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Structural characterization of the β-galactosidase from *Kluyveromyces lactis* and expression and directed evolution of β-galactosidases with high biotechnological interest

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El presente trabajo, **Structural characterization of the β-galactosidase from *Kluyveromyces lactis* and expression and directed evolution of β-galactosidases with high biotechnological interest** presentado por Don Ángel Pereira Rodríguez para aspirar al grado de Doctor en Biología, ha sido realizado bajo mi dirección en el Departamento de Biología Celular y Molecular de la Universidad de A Coruña.

Revisado el texto, estoy conforme con su presentación para ser juzgado.

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VºBº
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"La inspiración existe, pero tiene que encontrarte trabajando"

Pablo Picasso

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Contents

Contents

Abstract

β-galactosidases are hydrolase enzymes that catalyze the hydrolysis of β-galactosides into their monosaccharides. Due to this ability these proteins are very important in food, clinical and pharmaceutical industries.

In this thesis the *Kluyveromyces lactis* β-galactosidase was cloned in a *Saccharomyces cerevisiae* strain, expressed, purified, and crystallized. Its free state structure and its complex with the product galactose were determined to 2.75 and 2.8 Å, respectively. *K. lactis* β-galactosidase folds into 5 domains in a pattern conserved with other prokaryote enzymes solved for GH2 family, although two long insertions in domains 2 (264-274) and 3 (420-443) are unique and seem related to oligomerization and specificity. *K. lactis* β-galactosidase tetramer is an assembly of dimers, with higher dissociation energy for the dimers than for its assembly, which can explain that equilibrium exists in solution between the dimeric and tetrameric form of the enzyme.

On the other hand, a hybrid *K. lactis-Aspergillus niger* β-galactosidase was constructed, expressed and characterized. The hybrid protein between *K. lactis* and *A. niger* β-galactosidases increases the yield of the protein released to the growth medium and the modifications introduced in the construction conferred to the protein biochemical characteristics of biotechnological interest. The production of this hybrid *K. lactis - A. niger* β-galactosidase was also tested in a continuous immobilized culture using spent grains (a by-product of brewery industry) as an immobilizing material in an airlift fermenter.

Finally, different *A. niger* β-galactosidase constructions were expressed and studied in a *S. cerevisiae* strain and directed evolution techniques were applied to modify the optimal pH of the protein to a more neutral one.

Las β-galactosidasas son enzimas hidrolasas que catalizan la hidrólisis de β-galactósidos en sus monosacáridos correspondientes. Debido a esta capacidad, estas proteínas son muy importantes en las industrias alimentaria, farmacéutica y clínica.

En esta tesis, la β-galactosidasa de *K. lactis* fue clonada en una cepa de *Saccharomyces cerevisiae*, expresada, purificada y cristalizada. Su estructura en estado libre y la su complejo con el producto galactosa fueron determinadas a 2,75 y 2,8 Å, respectivamente. La β- galactosidasa de *K. lactis* está organizada en 5 dominios en un patrón que está conservado en otras enzimas procariotas de la familia GH2, aunque presenta dos inserciones largas en los dominios 2 (264-274) y 3 (420-443) que son únicas y parecen estar relacionadas con su oligomerización y especificidad. El tetrámero de la β-galactosidasa de *K. lactis* está formado por un par de dímeros, presentando una mayor energía de disociación la forma de dímero que su forma tetramérica, lo que puede explicar el por qué existe un equilibrio en solución entre la forma dimérica y tetramérica de la enzima.

Por otro lado, se ha construido, expresado y caracterizado una β-galactosidasa híbrida entre las β-galactosidasas de *K. lactis* - *Aspergillus niger*. La proteína híbrida entre las β-galactosidasas de *K. lactis* - *A. niger* aumenta el rendimiento de la proteína secretada al medio y las modificaciones introducidas en la construcción le han conferido características bioquímicas de interés biotecnológico. Se estudió además la producción de esta proteína híbrida en un cultivo continuo inmovilizado utilizando "spent grains" (un subproducto de la industria cervecera) como material de inmovilización en un fermentador tipo "Airlift".

Finalmente, se estudió la expresión y producción de la β-galactosidasa de *A. niger* en una cepa de *S. cerevisiae*, y se usaron técnicas de evolución dirigida para modificar el pH óptimo de la proteína hacia un pH más neutro.

As β-galactosidasas son hidrolasas, enzimas que catalizan a hidrólise dos β-galactósidos nos seus monosacáridos correspondentes. Debido a esta capacidade, estas proteínas son moi importantes na industria alimentaria, farmacéutica e clínica.

