kept in an incubation chamber for 36 h at 30 ^{o}C. The yeast was then activated in YPD liquid medium and incubated for 12-15 h, 200 rpm at 30 ^{o}C. An aliquot of the cells obtained after centrifugation (4000*g* for 5 min at 4 ^{o}C) was transferred to one liter of mineral medium (***11***) toward $O.D._{600\ nm}$ 0.1 (0.053 mg/mL). After incubation for 31 h, 200 rpm, at 30 ^{o}C, the biomass was separated by centrifugation (4000*g* for 5 min at 4 ^{o}C) and frozen (-20 ^{o}C) for enzyme extraction.

4.2.4.2. Extraction of intracellular enzyme

D. hansenii UFV-1 cells (15 g) were ground with liquid nitrogen and resuspended in 40 mL of 0.1 M sodium acetate buffer, pH 5, containing 0.25 % (by weight) of triton X-100. This mixture was submitted to a series of nitrogen freezing and thawing at 40 ^{o}C in a water

bath. It was then submitted to an ultrasonic bath (Branson, USA) for 10 min, and it was centrifuged (25,900*g* for 30 min at 4 °C). The supernatant was used as the source of intracellular enzyme.

4.2.4.3. Purification of intracellular α-galactosidase

The enzymatic extract was kept at -20 °C for 24 h (cryoprecipitation), thawed and the precipitate was removed by centrifugation (25,900*g* for 30 min at 4 °C). After this procedure, the supernatant was loaded onto a Sephadex G-150 size exclusion column (87.5 cm x 2.5 cm), equilibrated with 25 mM sodium acetate buffer, pH 5.5, at 4 °C. The proteins were eluted at a flow rate of 20 mL/h and 3.5 mL fractions were collected. Fractions containing α-galactosidase activity were pooled and loaded onto a DEAE-Sepharose anion exchange column (14.5 cm x 1.9 cm), equilibrated with 0.1 M sodium acetate buffer, pH 5.5, at 4 °C. Elution was performed at the flow rate of 40 mL/h, with a linear gradient formed with 150 mL of 0.1 M sodium acetate buffer and 150 mL of the same buffer containing 1 M NaCl. The active fractions were pooled and analyzed for purity by SDS-PAGE.

4.2.4.4. Enzyme assay

The activity of α-galactosidase was assayed by measuring the amount of *p*-nitrophenol (*p*-NP) released from the hydrolysis of *p*-nitrophenyl α-D-galactopyranoside (*p*NPαGal) as substrate. The standard reaction mixture contained 2 mM *p*NPαGal, 0.1 M sodium acetate buffer, pH 5.0, and the enzyme preparation (0.35 µg protein/mL) in a final volume of 1.0 mL. After incubation at 55 or at 40 °C for the purification assays, for 15 min, 1 mL of 0.5 M sodium carbonate was added to the mixture to stop the reaction. The absorbance of the mixture was then measured at 410 nm. The amount of *p*-NP released was calculated according to the standard curve (0-0.16 µmol *p*-NP).

The activities against maltose, gentiobiose, lactose, and melibiose were determined by the glucose-oxidase method (***15***). The production of reducing sugar was determined using the 3,5-dinitrosalicylate reagent (***16***), when sucrose, raffinose, and stachyose were used as substrates.

One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol of product per min under the assay conditions.

The data presented for all α-galactosidases activity determinations are mean values of triplicate assays in which the standard deviations values were always smaller than 10 %.

4.2.4.5. Protein determination

Protein concentration in the enzymatic extracts was determined by the BCA (bicinchonicic acid) method (***17***) with bovine serum albumin (BSA) as the standard.

4.2.4.6. Molecular mass determination

The molecular mass (*Mr*) of the purified enzyme was estimated by SDS-PAGE using a 12.5 % polyacrylamide gel (***18***). Molecular mass standards from Sigma Chemical Co. (St. Louis, MO, USA) were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhidrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20.1 kDa). After electrophoresis, the protein was visualized by silver staining (***19***).

Molecular mass of the native enzymes was determined using MALDI-TOF mass spectrometry where chromatographic fractions were dissolved in a satured α-cyano-4-hydroxycinnamic acid matrix solution 1:3 (w:w), spotted onto a MALDI target plate and dried at room temperature for 15 min. The average molecular mass was obtained in an AUTOFLEX III (Bruker^{TM}) in linear positive mode, with external calibration, using the Protein Calibration

Standard II for Mass Spectrometry calibration molecules: Trypsinogen $[M+ H]^{+}$= 23,982, Protein A $[M+ H]^{+}$= 44,613, Albumin-Bovine $[M+ H]^{+}$=66,431, Protein A $[M+ 2H]^{2+}$ = 22,306, Albumin-Bovine $[M+ 2H]^{2+}$= 33,216 (Bruker Daltonics). The Flex Analysis Software (Bruker Daltonics) was used to interpret mass spectra.

4.2.4.7. Amino acids analysis of *D. hansenii* UFV-1 α-galactosidases

Triplicate samples of purified *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases were hydrolyzed with 6 M HCl plus 0.1 % (v:v) phenol in vapor phase hydrolysis during 22 h at 110 ºC, and the amino acids were reacted with phenylisothiocianate to produce phenylthiocarbamyl derivatives. Amino acid standard (Pierce, Co, Rockford, IL, USA) and hydrolysis products were analyzed by RP-HPLC after derivatization (***20***).

4.2.4.8. Chromatofocusing

The α-galactosidase isoelectric point (*pI*) was estimated by chromatofocusing. Previously, the enzyme was dialyzed against start buffer, which contained 25 mM Bis-Tris/iminodiacetic acid, pH 6 (Amersham Pharmacia Biotech, Uppsala, Sweden). Around 100 µg of protein was applied onto chromatofocusing column chromatography Mono P HR 5/5 column (FPLC, Amersham Pharmacia Biotech) equilibrated with start buffer, pH 6. Elution was performed with 10 % (v/v) Polybuffer 74/iminodiacetic acid, pH 4 (Amersham Pharmacia Biotech), using a flow rate of 30 mL/h. The pH measurement of eluted fractions was performed by a potentiometer coupled to FPLC apparatus and using an isolated pH electrode. Ovalbumin (200 µg/mL) was used as standard (*pI* of 4.5).

4.2.4.9. Total carbohydrate determination

The carbohydrate content in the purified α-galactosidases was estimated using the phenol-sulfuric acid procedure (***21***). A glucose standard curve was plotted for each assay with glucose concentrations varying up to 70 μg/mL.

4.2.4.10. α-Galactosidases deglycosylation

Deglycosylation of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases was performed using N-Glycosidase F Deglycosylation Kit (New England Biolabs, Beverly, MA). Protein (200 μg) was incubated with 90 μL of 10X diluted denaturing buffer (5 % SDS and 10 % 2-mercatptoethnaol) at 100 °C for 10 min. After denaturation, were added 10 μL of 10X concentrated 50 mM sodium phosphate buffer, pH 7.5, 10 μL of 10 % Nonidet P40 and 2 μL of PNGase F (500,000 U/mL) purified of *Flavobacterium meningosepticu*. The reaction mixture was incubated at 37 °C for 31 h. Reaction products were separated by SDS-PAGE and visualized by silver staining.

4.2.4.11. *N*-terminal amino acid sequencing

The amino acid residue sequence of the intracellular α-galactosidase was determined by Edman degradation in a automated protein sequencer (Shimadzu PPSQ-21A, Shimadzu, Kyoto, Japan) coupled to a reverse-phase separation of PTH-amino acids in a WAKOSIL-PTH (4.6 x 250 mm) column (Wako, Osaka, Japan) at a flow rate 1 mL/min, with detection at 235 nm. Using the BLAST-P software, the obtained sequence was compared to known protein sequences.

4.2.4.12. Effect of pH and temperature on intracellular α-galactosidase activity

Influence of pH on the α-galactosidase activity was determined within the pH range from 3.0 to 8.0 by using the McIlvaine buffer (citric acid/sodium phosphate) at 55 °C (***22)***. The effect of the temperature on the enzyme activity on *p*NPαGal was determined in 0.1 M sodium acetate buffer, pH 5, at a temperature range of 25-80 °C.

For determination of α-galactosidase thermal stability, the enzyme was preincubated at various temperatures (40, 55 and 65 °C) in 0.1 M sodium acetate buffer, pH 5, for 0-48 h. After incubation, 2 mM *p*NPαGal was added and the remaining activity was measured by the standard method. Results of the analyses are presented as the mean ± SD for three measurements.

4.2.4.13. Substrate specificity

The enzyme was tested for its ability to hydrolyze various synthetic, natural, and polymeric substrates. The reaction mixtures contained 650 μL of 0.1 M sodium acetate buffer, pH 5, 70 μL of enzyme solution (0.35 μg protein/mL), and 250 μL of synthetic substrates (2 mM), or lactose, maltose, gentiobiose, stachyose, and sucrose (4 mM), or raffinose (30 mM), or melibiose (2 mM), or locust bean gum and guar gum solutions (1 %). The following synthetic substrates were employed: *p*NPβGal, 4-nitrophenyl β-D-galactopyranoside; *p*NPαGlc, 4-nitrophenyl α-D-glucopyranoside; *p*NPβX, 4-nitrophenyl β-D-xylopyranoside; *p*NPαA, 4-nitrophenyl α-D-arabinopyranoside; *p*NPαM, 2-nitrophenyl α-D-mannopyranoside; *o*NPβGlc, 2-nitrophenyl β-D-glucopyranoside; *o*NPβGal, 2-nitrophenyl β-D-galactopyranoside. The activities were measured under standard assay conditions at 55 °C. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

4.2.4.14. Effects of several compounds on intracellular α-galactosidase activity

The effect of ions, simple sugars and reducing agents on α-galactosidase activity was assayed by following the hydrolysis of *p*NPαGal. Enzyme samples were preincubated with each compound in 0.1 M sodium acetate buffer, pH 5, for 20 min at 55 ^{o}C. The data presented for all enzyme activity determinations are mean values ± SD of triplicate assays.

4.2.4.15. Kinetic properties

The intracellular α-galactosidase Michaelis-Menten constant (K_m) for substrate hydrolysis was calculated by nonlinear curve fitting of the data to the Michaelis-Menten plot. The substrate concentrations were 0.05 to 1.0 mM for *p*NPαGal; 5.0 to 120 mM for raffinose; 2.5 to 30 mM for stachyose, and 0.15 to 40 mM for melibiose. The α-galactosidases catalytic constants (K_{cat}), using *p*NPαGal substrate concentrations ranging from 0.4 to 2.4 mM were calculated by the direct linear method with Hanes-Woolf plot.

The inhibition constant (K_i) for galactose was calculated by the Dixon plot by using *p*NPαGal as substrate at the following concentrations: 0.05 to 1.0 mM. The concentrations of galactose were 0.5, 1.0, and 2.0 mM.

4.2.4.16. Treatment of soymilk with intracellular α-galactosidase

Soymilk prepared from dry seeds (5 mL) was incubated with either water or an aqueous solution of 10.5 U of purified α-galactosidase for 0, 2, 4 and 6 h under shaking (100 rpm) at 55 °C. Each reaction mixture was dried and the soluble sugars were extracted from 20-30 mg of lyophilized samples with organic solvents (***23***). The solvent was evaporated at 50 ^{o}C and the sugars resuspended in 1 mL of 80 % ethanol. The sugars were analyzed by HPLC system on a Shimadzu series 10A chromatograph using an analytical column [aminopropyl (-

NH_2)] eluted with an acetonitrile-water isocratic mixture (80:20 v:v) at 35 ^{o}C, at a flow rate of 1 mL/min. Individual sugars were automatically identified and quantified by comparison with retention times and known concentrations of standard sugars. Gentiobiose was used as an internal standard as it does not interfere with the other sugars and it is not found in soybean seeds.

4.2.5. Results and discussion

4.2.5.1. Production and purification of intracellular α-galactosidase

Debaryomyces hansenii UFV-1 grown in medium containing galactose as carbon source produced both extracellular (0.9 U/mL) and intracellular (1.5 U/mL) α-galactosidases. Highest specific activity of extracellular α-galactosidase was observed at 31 h of cultivation (11.9 U/mg). In this time, the dry cell mass was 3.4 mg/mL and the specific activity of intracellular α-galactosidase was 0.9 U/mg (0.51 U/mg cell). Therefore, the bioprocess was terminated after 31 h of cultivation. On the other hand, β-galactosidase and invertase presented only intracellular activity (data not shown). Several strains of yeasts are described to produce both intra and extracellular α-galactosidases (***24***). *Saccharomyces carlsbergensis* contains a soluble α-galactosidase localized inside the cell and an inducible form which is mainly secreted (**25**).

D. hansenii UFV-1 extracellular α-galactosidase has been purified and partially characterized (***11***). To investigate possible differences between *D. hansenii* UFV-1 internal and external α-galactosidases, the intracellular enzyme was compared to the extracellular one. The purification procedure for intracellular α-galactosidase is summarized in the **Table 3**. The specific activity of the enzyme increased from an average of 0.91 mmol L^{-1} min^{-1} $mg^{-1}$of protein in the crude extract to 263 mmol L^{-1} min^{-1} mg^{-1} after DEAE-Sepharose anion

exchange column. Thus, the resultant purification factor was 289 times with 45 % overall yield.

4.2.5.2. Molecular mass and isoelectric point

The protein elution profile and the corresponding enzyme activities are shown in **Figure 4**. The electrophoretic profile of the purified intracellular α-galactosidase in SDS-PAGE confirmed the presence of a single protein band, with an estimated molecular mass of 61 kDa (**Figure 5-lane 4**). Using MALDI-TOF mass spectrometry, the intracellular enzyme showed molecular mass of 54.84 ± 0.05 kDa. Molecular mass of approximately 60 kDa was reported for extracellular α-galactosidase from the same yeast using SDS-PAGE (***11***) and 54.50 ± 0.05 kDa determined by MALDI-TOF mass spectrometry. The molecular mass values estimated by SDS-PAGE from deglycosylated extracellular and intracellular enzymes were 36 and 40 kDa, respectively (**Figure 5-lanes 3 and 5**).

The isoelectric point (*pI*) of *D. hansenii* UFV-1 intracellular α-galactosidase was lower than the value obtained for the extracellular enzyme (4.15 vs 5.15). The *pI* values of *D. hansenii* UFV-1 α-galactosidases are in the common range (3.6-6.4), for α-galactosidases from widely different sources (***26***).

4.2.5.3. Carbohydrate content, amino acid composition and *N*-terminal sequence of α-galactosidases

The carbohydrate content of the *D. hansenii* UFV-1 intracellular enzyme quantified by phenol-sulfuric acid procedure was lower (34 %) when compared to the value obtained for the extracellular α-galactosidase (40 %) from the same yeast (***11***), from *Humicola* sp. (8.3 %) (***8***), and from *Thermomyces lanuginosus* CBS 395.62/b (5.3 %) (***27***).

Amino acid analysis from both *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases showed few variations in their contents (**Table 4**). *D. hansenii* UFV-1 intracellular α-galactosidase had the *N*-terminal amino acid sequence YENGLNLVPQMGWNSWNKFGXHI where X is most probably a Cys residue. Comparison of its *N*-terminal amino acid sequence with amino acid sequences from other α-galactosidases is shown in **Table 5**. The *N*-terminal amino acid sequence of *D. hansenii* UFV-1 intracellular α-galactosidase was identical to the sequence of the extracellular enzyme (***11***). The alignment of the *N*-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase (23 amino acid residues) with the sequence of *D. hansenii* CBS767 α-galactosidase showed five non-conservative changes (***28***). However, the N and V residues presented in *D. hansenii* UFV-1 α-galactosidase were conserved in other microbial α-galactosidases, as observed in the sequence of the *Magnaporthe grisea* 70-15 α-galactosidase (***29****)*. The K and I residues were conserved in the sequence of the *Schizosaccharomyces pombe* (***30***) (**Table 5**). The high similarity showed by the α-galactosidases presented in **Table 5**, which are classified as glycosyl hydrolases family 27 members, suggests that *D. hansenii* UFV-1 α-galactosidases also belongs to this family.

There are reports of production and characterization of both intracellular and extracellular fungi α-galactosidases, such as those from *Aspergillus nidulans* (***37***) and *Aspergillus tamarii* (***38****)*, showing similar or different enzymatic properties. Evidences suggest that intracellular and extracellular α-galactosidases from *D. hansenii* UFV-1 could be product of a single gene, because they have similar molecular mass and their *N*-terminal amino acid sequences are identical. However, there is a difference in the carbohydrate content between the intracellular (34 % by mass) and extracellular (40 % by mass) forms. These data suggest that *D. hansenii* UFV-1 α-galactosidases could exist as two glycoforms. The glycosylation level of a protein can affect its structure, stability and movement through the

secretory pathways. Thermostability and degree of glycosylation are two closely linked factors in a protein (***39***). The *D. hansenii* UFV-1 intracellular α-galactosidase showed smaller degree of glycosylation and smaller thermostability compared to the extracellular enzyme. The higher glycosylation level of extracellular enzyme could also be an important factor for its secretion.

4.2.5.4. Effect of pH and temperature on intracellular α-galactosidase activity

Substantial activity against *p*NPαGal was observed for intracellular enzyme preparation within a temperature range of 45–60 °C and pH range of 3.5–5.5 (**Figure 6**). The enzyme showed an optimum pH of 5 (**Figure 6A**) and an optimum temperature of 55 °C (**Figure 6B**). The slightly acidic pH optimum of α-galactosidase (4.6-6.2) is typical of some fungal glycosyl hydrolases. *D. hansenii* UFV-1 intracellular α-galactosidase kept about 80 % of its original activity after incubation for 8 h at 40 °C but only 30 % of its activity remained after incubation for 41 h at that temperature (**Figure 7**). At 55 °C, the enzyme maintained 85 and 40 % of its original activity after pre-incubation for 7 and 25 h, respectively. The half-life ($t_{1/2}$) values of *D. hansenii* UFV-1 intracellular α-galactosidase at 40, 55 and 65 °C were 39 h, 31 h, and 38 min, respectively. Similar results have been shown for *Humicola* sp. α-galactosidase (***8***), and higher $t_{1/2}$ values were determined for the *D. hansenii* UFV-1 extracellular α-galactosidase at 65 and 70 °C, which were 3 h, and 35 min, respectively. The thermostability of the enzymes is an important parameter for biotechnological applications. The stability exhibited by *D. hansenii* UFV-1 α-galactosidases in a broad range of temperature indicates that these enzymes could be used for industrial applications, especially for processing of GO in soybean products.

4.2.5.5. Substrate specificity

The substrate specificities of *D. hansenii* UFV-1 intracellular α-galactosidase were investigated by using synthetic substrates, galactose-containing oligosaccharides, and polymers. Under the experimental conditions, the enzyme was highly selective, showing absolute specificity for α-galactosyl bond. This enzyme hydrolyzed *p*NPαGal (2.49 U/mL ± 0.03) but showed no activity for other synthetic substrates such as glucosidic derivatives or for β-linked glycosides. These results indicate that the configurations at C1 and C4 atoms of the substrates are very important for their interaction with the catalytic site. When assayed against natural substrates, the enzyme hydrolyzed α-galactosyl linkage in the oligosaccharides, and showed highest activity for stachyose (3.19 U/mL ± 0.01) followed by raffinose (2.28 U/mL ± 0.01). The enzyme was able to hydrolyze polymers, such as locust bean gum and guar gum (0.55 U/mL ± 0.01) and (0.55 U/mL ± 0.02), respectively, suggesting its potential industrial application for modification of gelling and rheological properties of the polysaccharides. Our results demonstrated that *D. hansenii* UFV-1 α-galactosidases can hydrolyze exclusively α-D-galactosides in natural, synthetic, and polymeric substrates (***11***).

4.2.5.6. Effects of several compounds on intracellular α-galactosidase activity

The effect of various mono- and divalent metal ions, monosaccharides, and reducing and nonreducing oligosaccharides on the activity of the *D. hansenii* UFV-1 intracellular α-galactosidase was examined under the standard enzyme assay conditions (**Figure 8**). The enzyme lost 70 % of its activity in the presence of Ag(I) and became completely inactive in the presence of Cu(II). Participation of carboxyl groups and/or histidine imidazolium groups in the catalytic action is supposed on the basis of the inhibitory effect (***40***). On the other hand, the activity of *Ganoderma lucidum* α-galactosidase (***41***) seemed to be slightly stimulated by

Cu(II) and decreased to 32 % of its original activity by addition of Ag(I). The *D. hansenii* UFV-1 intracellular α-galactosidase activity presented very low or no inhibition by EDTA, Mg(II), iodoacetamide, Na(I), K(I), Ca(II), β-mercaptoethanol, raffinose, maltose, sucrose, D-glucose, lactose, stachyose, and D-mannose. The enzyme activity against *p*NPαGal was partially decreased by D-galactose and by melibiose (**Figure 8**). D-Galactose inhibited the *D. hansenii* UFV-1 intracellular enzyme acompetitively and the K_i value determined by the Dixon plot was 0.70 mM. On the one hand, competitive inhibition by the same substrate has been reported for *Candida javanica* α-galactosidase (***42***). On the other hand, the *D. hansenii* UFV-1 extracellular enzyme showed noncompetitive inhibition by galactose (***11***).

4.2.5.7. Kinetic properties

The lowest K_m value determined for all the substrates assayed was obtained for *p*NPαGal (0.32 mM). On the other hand, for the natural substrates, the lowest K_m value was determined for the hydrolysis of melibiose (2.12 mM). The smaller K_m value for stachyose (10.8 mM) compared to the value for raffinose (32.8 mM) indicates that the *D. hansenii* UFV-1 intracellular α-galactosidase has a relatively large substrate site (**Table 6**). This is very interesting for industrial purposes as the amount of stachyose in soybean products is higher than that of raffinose. Activities on aryl α-D-galactosides as well as on melibiose, raffinose, and stachyose are exhibited by several α-galactosidases but most of these enzymes present a higher affinity for the trisaccharide raffinose than for the tetrasaccharide stachyose (***43***). Also, the K_m values calculated for the *D. hansenii* UFV-1 extracellular α-galactosidase (***11***) with the same substrates (melibiose, raffinose and stachyose) were close to the values determined in this work for the intracellular enzyme (**Table 6**). The K_m values of *Humicola* sp. intracellular α-galactosidase, determined for *p*NPαGal, raffinose, and stachyose were 0.28, 1.45 and 1.42 mM, respectively (***8***). The *D. hansenii* UFV-1 intracellular α-galactosidase presented smaller

K_{cat} value for *p*NPαGal when compared to the extracellular enzyme (3.29 vs 7.16 s^{-1}) as calculated by Hanes-Woolf plot.