Nesta tese, a β-galactosidasa de *K. lactis* foi clonada nunha cepa de *Saccharomyces cerevisiae*, expresada, purificada e cristalizada. As estruturas en estado libre e no complexo co produto galactosa foron determinadas a 2,75 e 2,8 Å, respectivamente. A β-galactosidasa de *K. lactis* está organizada en 5 dominios nun patrón conservado noutras enzimas procariotas da familia GH2, pero ademáis presenta dúas inserciónss longas nos dominios 2 (264-274) e 3 (420-443) que son únicas e parecen estar relacionadas coa súa oligomerización e especificidade. O tetrámero da β-galactosidasa de *K. lactis* está formado por un par de dímeros, tendo unha maior enerxía de disociación a forma de dímero que a súa forma tetramérica, o que pode explicar o por qué do equilibrio existente en solución entre a forma dimérica e a forma tetramérica.

Por outra banda, construiuse unha proteína híbrida entre as β-galactosidasas de *K. lactis* e *Aspergillus niger*, que foi expresada e caracterizada. A proteína híbrida entre as β-galactosidasas de *K. lactis – A. niger* aumenta o rendemento da proteína secretada ao medio e coas modificaciónss introducidas na construción conseguíronse características bioquímicas de gran interese biotecnolóxico. Estudouse ademáis a produción da β-galactosidasa híbrida *K. lactis-A. niger* nun cultivo continuo inmovilizado nun fermentador tipo "Airlift" usando como material de inmobilización "spent grains" (un subproduto da industria cervexeira).

Finalmente, foi estudada a expresión e produción da β-galactosidasa de *A. niger* nunha cepa de *S. cerevisiae*, e usáronse técnicas de evolución dirixida para modificar o pH óptimo da proteína hacia un pH máis neutro.

Introduction

1. β-galactosidases

a. What are they? Small summary of E.coli, *K. lactis* and *A. niger* β-galactosidase.
b. Why β-galactosidase from *K. lactis*?
c. Why β-galactosidase from *A. niger*?
d. Which families?
e. Applications?
    i. Cheese Whey
    ii. Pharmaceutical/Medical Industry
    iii. Other Agrofood issues

2. Three-dimensional analysis

3. Directed evolution

β-galactosidases

β-galactosidases (sometimes called lactases) are hydrolase enzymes that catalyze the hydrolysis of β-galactosides into their monosaccharides.

There are β-galactosidases from prokaryotes to eukaryotes (including humans), and the first sequenced β-galactosidase was the *Escherichia coli* β-galactosidase (Fowler and Zabin 1970) with 1,024 amino acids[1].

This could be stated as the first step in a prolific run into the study of the β-galactosidases, and it was and it is at present a model for the rest of the β-galactosidases.

Twenty-four years passed until the three-dimensional β-galactosidase structure was found (Jacobson *et al.* 1994), and the information was essential to continue studying the β-galactosidases from other organisms like the *Kluyveromyces lactis* β-galactosidase which had been sequenced only two years before (Poch *et al.* 1992).

1.1 Most important β-galactosidases

The most important β-galactosidases due to its biotechnology potential are:

1. *Escherichia coli* β-galactosidase (hereafter *E. coli* β-galactosidase)
2. *Kluyveromyces* lactis β-galactosidase (hereafter *K. lactis* β-galactosidase
3. *Aspergillus* niger β-galactosidase (hereafter *A. niger* β-galatosidase)

1. *E. coli* β-galactosidase (EC 3.2.1.23 - P00722)

As it was stated before, its sequence was published in 1970 *(Fowler and Zabin 1970)* and revealed that the β-galactosidase was composed by 1024[1] amino acids and a molecular weight of 116.000 KDa[1].

The most important following studies in the *E. coli* β-galactosidase (Langley *et al.* 1975; Cupples *et al.* 1990; Jacobson *et al.* 1994; Roth and Huber 1996; Roth and Huber 1996; Huber *et al.* 2001; Juers *et al.* 2001; Huber *et al.* 2003; Juers *et al.* 2003; Roth *et al.* 2003; Spiwok *et al.* 2004; Matthews 2005; Juers *et al.* 2009; Lo *et al.* 2009) determined that the protein is a tetramer of 464,000 KDa, and each monomer contains five domains, the third of which is an eight-stranded α/β barrel that comprises much of the active site.