4.2.5.8. Hydrolysis of oligosaccharides in soymilk by intracellular α-galactosidase

The hydrolysis of galacto-oligosaccharides (GO) by *D. hansenii* UFV-1 intracellular α-galactosidase in soymilk is displayed in **Table 7.** The reaction mixture initially contained sucrose, raffinose, and stachyose at the concentrations 3.35, 1.50, and 3.30 % (w/v), respectively (**Table 7**). After 2 h of incubation with the enzyme, a reduction of 63 and 100 % was observed in the amounts of raffinose and stachyose, respectively. After incubation for 4 and 6 h, the amount of raffinose was reduced by 28 and 73 %, respectively, while the sucrose concentration rose to 6.74 % (**Table 7**). No oligosaccharide hydrolysis was detected in control tubes without enzyme. The ability of the enzyme to hydrolyze stachyose and raffinose is of particular interest for biotechnological applications. The high ability of *D. hansenii* UFV-1 intracellular α-galactosidase to hydrolyze stachyose is a desirable feature for industrial purposes as the concentration of this sugar in soy derived products is higher than that of raffinose. As the enzyme preparation showed no invertase activity, our results indicate that *D. hansenii* UFV-1 intracellular α-galactosidase acts on GO present in soymilk. Many α-galactosidases from microorganisms have been used to degrade GO in soymilk (***8,44***). However, the potential enzymes suggested for this purpose are generally produced by microorganisms that do not have the GRAS (generally recognized as safe) status. *Debaryomyces hansenii* is a yeast species frequently found in protein-rich fermented products, such as sausages and cheeses, where it contributes to the development of special flavors of those products (***45***). There should be no restriction regarding safeness for the use of this microorganism in food processing. The high yield of *D. hansenii* UFV-1 intracellular α-

galactosidase, its acidic optima pH and its acceptable heat stability may prove useful in industrial applications for degradation of GO from soymilk and from other soy products. The possibility of a combined use of both *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases could enhance the enzyme yield, making more economically viable the process for GO hydrolysis in soybean products.

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4.2.6. Literature cited

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Tables

Table 3 - Summary of the purification steps of *D. hansenii* UFV-1 intracellular α-galactosidase.

Purification step	Protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification factor	Recovery (%)
Crude extract	64.5	59.0	0.91	1.00	100
Cryoprecipitation	58.3	54.7	0.94	1.03	93
Sephadex G-150	1.24	46.7	37.7	41.4	79
DEAE-Sepharose	0.10	26.3	263.0	289.0	45

[a] One unit (U) of enzyme activity is defined as the amount of enzyme that released 1µmol of *p*-nitrophenol per minute.

Table 4 - Amino acid composition of *D. hansenii* UFV-1 α-galactosidases.

Amino acid	Intracellular	Extracellular
	mol/mol	mol/mol
Asx	63.6	48.8
Glx	42.8	44.2
Ser	30.2	38.3
Gly	37.1	44.3
His	6.1	12.2
Arg	10.4	10.5
Thr	22.3	18.8
Ala	20.2	22.7
Pro	13.5	12.4
Tyr	15.3	9.9
Val	20.2	15.9
Met	4.1	3.2
Cys	nd.	nd.
Ile	21.9	16.2
Leu	23.7	17.5
Phe	12.9	9.5
Lys	22.1	15.4
Total	366.5	339.7

*Cys was not detected (nd.); * Trp was not determined due to hydrolysis instability;
*mol/mol was calculated as 36 kDa for extracellular and 40 kDa for intracellular α-galactosidase;
*Asx and Glx correspond to sum of Aspartic acid/Asparagine and Glutamic acid/Glutamine;
*Average standard deviation for triplicate analysis was less than 8 %.

Table 5 - *N*-terminal sequence alignment of *D. hansenii* UFV-1 intracellular α-galactosidase with other microbial α-galactosidases.

Organism		Sequence		Reference
Debaryomyces hansenii UFV-1 (intracellular)		**YENGLNLVPQMGWNSWNKFGXHI**		This study
Debaryomyces hansenii UFV-1 (extracellular)		**YENGLNLVPQMGWN**		(11)
Debaryomyces hansenii CBS767	21	**YENGL**GLT**PQMGWNSWN**IY**GCDI**	43	(28)
Magnaporthe grisea 70-15	36	**NGLNLVPQMGWN**N**WN**AYH**C**DV	56	(29)
Schizosaccharomyces pombe	27	**NGL**GLK**PQMGWNSWNK**YA**CDI**	47	(30)
Zygosaccharomyces mrakii	24	**NGL**GLT**PQMGWN**N**WN**T**F**A**C**NV	44	(31]
Saccharomyces cerevisiae	24	**NGL**GLT**PQMGW**DS**WN**T**F**A**C**DV	35	(32)
Saccharomyces mikcatae	24	**NGL**GLT**PQMGW**DN**WN**T**F**A**C**DV	44	(33)
Emericella nidulans	26	DD**GL**ART**PQMGWN**TYNQYN**C**	45	(34)
Aspergillus oryzae	24	D**G**VGRL**P**AL**GWN**T**WN**A**FGC**D**I**	44	(35)
Aspergillus fumigatus Af293	267	D**NGL**ART**PQMGWNS**YNYYS**C**	286	(36)

*Conserved amino acid residues in all sequences are shown in bold. "X" is most probably a Cys residue.

Table 6 - K_m, K_{cat} and K_{cat}/K_m values determined for *D. hansenii* UFV-1 intracellular α-galactosidase.

Substrates	K_m (mM)	$K_{cat}(s^{-1})$	$K_{cat}/K_m(mM.s)^{-1}$
*p*NPαGal	0.32	3.29	10.28
Melibiose	2.12	0.03	0.01
Stachyose	10.8	6.05	0.56
Raffinose	32.8	4.12	0.13

Table 7 - Hydrolysis of oligosaccharides present in soymilk by intracellular α-galactosidase from *D. hansenii* UFV-1.

	Content (%) ± SD	
Incubation time (h)	**Raffinose**	**Stachyose**
0	1.50 ± 0.02	3.30 ± 0.01
2	0.56 ± 0.03	0.0
4	1.08 ± 0.01	0.0
6	0.41 ± 0.02	0.0

Figures

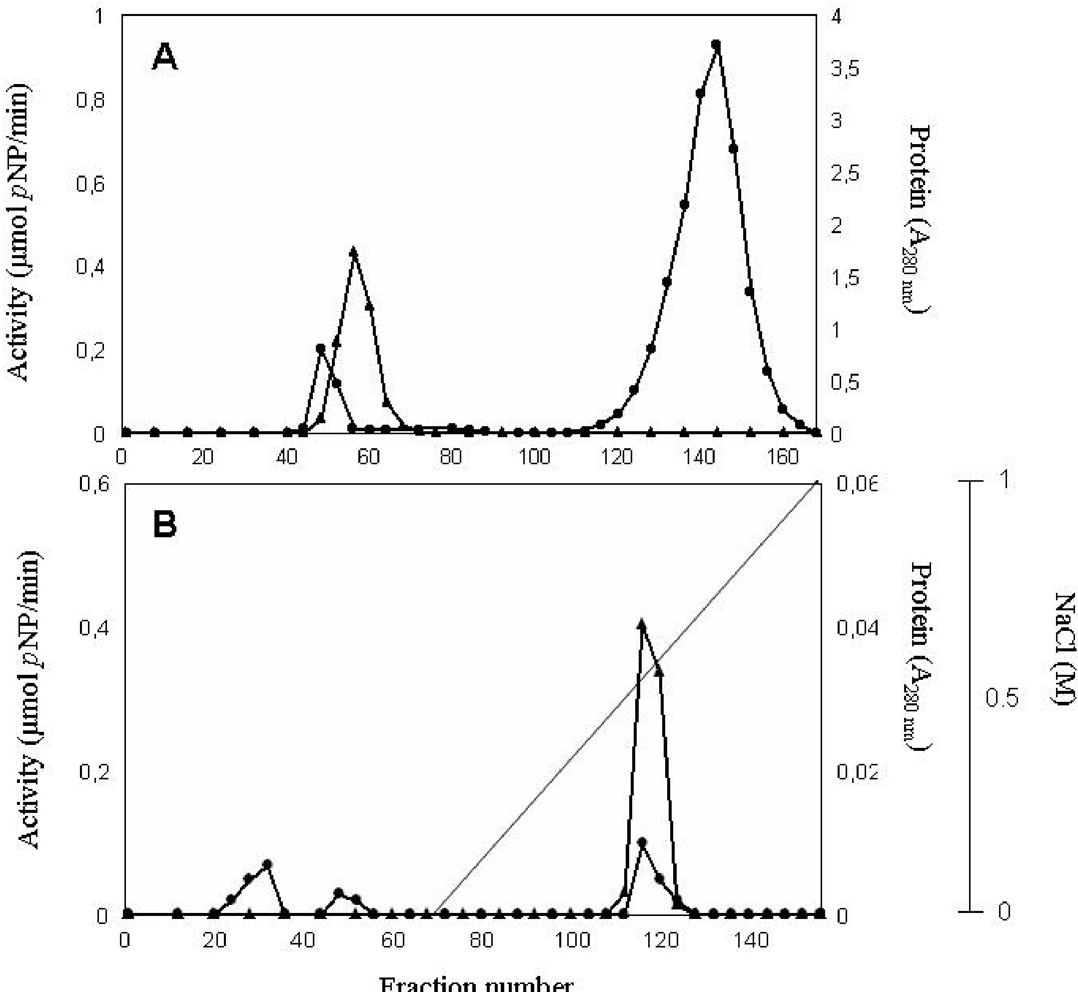

Figure 4 - Elution profile of the intracellular α-galactosidase from *D. hansenii* UFV-1 on a Sephadex G-150 column (**A**) and DEAE-Sepharose column (**B**). α-Galactosidase activity (▲); protein (●) and NaCl gradient (–).

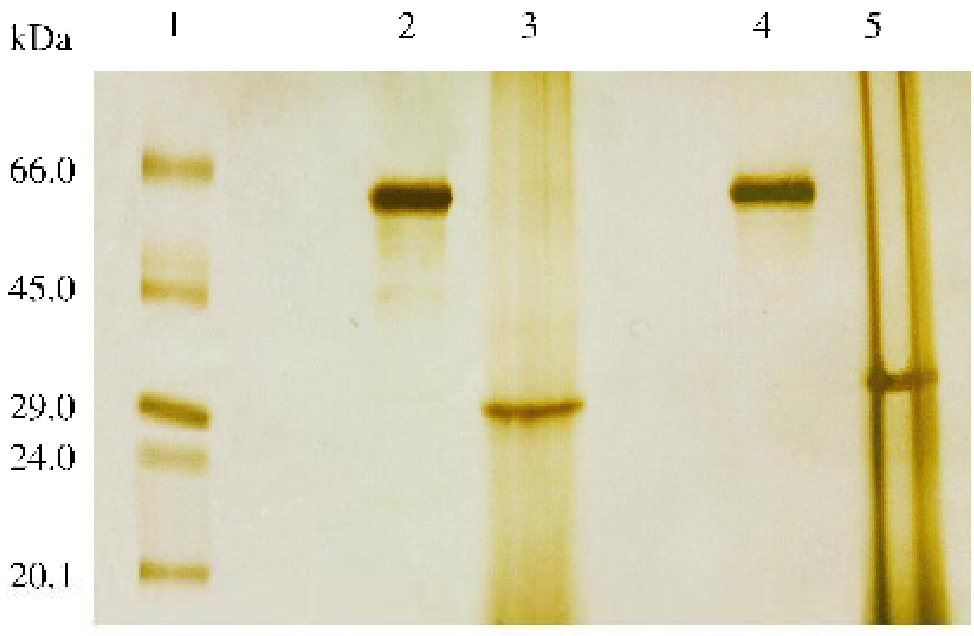

Figure 5 - SDS-PAGE (12.5 %) of purified and deglycosylated *D. hansenii* UFV-1 α-galactosidases. **Lane 1**: molecular mass standards; **lane 2**: purified extracellular enzyme; **lane 3:** deglycosylated extracellular enzyme; **lane 4**: purified intracellular enzyme; and **lane 5**: deglycosylated intracellular enzyme. Protein gel was stained with silver nitrate (***19***).

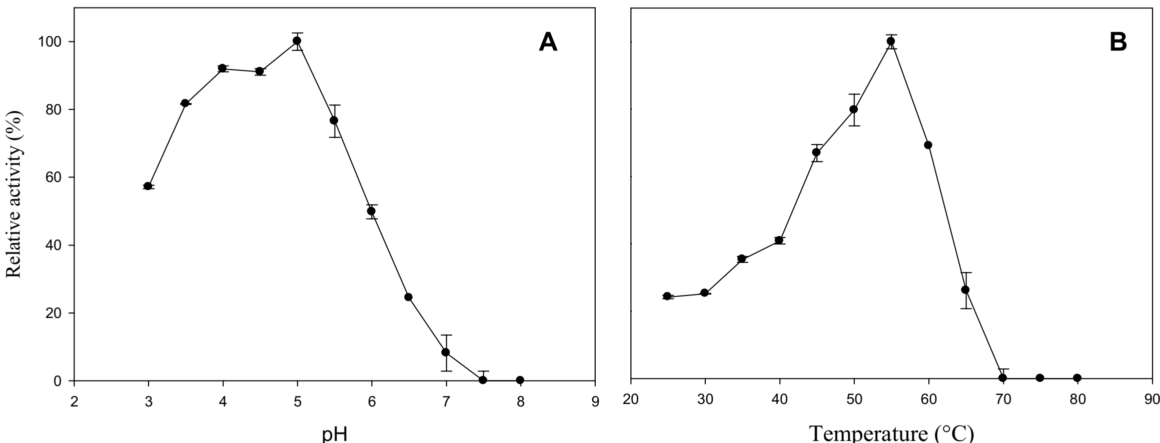

Figure 6 - pH (**A**) and temperature (**B**) effects on the activity of the *D. hansenii* UFV-1 intracellular α-galactosidase.

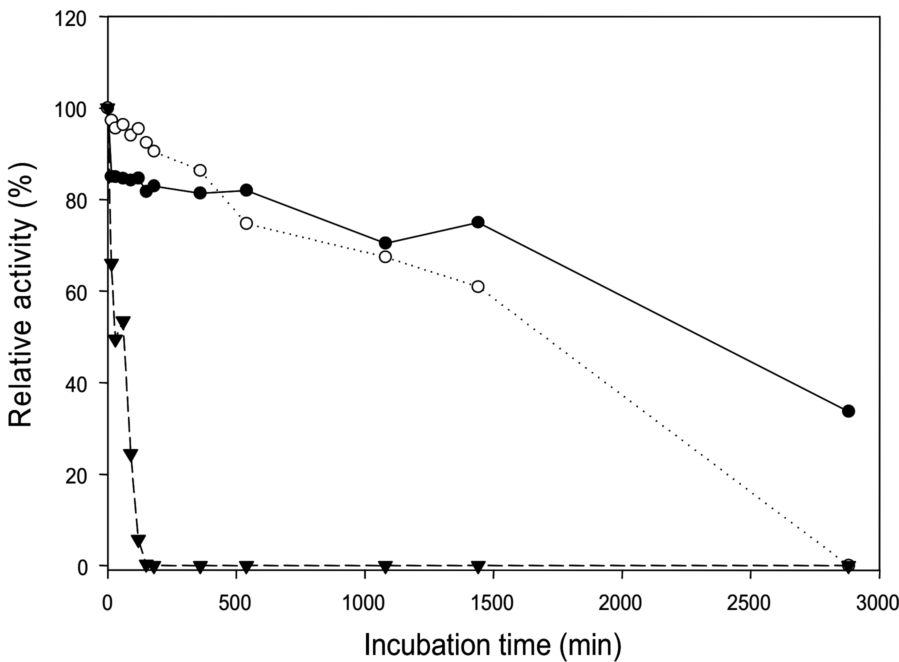

Figure 7 - Effect of temperature on the stability of the *D. hansenii* UFV-1 intracellular α-galactosidase. Enzyme preparations were preincubated for 48 h at 40 °C (●); 55 °C (o) and 65 °C (▼).

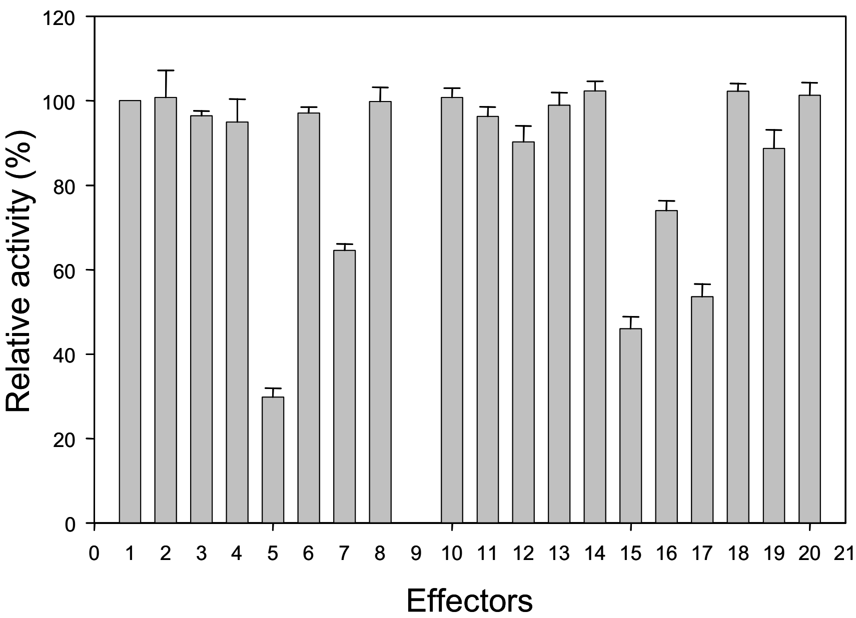

Figure 8 - Effect of EDTA (**2**); $MgCl_2$ (**3**); Iodoacetamide (**4**); $AgNO_3$ (**5**); NaCl (**6**); SDS (**7**); KCl (**8**); $CuSO_4$ (**9**); $CaCl_2$ (**10**); β-mercaptoethanol (**11**); Raffinose (**12**); Maltose (13); Sucrose (**14**); Melibiose (**15**); D-Glucose (**16**); D-Galactose (**17**); D-Mannose (**18**); Lactose (**19**); Stachyose (**20**) on *D. hansenii* UFV-1 intracellular α-galactosidase, and (**1**) without effectors. The final concentration of all effectors was 2 mM.

Capítulo 3

Evaluation of *Debaryomyces hansenii* UFV-1 α-Galactosidases Inhibitory Activity of some α-D-Galactopyranoside Derivatives

Evaluation of *Debaryomyces hansenii* UFV-1 α-Galactosidases Inhibitory Activity of some α-D-Galactopyranoside Derivatives

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4.3.1. Abstract

α-D-Galactopyranosides were synthesized and their inhibitory activities towards the *Debaryomyces hansenii* UFV-1 extracellular and intracellular α-galactosidases were evaluated. Methyl α-D-galactopyranoside was the most potent inhibitor compared to the others tested, with *K*i values of 0.82 and 1.12 mM, for extracellular and intracellular enzymes, respectively. These results indicate that the presence of hydroxyl group in the C-6 position is important for the recognition by *D. hansenii* UFV-1 α-galactosidases. The glycoproteins were completely hydrolyzed with 6 M HCl at 80 °C, releasing the monosaccharides after 2 h of incubation. The presence of galactose and mannose was observed in the extracellular α-galactosidase and xylose in the intracellular enzyme.

4.3.2. Keywords

α-Galactosidases; *Debaryomyces hansenii* UFV-1; Galactosides derivatives; Inhibitory activity; Glycoprotein; Sugar composition.

4.3.3. Introduction

Numerous glycosidases have been isolated and fully characterized and are thus classified into families and groups according to their action mechanisms (***1***). Generally, different types of *exo*-glycosidases are distinguished on the basis of specificity of their glycon structure substrates, and their substrate specificities are expressed as their relative activities against substrates with various aglycons. Several studies have reported that hydrolytic reactions with analogous deoxy of the appropriate glycon can be catalyzed by several D-glucosidases, but the tolerance of the active site to deoxygenation position varies between enzymes from different sources (***2,3,4,5)***. Despite the widespread use of iminosugars as transition-state analogous inhibitors of glycosidases (***6***), inhibitors based on modifications of conventional carbohydrates are still valuable.

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) have gained considerable attention as an industrial catalyst, due to their potential biotechnological applications (***7,8***). These glycosyl hydrolases are widely distributed in microorganisms, plants and animals (***9,10,11,12***). Galactosidase catalyzes the hydrolysis of the terminal nonreducing α-galactosyl residues from various α-galactosides (***13)***. The production of α-galactosidases from *Debaryomyces hansenii* UFV-1 was recently reported when cultivated in a medium containing galactose as carbon source (***14***). *Debaryomyces hansenii* is a yeast frequently found in protein-rich fermented products, such as sausages and cheeses (***15,16)***.

The aim of the present study was to evaluate the influence of substituents in the pyranosidic ring of some α-D-galactopyranosides derivatives on the activity of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidase isoforms. The compounds methyl 6-*O*-(4-toluenesulfonyloxy)-α-D-galactopyranoside (**2**), and methyl 6-azido-6-deoxy-α-D-galactopyranoside (**3**) are derivatives of methyl α-D-galactopyranoside (**1)** that present substituents of different chemical nature in the C-6 position (a bulky lipophilic group and a

small hydrophilic group, respectively) where synthesized in order to be compared to the parent galactoside derivative (**1**). Thus, the importance of the hydroxyl group in the position C-6, as well as the effect of the groups with distinct properties could be evaluated concerning the recognition of the enzymatic active sites. The galactopyranoside derivative **4** (*N*-acetyl-2-azido-1,2-dideoxy-α-D-galactopyranosylamine) was synthesized to evaluate the effect of simultaneous modifications in anomeric carbon and its vicinal C-2. The compounds 4-nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside (**5**) and 4-(acetamido)phenyl α-D-galactopyranoside (**6**), were prepared to be assayed as substrates or inhibitors of the glycosyl hydrolase isoforms. The aromatic galactoside **5** is a derivative of the chromogenic substrate 4-nitrophenyl α-D-galactopyranoside (*p*NPαGal**)** that presents an azido group in the C-6 position (as the compound **3)**. This compound was prepared to evaluate whether the presence of the azido group influences the α-galactosidase activity, compared to the parent substrate. Preliminary experiments carried out with partially purified β-glycosidase from *Biomphalaria glabrata* (unpublished data), showed that identical substitution in 4-nitrophenyl-β-D-glucopyranoside led to the corresponding 6-azido derivative, that was an enzymatic inhibitor with a K_i value of 1.0 mM. Finally, aromatical galactoside **6** is a derivative of 4-nitrophenyl-α-D-glucopyranoside where the nitro group was reduced and acetylated with the aim of evaluating the capability of the α-galactosidases to hydrolyze aryl α-D-galactopyranosides with electron-releasing groups instead of electron-withdrawing ones.