The five domains are represented in Figure 1:

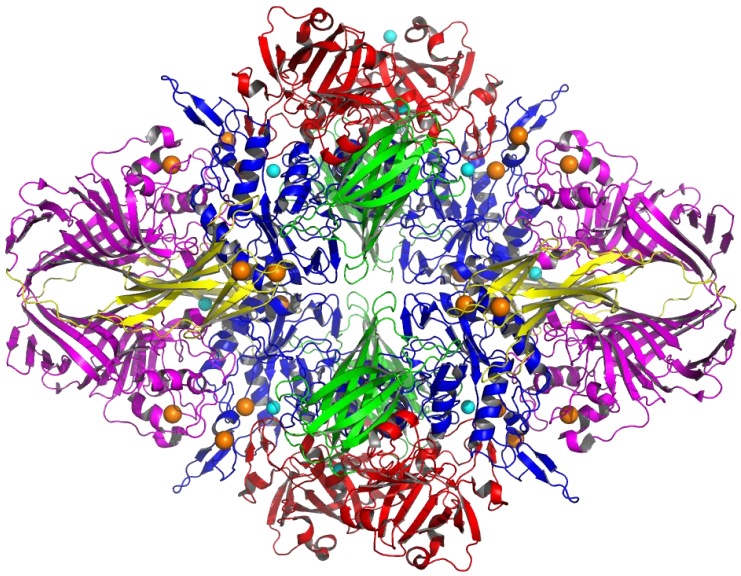

Figure 1: *E. coli* β-galactosidase (Domain 1 (1-217) in red, Domain 2 (218-334) in green, Domain 3 (335-627) in blue, Domain 4 (628-736) in yellow and Domain 5 (737-1023) in magenta). Orange and blue spheres are Na and Mg ions respectively.

As can be seen in the Figure 1, in the tetramer the four monomers are grouped around three mutually-perpendicular two-fold axes of symmetry.

β-Galactosidase has two catalytic activities. First, it hydrolyzes the disaccharide lactose to galactose plus glucose. Second, it converts lactose to another disaccharide, allolactose, which is the natural inducer for the *lac* operon.

a. How it works

In summary, substrates initially bind in a "shallow" mode, subsequently moving deeper into the active site so that the glycosidic oxygen is close enough to be protonated by Glu-461 (general acid catalysis) and the galactosyl anomeric carbon is close enough to contact the nucleophile, Glu-537. A carbocation-like transition state forms that collapses into an a-galactosidic bond between the carboxyl of Glu-537 and the C1 of galactose. This first step of the reaction is called galactosylation (the enzyme becomes galactosylated).

Upon glycosidic bond cleavage, the first product normally diffuses away. Water or an acceptor with a hydroxyl group then enters and is activated by Glu-461 via general base catalysis. The galactosyl moiety is released to this

molecule via a second carbocation-like transition state (the two transition states are thought to be similar) to form free galactose or an adduct having a galactosidic bond with the acceptor. If the reaction is with water is called degalactosylation and if the reaction is with an acceptor is called transgalactosylation.

β-Galactosidase requires $\underline{Mg^{2+}}$ or $\underline{Mn^{2+}}$ for full catalytic activity, but the exact role of this ion in catalysis is unclear. The active site also includes a monovalent cation (usually either Na^+ or K^+) important for activity, which directly ligates the galactosyl O_6 hydroxyl during catalysis. The two ion sites are situated a few A° apart in the active site, both very near to an interface between two domains of the protein.

Crystal structure and site directed mutagenesis experiments have shown that His-418, along with **Glu-416** and **Glu-461** (the **acid/base catalyst**) are ligands to the Mg^{2+} at the active site. Besides ligating the Mg^{2+} ion, His-418 is one of several residues that together form an opening that guides substrates into the binding site , and it is thought to contact the aglycone moeity of the substrate, pointing to a possible role in the formation of allolactose. His-418 is also close to Glu-461, and so very likely directly impacts the properties of that important catalytic residue.

2. *K. lactis* β-galactosidase (EC 3.2.1.21 - P00723)

Its sequence was published in 1992 (Poch *et al.* 1992) and revealed that the β-galactosidase was composed by 1025 amino acids and a molecular weight of 117.618 KDa.

At present, no three-dimensional structure has been published by any research group, despite of the importance of this protein in agrofood and pharmaceutical/medical industries[2].

The optimal pH of the enzyme is neutral (close to 7), and is considered as GRAS (Generally Recognized/Regarded As Safe) by the FDA (American Food and Drug Administration).

It is because is produced by an eukaryotic organism, and the fact of the optimum pH close to the neutrality, what makes this strain very important in biotechnology and the perfect candidate to grow in cheese whey, which is a by-product of the cheese factories all over the world (especially in small ones).