Here, the assays of synthetic aryl and alky glycosides as inhibitors of extracellular and intracellular isoforms of *Debaryomyces hansenii* UFV-1 α-galactosidases were reported.

4.3.4. Experimental

4.3.4.1. Chemicals

The ***p*NPαGal** (4-nitrophenyl α-D-galactopyranoside) substrate was obtained from Sigma Chemical Co. Compound **1**, methyl α-D-galactopyranoside, was purchased from Aldrich. Compounds **2**, methyl 6-*O*-(4-toluenesulfonyloxy)-α-D-galactopyranoside, **3,** methyl 6-azido-6-deoxy-α-D-galactopyranoside and **6**, 4-(acetamido)phenyl α-D-galactopyranoside were prepared according to described procedures (***17,18,19***). Compound **4** (*N*-acetyl-2-azido-1,2-dideoxy-α-D-galactopyranosylamine) was obtained by the Zemplén´s reaction (MeONa/MeOH) (***20)*** from the corresponding peracetate galactoside derivative (***21***). Compound **5** (4-nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside) was prepared from 4-nitrophenyl α-D-galactopyranoside in four steps, namely, regioselective tosylation in the C-6 position, peracetylation, tosylate displacement with sodium azide in DMF and deacetylation (Zemplén´s reaction). The new compounds were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy.

4.3.4.2. α-Galactosidase assay

Debaryomyces hansenii UFV-1 α-galactosidases purified by two-step chromatography (***14)*** were used in this study. Hydrolytic activity of α-galactosidases was determined by measuring the release of *p*-nitrophenol (*p*-NP) from 4-nitrophenyl α-D-galactopyranoside (*p*NPαGal) after incubation at the following conditions: 650 µL of 0.1 M sodium acetate buffer, pH 5, 100 µL of enzyme solution (1.02 µg protein/mL) and 250 µL of 2 mM *p*NPαGal at 60 and 55 °C for the extracellular and intracellular α-galactosidases, respectively. The amount of *p*-NP released was determined at 410 nm and calculated according to the standard curve (0-0.16 µmol *p*-NP).

One enzyme unit (U) was defined as the amount of enzyme which released 1 μmol of product per minute under the assay conditions.

4.3.4.3. Inhibitory activity determination (K_i)

The inhibition constants (K_i) of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases for each galactoside derivative (**Figure 9**) were calculated from the Dixon plot. The *p*NPαGal concentrations were 0.4, 0.8, 1.2, 1.6, and 2.0 mM and the inhibitors concentrations (galactoside derivatives) were 0.5, 1.0 and 2.0 mM.

4.3.4.4. Determination of protein concentration

Protein concentration in the enzymatic preparations was determined by the Coomassie Blue binding method (Bio-Rad Protein Assay) with bovine serum albumin (BSA) as a standard (***22***).

4.3.4.5. Eletrophoretic analysis for glycoproteins

D. hansenii UFV-1 extracellular and intracellular α-galactosidases (20 μg) applied in 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were stained with periodic acid of Schiff (PAS) for glycoprotein determination (***23***). The glycoprotein standard (Pharmacia) used was ovalbumin (45 kDa).

4.3.4.6. Hydrolysis of glycoproteins and monosaccharide analysis

D. hansenii UFV-1 extracellular and intracellular α-galactosidases samples (10 μg) were incubated with 6 M HCl at 80 °C for 2 h. The aliquots were dried, dissolved in distilled water and redried to obtain acid free sugars residues (***24***). Identification and quantification of monosaccharides in *D. hansenii* UFV-1 α-galactosidases were obtained by HPLC on a

Shimadzu series 10A chromatograph using a Shim-Pack SCR-101P column (30 cm x 0.79 cm, Shimadzu). The monosaccharides were eluted with water at 80 ^{o}C, at a flow rate of 0.5 mL/min. Individual sugars were automatically identified and quantified by comparison with the standard retention times and sugar concentrations. The standard sugars (1 mg/mL) were galactosamine (Galn), glucose (Glu), xylose (Xyl), galactose (Gal) and mannose (Man).

4.3.5. Results and discussion

Based on the results presented in **Table 8**, the modifications of the galactopyranoside ring had significant influence in the interaction of these substances with the *Debaryomyces hansenii* UFV-1 α-galactosidases studied. None of analyzed derivatives were substrates for the enzymes, being therefore, treated as inhibitors.

The methyl α-D-galactopyranoside (**1)** compound was the most potent inhibitor of the studied compounds (**Figure 9**), presenting comparable inhibitory activity to the natural sugar melibiose, as described in a previous study (***14***). Since galactoside **1** is a slightly better inhibitor than D-galactose, whose observed K_i value for the *D. hansenii* UFV-1 extracellular isoform was 2.7 mM (***14***), the hydrogen bond donor capability of anomeric center can be removed with no deleterious consequence for the inhibitory activity. This is confirmed by the similar K_i values for melibiose and galactoside **1**. It is important to point out that inhibition is acompetitive for galactoside **1** but it is noncompetitive for D-galactose (***14***).

The substitution of compound **1**, in the C-6 position in replace of compounds **2** and **3**, led to a significant reduction of inhibitory activity, indicating that the hydroxyl group in C-6 is important for the recognition of the *D. hansenii* UFV-1 α-galactosidases active sites (**Table 8**). There are no similar studies in literature concerning inhibition activity, but the presence of three hydroxyl groups (C-3, C-4, and C-6 positions) in the *p*NPαGal substrate is necessary for the derivatives to be *Aspergillus niger* α-galactosidase substrates (***5***).

When comparing compounds **2** and **3**, it is observed that derivative **2** is slightly more potent that **3**. This difference can be attributed to the more lipophilic character of compound **2**, resulting in more efficient desolvatation when this galactoside derivative is transferred to the enzymatic active site (**Table 8**). The same occurred with *D. hansenii* UFV-1 extracellular α-galactosidase, where galactose and melibiose inhibition were found to be noncompetitive, with a lower K_i value (1.12 mM) for the disaccharide (***14***).

The galactoside derivative **4** was also a poor inhibitor with K_i values comparable to those observed for derivative **3**. Thus, the simultaneous modifications in the C-1 and C-2 positions were deleterious for the recognition of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases (**Table 8**). The importance of the hydroxyl group in the C-2 position has also been reported for α-galactosidases of green coffee beans and *Mortierella vinacea* against *p*NPαGal deoxy derivatives, though it is less important for the activity of the enzyme of *Aspergillus niger* (***5***). Thus, the presence of the azido group in the C-2 position of the derivative **4** could explain the high value of K_i of this substance when compared to compound **1.** However, the possible influence of the acetamido group in the C-1 position cannot be discarded, especially because derivatives with classical glycoside linkage, such as melibiose and compound **1** are good inhibitors (**Table 8**). Inhibition of *Talaromyces flavus* α-galactosidase activity was observed in the presence of 2 mM α-D-galactopyranosyl azido (75 %) and D-galactose (51 %) with *K*i values of 0.25 and 0.38 mM, respectively (***25***).

In the series of the aromatic galactosides, it was observed that a variation of the substituents had significant influence on the behavior of these substances, when compared to the α-galactosidase chromogenic substrate, *p*NPαGal. The substitution of the azido group for the hydroxyl group in the C-6 position of *p*NPαGal (compound **5**) resulted in the abolition of enzymatic activity (**Table 8**). Considering that it was not possible to determine the K_i values of both α-galactosidase isoforms for compound **5** at the assay conditions, it seems plausible to

assume that this substance is not recognized by the enzymes, evidencing once more the importance of the hydroxyl group in the C-6 position. These results also suggest that improvements of inhibitory capacity of derivative **1** must be based on alkyl glycosides instead of aryl glycosides, probably for sterical reasons.

Galactoside **6** was an inhibitor, however less potent than **1**, confirming the previous suggestion (**Table 8**). Galactoside **6** is an inhibitor while *p*NPαGal is a substrate, probably because the aglycon of the **6** is a worse leaving group (4-acetamidophenol) than that of *p*NPαGal (4-nitrophenol), due to its the electron-releasing character. However, it is important to consider that the recognition of the *N*-acetyl group by the enzymatic active site may contribute to its recognition as an inhibitor.

It is interesting to note that different inhibition types for the extracellular and intracellular isoforms are observed for **3** and **6** compounds (**Table 8**). This observation is relevant as additional evidence of the presence of distinctive isoforms, despite their identical *N*-terminal sequences and molecular mass. It is also promising that the α-D-galactopyranoside derivatives investigated here can be lead compounds to develop specific inhibitors for each α-galactosidase isoform through further chemical modification.

Glycosylation is a major post-translational modification found on cytoplasmic, especially in secreted and membrane proteins (***26,27***). Oligosaccharides occupy a large part of glycoproteins and can modify the structure, dynamics, thermodynamic stability and functional activity of their conjugate polypeptides. Thus, it is important to characterize the structural basis of a glycoprotein function. In **Figure 10**, it was observed that both *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases are glycoproteins. As show in **Table 9**, under conditions of 6 M HCl at 80 °C for 2 h, the sugars present in the *D. hansenii* UFV-1 α-galactosidases were released and analyzed in HPLC. The results indicated that both α-galactosidase isoforms have differences in their monosaccharide compositions. The presence

of galactose and mannose in the extracellular enzyme and xylose in the intracellular enzyme was also observed (**Table 9**). Nevertheless, it is important to point out that these differences in the glycosidic portion of *D. hansenii* UFV-1 α-galactosidases affect the active site vicinity as the results of the galactopyranoside inhibitors clearly show. The construction of a novel two-dimensional (2-D) array was described (***28***) for rapid and simultaneous determination of *N*- and *O*-glycan sequences and their glycan moieties on possible glycosylation sites, using MALDI-TOF MS analyses.

The previous characterization of *Debaryomyces hansenii* UFV-1 α-galactosidases (***14***) and these studies of inhibitory activity with α-D-galactopyranoside derivatives and glycosylation allow for future cloning works, over-expression and sequencing of the entire gene coding of the enzyme. By homology modeling with known α-galactosidases structures (***29,30***) these studies aim to provide structural understanding of the *Debaryomyces hansenii* UFV-1 α-galactosidase.

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4.3.6. References

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Figures

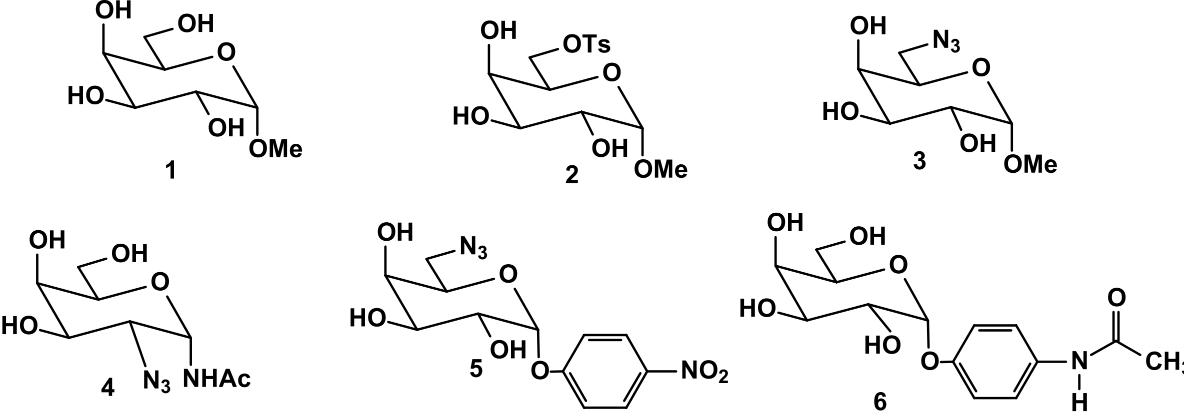

Figure 9 – α-D-Galactopyranoside derivatives: methyl α-D-galactopyranoside (**1**); methyl 6-*O*-(4-toluenesulfonyloxy)-α-D-galactopyranoside (**2**); methyl 6-azido-6-deoxy-α-D-galactopyranoside (**3**); *N*-acetyl-2-azido-1,2-dideoxy-α-D-galactopyranosylamine (**4**); 4-nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside (**5**); 4-(acetamido)phenyl α-D-galactopyranoside (**6**).

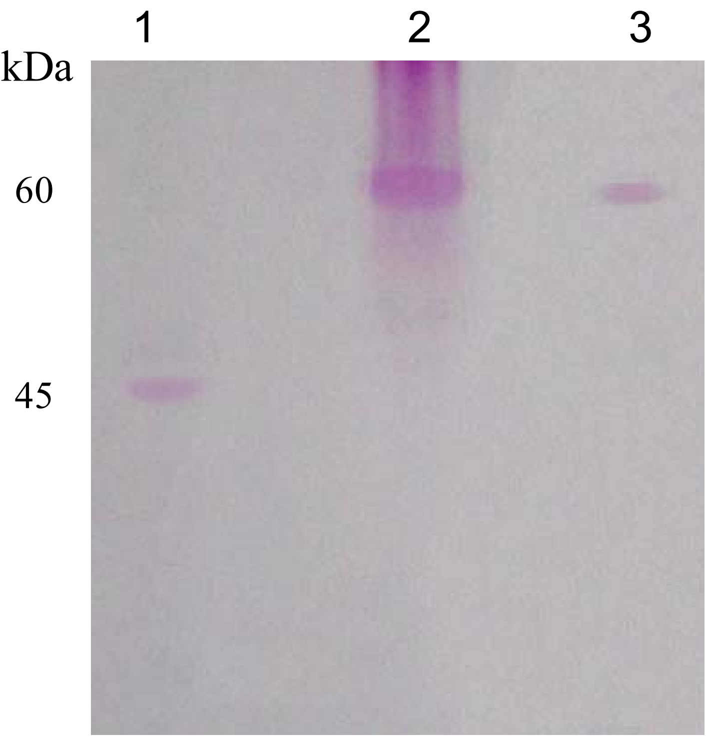

Figure 10 – SDS-PAGE (12.5 %) of *D. hansenii* UFV-1 purified α-galactosidases. Ovalbumin (**1**); extracellular enzyme (**2**) and intracellular enzyme (**3**), stained with periodic acid of Schiff (PAS).

Tables

Table 8 - Inhibitory activity (K_i) of α-D-galactopyranoside derivatives against *D. hansenii* UFV-1 α-galactosidases.

Compound	Inhibition		K_i (mM)	
	Extracellular	**Intracellular**	**Extracellular**	**Intracellular**
1	Acompetitive	Acompetitive	0.82 ± 0.01	1.12 ± 0.03
2	Competitive	Competitive	6.00 ± 0.02	2.33 ± 0.02
3	Acompetitive	Competitive	9.35 ± 0.02	2.45 ± 0.01
4	Noncompetitive	Noncompetitive	7.37 ± 0.04	15.7 ± 0.02
5	*ND	*ND	> 20	> 20
6	Competitive	Noncompetitive	3.03 ± 0.03	5.53 ± 0.03

*ND: not determined under assay conditions used.

Table 9 – Summary of the acid hydrolysis (6 M HCl; 80 °C; 2 h) of *D. hansenii* UFV-1 α-galactosidases. The data are the average from three independent assays.

Sugars	**Observed (µg sugar/µg protein)**	
	Extracellular	**Intracellular**
Galactosamine	*nd	*nd
Glucose	*nd	*nd
Xylose	*nd	0.20 ± 0.02
Galactose	0.15 ± 0.02	*nd
Mannose	0.26 ± 0.04	*nd

*nd: not detected under assay conditions used.

Capítulo 4

Comparative Spectroscopic, Thermodynamic and Kinetic Properties of *Debaryomyces hansenii* UFV-1 α-Galactosidases

Comparative Spectroscopic, Thermodynamic and Kinetic Properties of *Debaryomyces hansenii* UFV-1 α-Galactosidases

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4.4.1. Abstract

Spectroscopic, kinetic and thermodynamic properties were determined for *D*ebaryomyces *hansenii* UFV-1 extracellular and intracellular α-galactosidases. The effects of pH and temperature on the structure of the *D. hansenii* α-galactosidases were investigated using Circular Dichroism (CD). *D. hansenii* α-galactosidases showed very similar secondary structure compositions (α-helix, β-sheet parallel and β-turn). Differential Scanning Calorimetry (DSC) was employed for the determination of some thermodynamic parameters during protein denaturation. Thermal denaturation reversibility was not immediately observed for *D. hansenii* UFV-1 α-galactosidases; however it occurred as a thermodynamically controlled process. Extracellular α-galactosidase, at pH 5.5, showed a T_m value lower (86.1 °C) when compared to the intracellular enzyme (87.3 °C). The cooperativity relative index for intracellular α-galactosidase ($\Delta T_{1/2}$ = 5.0 ºC) was lower than that of the extracellular enzyme ($\Delta T_{1/2}$ = 5.6 ºC). The related Arrhenius activation energy for extracellular and intracellular α-galactosidase from *D. hansenii* (88 and 95 KJ/mol, respectively) is correspondingly high and indicates a considerable transient chemical change. Thus, the CD and DSC data suggest that the *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases have different and discrete behaviors although they possess similar secondary structure.

4.4.2. Keywords

Debaryomyces hansenii UFV-1; α-Galactosidases; Circular Dichroism; Differential Scanning Calorimetry; Activation energy.

4.4.3. Introduction

Many proteins express varying degrees of stability when subjected to heat denaturation. Resistance to structure loss and/or function of a particular protein due to temperature-induced denaturation is a measure of its stability [**1**]. The thermodynamic stability of a protein is a balance between large stabilizing forces derived from noncovalent intramolecular interactions and large destabilizing forces, primarily chain conformational entropy. Typical intramolecular interactions (hydrogen bonds, salt bridges and hydrophobic interactions) contribute to from -2 to -20 KJ/mol. Thus, the stability of the folded state can be affected by relatively minor changes in primary structure that leads to the optimization of hydrophobic and hydrophilic stabilizing interactions [**1**]. In particular, it has been recognized that an increased number of salt bridges, often organized in networks, play a predominant role in the stabilization of proteins from higher thermophiles [**2**].

Trends commonly associated with elevated thermostability in proteins include relatively small solvent-exposed surface area, increased packing density that reduces cavities in the hydrophobic core, optimization of hydrophobic interactions, decreased length of surface loops and hydrogen bonds between polar residues [**3,4**]. Other factors affecting protein stability are conformational flexibility [**5**], glycosylation [**6**], protein-protein interactions [**7**], and the presence of disulfide bonds [**8**]. Disulfide bonds are believed to increase the stability of the native state of proteins by decreasing the conformational entropy of the unfolded state due to the conformational constraints imposed by the cross-links (i.e. increasing the free energy of the unfolded state). The question of how some proteins achieve extreme thermal stability is important, because of their potential industrial and practical applications, and the fundamental physical and chemical processes involved. Probing the conformation behavior of a single-chain polypeptide at high temperatures provides insight into the different forces

involved in the stabilization of a protein [**9**]. Previous studies with *D. hansenii* UFV-1 α-galactosidases have demonstrate its. [**10**].

α-D-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) have been isolated from various sources, including bacteria, fungi, plants and animals [**11,12,13,14**]. Generally, α-galactosidases hydrolyze a variety of simple α-D-galactosides as well as more complex molecules, such oligosaccharides and polysaccharides [**15**]. In humans, α-galactosidase is a lysosomal exoglycosidase that cleaves the terminal α-galactose residues from glycolipids and glycoprotein. Mutations in the α-galactosidase gene cause incomplete degradation of carbohydrates, resulting in Fabry disease [**16**]. In plants, galactomannan is one of the major storage polysaccharides in seeds, and α-galactosidase is one of the key enzymes for degradation of cell wall galactomannan during germination [**17**]. Raffinose and stachyose in beans are known to cause flatulence, and α-galactosidase has the potential to alleviate these symptoms [**18,19,20**]. Furthermore, in the sugar-producing industry, sucrose yield can be improved by α-galactosidase-mediated elimination of raffinose and prevention of beet sugar crystallization [21]. In previous studies, an expressive amount of extracellular and intracellular α-galactosidases were produced by *Debaryomyces hansenii* UFV-1 [**10**]. *D. hansenii* is a yeast frequently found in protein-rich fermented products, such as sausages and cheeses [**22,23**].

In the present investigation, the influence of temperature and pH on the structure of *D. hansenii* UFV-1 α-galactosidases by Circular Dichroism (CD) and Differential Scanning Calorimetry (DSC) was investigated. DSC providing qualitative information on the protein thermodynamic properties [**24**].

4.4.4. Materials and Methods

4.4.4.1. Circular Dichroism spectroscopy

Purified extracellular and intracellular α-galactosidases (0.2 mg/mL) from *D. hansenii* UFV-1 in 5 mM sodium acetate buffer, pH 5.5, were analysed through far-UV CD spectra in the 190-260 nm range, at different temperatures (20-80 °C). CD measurements were also obtained for the extracellular enzyme (0.2 mg/mL) in 5 mM Mcllvaine buffer (citric acid/sodium phosphate) [**25**], at different pH values (3-7) and temperatures (20-80 °C). A Jasco J-810 spectropolarimeter was used for the CD measurements, utilizing a 1 mm path-length cell. At each temperature or pH, the buffer spectrum was subtracted from the corresponding protein spectrum. Each spectrum represented the average of 10 accumulations recorded between wavelengths of 190 and 260 nm, with band pitch of 0.5 nm, a response time of 4 s, high sensitivity and scan speed 100 nm/min. The CD intensities were expressed as molar ellipticity (deg.cm^2/dmol). Percentages of the different secondary structures (α-helix, β-sheet, β-turn and random coil) were estimated using the CDNN program [**26**].

4.4.4.2. Differential Scanning Calorimetry (DSC)

DSC measurements were performed using a Microcal (Northampton, MA, USA) VP-DSC model scanning microcalorimeter. Proteins were dissolved in 50 mM sodium acetate buffer, pH 5.5, and the proteins concentrations were determined by the Bradford method [**27**]. *D. hansenii* UFV-1 extracellular α-galactosidase was subjected to the calorimeter at 0.75 mg/mL and the intracellular enzyme at 0.47 mg/mL, due to individual aggregation propension of proteins at specific calorimetry conditions. The samples were degassed at low pressure for 30 min, placed in the microcalorimeter cell (0.5 mL) and scanned relative to the reference buffer over the temperature range of 20 to 100 ºC at a scan rate of 60 ºC/h and at a constant

pressure of 30 psi. Buffer versus buffer baseline scans were determined and subtracted from transition scans prior to normalization and analysis of protein denaturation [**28**]. Finally, the values of the excess heat capacity were obtained after subtraction of the baseline [**29**]. Raw data from the DSC runs were curve fitted using the Microcal Origin™ DSC (version 4.1) software.

4.4.4.3. Scan rate effect on T_m thermodynamics parameters

Buffered solutions of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases and a reference (cell containing 50 mM sodium acetate buffer, pH 5.5) were scanned from 20 to 100 ºC at two different scan rates (30 and 60 ºC/h). The calorimetric experiments were performed under a pressure of 30 psi. The comparison of T_m values obtained from different scan rates defined wether the process was thermodynamically or kinetically driven.

4.4.4.4. Determination of activation energy (E_a)

The activation energy of the *p*NPαGal substrate (0.4-1.6 mM) was determined with the extracellular and intracellular α-galactosidases from *D. hansenii* UFV-1 using the Arrhenius plot ($\log V_{máx}$ vs 1/T) in the temperature ranges of 30-60 °C and 35-65 °C, respectively. The $V_{máx}$ values were obtained using Hanes-Woolf (S/V x S) plot at different temperatures, and the slope was determined ($-E$a/2.3R), where R is the Gas Constant (8.31 J/mol K).

4.4.5. Results and discussion

4.4.5.1. Circular Dichroism studies

Circular Dichroism (CD) is being increasingly recognized as a valuable technique for examining the structure of proteins in solution [**30**].

Figure 11 shows the comparative far-UV CD spectra of the *D. hansenii* UFV-1 extracellular (**Figure 11A**) and intracellular (**Figure 11B**) α-galactosidases (0.2 mg/mL) in sodium acetate buffer, pH 5.5, at different temperatures. As seen in **Figure 11A**, the extracellular enzyme shows a single minimum around 200 nm at 20 °C. At elevated temperatures (75 °C), the intensity of this band is reduced and red-shifted to approximately 204 nm. Furthermore, there is an appearance of shoulder-like regions at 214 and 222 nm. The intracellular enzyme shows a single minimum near 213 nm at 70 °C and the presence of positive bands at 195 nm (**Figure 11B**). In this figure, the appearance of an isosbetic point, when compared with the extracellular enzyme (**Figure 11A**) can also be observed. A CD spectrum with a strong negative band near 200 nm, and some weak bands between 220 and 230 nm that can be either positive or negative signs are associated with oligo- and polypeptides belonging to the unordered protein class [**31**]. Deconvoluted CD data were obtained using the CDNN program and the *D. hansenii* UFV-1 α-galactosidases showed very similar secondary structure compositions (α-helix, β-sheet paralel and β-turn) (**Figure 12**). In the extracellular α-galactosidase (**Figure 12A**) greater β-sheet antiparalel content was observed whereas in the intracellular enzyme the random coil was predominant (**Figure 12B**). The β-sheet antiparalel content in the extracellular enzyme decreased when was increased the temperature while no alteration was shown in the intracellular enzyme (**Figure 12**).

4.4.5.2. Effect of pH and temperature on the secondary structure of *D. hansenii* UFV-1 extracellular α-galactosidase monitored by CD

CD is an excellent spectroscopic technique for observing the unfolding and folding of proteins as a function of temperature [**32**]. In order to understand the *D. hansenii* UFV-1 extracellular α-galactosidase behavior as a function of pH and temperature, CD spectra of this protein were measured over a wide range of pH values (3-7), in Mclvaine buffers, at different temperatures (20-80 °C). As shown in **Figure 13**, the extracellular enzyme did not show dependence on pH. These data indicate that the secondary structures of this protein are not significantly affected by the solution acidity, suggesting that these enzymes preserve their structure at acidic pH levels. However, the increase of temperature in different pH values induced a slightly reduction of the regular conformations, observed both in the Mclvaine buffer (**Figure 13**) and in the sodium acetate buffer (**Figure 11**). These data suggest that the *D. hansenii* UFV-1 extracellular α-galactosidase is stable in the buffers tested.

4.4.5.3. Scan rate effect on DSC thermograms

When equilibrium between folded and unfolded molecules is approached on a longer time scale than the scan rate, kinetics effects must be taken into account when interpreting the experimental results. Nevertheless, such condition validity was checked thoroughly only in a few cases [**33,34,35**]. This test, however, is of utmost importance to adopt a correct interpretation method for the calorimetric results. The process can be considered under thermodynamic equilibrium or under kinetic control depending on the heating rate effects on the transition parameter T_m . The data shown in **Table 10** proves that the entire denaturation process is not scan-rate-dependent, because the T_m variations are very small when the scan rate is increased. These results indicate that for both extracellular and intracellular α-galactosidases from *D. hansenii* UFV-1, the calorimetric parameters are independent of the

heating rate (**Table 10**). Thus, thermal denaturation occurs as a thermodynamically controlled process [**33,36**] and can be considered a process under thermodynamic equilibrium [**37,38**].

4.4.5.4. Thermal Denaturation

The thermogram for both extracellular and intracellular α-galactosidases from *D. hansenii* UFV-1 in buffered solutions showed a single symmetric endothermic transition peak that is typical for protein denaturation [**33**]. These curves are the results from the subtraction of the baseline and then the heat capacity difference between the denatured and native states followed by normalization of protein concentration and fitting of the resultant curve in two state model transitions.

A second heating of the sample solution, which should show a similar transition peak to the one observed in the first heating curve, generally confirms the reversibility of the denaturation process as defined in *equation 1*.

100 % x ($\Delta H_{cal\ (consecutive\ scan)} / \Delta H_{cal\ (first\ scan)}$) = % of reversibility (*equation 1*).

The thermal denaturation reversibility was low for both extracellular and intracellular α-galactosidases from *D. hansenii* UFV-1 (**Figure 14**).

The denaturation of extracellular and intracellular α-galactosidases, at pH 5.5, presented the following T_m values: 86.1 ± 0.1 ºC and 87.3 ± 0.1 ºC, respectively. The extracellular α-galactosidase T_m value was lower in comparison with the intracellular enzyme (**Figure 15**).

The cooperativity relative index [**24,39**] for intracellular α-galactosidase (($\Delta T_{1/2}$ = 5.0 ± 0.1 ºC) was lower compared to the extracellular enzyme (($\Delta T_{1/2}$ = 5.6 ± 0.1 ºC). The RCI analysis is performed by knowing that as $\Delta T_{1/2}$ (width at half height) decreases, the more cooperative is the molecule during the thermal transition. According to the experimental data, the extracellular isoform was less cooperative during the thermal transition (**Figure 15**).

The calorimetric measurements performed for *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases showed an irreversible and thermodynamically driven thermal transition. The *D. hansenii* UFV-1 extracellular α-galactosidase showed to be less stable and cooperative when compared to the intracellular isoform, during the thermal transition.

4.4.5.5. Activation Energy (E_a)

Figure 16 shows the Arrhenius plots for the hydrolysis reactions of *p*NPαGal substrate at different temperatures with *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases. The substrate concentrations were 0.4 to 1.6 mM and the temperatures used were 30-60 °C for extracellular α-galactosidase and 35-65 °C for intracellular enzyme. In the temperatures 70 and 75 °C, the speeds decreased quickly and these values were not used. Thus, the calculated activation energies of the extracellular and intracellular α-galactosidases were 88 and 95 KJ/mol, respectively (**Figure 16**). These values were higher when compared with α-Gal I and α-Gal II from *Penicillium griseoroseum*, 40.1 e 48.7 kJ/mol, respectively (unpublished data), and smaller when compared with activation energy of *Bacillus stearothermophillus* α-galactosidase, 97.8 KJ/mol [**40**].

Although the *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases had presented similar properties, as molecular mass, *N*-terminal amino acid sequences [**10**] and secondary structures, some thermodynamic and kinetic differences had been observed. These data indicate that *D. hansenii* UFV-1 α-galactosidases coud exist as two isoforms.

Acknowledgements

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4.4.6. References

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Figures

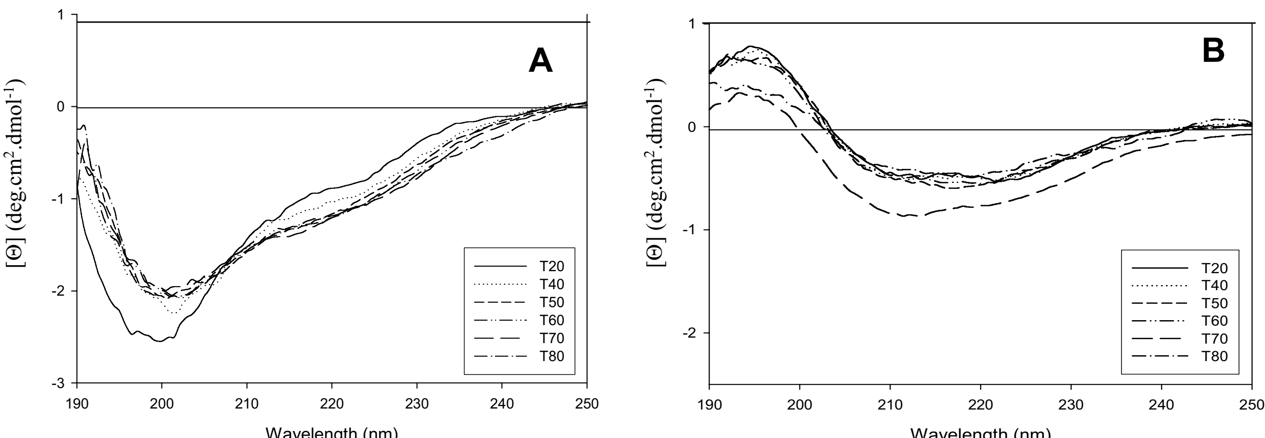

Figure 11 - Effect of temperature on the conformation of *D. hansenii* UFV-1 α-galactosidases. Far-UV CD spectra of the extracellular enzyme (**A**) and intracellular enzyme (**B**), in 5 mM sodium acetate buffer, pH 5.5.

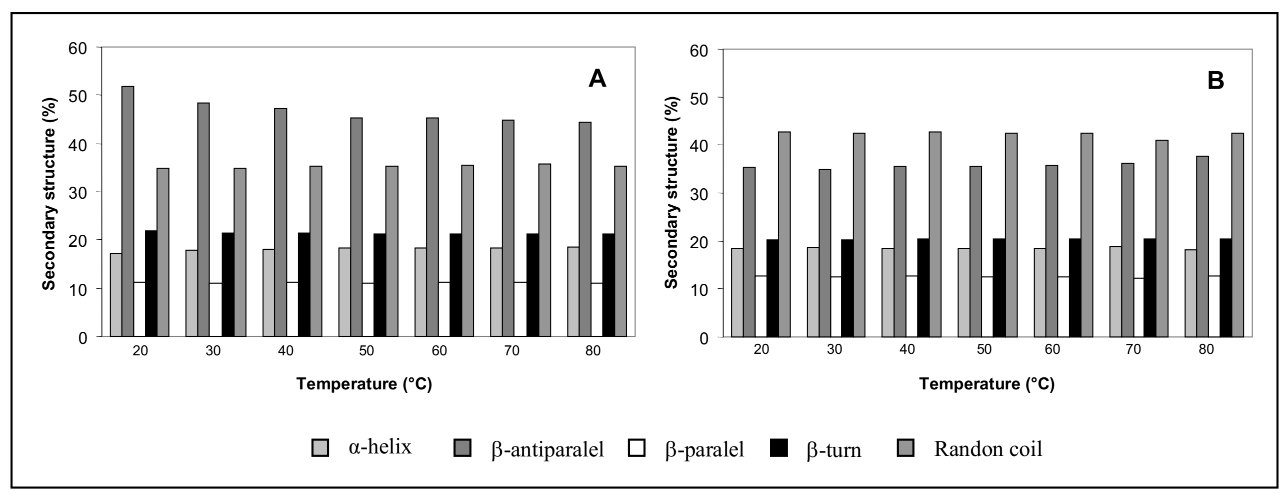

Figure 12 - Secondary structure (%) of *D. hansenii* UFV-1 extracelllular (**A**) and intracellular (**B**) α-galactosidases, in 5 mM sodium acetate buffer, pH 5.5, at different temperatures.

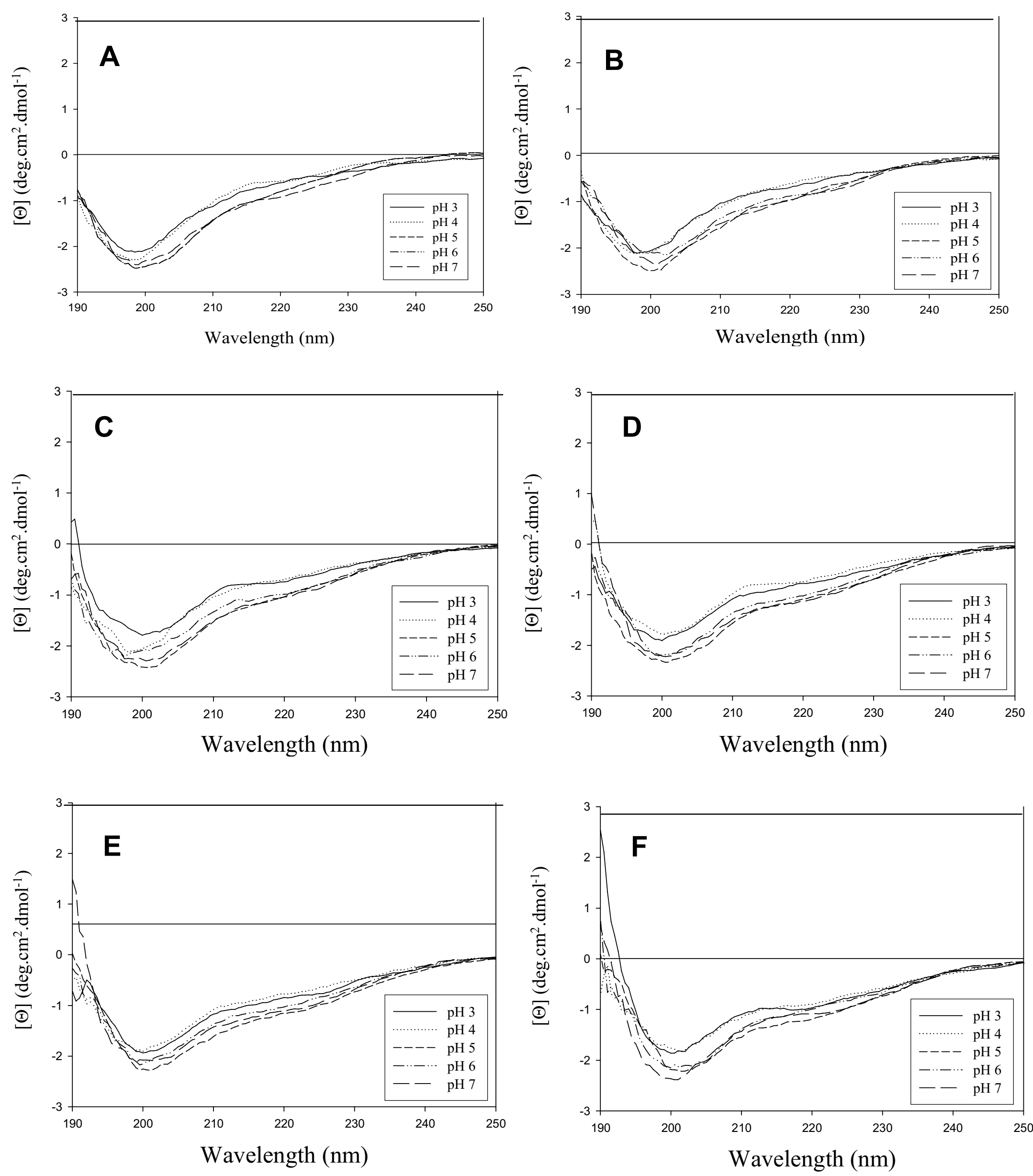

Figure 13 - CD spectra of *D. hansenii* UFV-1 extracellular α-galactosidase in different pH (Mclvaine buffer). The protein was progressively heated: 30 °C (**A**), 40°C (**B**), 50 °C (**C**), 60 °C (**D**), 70 °C (**E**), and 80 °C (**F**).

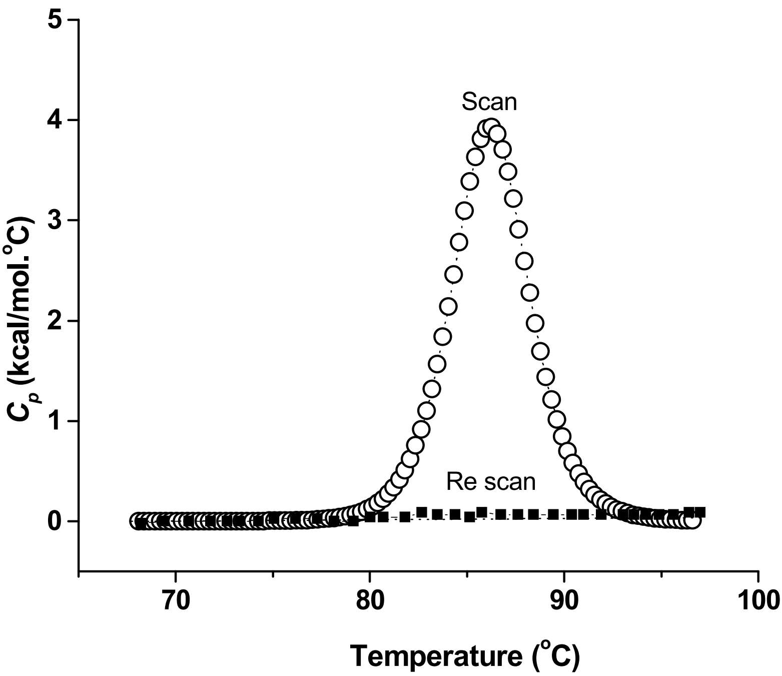

Figure 14 - Reversibility test for *D. hansenii* UFV-1 extracellular α-galactosidase in 50 mM sodium acetate, pH 5.5, in the temperature range of 70-100 °C The first scan (O) and consecutive scan run or re scan (■).

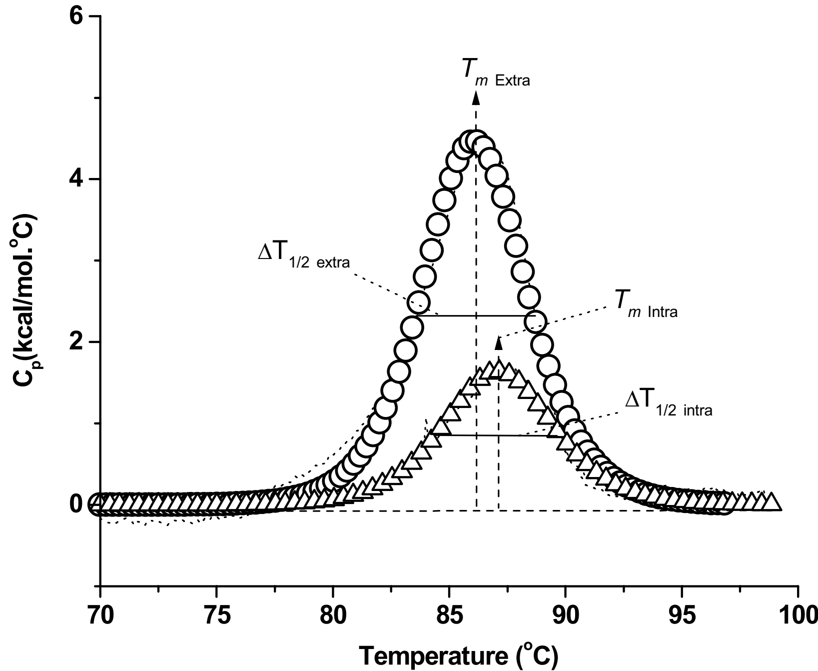

Figure 15 - Cooperativity Relative Index ($\Delta T_{1/2}$) and T_m calculation for *D. hansenii* UFV-1 extracellular (O) and intracellular (Δ) α-galactosidases, in 50 mM sodium acetate buffer, pH 5.5, submitted at scan rate 60 ºC/h in the temperature range of 70-100 °C. The lines (o and Δ) shows the processed data after subtraction of the baseline and normalization of protein concentration and fitting for two state model. T_m is the temperature midpoint of transition for the enthalpy change that occurs when the protein goes from the native to denatured form.

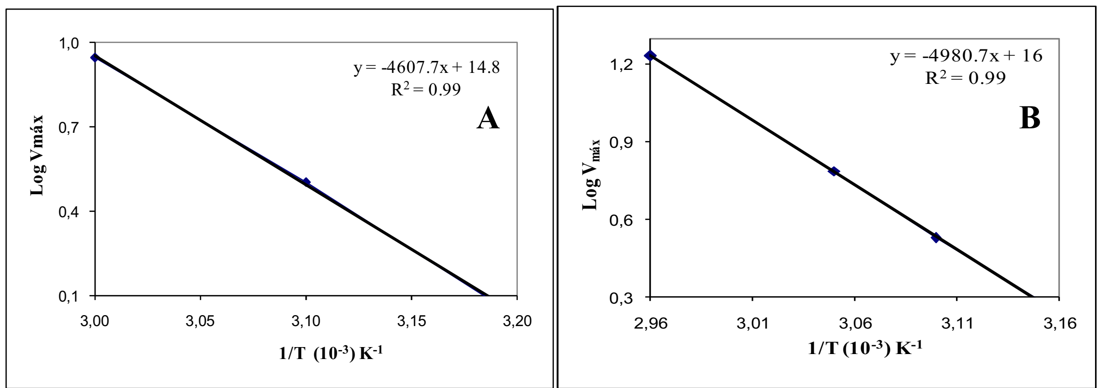

Figure 16 - Arrhenius plots for *p*NPαGal substrate hydrolysis at 30-60 °C for *D. hansenii* UFV-1 extracellular α-galactosidase (**A**); and at 35-65 °C for intracellular enzyme (**B**).