The most impostant studies about an approximation to the protein structure of the β-galactosidase are in temporary order: a comparison with prokaryotic enzymes and secondary structure analysis (Poch *et al.* 1992), (Athes *et al.* 1998) who studied the ifluence of polyols on the structural properties of *Kluyveromyces lactis* β-galactosidase under high hydrostatic pressure, (Becerra *et al.* 1998) who find out that dimeric and tetrameric form of the β-galactosidase are active, and finally (Tello-Solis *et al.* 2005) who discover and presented the secondary structure of the β-galactosidase using circular dichroism.

In the purification of the protein all began with the first purification of (Dickson *et al.* 1979).

Later the works of (Dickson *et al.* 1979; Becerra *et al.* 1998; Becerra *et al.* 1998) with different purification techniques improve the quality of the purification.

About the production and applications in the eighties of the past centrury, Solomons studied the effect of lactose and β-galactosidases in humans (Solomons *et al.* 1985; Solomons *et al.* 1985), and Sreekrishna constructed the first strains capable of grow in lactose (Sreekrishna and Dickson 1985).

In the nineties, the first prolific studies about the K. lactis production, and the production of ethanol from lactose arise (de Figueroa *et al.* 1990; Becerra *et al.* 1997; Kim *et al.* 1997; Rubio-Texeira *et al.* 1998).

From 2000 to present a great number of papers show the potential of the *K. lactis* β-galactosidase production and its uses to produce ethanol from cheese whey (Becerra *et al.* 2001; Becerra *et al.* 2001; Domingues *et al.* 2001; Rubio-Texeira *et al.* 2001; Becerra *et al.* 2002; Kim *et al.* 2003; Panuwatsuk and Da Silva 2003; Ramirez Matheus and Rivas 2003; Becerra *et al.* 2004; Jurascik *et al.* 2006; Clop *et al.* 2008; Guimaraes *et al.* 2008; Guimaraes *et al.* 2008; Guimaraes *et al.* 2008; Ornelas *et al.* 2008; Guimarães *et al.* 2010; Oliveira *et al.* 2011).

3. *A. niger* β-galactosidase (EC 3.2.1.3 - P29853.2)

The first research with information of its structure was published at 1979 (Widmer and Leuba 1979), and the minimum expression of the protein was determined in 124.000 KDa. The pH optimum oscillate between 2.5 and 4.0.

Due to the acid optimum pH of this protein, and the fact that is excreted naturally by the fungus, make this protein a good candidate to use in biotechnology applications.

Some of the most important studies related to the heterologous expression of this protein are summarized in table 1 extracted from (Oliveira *et al.* 2011).

1.2. Families

On the basis of their sequence, β-galactosidases are classified in CAZy (Cantarel *et al.* 2009) within families 1, 2, 35 and 42 of glycosyl hydrolases. Those from eukaryotic organisms are grouped into family 35 with the only exceptions of *K. lactis* (P00723) and *K. marxianus* (Q6QTF4) β-galactosidases (99% identity), which belong to the family 2 together with the prokaryotic β-galactosidases from *E. coli* and *Arthrobacter sp.* Whereas the structures of these last two prokaryotic enzymes have been determined (Juers *et al.* 2000; Skalova *et al.* 2005), none of the eukaryotic β-galactosidase structures has been reported to date. Although their sequence homology with the prokaryotic enzymes is significant (48% vs. *E. coli* and 47% vs. *Arthrobacter*) there are many differences, particularly some long insertions and deletions, which can play an important role in protein stability and in substrate recognition and specificity.

1.3. Applications

β-galactosidases are used in different applications, but can be summarized in three most important fields:

a. Cheese whey valorisation

b. Pharmaceutical/Medical Industry

c. Other Agrofoods issues

Table 1

Source of enzyme	Expression host/plasmid	Media and culture conditions	Extracellular recombinant β-galactosidase activity	References
A. niger	Mauri distiller's yeast/pVK1.1	Modified Dw medium+10% lactose (2-L bioreactor)	10 U/mL	(Ramakrishnan and Hartley 1993)
A. niger	Brewer's yeast W204-FLO1L/ pET13.1+*lacA* cassette (pLD1)	SSlactose 2% (shake flasks)	17 U/mL	(Domingues *et al.* 2000)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	SSlactose 1% (Shake Flask)	350 U/mL	(Domingues *et al.* 2002)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	SSlactose 5% (2-L Bioreactor)	2000 U/mL	(Domingues *et al.* 2002)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	SSlactose 10%	5096 U/mL	(Domingues *et al.* 2002)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	Cheese whey permeate 5% lactose	2635 U/mL	(Domingues *et al.* 2002)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	SSlactose 15%+1.5% YE (10-L bioreactor)	7350 U/mL	(Domingues *et al.* 2004)
A. niger	*S. cerevisiae* NCYC869-A3/pVK1.1	SSlactose 5% (6-L airlift bioreactor)	Maximum: 3250 U/mL (D=0.4 h−1)	(Domingues *et al.* 2005)
A. niger	*S. cerevisiae* NCYC869/pδ-neo+lacA cassette	SSlactose 5% (6-L airlift bioreactor)	Maximum: 2754 U/mL (D=0.1 h−1)	(Oliveira *et al.* 2007)
K. lactis* and *A. niger	*K. lactis* MW 190-9B/pSPGK1-LAC4; pSPGK1-LAC4-LACA	Culture medium is not described (shake flasks)	*K. lactis*/pSPGK1-LACA: up to 100 U/mL	(Rodriguez *et al.* 2006)
K. lactis* and *A. niger	*K. lactis* MW 190-9B/pSPGK1-LAC4; pSPGK1-LAC4-LACA	Culture medium is not described (shake flasks)	*K. lactis*/pSPGK1-LAC4-LACA: up to 100–200 U/mL	(Rodriguez *et al.* 2006)

a. Cheese whey valorisation

Cheese whey is a subproduct resulted in the production of cheese, after the curdle separation.

It has a yellow-green colour, an elevated COD and BOD, due to the great quantities of nutrients it retains. Because of that it has to be treated after the elution to the drains.

There are two types of cheese whey:

Sweet whey: from cheeses that were produced by enzymatic coagulation. pH=6,5-7

Acid whey: from cheeses that were produced by acid coagulation. pH=4,5

The composition of cheese whey is not always the same because it depends in the cheese master and how he/she make the cheese (the pastry could be washed more or less, it could have more or less salt, depends on the type of milk:origin, land, etc), but it could be made an average like follows:

Lactose	50 g/L
Proteins	7,5 g/L
Lipids	5 g/L
N/NH_4	0,031g/L
Lactic Acid, Vit. B, others	-
QOD	79 g/L
BOD	30.000-60.000 ppm
Dry extract	6-7%
Salts	8-10% Dry extract
Phosphates	0,39 g/L

There are a high variety of cheese whey uses: SCP (Single Cell Protein), biosurfactants production, biopolymers production, lactic acid production, bacteriocines production, biogas production, production of recombinant proteins, GOS and bioethanol production

b. Pharmaceutical/Medical Industry

On one handfor pharmaceutical industry, interest in β-galactosidases is due to the reproducibility of their activities which are used in a lot of commercial kits

as a reporter gene and so to reduce the problems associated to the lactose intolerance (Solomons *et al.* 1985; Solomons *et al.* 1985; Vesa *et al.* 2000; Bhatnagar and Aggarwal 2007; Ibrahim *et al.* 2009; O'Connell and Walsh 2009).

On the other hand, the medical industry interest is due lactose intolerance in humans affects to over 70% of the world's adult population (Oliveira *et al.* 2011), which typical symptoms are abdominal pain, gas, nausea and diarrhea.

c. Other Agrofoods issues

In the food industry β-galactosidase is also used to enhance the sweetness of lactose for example in desserts like ice-creams, cakes, etc.

2. Protein Structure Analysis

Determine the 3D protein structure is essential because the function of a biological macromolecule is related to its 3D shape, so knowledge about the structure is essential to understand the function.
The 3D protein structure benefits:

1. Understanding biological processes at the atomic level
2. Study interactions among proteins and/or other molecules
3. Design of specific inhibitors/activators for a protein – drug discovery

The three most important strategies to determine the protein structure are: Nuclear Magnetic Resonance (NMR), X-Ray Crystallization (XRC) and cryo-Electron Microscopy (EM).

NMR is based in the concept that NMR active nuclei absorb electromagnetic radiation at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field.

The advantages of NMR over XRC are:

1. Can be performed in the solution-state, so the structures may be more physiologically relevant.
2. Some proteins do not give diffraction-quality crystals.
3. Provides dynamics and other information: internal mobility, flexibility, order-disorder, hydrogen exchange rates, pKa values, binding constants, conformational exchange rates, …

Disadvantages of NMR over XRC are:

1. Molecular weight limitations: 50 kDa for complete structure determination and 100 kDa for local or partial analysis.
2. Stable-isotope enrichment usually required: need efficient bacterial expression system.
3. Structure determination methods more time consuming, difficult …

XRC is a method of determining the arrangement of atoms within a crystal, in which a beam of X-rays strikes a crystal and diffracts into many specific directions. The crystal is an ordered solid in where a basic organizational unit is repeated (Lesk 2001).
From the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and various other information.