Tables

Table 10 - Scan rate effect on T_m thermodynamics parameter in the *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases.

Scan rate (ºC/h)	**T_m (ºC) Extracellular**	**T_m (ºC) Intracellular**
30	86. 2 ± 0.1	87.5 ± 0.1
60	86.1 ± 0.2	87.3 ± 0.1

Capítulo 5

Testes de Cristalização por Difusão de Vapor da α-Galactosidase Extracelular de *Debaryomyces hansenii* UFV-1

Testes de Cristalização por Difusão de Vapor da α-Galactosidase Extracelular de *Debaryomyces hansenii* UFV-1

4.5.1. Resumo

A cristalização é considerada uma das principais etapas na determinação da estrutura de macromoléculas biológicas. A busca pelas circunstâncias apropriadas para o crescimento de cristais permanece empírica, particularmente, a seleção das condições iniciais de cristalização. O método da matriz esparsa usando "kits" comerciais disponíveis tem se tornado a maneira mais popular na determinação das condições preliminares de cristalização de macromoléculas. As condições testadas para a cristalização da α-galactosidase extracellular de *D. hansenii* UFV-1 foram os "Kits" 1 e 2 (*Crystal Screen)*, usando a técnica da difusão de vapor em gota suspensa. Em algumas condições de cristalização, houve aparecimento de cristais, entretanto, os padrões de difração indicaram a formação dos sais usados na cristalização.

4.5.2. Palavras chaves

Debaryomyces hansenii UFV-1; α-Galactosidase; Cristalização; Matriz esparsa; Difusão de vapor.

4.5.3. Introdução

A determinação da estrutura tridimensional de proteínas e peptídeos constitui um dos passos mais complexos e importantes no processo de caracterização destas macromoléculas. Com o desenvolvimento de técnicas capazes de revelar a disposição espacial dos átomos de uma proteína podem-se identificar com precisão quais aminoácidos estão envolvidos no mecanismo catalítico e quais as transformações que ocorrem em uma enzima quando ligada aos seus efetores (HWA; POLIKARPOV, 1999). A elucidação da estrutura tridimensional de proteínas também contribui para um melhor entendimento dos princípios químicos e físicos que regulam as mais variadas interações entre diferentes moléculas, o processo de enovelamento de proteínas e a manutenção de sua estrutura terciária (TINOCO; SAWER; WANG, 1995).

A cristalografia é o mais poderoso método utilizado para descrever uma molécula de proteína, por meio do posicionamento preciso de cada um dos seus átomos (HWA; POLIKARPOV, 1999). O processo consiste de seis passos básicos: obtenção do cristal da proteína, obtenção do padrão de difração, análise computacional do padrão de difração, determinação inicial da estrutura da proteína, refinamento e determinação final da estrutura (PUSEY *et al*., 2005). A cristalização é o passo limitante no processo de determinação da estrutura de proteínas (DeLUCAS *et al*.; PUSEY *et al*., 2005).

As proteínas são moléculas que, apesar de apresentarem uma estrutura básica, são extremamente flexíveis e devido a isto, possuem uma grande sensibilidade às modificações que ocorrem no meio que as rodeiam. Vários fatores afetam a cristalização de uma proteína, incluindo a pureza, a concentração, a forma, a natureza da proteína, o pH do meio, a temperatura, o agente precipitante, a presença de aditivos, o tampão utilizado, entre outros (PUSEY *et al*., 2005). Para obtenção de cristais de qualquer composto, as moléculas ou íons têm que alcançar um estado de supersaturação, termodinamicamente instável no qual pode se

desenvolver numa fase amorfa ou cristalina. Tal estado de supersaturação pode ser alcançado pela evaporação lenta do solvente ou pela alteração de alguns parâmetros, como temperatura, pH, força iônica, adição de agentes precipitantes, aditivos, entre outros. Uma vez que, a formação de cristais é favorecida pela interação entre a proteína e o solvente. A estabilidade das macromoléculas biológicas em solução baseia-se na competição das interações solvente-soluto com interações intramoleculares, necessárias para manter a estrutura da macromolécula (VON HIPPEL; SCHLEICH, 1969).

As proteínas são moléculas que apresentam alta flexibilidade e inúmeras composições e formas, exigindo, portanto, condições muito específicas para a sua cristalização, que variam entre diferentes proteínas. A melhor condição de cristalização para uma determinada proteína deve ser obtida por meio de testes, onde a proteína é exposta a uma série de variáveis que afetam a cristalização, inicialmente aleatórias (PUSEY *et al.*, 2005). Em média, algumas centenas de experimentos de cristalização são realizadas para obtenção de cristais (SEGELKE, 2001). Jankarik; Kim (1991) analisaram uma série de condições de cristalização para diferentes proteínas, determinando-se tampões, aditivos, pH e agentes precipitantes, que apresentaram melhor desempenho em processos de cristalização. Com base neste estudo, os autores propuseram a utilização de 50 condições diferentes de cristalização, que teoricamente, garantiriam uma maior probabilidade de obtenção de cristais. Este método, conhecido como método da matriz esparsa é um dos mais utilizados em experimentos de cristalização de uma macromolécula (DeLUCAS *et al.*; PUSEY *et al.*, 2005). No entanto, na prática a utilização deste método não garante sucesso no processo de cristalização de uma proteína desconhecida (JANCARIK; KIM, 1991; PUSEY *et al.*, 2005).

Atualmente, vários "kits" são disponíveis para os testes iniciais de cristalização de uma macromolécula, dentre os quais, "Crystal Screens" da *Hampton Research*, "Wizard Screens" da *Molecular Dimensions*, entre outros.

O grande desenvolvimento nas áreas de nanotecnologia e robótica permitiu o desenvolvimento de equipamentos extremamente sensíveis e rápidos, capazes de avaliar uma grande quantidade de condições de cristalização em tempo mínimo, quando comparados aos testes realizados manualmente.

Nos últimos anos, a maioria das pesquisas realizadas na determinação de estruturas de proteínas tem concentrado esforços para o desenvolvimento de técnicas, que facilitem a obtenção de cristais de proteínas (DeLUCAS *et al.*; PUSEY *et al.*, 2005). A técnica mais adotada para a cristalização de proteínas tem sido a de difusão de vapor em gota suspensa. Neste processo, uma gota de solução tampão contendo a proteína a ser cristalizada, além dos agentes de cristalização e aditivos é equilibrada contra um reservatório contendo uma solução do mesmo agente de cristalização numa concentração superior àquela dos agentes na gota, em um ambiente vedado (DUCRUIX; GIEGÉ, 1992). O equilíbrio prossegue por meio da difusão das espécies voláteis (água e solvente orgânico) da gota para o reservatório, até que a pressão de vapor na gota se iguale àquela do reservatório. Se o equilíbrio ocorrer por meio de troca da água da gota para o reservatório, isto leva a uma diminuição do volume da gota e conseqüentemente a um aumento na concentração de todos os constituintes da gota de cristalização. Para espécies químicas com uma pressão de vapor maior que a pressão de vapor da água, a troca ocorre do reservatório para a gota.

O objetivo principal dos experimentos aqui relatados foi testar condições que favoreçam o crescimento de cristais da proteína α-galactosidase extracelular de *D. hansenii* UFV-1 utilizando a técnica da difusão de vapor em gota suspensa.

4.5.4. Material e Métodos

4.5.4.1. Preparo da α-galactosidase extracelular de *D. hansenii* UFV-1

A enzima α-galactosidase extracelular de *D. hansenii* UFV-1 foi purificada conforme Viana *et al.* (2006). Posteriormente, a proteína foi dialisada em tampão acetato de sódio, 2 mM, pH 5, a 4 °C e concentrada, utilizando-se uma membrana de ultrafiltração polietersulfonada (10 kDa) para 8 a 10 mg/mL.

4.5.4.2. Determinação do teor protéico nos extratos enzimáticos

Os teores protéicos nas soluções enzimáticas da α-galactosidase foram determinados pelo método colorimétrico do ácido bicinconínico (BCA), utilizando albumina sérica bovina (BSA) como padrão (SMITH *et al*., 1985).

4.5.4.3. Teste de armazenamento da α-galactosidase

Amostras (5 mL) de α-galactosidase extracelular de *D. hansenii* UFV-1 após a última etapa de purificação (troca-iônica) foram dessalinizadas de formas diferentes: algumas foram dialisadas em água destilada, outras dialisadas em tampão acetato de sódio 2 mM, pH 5 e outras submetidas a Sephadex G-25. As amostras foram armazenadas em diferentes temperaturas (4; -5; -17; -20 e -30 °C) durante o período de quatro meses, em intervalos de tempos definidos (10, 20, 30, 60 e 120 dias). As atividades da α-galactosidase foram determinadas para verificar a melhor condição de armazenamento desta enzima.

4.5.4.4. Determinação da atividade da α-galactosidase

As misturas de reações continham 700 µL de tampão acetato de sódio 100 mM, pH 5, 250 µL de solução *p*NPαGal 2 mM e 50 µL (1:10) do extrato enzimático. As reações foram

conduzidas por 15 min em banho-maria a 60 ºC e interrompidas pela adição de 1 mL de solução de Na_2CO_3 0,5 M. A quantidade de *p*-nitrofenol (*p*-NP) liberada foi determinada a 410 nm. Uma unidade de enzima (U) foi definida como a quantidade de enzima que libera 1 μmol de *p*-NP por minuto, nas condições de ensaio.

4.5.4.5. Preparo das lamínulas utilizadas para cristalização de proteínas

Inicialmente, as lamínulas utilizadas para os testes de cristalização de proteínas foram silanizadas com o objetivo de aumentar a aderência entre a amostra e a superfície da lamínula. Este processo consiste em:

- Lavá-las com água destilada e detergente, esfregando uma de cada vez;
- Lavá-las com água destilada 5 vezes;
- Colocá-las no ultrassonicador por 30 minutos;
- Lavá-las novamente com água destilada 3 vezes;
- Colocá-las em solução de silano (1:100) por 1 minuto;
- Lavá-las com água destilada uma vez;
- Colocá-las em suporte apropriado para secar em estufa a 37 °C.

4.5.4.6. Técnica da difusão de vapor em gota suspensa

A técnica utilizada para a cristalização da α-galactosidase foi a difusão de vapor em gota suspensa ("*Hanging drop*"). Uma pequena gota (1-10 μL) de uma solução contendo a proteína é misturada à solução precipitante numa lamínula, sendo esta colocada invertida sobre o reservatório contendo a solução precipitante (**Figura 17**). A solução do reservatório é livre de proteína e possui uma concentração maior do que àquela da gota. Como a pressão de vapor da gota é menor do que a pressão de vapor da solução do reservatório, a mesma deve

perder água até que o equilíbrio seja alcançado. Então, haverá um aumento da concentração dos componentes da gota causando nucleação e cristalização da proteína (WIENCEK, 1999).

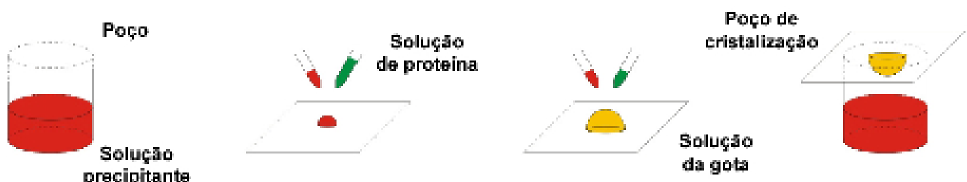

Figura 17 - Esquema do experimento de cristalização de proteínas pela técnica da difusão de vapor (método da gota suspensa).

Durante os experimentos de cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1, utilizamos essencialmente o procedimento de matriz esparsa em experimentos de difusão de vapor em gota suspensa. Estes experimentos foram realizados em caixas próprias (caixas Linbro) (**Figura 18**).

Figura 18 - Exemplo de uma caixa Linbro utilizada para a cristalização de proteínas.

4.5.4.7. Condições testadas para cristalização da α-galactosidase

As condições testadas para cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1 foram:

- "Kit" 1: "*Crystal Screen"* (HR2-110), com 50 diferentes condições (**Tabela 11**);
- "Kit" 2: "*Crystal Screen"* (HR2-112), com 48 diferentes condições (**Tabela 12**);
- Variação da condição 23 do "Kit" 2: 10 % dioxano, 0,1 M MES, pH 6,5 e 1,6 M sulfato de amônio: dioxano (2, 4, 8 e 12 %); 0,1 M MES, pH 6,5 e sulfato de amônio (0,6; 0,8; 1,0; 1,4 e 1,6 M);
- Variação da condição 11 do "Kit" 1: 0,1 M citrato de sódio, pH 5,6 e 1 M fosfato de amônio: 0,1 M citrato de sódio pH 5,6 e fosfato de amônio (0,4; 0,6; 0,8; 1,2; 1,4 e 1,6 M);
- Variação da condição 49 do "Kit" 1: 1 M sulfato de lítio e 2 % PEG 8000: sulfato de lítio (0,5; 0,75; 1,0 e 1,25 M) e PEG 8000 (2; 6; 10; 14; 18 e 22 %);
- Variação da condição 14 do "Kit" 1: 0,2 M cloreto de cálcio, 0,1 M HEPES-Na, pH 7,5 e 28 % PEG 400: 0,2 M cloreto de cálcio, 0,1 M HEPES-Na, pH 7,5 e PEG 400 (16; 20; 24; 28; 32 e 36 %) ou PEG 400 (31 a 36 %);
- 0,2 M cloreto de cálcio, 0,1 M HEPES-Na, pH 7,5 e PEG 1000 (16; 20; 24; 28; 32 e 36 %);
- 0,2 M cloreto de cálcio, 0,1 M acetato de sódio pH 5,0 e PEG 400 (31 a 36 %);
- 0,1 M HEPES-Na pH 7,5 e PEG 400 (32 e 33 %);
- 2-propanol 5 %, 0,1 M acetato de sódio, pH 4,5 e 0,1 M sulfato de amônio;
- 5 % PEG 4000, 0,1 M Tris-HCl e 0,2 M sulfato de amônio;
- Citrato de sódio (0,05; 0,03 e 0,01 M) ou tampão Mclvaine (ácido cítrico/fosfato de sódio), pH 5 e fosfato de amônio (0,4; 0,8; 1,0; 1,2; 1,4 e 1,6 M);
- Tampão Mclvaine, pH 5, diluído 10 vezes ou água Milli-Q, PEG 8000 (18; 19 e 20 %) e sulfato de lítio (0,25; 0,5; 0,75 e 1,0 M).

Em todas as condições citadas acima, a gota era formada pela mesma condição do reservatório (1µL proteína + 1µL solução precipitante).

- PEG 4000 25 % preparado em fosfato de sódio (10; 20; 50; 80 e 100 mM) nos pH 4,4; 5,0; 6,0 e 7,0; a solução precipitante era formada de PEG 4000 16% preparado em 0,1 M fosfato de sódio pH 5. Foram utilizados 4 µL proteína (1,5 mg/mL) + 3 µL solução precipitante e 3 µL proteína (6 mg/mL) + 1 µL solução precipitante.
- PEG 4000 25 % preparado em acetato de sódio (10; 20; 40 e 50 mM) nos pH 4,6; 4,8 e 5,0; a solução precipitante era formada de PEG 4000 25 % preparado em 0,1 M fosfato de sódio (pH 4,6; 4,8 e 5). Foram utilizados 8 µL proteína (3 mg/mL) + 3 µL solução precipitante em cada pH.
- PEG 8000 25 % preparado em acetato de sódio (10; 20; 40 e 50 mM) nos pH 4,6; 4,8; 5,0 e 5,2; a solução precipitante era formada de PEG 8000 16% preparado em 0,1 M fosfato de sódio (pH 4,6; 4,8; 5,0 e 5,2). Foram utilizados 3 µL proteína (5 mg/mL) + 2 µL solução precipitante em cada pH.

As amostras para a cristalização da α-galactosidase foram armazenadas à 18 °C e visualizadas por microscópio eletrônico diariamente, para verificação do aparecimento de cristais.

Tabela 11 – "Kit" 1: "*Crystal Screen"*

1. 0.02 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
2. 0.4 M Potassium sodium tartrate tetrahydrate
3. 0.4 M Ammonium phosphate monobasic
4. 0.1 M TRIS hydrochloride pH 8.5, 2.0 M Ammonium sulfate
5. 0.2 M Sodium citrate tribasic dihydrate, 0.1 M HEPES sodium pH 7.5, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
6. 0.2 M Magnesium chloride hexahydrate, 0.1 M TRIS hydrochloride pH 8.5, 30% w/v Polyethylene glycol 4,000
7. 0.1 M Sodium cacodylate trihydrate pH 6.5, 1.4 M Sodium acetate trihydrate
8. 0.2 M Sodium citrate tribasic dihydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% v/v 2-Propanol
9. 0.2 M Ammonium acetate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 30% w/v Polyethylene glycol 4,000
10. 0.2 M Ammonium acetate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% w/v Polyethylene glycol 4,000
11. 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 1.0 M Ammonium phosphate monobasic
12. 0.2 M Magnesium chloride hexahydrate, 0.1 M HEPES sodium pH 7.5, 30% v/v 2-Propanol
13. 0.2 M Sodium citrate tribasic dihydrate, 0.1 M TRIS hydrochloride pH 8.5, 30% v/v Polyethylene glycol 400
14. 0.2 M Calcium chloride dihydrate, 0.1 M HEPES sodium pH 7.5, 28% v/v Polyethylene glycol 400
15. 0.2 M Ammonium sulfate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% w/v Polyethylene glycol 8,000
16. 0.1 M HEPES sodium pH 7.5, 1.5 M Lithium sulfate monohydrate
17. 0.2 M Lithium sulfate monohydrate, 0.1 M TRIS hydrochloride pH 8.5, 30% w/v Polyethylene glycol 4,000
18. 0.2 M Magnesium acetate tetrahydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 20% w/v Polyethylene glycol 8,000
19. 0.2 M Ammonium acetate, 0.1 M TRIS hydrochloride pH 8.5, 30% v/v 2-Propanol
20. 0.2 M Ammonium sulfate, 0.1 M Sodium acetate trihydrate pH 4.6, 25% w/v Polyethylene glycol 4,000 21. 0.2 M Magnesium acetate tetrahydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
22. 0.2 M Sodium acetate trihydrate, 0.1 M TRIS hydrochloride pH 8.5, 30% w/v Polyethylene glycol 4,000
23. 0.2 M Magnesium chloride hexahydrate, 0.1 M HEPES sodium pH 7.5, 30% v/v Polyethylene glycol 400
24. 0.2 M Calcium chloride dihydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 20% v/v 2-Propanol
25. 0.1 M Imidazole pH 6.5, 1.0 M Sodium acetate trihydrate
26. 0.2 M Ammonium acetate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
27. 0.2 M Sodium citrate tribasic dihydrate, 0.1 M HEPES sodium pH 7.5, 20% v/v 2-Propanol
28. 0.2 M Sodium acetate trihydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% w/v Polyethylene glycol 8,000
29. 0.1 M HEPES sodium pH 7.5, 0.8 M Potassium sodium tartrate tetrahydrate
30. 0.2 M Ammonium sulfate, 30% w/v Polyethylene glycol 8,000
31. 0.2 M Ammonium sulfate, 30% w/v Polyethylene glycol 4,000
32. 2.0 M Ammonium sulfate
33. 4.0 M Sodium formate
34. 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Sodium formate
35. 0.1 M HEPES sodium pH 7.5, 0.8 M Sodium phosphate monobasic monohydrate, 0.8 M Potassium phosphate monobasic
36. 0.1 M TRIS hydrochloride pH 8.5, 8% w/v Polyethylene glycol 8,000
37. 0.1 M Sodium acetate trihydrate pH 4.6, 8% w/v Polyethylene glycol 4,000
38. 0.1 M HEPES sodium pH 7.5, 1.4 M Sodium citrate tribasic dehydrate
39. 0.1 M HEPES sodium pH 7.5, 2% v/v Polyethylene glycol 400, 2.0 M Ammonium sulfate
40 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 20% v/v 2-Propanol, 20% w/v Polyethylene glycol 4,000
41. 0.1 M HEPES sodium pH 7.5, 10% v/v 2-Propanol, 20% w/v Polyethylene glycol 4,000
42. 0.05 M Potassium phosphate monobasic, 20% w/v Polyethylene glycol 8,000
43. 30% w/v Polyethylene glycol 1,500
44. 0.2 M Magnesium formate dehydrate
45. 0.2 M Zinc acetate dihydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 18% w/v Polyethylene glycol 8,000
46. 0.2 M Calcium acetate hydrate, 0.1 M Sodium cacodylate trihydrate pH 6.5 , 18% w/v Polyethylene glycol 8,000
47. 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Ammonium sulfate
48. 0.1 M TRIS hydrochloride pH 8.5, 2.0 M Ammonium phosphate monobasic
49. 1.0 M Lithium sulfate monohydrate, 2% w/v Polyethylene glycol 8,000
50. 0.5 M Lithium sulfate monohydrate, 15% w/v Polyethylene glycol 8,000

Tabela 12 – "Kit" 2: "*Crystal Screen"*

1. 10% w/v Polyethylene glycol 6000; 2.0 M Sodium chloride
2. 0.5 M Sodium chloride, 0.01 M Magnesium chloride hexahydrate, 0.01 M Hexadecyltrimethylammonium bromide
3. 25% v/v Ethylene glycol
4. 35% v/v 1,4-Dioxane
5. 2.0 M Ammonium sulfate, 5% v/v 2-Propanol
6. 1.0 M Imidazole pH 7.0
7. 10% w/v Polyethylene glycol 1,000, 10% w/v Polyethylene glycol 8,000
8. 1.5 M Sodium chloride, 10% v/v Ethanol
9. 0.1 M Sodium acetate trihydrate pH 4.6, 2.0 M Sodium chloride
10. 0.2 M Sodium chloride, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
11. 0.01 M Cobalt (II) chloride hexahydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 1.0 M 1,6-Hexanediol
12. 0.1 M Cadmium chloride hydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v Polyethylene glycol 400
13. 0.2 M Ammonium sulfate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% w/v Polyethylene glycol monomethyl ether 2,000
14. 0.2 M Potassium sodium tartrate tetrahydrate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2.0 M Ammonium sulfate
15. 0.5 M Ammonium sulfate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 1.0 M Lithium sulfate monohydrate
16. 0.5 M Sodium chloride, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2% v/v Ethylene imine polymer
17. 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 35% v/v tert-Butanol
18. 0.01 M Iron (III) chloride hexahydrate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 10% v/v Jeffamine M-600
19. 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2.5 M 1,6-Hexanediol
20. 0.1 M MES monohydrate pH 6.5, 1.6 M Magnesium sulfate heptahydrate
21. 0.1 M Sodium phosphate monobasic monohydrate, 0.1 M Potassium phosphate monobasic, 0.1 M MES monohydrate pH 6.5, 2.0 M Sodium chloride
22. 0.1 M MES monohydrate pH 6.5, 12% w/v Polyethylene glycol 20,000
23. 1.6 M Ammonium sulfate, 0.1 M MES monohydrate pH 6.5, 10% v/v 1,4-Dioxane
24. 0.05 M Cesium chloride, 0.1 M MÊS monohydrate pH 6.5, 30% v/v Jeffamine M-600
25. 0.01 M Cobalt (II) chloride hexahydrate, 0.1 M MES monohydrate pH 6.5, 1.8 M Ammonium sulfate
26. 0.2 M Ammonium sulfate, 0.1 M MES monohydrate pH 6.5, 30% w/v Polyethylene glycol monomethyl ether 5,000
27. 0.01 M Zinc sulfate heptahydrate, 0.1 M MES monohydrate pH 6.5, 25% v/v Polyethylene glycol monomethyl ether 550
28. 1.6 M Sodium citrate tribasic dihydrate pH 6.5
29. 0.5 M Ammonium sulfate, 0.1 M HEPES pH 7.5, 30% v/v (+/-)-2-Methyl-2,4-pentanediol
30. 0.1 M HEPES pH 7.5, 10% w/v Polyethylene glycol 6,000, 5% v/v (+/-)-2-Methyl-2,4-pentanediol
31. 0.1 M HEPES pH 7.5, 20% v/v Jeffamine M-600
32. 0.1 M Sodium chloride, 0.1 M HEPES pH 7.5, 1.6 M Ammonium sulfate
33. 0.1 M HEPES pH 7.5, 2.0 M Ammonium formate
34. 0.05 M Cadmium sulfate hydrate, 0.1 M HEPES pH 7.5, 1.0 M Sodium acetate trihydrate
35. 0.1 M HEPES pH 7.5, 70% v/v (+/-)-2-Methyl-2,4-pentanediol
36. 0.1 M HEPES pH 7.5, 4.3 M Sodium chloride
37. 0.1 M HEPES pH 7.5, 10% w/v Polyethylene glycol 8,000, 8% v/v Ethylene glycol
38. 0.1 M HEPES pH 7.5, 20% w/v Polyethylene glycol 10,000
39. 0.2 M Magnesium chloride hexahydrate, 0.1 M Tris pH 8.5, 3.4 M 1,6-Hexanediol
40 0.1 M Tris pH 8.5, 25% v/v tert-Butanol
41. 0.01 M Nickel (II) chloride hexahydrate, 0.1 M Tris pH 8.5, 1.0 M Lithium sulfate monohydrate
42. 1.5 M Ammonium sulfate, 0.1 M Tris pH 8.5, 12% v/v Glycerol
43. 0.2 M Ammonium phosphate monobasic, 0.1 M Tris pH 8.5, 50% v/v (+/-)-2-Methyl-2,4-pentanediol
44. 0.1 M Tris pH 8.5, 20% v/v Ethanol
45. 0.01 M Nickel (II) chloride hexahydrate, 0.1 M Tris pH 8.5, 20% w/v Polyethylene glycol monomethyl ether 2,000
46. 0.1 M Sodium chloride, 0.1 M BICINE pH 9.0, 20% v/v Polyethylene glycol monomethyl ether 550
47. 0.1 M BICINE pH 9.0, 2.0 M Magnesium chloride hexahydrate
48. 0.1 M BICINE pH 9.0, 2% v/v 1,4-Dioxane, 10% w/v Polyethylene glycol 20,000

4.5.5. Resultados e Discussão

Para verificar a melhor condição de armazenamento da α-galactosidase extracelular de *D. hansenii* UFV-1, foi feito um teste com a proteína depois de purificada, sendo a mesma, dessalinizada de formas diferentes e armazenada em várias temperaturas, durante o período de quatro meses. A atividade (U/mL) da α-galactosidase foi medida após sua purificação e comparada com as atividades após cada período de armazenamento nas diferentes condições (**Tabela 13**).

Tabela 13 - Atividades da α-galactosidase extracelular de *D. hansenii* UFV-1 armazenada em condições diferentes durante quatro meses.

	Atividade de α-galactosidase (U/mL)														
Dias	**4 °C**			**-5 °C**			**-17 °C**			**-20 °C**			**-30 °C**		
	D_H	D_T	**S**	D_H	D_T	**S**	D_H	D_T	**S**	D_H	D_T	**S**	D_H	D_T	**S**
10	1,20	1,57	1,13	1,22	1,33	0,80	1,13	1,41	1,11	0,72	1,46	0,89	0,48	1,37	0,80
20	1,16	1,52	0,88	1,19	1,31	0,73	1,12	1,39	0,87	0,45	1,43	0,87	0,09	1,32	0,60
30	1,08	1,31	0,80	1,03	1,16	0,66	0,98	1,38	0,61	0,20	1,35	0,50	0,0	0,89	0,53
60	1,02	1,17	0,65	0,90	1,06	0,62	0,92	1,11	0,49	0,15	0,90	0,29	0,0	0,88	0,50
120	0,81	1,06	0,62	0,78	0,86	0,56	0,91	1,03	0,27	0,02	0,85	0,0	0,0	0,74	0,42

*D_H: diálise em água; D_T: diálise em tampão; **S**: Sephadex G-25.
Atividade da α-galactosidase após purificação: **1,83 U/mL**

De acordo com os resultados apresentados na **Tabela 13**, observamos que a melhor condição de dessalinização da α-galactosidase depois de purificada foi a diálise em tampão acetato de sódio 2 mM, pH 5, onde houve menor perda da atividade da proteína. Após 10 dias de armazenamento da proteína dialisada em tampão, nas temperaturas de 4, -5, -17, -20 e -30 °C, observamos uma perda da atividade de 14, 27, 23, 20 e 25 %, respectivamente. Portanto, a melhor condição de armazenamento da enzima dialisada em tampão, por períodos curtos (10 dias), seria à temperatura de 4 °C. Observamos que, durante períodos maiores de armazenamento da proteína (120 dias), ocorreu uma perda maior da atividade da α-galactosidase, 54 e 60 % em temperaturas menores, conforme observado em -20 e -30 °C,

respectivamente. As temperaturas melhores para armazenamento da α-galactosidase por períodos maiores (4 meses) foram 4 e -17 °C. Caso a proteína seja utilizada diariamente, a melhor temperatura de armazenamento seria 4 °C e, no caso de maiores quantidades de proteína, a melhor temperatura para estocá-la seria a -17 °C. O ideal seria utilizar a proteína assim que fosse purificada, para evitar possíveis contaminações, além da perda da atividade da mesma com o tempo.

Durante os experimentos de cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1 utilizamos a α-galactosidase purificada, além de reagentes químicos de qualidade durante o preparo das soluções. Wooh *et al.* (2003), fizeram uma comparação entre três "kits" comerciais de cristalização utilizando o procedimento de matriz esparsa em experimentos de difusão de vapor em gota suspensa, com 19 proteínas diferentes. Eles observaram diferenças ente dois desses "kits" com uma mesma formulação, com relação ao aparecimento e crescimento de cristais. Algumas dessas diferenças foram devido à fonte dos reagentes químicos e aos procedimentos usados no preparo das formulações. Esta diferença enfatiza a importância da preparação, pureza e qualidade dos componentes de cristalização utilizados na indução do crescimento dos cristais.

Inicialmente, para os experimentos de cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1 foram testados os "Kits" 1 e 2 (*Crystal Screen*) **Tabelas 11 e 12**, utilizando-se a proteína na concentração de 10 mg/mL. Após 3 dias de armazenamento das caixinhas de cristalização a 18 °C, observou-se o aparecimento de microcristais na condição 23 do "Kit" 2, após 10 dias de armazenamento nas condições 11, 16 e 20 do "Kit" 1 e, após 15 dias de armazenamento na condição 49 do "Kit" 1. Com isso, resolvemos variar dentro de determinada condição as concentrações de alguns reagentes envolvidos. Primeiramente, variamos as concentrações de dioxano (2, 4, 8 e 12 %) e sulfato de amônio (0,6; 0,8; 1,0; 1,4 e

1,6 M) da condição 23 do “Kit” 2 e mantivemos o tampão (0,1 M MES, pH 6,5). Os cristais permaneceram do mesmo tamanho.

Em seguida, variamos as concentrações de fosfato de amônio (0,4; 0,6; 0,8; 1,2; 1,4 e 1,6 M) da condição 11 do “Kit” 1 e mantivemos o tampão (0,1 M citrato de sódio, pH 5,6). Houve aparecimento de um cristal após 8 meses de armazenamento na condição com 0,8 M de fosfato de amônio. O cristal foi levado ao LNLS (Laboratório Nacional de Luz Síncroton) em Campinas para verificar se era de proteína. Infelizmente, o padrão de difração correspondeu a um sal (**Figura 19**).

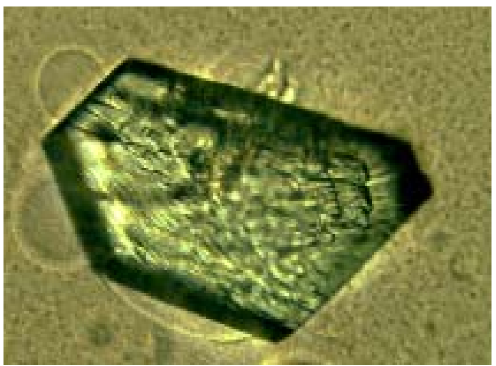

Figura 19 – Cristal formado na condição contendo 0,8 M de fosfato de amônio e 0,1 M de citrato de sódio, pH 5,6.

Na condição 49 do “Kit” 1, variamos a concentração de sulfato de lítio (0,5; 0,75; 1,0 e 1,25 M) e PEG 8000 (2; 6; 10; 14; 18 e 22 %). Após 3 dias de armazenamento, houve o aparecimento de um cristal na condição 0,5 M de sulfato de lítio e PEG 8000 18 %. Fizemos uma nova caixinha, com as concentrações de PEG 8000 (18; 19 e 20 %) e sulfato de lítio nas mesmas concentrações anteriores, completando o volume dos poços com água Milli-Q ou tampão Mclvaine, pH 5, diluído 10 vezes. Continuou aparecendo cristal na mesma condição

descrita anteriormente. Levamos ao LNLS em Campinas para verificar se o cristal era de proteína. Infelizmente, o padrão de difração também correspondeu a um sal (**Figura 20**).

Figura 20 – Cristal formado na condição contendo 0,5 M de sulfato de lítio e 18 % de PEG 8000.

Variando as concentrações de PEG 400 (16 a 36 %) da condição 14 do “Kit” 1 e mantendo o cloreto de cálcio 0,2 M e o HEPES-Na 0,1 M, pH 7,5, houve o aparecimento de cristais em todas as condições testadas, após 5 dias de armazenamento. Com isso, fizemos novas caixinhas variando o PEG 400 (31 a 36 %) e apareceram novamente cristais em todas as condições. Utilizamos o PEG 1000 (16; 20; 24; 28; 32 e 36 %), com 0,2 M de cloreto de cálcio e 0,1 M HEPES-Na, pH 7,5 e não houve aparecimento de cristais. Testamos novamente outras condições com o PEG 400 (31 a 36 %), 0,2 M de cloreto de cálcio e 0,1 M de acetato de sódio, pH 5. Após 24 h de armazenamento, novamente apareceram cristais em todas as condições. Fizemos condições sem o cloreto de cálcio, apenas com PEG 400 (32 e 33 %) e HEPES-Na, pH 7,5, para verificar se o cristal era de sal. Não houve aparecimento de cristais. Mesmo assim levamos os cristais ao LNLS e confirmamos pelo padrão de difração cristais de sal (**Figura 21**).

Figura 21 – Cristal formado na condição contendo 0,2 M de cloreto de cálcio, 0,1 M de HEPES-Na, pH 7,5 e 32 % de PEG 400.

Nas demais condições testadas, não houve crescimento de cristais e, em algumas delas apareceram apenas microcristais.

DeLucas *et al.* (2005), citam em sua revisão o desenvolvimento de um sistema automatizado (Nano Screem ^{TM}) capaz de montar 400 experimentos de cristalização em 1 hora, utilizando cerca de 20 nL de solução de proteína em cada teste. Tal sistema pode montar mais de 1000 experimentos de cristalização consumindo menos de 600 μg de proteínas (10 μg/mL), abrangendo um número muito maior de condições de cristalização. A este sistema é acoplado um robô (Cristal Score ^{TM}), o qual se vale de potentes microscópios que são utilizados para monitorar quais são as condições de cristalização que apresentam maior êxito. Este sistema é baseado no modelo que incorpora fatores cujos níveis são matematicamente balanceados (método do fatorial incompleto). Cada possível nível de um fator é amostrado em igual número de vezes. Por exemplo, em 360 experimentos, cada um dos seis precipitantes é amostrado 60 vezes. Combinações binárias são também balanceadas. Acima de 30 combinações, estas são distribuídas randomicamente. Um programa estatístico de computador

é utilizado para construir as matrizes balanceadas que codificam os experimentos. Este modelo balanceado facilita a determinação de quais níveis de um fator são mais susceptíveis para cristalização.

Comparando-se os métodos de matriz esparsa e fatorial, com relação à habilidade de cristalizar proteína, sugere-se que, o método do fatorial geralmente resulta em mais condições por proteína que favorecem o crescimento de cristais (JANKARIK; KIM, 1991). Uma outra proposta de técnica de cristalização que vem sendo usada com resultados satisfatórios é a cristalização em capilar ou contradifusão, sendo proposta primeiramente por Carter, C. W. J; Carter, C. W. (1979). A contradifusão consiste em carregar um capilar muito fino (0,2 mm) com uma solução protéica e equilibrar esta solução contra um reservatório contendo uma solução de agentes cristalizantes, tendo um gel como interface (**Figura 22**).

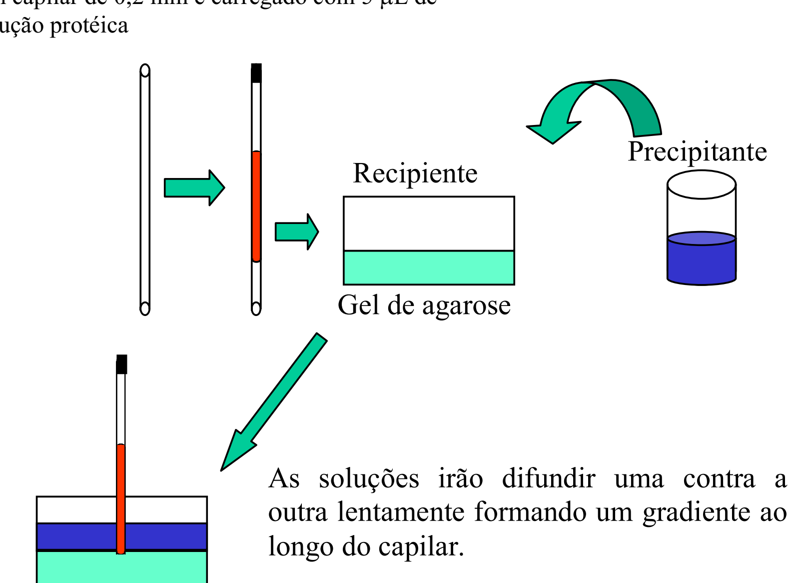

Figura 22 - Cristalização de proteínas pela técnica da contradifusão.

Gradativamente, devido ao efeito de concentração, as amostras irão difundir uma contra outra, porém, de forma muito lenta, pois o gel não permite uma difusão rápida. Tal fenômeno permite a formação de um gradiente de concentração dos agentes precipitantes ao longo do capilar e com isto, haverá uma região do capilar com condições ótimas para a cristalização (**Figura 22**).

Estudos cristalográficos e de difração de raios-X foram realizados em α-galactosidases de arroz (FUJIMOTO *et al*, 2003) e de *Trichoderma reesei* (GOLUBEV *et al*, 2004), utilizando a técnica da difusão de vapor. Esses estudos mostraram que a α-galactosidase de arroz consiste de um domínio catalítico com estrutura em $(\beta/\alpha)_8$-barril e o domínio C-terminal é constituído de oito folhas β, contendo um motivo chave-grega (FUJIMOTO *et al.*, 2003). O modelo cristalográfico da α-galactosidase de *Trichoderma reesei* consiste de dois domínios, um domínio catalítico *N*-terminal $(\beta/\alpha)_8$-barril e um domínio *C*-terminal, formado de estruturas β antiparalelas. Esta proteína contém quatro sítios de *N*-glicosilação localizados no domínio catalítico. De modo similar à α-galactosidase de arroz, a enzima de *Trichoderma reesei* liga-se na fenda do sítio ativo localizado no centro do barril do domínio catalítico (GOLUBEV *et al*, 2004).

As análises dos complexos α-galactosidases-galactoses revelaram os resíduos do sítio ativo e forneceram uma base estrutural para a identificação do possível mecanismo de reação enzimática (FUJIMOTO *et al.*, 2003; GOLUBEV *et al.*, 2004). Foram realizados estudos cristalográficos e de difração de raios-X por Garman; Garboczi (2004) de uma α-galactosidase humana que apresentou estrutura tridimensional em homodímero, com cada monômero contendo um domínio $(\beta/\alpha)_8$ com um sítio ativo e um domínio β antiparalelo. Também se encontra depositado no “Joint Center For Structural Genomics (Jcsg), 9-Jun-05” a estrutura com resolução de 2,34 Angstrons de uma α-galactosidase de *Thermotoga maritima*.

Futuramente, outras formas de cristalização da α-galactosidase extracelular de *D. hansenii* UFV-1 poderão ser testadas, como a técnica da contradifusão. Alternativas seriam utilizar a α-galactosidase desglicosilada, e até mesmo fazer a clonagem e a super-expressão desta proteína.

4.5.6. Referências Bibliográficas

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5. DISCUSSÃO GERAL

A levedura *Debaryomyces hansenii* UFV-1 produz α-galactosidases extracelular e intracelular quando cultivada em diferentes meios com várias fontes de carbono. A produção da massa celular nem sempre está associada à atividade enzimática, conforme observado no meio YP com lactose como fonte de carbono após 48 h de cultivo, e no meio mineral MM com melibiose após 27 h (Figura 1 e Tabela 1). Esta diferença está relacionada à capacidade da levedura utilizar determinado açúcar e ao mecanismo de indução da atividade das α-galactosidases, sendo este último ainda pouco conhecido. Lactose e galactose foram as fontes de carbono que induziram maiores produções de massas celulares e maiores atividades de α-galactosidases de *D. hansenii* UFV-1, nas condições de ensaio testadas. . Uma vez que a lactose foi um excelente indutor de α-galactosidases extracelular e intracelular, é possível utilizar também como meio de cultura, o soro de queijo (dados não mostrados) que é um subproduto das indústrias de laticínios, e na maioria das vezes, é descartado. Com isso, teríamos uma combinação de alta produtividade com baixo custo de produção das α-galactosidases.

Caracterizações bioquímicas, cinéticas, termodinâmicas e estruturais foram feitas com as α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1, mostrando algumas diferenças entre elas.

As α-galactosidases apresentam massas moleculares (por MALDI-TOF e SDS-PAGE) semelhantes, sequências do terminal amínico idênticas, e pequenas variações na composição de aminoácidos (Asx, His, Tyr, Ile, Phe), indicando que estas enzimas são possíveis isoformas.

Galactose inibe a α-galactosidase extracelular acompetitivamente, ou seja, o inibidor não se liga à enzima livre, enquanto inibe não-competitivamente a enzima intracelular, indicando que, o inibidor se liga tanto à enzima livre como ao complexo enzima-substrato. Tipos diferentes de inibições para as α-galactosidases de *D. hansenii* UFV-1 também são observados quando ensaiadas com os derivados α-D-galactopiranosídeos, 6-azido-6-desoxi-α-D-galactopiranosídeo de metila e 4-(acetamido)fenil α-D-galactopiranosídeo, que neste caso, mostra a importância da hidroxila na posição C-6 e do grupo NO_2 nos compostos, para o reconhecimento pelas α-galactosidases. As α-galactosidases também são inativadas por Cu^{+2} e têm suas atividades reduzidas na presença da Ag^{+1}, o que sugere a participação dos grupos carboxil e/ou imidazol da histidina durante a ação catalítica.

Por Dicroísmo Circular (CD), observa-se que as α-galactosidases são estáveis, nas condições com tampão acetato de sódio, pH 5,5 e com tampão Mclvaine, na faixa de pH 3 a 7. As enzimas mostram pequenas variações na composição de suas estruturas secundárias, nas temperaturas de 20 a 80 °C. Comparando-se estes resultados com o efeito da temperatura na estabilidade da α-galactosidase intracelular de *D. hansenii* UFV-1, em tampão acetato de sódio, pH 5, observa-se que à 65 °C a meia-vida desta enzima é de 38 minutos, diferente do resultado revelado por CD, visto que nesta temperatura, a enzima ainda mantém sua conformação estável. Isto ocorre porque, quando se analisa o efeito da temperatura na

estabilidade da α-galactosidase usando CD, no UV distante, avaliam-se estruturas secundárias da proteína (α-hélice, folhas β, voltas β e estrutura desordenada), enquanto que, o efeito da temperatura na atividade da enzima está relacionado com a sua estrutura terciária. Provavelmente, ocorreu uma mudança no sítio ativo da enzima, fazendo com que a mesma perdesse sua atividade catalítica.