In an X-ray diffraction measurement, a crystal is mounted on a goniometer and gradually rotated while being bombarded with X-rays, producing a diffraction pattern of regularly spaced spots known as reflections. The two-dimensional images taken at different rotations are converted into a three-dimensional model of the density of electrons within the crystal using the mathematical method of Fourier transforms, combined with chemical data known for the sample.

EM is the most innovative of the fourth methods, and allows the structure determination of macromolecules and biological aggregates at molecular resolution (7 to 30 Å) up to near atomic resolution (2-3 Å) that have not been stained or fixed in any way, showing them in their native environment.

The advantages over XRC and NMR are:

1. There is no limit to the size of the structure in study, so large and complex structures which cannot be studied with the mentioned strategies can be solved with EM (for ex. membrane proteins).
2. Relatively small sample amounts
3. Cryo methods allow the observation of the molecules in their aqueous native state, close to physiological conditions.

3. Directed Evolution

Engineering the specificity and properties of enzymes and proteins within rapid time frames has become feasible with the advent of directed evolution. In the absence of detailed structural and mechanistic information, new functions can be engineered by introducing and recombining mutations, followed by subsequent testing of each variant for the desired new function. A range of methods are available for mutagenesis, and these can be used to introduce mutations at single sites, targeted regions within a gene or randomly throughout the entire gene. In addition, a number of different methods are available to allow recombination of point mutations or blocks of sequence space with little or no homology. Currently, enzyme engineers are still learning which combinations of selection methods and techniques for mutagenesis and DNA recombination are most efficient. Moreover, deciding where to introduce mutations or where to allow recombination is actively being investigated by combining experimental and computational methods. These techniques are already being successfully used for the creation of novel proteins for biocatalysis and the life sciences (Williams *et al.* 2004).

Principally, the directed evolution methods are divided in recombinative and non-recombinative methods.

Non-recombinative methods generally create diversity via point mutation and include the directed substitution of single amino acids, the insertion or deletion of more than one amino acid, for example by cassette mutagenesis, and random mutagenesis across the whole gene.

The simplest, and still a popular, method of choice for introducing diversity is Error Prone – PCR (EP-PCR). The mutation rate can be adjusted so that, usually, an average of 1–2 amino acid mutations is introduced per gene product (Moore *et al.* 1997).

It is generally accepted that using a low mutation rate increases the probability of discovering beneficial mutations, since most random mutations are either neutral or deleterious.

EP-PCR at low mutation rate suffers from one drawback: there is an inherent bias introduced since on average only 5.6 amino acids per codon can be accessed given the substitution of a single nucleotide. In addition, the inherent bias of polymerases further reduces the diversity that can be accessed by EP-PCR.

These problems can be overcome by the use of gene site saturation mutagenesis (GSSM).

GSSM is a method that uses sets of degenerate primers to introduce all 19 amino acid substitutions at every position of the gene to produce every possible single amino acid mutant (DeSantis *et al.* 2003).

In the case of recombinative methods DNA shuffling is still the most popular method of recombining DNA, whether homologous genes from different sources are being recombined, or for the recombination of point mutations.

Briefly, the original DNA shuffling technique (Stemmer 1994; Stemmer 1994)involves the controlled fragmentation of the source DNA using DNase I, followed by a primer-less, reassembly PCR reaction, which gradually produces full-length recombined sequences. Finally, the small amount of fulllength gene present in the reassembly reaction is amplified by a standard PCR reaction in the presence of flanking primers.

Other recombinative method is the Staggered Extension Process (StEP) which uses a simple PCR reaction with very short elongation times; recombination occurs where partially elongated strands melt and anneal to a new template, producing a crossover (Zhao *et al.* 1998).

One more recombinative method is Random Chimeragenesis on Transient Templates (RACHITT), which is similar to DNA shuffling but requires many more experimental steps; however the method does produce a much larger number of crossovers than basic DNA shuffling (Coco *et al.* 2001).

Two other also known recombinative methods are Synthetic Shuffling and Assembly of Designed Oligonucleotides (ADO): They use of entirely synthetic oligonucleotides that result in the production of fulllength genes with defined crossover points and composition. Both techniques result in

significantly increased recombination frequency and have been validated experimentally (Zha *et al.* 2003).

There are two last recombinative methods, the first is the Incremental Truncation for the Creation of HYbrid enzymes (ITCHY) which involves the direct ligation of truncated N- and C-terminal fragments of two genes, removing the requirement of homology (Ostermeier *et al.* 1999). Crossovers occur at random positions, and the initial products only contain a single crossover. DNA shuffling of the ITCHY products can generate products with multiple crossovers, and plasmid systems are available for selection of only in frame ligation products.

The second is a computational algorithm (SCHEMA) which identifies optimal crossover points using structural information to identify sites with minimal interaction with the rest of the protein (Voigt *et al.* 2002).

Once the directed evolution method was chosen and applied, the following step is to decide the optimum screening strategy, which is the most critical step in the directed evolution, and usually is a bottleneck.

There are different screening strategies, but the most common ones are summarized in this review (Arnold F H *et al.* 2003).

As reviewed, the directed evolution methods make to evolve easily and fastly proteins (principally enzymes), which is really important in biotechnology to solve problems like the cheese whey.

Different techniques have been applied to develop strains capable of secrete a directed evolution β-galactosidase, that makes the strain able to grow in cheese whey and consume the nutrients which make it toxic to environment.

[1] Fowler *et. al* think initially that the *E.coli* β-galactosidase was composed of 1171 residues and has a molecular weight of 135 KDa, but future experiments demonstrate that it is composed of 1024 amino acids and its molecular weight is 116KDa.

[2] In the chapter 3of this thesis, the three-dimensional structure is presented and discussed.

Objectives

The aim of this thesis is to determine and analyze the structure of the *Kluyveromyces lactis* β-galactosidase and express and apply directed evolution techniques in β-galactosidases with high biotechnological potential, and specifically:

1. Expression, purification and crystallization of the *Kluyveromyces lactis* β-galactosidase.

2. Structural characterization ot the *Kluyveromyces lactis* β-galactosidase.

3. Development of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase.

    a. Construction, and biochemical analysis of the hybrid enzyme
    b. Production of the hybrid enzyme in a continuous culture

4. Development of a *Saccharomyces cerevisiae* strain which expresses the *Aspergillus niger* β-galactosidase and application of directed evolution methods to modify the optimum pH of the enzyme.

Outline of this thesis

β-galactosidases are probably one of the most important proteins in terms of biotechnology potential due to their applications in Pharmacy, Medicine, Agrofood Industries, etc.

Within the group of β-galactosidases, the most interesting ones, due to its biological and chemical properties are *Escherichia coli*, *Kluyveromyces lactis* and *Aspergillus niger* β-galactosidases.

The first one, the *Escherichia coli* β-galactosidase, has been very thoroughly studied, so this thesis deals with the study of the other two β-galactosidases, focusing more in the *K. lactis* β-galactosidase due to its use in sweet cheese whey which is the most abundant in Galicia.

Chapter 1 presents the details of a full-factorial design used to find conditions for growing good-quality crystals of *K. lactis* β-galactosidase. The application of a full-factorial approach to protein crystallization could reduce substantially the number of crystallization trials. The method is based on a factorial approach to experimental design, permits the assay of a large number of crystallization conditions with as few experiments as possible. This is accomplished by varying more than one factor at a time in a given experiment; this saves material, and from the analysis of the results, it is possible to readily determine the factors that are critical for crystallization.

Chapter 2 is about the expression, purification, optimization of crystallization, and diffraction of crystals to obtain the *K. lactis* β-galactosidase structure. This protein has been expressed and purified in yeast for the crystallization trials. However, even optimization of the best crystallization conditions yielded crystals with poor diffraction quality that precluded further structural studies. Finally, thanks to the streak seeding technique, the crystal quality was improved and a complete diffraction data set was collected at 2.8 Å resolution.

Chapter 3 describes X-ray crystallographic studies and an analysis of *K. lactis* β-galactosidase. β-galactosidase sequences can be deduced from various databases, and these can be classified into four different glycoside hydrolase (GH) families 1, 2, 35, and 42, based on functional similarities. Those from eukaryotic organisms are grouped into family 35 with the only exceptions of *K. lactis* and *K. marxianus* β-galactosidases (99% identity), which belong to the family 2 together with the prokaryotic β-galactosidases from *Escherichia coli* and *Arthrobacter sp.* Whereas the structures of these last two prokaryotic enzymes have been determined, none of the eukaryotic β-galactosidase structures has

been reported. In fact, to date, the X-ray crystal structures of eight different microbial β-galactosidases are available in the PDB, although none of the enzymes with solved structures is known to be used in food processing. Here it is reported the three-dimensional structure at 2.75 Å resolution and the complex structure with galactose at 2.8 Å resolution of the β-galactosidase from *K. lactis*, one of the most important and widely used enzymes of the food industry.