Um dos fatores que afeta a estabilidade das proteínas é a glicosilação, ou seja, resíduos de açúcar adicionados à proteína aumentam a estabilidade do seu estado nativo, uma vez que, ocorre uma redução da entropia conformacional do estado desenovelado. A alta estabilidade conformacional das α-galactosidases de *D. hansenii* UFV-1 testadas em diferentes tampões pela técnica de CD confirmam a glicosilação da proteína. Observa-se a presença de galactose e manose na α-galactosidase extracelular de *D. hansenii* UFV-1 e xilose na enzima intracelular, revelando uma diferença no tipo de carboidrato encontrado nas enzimas em função da localização celular. A glicosilação é um evento da via de secreção de proteínas, com a α-galactosidase extracelular apresentando maior conteúdo de carboidratos (40 %) que a enzima intracelular (34 %).

A α-galactosidase extracelular de *D. hansenii* UFV-1 apresenta maior termoestabilidade comparada com a enzima intracelular, além de maior constante de especificade (K_{cat}/K_m) para o substrato *p*NPαGal, que relaciona a eficiência catalítica da enzima com afinidade pelo substrato. Observa-se menor valor da constante de Michaelis-Menten (K_m) para o substrato estaquiose comparado com rafinose, mostrando uma maior afinidade da α-galactosidase pelo tetrassacarídeo, visto que na soja e seus derivados o teor de estaquiose é maior que o teor de rafinose.

As α-galactosidases de *D. hansenii* UFV-1 são eficientes na hidrólise de GO no extrato hidrossolúvel da soja, observando total redução de rafinose e estaquiose após 4 h de incubação a 60 °C com a α-galactosidase extracelular, e uma redução de 73 % no teor de

rafinose e 100 % do teor de estaquiose com a enzima intracelular, após 6 h de incubação a 55 °C. Comparando-se os resultados da α-galactosidase intracelular com as células de *D. hansenii* UFV-1 permeabilizadas com etanol, não foi observada diferença no tempo e porcentagem de hidrólise dos açúcares não digeríveis, rafinose e estaquiose. Isto mostra que, numa indústria de alimentos, poderia ser utilizada a própria levedura permeabilizada para a hidrólise dos GO, evitando-se os processos de obtenção e purificação da enzima intracelular. Entretanto, a baixa atividade específica da biomassa bruta e o risco de introduzir sabores estranhos ao produto devem ser avaliados. A termoestabilidade das enzimas é importante para aplicações industriais. De acordo com os resultados obtidos neste trabalho, observa-se um valor de T_m (temperatura onde 50 % da proteína está na forma desnaturada e 50 % está na foma nativa) elevado para as α-galactosidases, na faixa de 80 °C, calculado por DSC, mostrando que, estas enzimas podem ser utilizadas na indústria de alimentos, para redução de GO da soja e seus derivados. Além disso, os dados de ponto isoelétrico (*pI*) encontrados para as α-galactosidases, 5,15 e 4,15 para as enzimas extracelular e intracelular, respectivamente, favorecem a aplicação destas enzimas na indústria, pois o extrato hidrossolúvel da soja tem pH em torno de 6,5, não colocando em risco a solubilidade do biocatalizador durante a hidrólise dos GO.

Conclui-se dos resultados obtidos para ambas α-galactosidases de *D. hansenii* UFV-1, que a forma mais adequada da enzima para utilização industrial é a extracelular, uma vez que a mesma apresenta maior termoestabilidade e maior eficiência na hidrólise dos GO comparada com a enzima intracelular. Além disso, é de fácil obtenção e purificação, requerendo apenas duas etapas básicas de cromatografias (Gel filtração e Troca-iônica).

6. CONCLUSÕES

- O estudo comparativo entre as α-galactosidases extracelular e intracelular de *Debaryomyces hansenii* UFV-1 mostrou que, estas enzimas são glicoproteínas de massas moleculares 54,5 e 54,8 kDa, respectivamente, sendo que, a forma extracelular apresentou maior conteúdo de carboidratos (40 %), comparada com a enzima intracelular (34 %).
- As α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1 desglicosiladas apresentaram massas moleculares 36 e 40 kDa, respectivamente, estimadas por SDS-PAGE.
- As α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1 apresentaram pontos isoelétricos 5,15 e 4,15, respectivamente, seqüências de *N*-terminal idênticas, similares com α-galactosidases de diversos microrganismos.
- A análise da composição de aminoácidos das α-galactosidases de *D. hansenii* UFV-1 mostrou pequenas variações em seus conteúdos.

- A α-galactosidase extracelular de *D. hansenii* UFV-1 apresentou maior constante de especificidade K_{cat}/K_m, 23,9 $(mM.s)^{-1}$, comparada com a enzima intracelular, 10,3 $(mM.s)^{-1}$para o substrato *p*NPαGal.
- A α-galactosidase intracelular de *D. hansenii* UFV-1 foi eficiente na redução dos GO presentes em produtos derivados da soja, podendo ser utilizada industrialmente para o processamento desses açúcares.
- O derivado α-D-galactopiranosídeo de metila foi o inibidor mais potente comparado aos demais utilizados, com valores de K_i 0,82 e 1,12 mM, para as α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1, respectivamente.
- A presença da hidroxila na posição C-6 dos galactopiranosídeos é importante para o reconhecimento pelas α-galactosidases de *D. hansenii* UFV-1.
- As modificações nas posições C-1 e C-2 do derivado 4-nitrofenil 6-azido-6-desoxi-α-D-galactopiranosídeo foram deletérias para o reconhecimento pelas α-galactosidase de *D. hansenii* UFV-1.
- Tipos diferentes de inibição para as α-galactosidases de *D. hansenii* UFV-1 foram observadas para os compostos 6-azido-6-desoxi-α-D-galactopiranosídeo de metila e 4-(acetamido)fenil α-D-galactopiranosídeo.
- As glicoproteínas foram hidrolisadas com 6 M HCl a 80 °C, liberando completamente os monossacarídeos após 2 h de incubação. A α-galactosidase extracelular apresentou galactose e manose, enquanto a enzima intracelular somente xilose, nas condições de ensaio testadas.
- As α-galactosidases de *D. hansenii* UFV-1 apresentaram similaridade na composição de estruturas secundárias (α-hélice, folhas β-paralela e volta β) por Dicroísmo Circular (CD).

- A α-galactosidase extracelular de *D. hansenii* UFV-1 apresentou maior conteúdo de folhas β antiparalelas, enquanto na enzima intracelular predominou estrutura secundária desordenada.
- A Calorimetria de Varredura Diferencial (DSC) mostrou ser uma ferramenta útil e com alta resolução, capaz de detectar pequenas diferenças termodinâmicas entre as α-galactosidases de *D. hansenii* UFV-1.
- Medidas calorimétricas (DSC) realizadas com as α-galactosidases de *D. hansenii* UFV-1 mostraram uma transição térmica irreversível e termodinamicamente dirigida.
- O valor de T_m da α-galactosidase extracelular de *D. hansenii* UFV-1 em pH 5,5, foi menor (86,1 °C) comparado com a enzima intracelular (87,3 °C), determinados por DSC.
- A α-galactosidase extracelular de *D. hansenii* UFV-1 foi menos estável e cooperativa, durante a transição térmica, em comparação com a enzima intracelular.
- Em algumas condições de cristalização, houve aparecimento de cristais, entretanto, os padrões de difração indicaram a formação dos sais usados na cristalização.

APÊNDICES

APÊNDICE A – Capítulo 1

Tabela 1A - Atividade de α-galactosidase extracelular (U/mg proteína) no meio de cultura YP de *D. hansenii* UFV-1 com diferentes fontes de carbono, a 30 ^oC, por vários tempos.

	Açúcares					
Tempo (h)	**Glicose**	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	1,58	1,37	3,90	2,63	0,155	12,46
27	1,24	1,54	4,32	1,45	0,212	3,63
36	2,34	6,62	10,20	3,26	0,207	3,95
48	3,05	4,93	5,02	3,97	0,232	3,56

1U: 1µmol de *p*-NP formado por minuto.

Tabela 2A - Atividade de α-galactosidase extracelular (U/mg célula) no meio de cultura YP de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 ^oC, por vários tempos.

	Açúcares					
Tempo (h)	**Glicose**	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,009	0,029	0,053	0,010	0,007	0,037
27	0,034	0,035	0,144	0,037	0,021	0,104
36	0,031	0,042	0,083	0,047	0,015	0,118
48	0,103	0,071	0,176	0,148	0,043	0,034

1U:1µmol de *p*-NP formado por min.

Tabela 3A - Atividade de α-galactosidase extracelular (U/mg proteína) no meio mineral com extrato de levedura (MME) de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 ^oC, por vários tempos.

	Açúcares					
Tempo (h)	**Glicose**	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,750	2,258	1,77	0,700	0,194	1,53
27	4,267	4,20	10,79	4,118	2,98	7,64
36	3,143	8,071	6,26	8,294	2,42	6,67
48	4,733	8,750	8,61	6,478	2,15	10,63

1U: 1µmol de *p*-NP formado por min.

Tabela 4A - Atividade de α-galactosidase extracelular (U/mg célula) no meio mineral com extrato de levedura (MME) de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 °C, por vários tempos.

Tempo (h)	Açúcares					
	Glicose	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,009	0,036	0,054	0,010	0,019	0,051
27	0,024	0,023	0,094	0,046	0,039	0,052
36	0,025	0,039	0,101	0,050	0,066	0,059
48	0,027	0,042	0,189	0,061	0,100	0,061

1U: 1µmol de *p*-NP formado por min.

Tabela 5A - Atividade de α-galactosidase extracelular (U/mg célula) no meio mineral (MM) de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 °C, por vários tempos.

Tempo (h)	Açúcares					
	Glicose	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,053	0,192	0,288	0,063	0,246	0,181
27	0,095	0,115	0,088	0,022	0,001	0,005
36	0,118	0,004	0,079	0,000	0,001	0,021
48	0,036	0,008	0,566	0,003	0,023	0,240

1U: 1µmol de *p*-NP formado por min.

Tabela 6A: Atividade de α-galactosidase intracelular (U/mg célula) no meio de cultura YP de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 °C, por vários tempos.

Tempo (h)	Açúcares					
	Glicose	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,059	0,094	0,087	0,106	0,172	0,142
27	0,240	0,360	0,441	0,370	0,476	0,515
36	0,404	0,462	0,542	0,547	0,404	0,417
48	0,451	0,630	0,746	0,918	0,338	0,746

1U:1µmol de *p*-NP formado por min.

Tabela 7A - Atividade de α-galactosidase intracelular (U/mg célula) no meio mineral com extrato de levedura (MME) de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 °C, por vários tempos.

	Açúcares					
Tempo (h)	**Glicose**	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,058	0,198	0,158	0,323	0,076	0,153
27	0,332	0,247	0,494	0,671	0,241	0,282
36	0,435	0,256	0,473	0,536	0,185	0,283
48	0,265	0,265	0,775	0,572	0,463	0,322

1U: 1μmol de *p*-NP formado por min.

Tabela 8A - Atividade de α-galactosidase intracelular (U/mg célula) no meio mineral (MM) de *D. hansenii* UFV-1, com diferentes fontes de carbono, a 30 °C, por vários tempos.

	Açúcares					
Tempo (h)	**Glicose**	**Sacarose**	**Lactose**	**Galactose**	**Melibiose**	**Rafinose**
0	0,0	0,0	0,0	0,0	0,0	0,0
12	0,339	0,342	0,169	0,414	0,449	0,276
27	0,369	0,496	0,517	0,270	0,086	0,443
36	0,579	0,223	1,420	0,398	0,149	0,764
48	0,421	0,419	1,159	0,209	0,361	1,635

1U: 1μmol de *p*-NP formado por min.

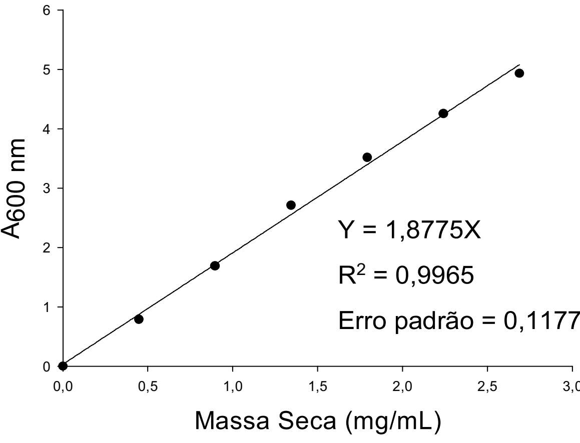

Figura 1A – Curva de calibração utilizada na definição da massa seca correspondente a uma unidade de absorbância (A_{600}).

APÊNDICE B – Capítulo 2

Tabela 1B – Hidrólise de vários substratos com a α-galactosidase intracelular de *D. hansenii* UFV-1.

Substrato	**Concentração***	**Atividade (U/mL) ± SD**
*p*NPαGal[b]	0.5	2.49 ± 0.03
*p*NPβGal[b]	0.5	0.0
*p*NPαGlc[b]	0.5	0.0
*p*NPβXil[b]	0.5	0.0
*p*NPαMan[b]	0.5	0.0
*p*NPαAra[b]	0.5	0.0
*o*NPβGal[b]	0.5	0.0
*o*NPβGlc[b]	0.5	0.0
Sacarose	10	0.0
Estaquiose	10	3.19 ± 0.01
Rafinose	30	2.28 ± 0.01
Melibiose	2	0.002
Gentiobiose	10	0.0
Maltose	10	0.0
Lactose	10	0.0
Goma de alfarroba	1	0.55 ± 0.01
Goma Guar	1	0.55 ± 0.02

*Concentrações em mM, exceto Goma de alfarroba e Goma Guar (%).

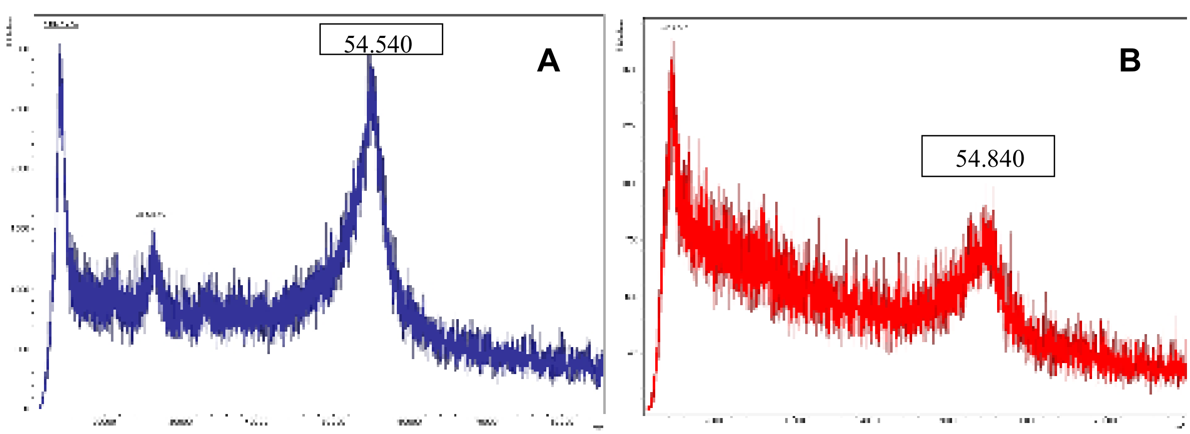

Figura 1B – Cromatogramas das massas moleculares das α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1 determinadas por espectrometria de massa (MALDI-TOF).

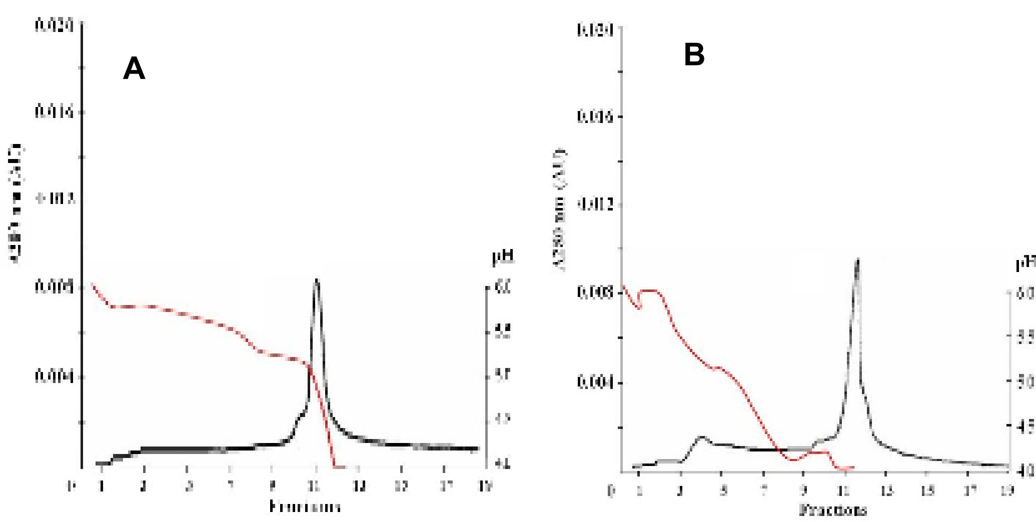

Figura 2B – Gráficos dos pontos isoelétricos (*pI*) determinados por cromatofocalização das α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1.

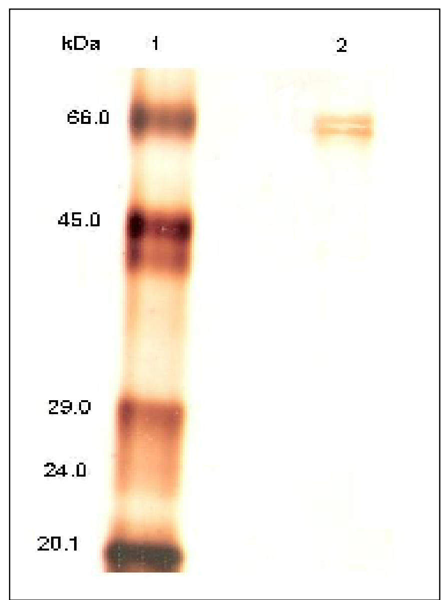

Figura 3B - SDS-PAGE (8,5 – 12,5 %) das α-galactosidases extracelular e intracelular de *D. hansenii* UFV-1. **Linha 1**: padrões de massas moleculares conhecidas; **Linha 2**: Enzimas purificadas aplicadas juntas.

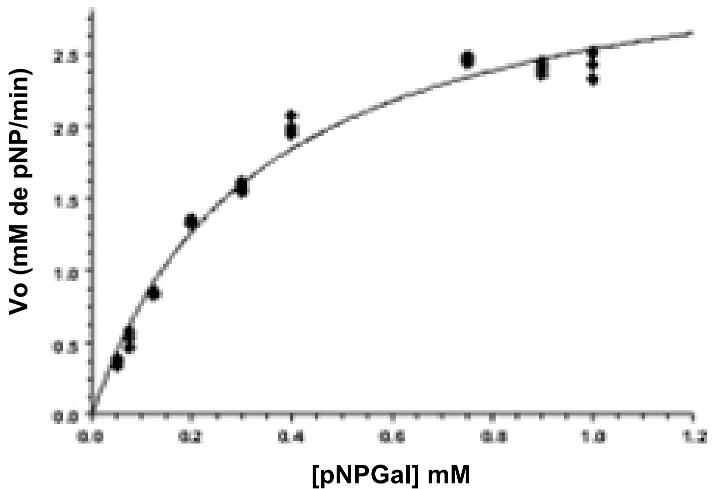

Figura 4B - Efeito da concentração do substrato *p*NPαGal na velocidade da reação catalisada pela α-galactosidase intracelular de *D. hansenii* UFV-1.

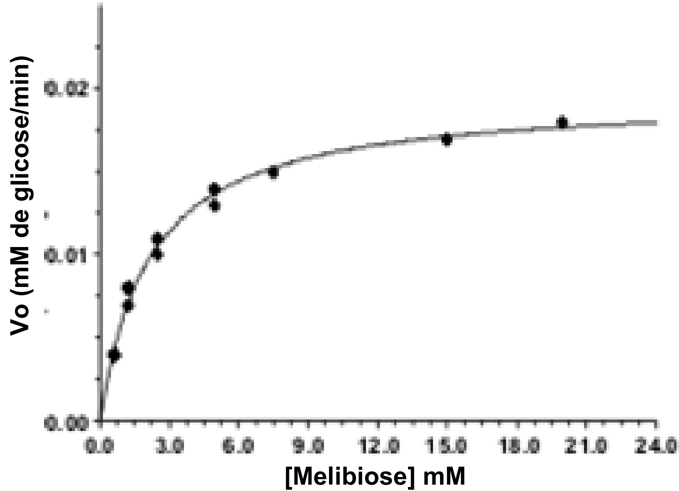

Figura 5B - Efeito da concentração do substrato melibiose na velocidade da reação catalisada pela α-galactosidase intracelular de *D. hansenii* UFV-1.

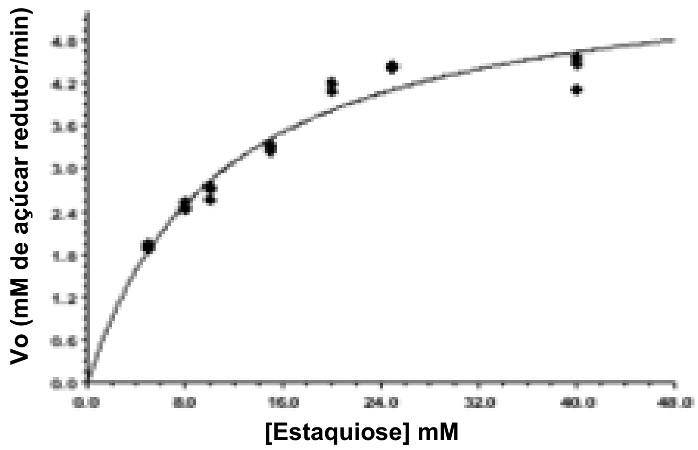

Figura 6B - Efeito da concentração do substrato estaquiose na velocidade da reação catalisada pela α-galactosidase intracelular de *D. hansenii* UFV-1.

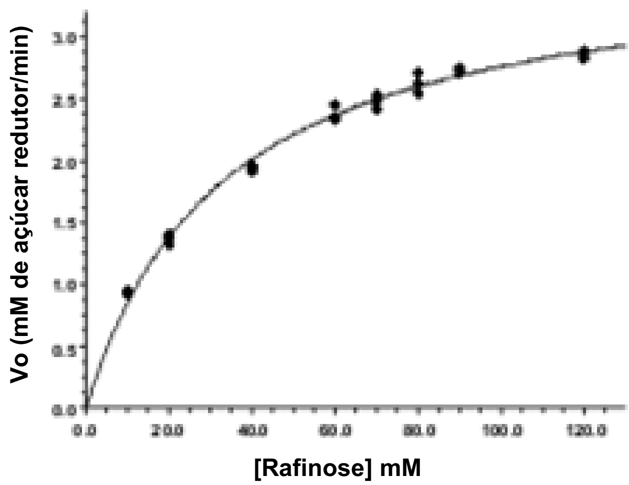

Figura 7B - Efeito da concentração do substrato rafinose na velocidade da reação catalisada pela α-galactosidase intracelular de *D. hansenii* UFV-1.

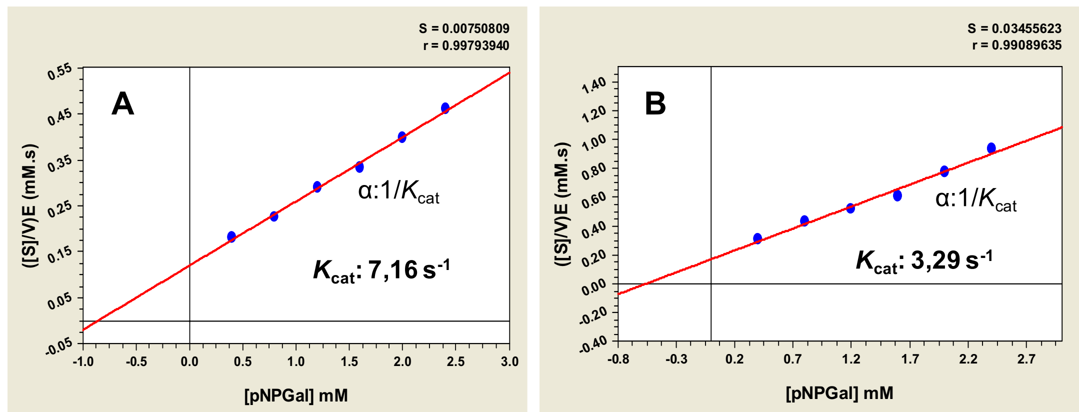

Figura 8B – Constantes catalíticas (K_{cat}) das α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1 com o substrato *p*NPαGal calculadas pelo gráfico de Hanes-Woolf.

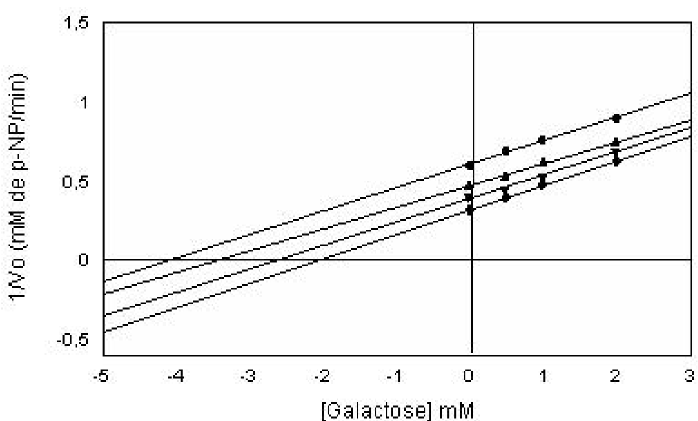

Figura 9B – Gráfico de Dixon para um inibidor acompetitivo, galactose (0,5; 1,0 e 2,0 mM) com a α-galactosidase intracelular de *D. hansenii* UFV-1 na presença do substrato *p*NPαGal nas concentrações de [0,2] (●); [0,3] (▲); [0,4] (▼) e [1,0] (◆).

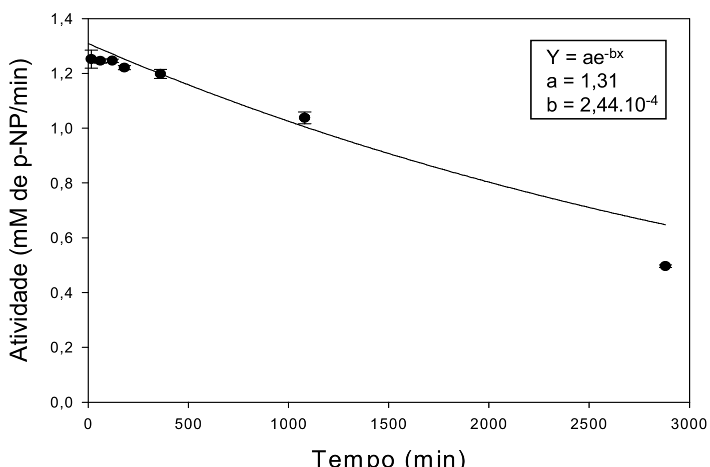

Figura 10B - Meia-vida a 40 °C da α-galactosidase intracelular de *D. hansenii* UFV-1.

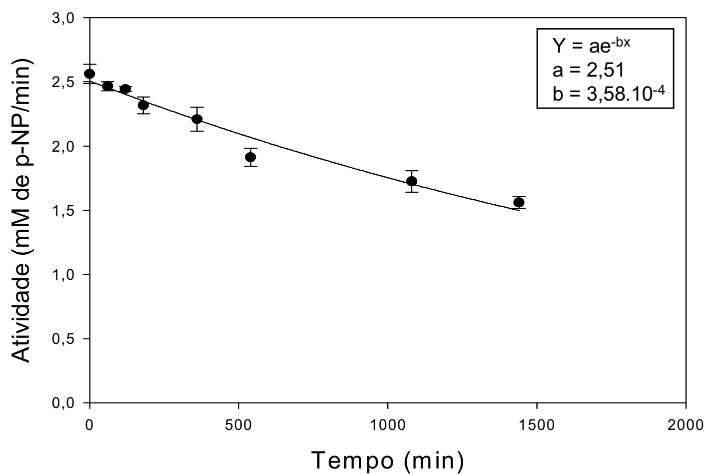

Figura 11B - Meia-vida a 55 °C da α-galactosidase intracelular de *D. hansenii* UFV-1.

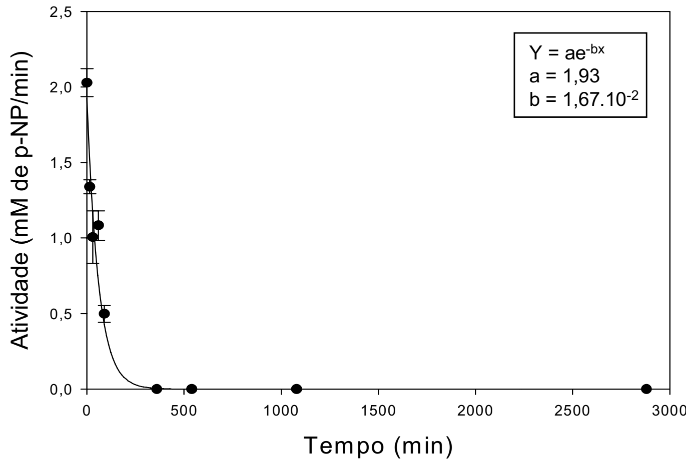

Figura 12B - Meia-vida a 65 °C da α-galactosidase intracelular de *D. hansenii* UFV-1.

APÊNDICE C – Capítulo 3

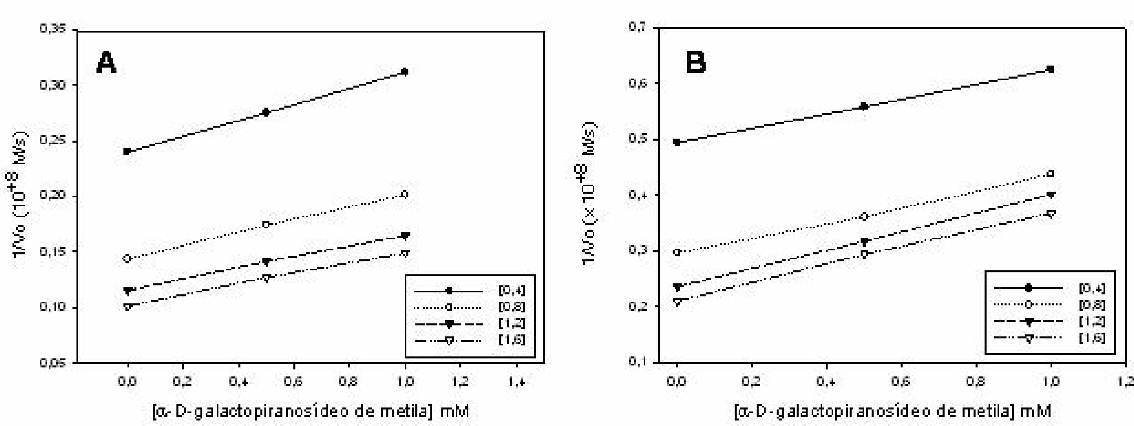

Figura 1C – Gráficos de Dixon para um inibidor acompetitivo, α-D-galactopiranosídeo de metila (0,5; 1,0 e 2,0 mM) com as α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1 na presença do substrato *p*NPαGal, nas concentrações de 0,4; 0,8; 1,2 e 1,6 mM.

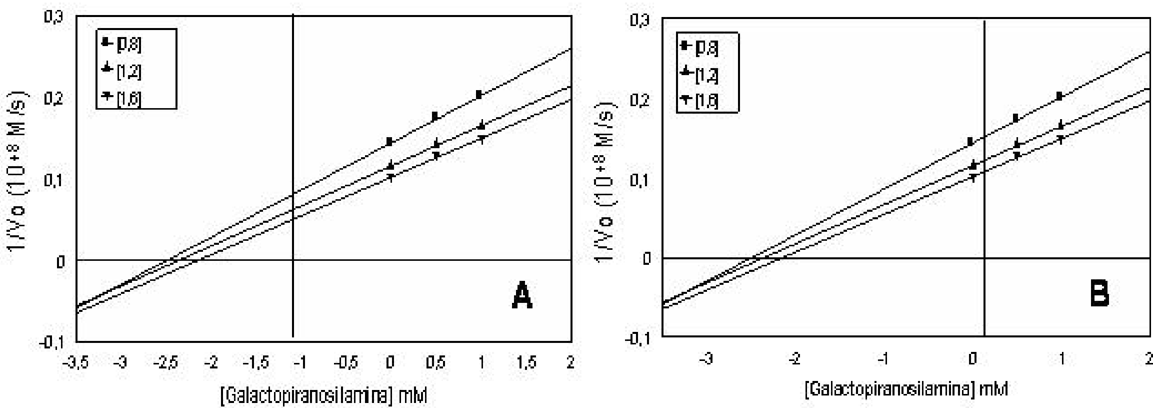

Figura 2C – Gráficos de Dixon para um inibidor não-competitivo, *N*-acetil-2-azido-1,2-didesoxi-α-D-galactopiranosilamina (0,5; 1,0 e 2,0 mM) com as α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1 na presença do substrato *p*NPαGal, nas concentrações de 0,8; 1,2 e 1,6 mM.

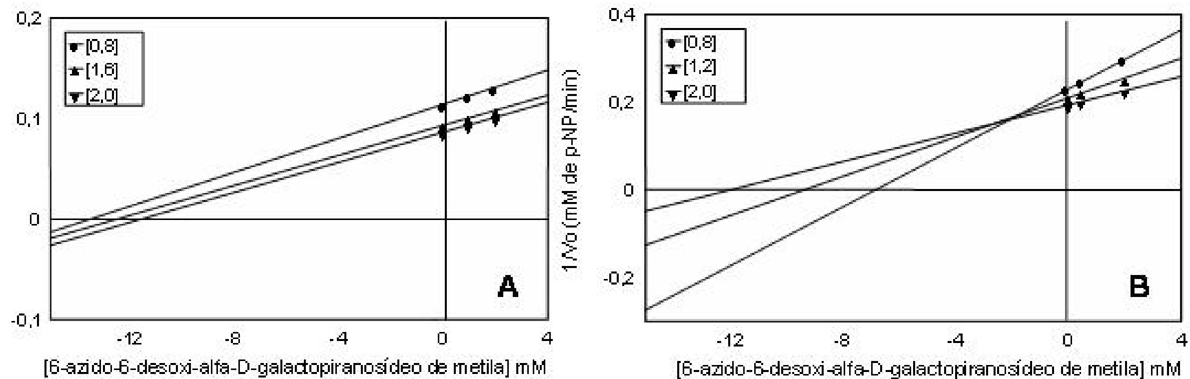

Figura 3C – Gráficos de Dixon para um inibidor acompetitivo (**A**) e competitivo (**B**), 6-azido-6-desoxi-α-D-galactopiranosídeo de metila (0,5; 1,0 e 2,0 mM) com as α-galactosidases extracelular (**A**) e intracelular (**B**) de *D. hansenii* UFV-1 na presença do substrato *p*NPαGal em diferentes concentrações.

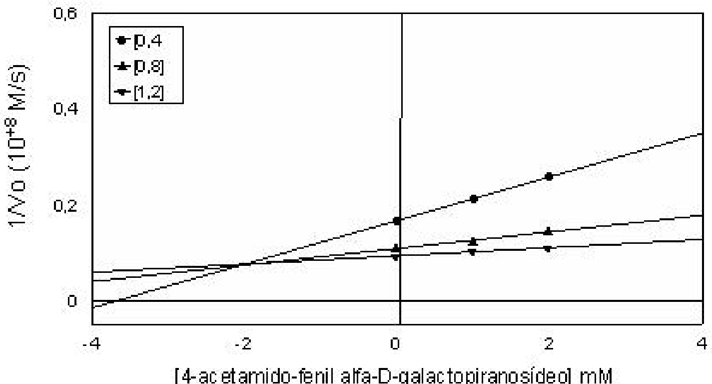

Figura 4C – Gráfico de Dixon para um inibidor competitivo, 4-(acetamido)fenil α-D-galactopiranosídeo (0,5; 1,0 e 2,0 mM) com a α-galactosidase extracelular de *D. hansenii* UFV-1 na presença do substrato *p*NPαGal, nas concentrações de 0,4; 0,8 e 1,2 mM.

APÊNDICE D – Capítulo 4

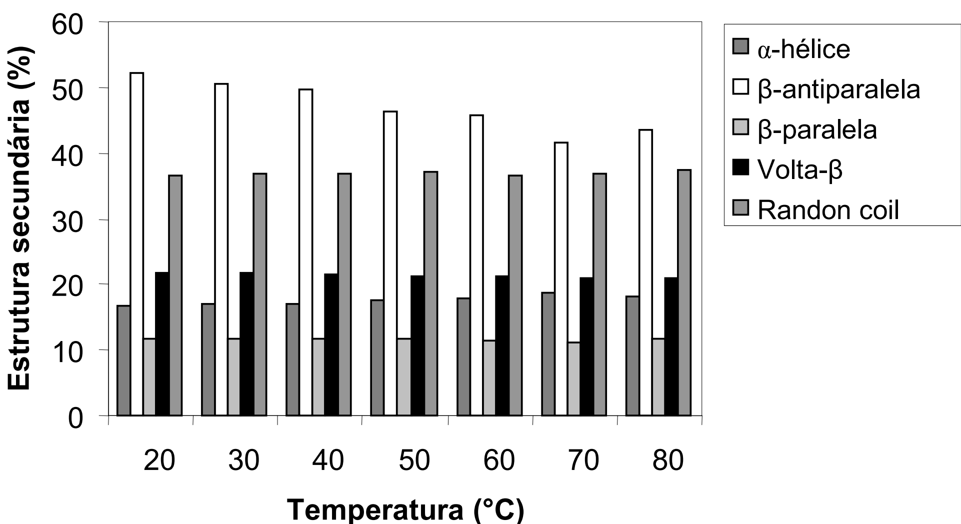

Figura 1D – Estruturas secundárias (%) da α-galactosidase extracelular de *D. hansenii* UFV-1 em diferentes temperaturas, pH 3,0.

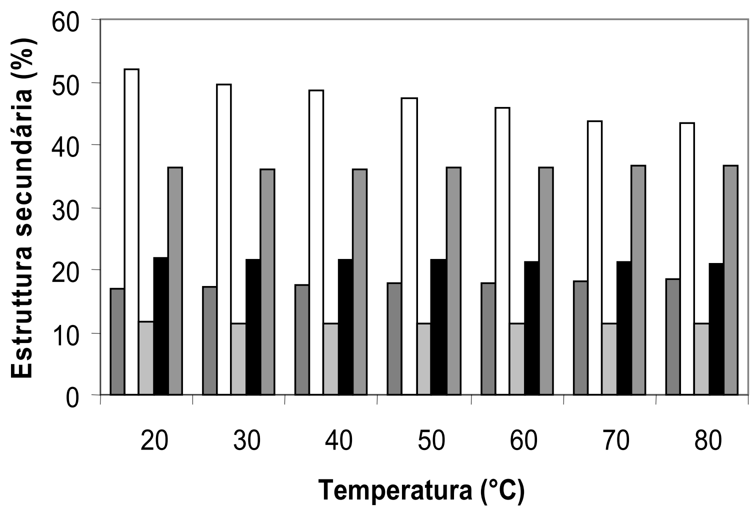

Figura 2D – Estruturas secundárias (%) da α-galactosidase extracelular de *D. hansenii* UFV-1 em diferentes temperaturas, pH 4,0.

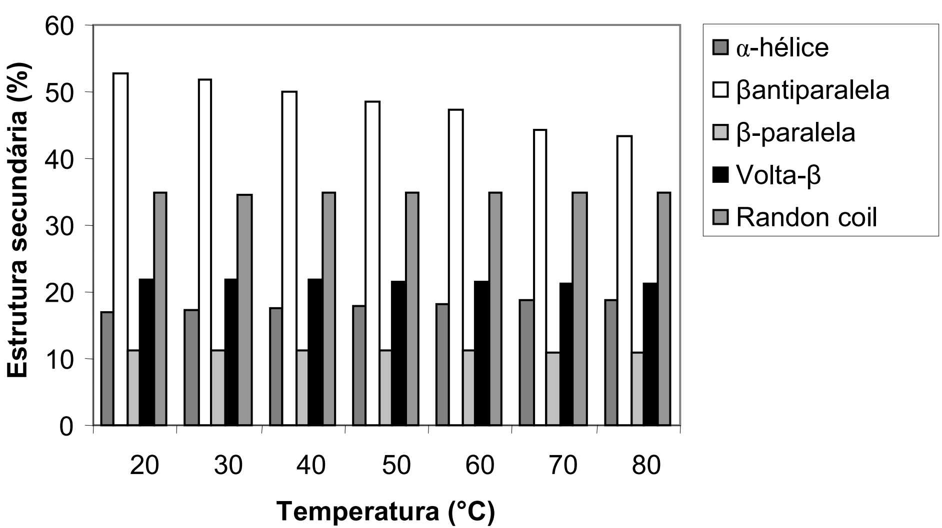

Figura 3D – Estruturas secundárias (%) da α-galactosidase extracelular de *D. hansenii* UFV-1 em diferentes temperaturas, pH 5,0.

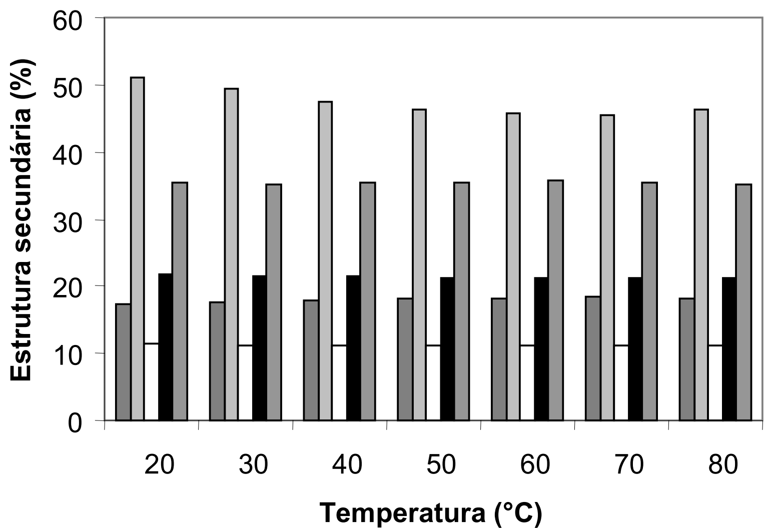

Figura 4D – Estruturas secundárias (%) da α-galactosidase extracelular de *D. hansenii* UFV-1 em diferentes temperaturas, pH 6,0.

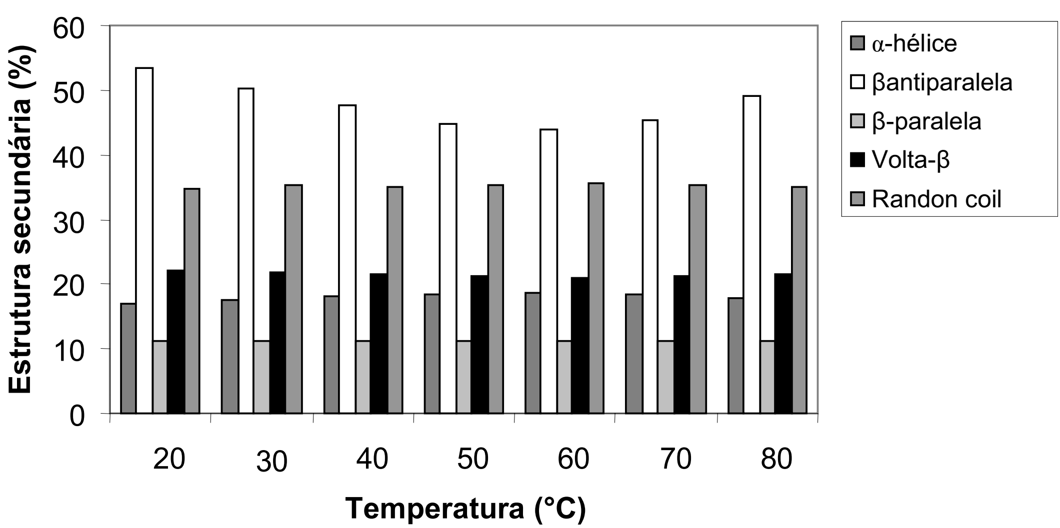

Figura 5D – Estruturas secundárias (%) da α-galactosidase extracelular de *D. hansenii* UFV-1 em diferentes temperaturas, pH 7,0.