Chapter 4 shows the construction and analysis of a two hybrid proteins from the β-galactosidase of *K. lactis*, intracellular, and its *A. niger* homologue that is extracellular; and the production of the hybrid *K. lactis-A. niger* β-galactosidase in a continuous immobilized culture. A hybrid protein between K. lactis and *A. niger* β-galactosidases was constructed that increases the yield of the protein released to the growth medium. Modifications introduced in the construction, besides to improve secretion, conferred to the protein biochemical characteristics of biotechnological interest. This hybrid β-galactosidase showed an optimal pH around the neutrality and it had a better stability at high temperatures compared with the wild protein from yeast. Different culture mediums were assayed to scale-up the production of this hybrid β-galactosidase. The use of spent grains (a by-product of brewery industry) as an immobilizing material (in which the yeast can grow inside) to stabilize the production of the protein, was checked too.

Chapter 5 presents the construction, expression and analysis of three new recombinant *Saccharomyces cerevisiae* strains expressing *A. niger* β-galactosidase, and the directed evolution of the enzyme to modify its optimum pH. The development of *S. cerevisiae* strains with the capability of metabolizing lactose is an important biotechnological objective, and here it is described the construction of new recombinant *S. cerevisiae* strains which was able to express and secrete the extracellular and thermostable β-galactosidase from *A. niger* up to 90% of the total β-galactosidase activity into the growth medium. Finally using a directed evolution technique, mutations in the enzyme were done, in order to modify its optimum pH.

CHAPTER 1

Kluyveromyces lactis β-galactosidase crystallization using full-factorial experimental design

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SUMMARY

Kluyveromyces lactis β-galactosidase is an enzyme with numerous applications in the environmental, food and biotechnological industries. Despite of its biotechnological interest, its three-dimensional structure has not yet been determined. The growth of suitable crystals is an essential step in the structure determination of a protein by X-ray crystallography. At present, crystals are mostly grown using trial-and-error procedures since their growth often depends on the combination of many different factors. Testing the influence on crystallization of even only a small number of these factors requires many experimental set-ups and large amounts of protein. In the present work, a full-factorial design has been used in order to find conditions for obtaining good-quality crystals of *K. lactis* β-galactosidase. With this full-factorial method protein crystals have been obtained.

INTRODUCTION

The microbial lactase or β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from the yeast Kluyveromyces lactis, the enzyme which is responsible for the hydrolysis of lactose into glucose and galactose, has outstanding biotechnological interest. Therefore it has attracted the attention of researchers and industries because of its important applications in the fields of medicine (treatment of lactose intolerance), food technology (to prevent lactose crystallization and increase its sweetening power) and the environment (cheese whey utilization). Although much of the work on *K. lactis* β-galactosidase has dealt with the production (Becerra *et al.* 1997; Becerra *et al.* 2001; Becerra *et al.* 2002; Becerra *et al.* 2004), the use (Becerra *et al.* 2003) and biochemical characterization (Athes *et al.* 1998; Becerra *et al.* 1998; Tello-Solis *et al.* 2005), to the best of our knowledge, very little has been reported about its structure (Tello-Solis *et al.* 2005).

The growth of suitable protein crystals is an essential step in the structure determination of a protein by X-ray crystallography. At present, crystals are mostly grown using trial-and-error procedures, whereby various factors: pH, temperature, salt concentration, etc, are systematically varied until crystals are obtained. Usually, in these experiments, the different factors are varied only over a narrow range of values. The use of this method often requires large amounts of material and is frequently time consuming.

Carter and Carter (Tello-Solis *et al.* 2005) demonstrated that the application of a full-factorial approach to protein crystallization could reduce substantially the number of crystallization trials. Their method, which is based on a factorial approach to experimental design (Fisher 1942), permits the assay of a large number of crystallization conditions with as few experiments as possible. This is accomplished by varying more than one factor at a time in a given experiment; this saves material, and from the analysis of the results, it is possible to readily determine the factors that are critical for crystallization. We present here the details of a full-factorial design to find conditions for growing good-quality crystals of *K. lactis* β-galactosidase.

MATERIAL AND METHODS

Strains and culture conditions

The following strains were used: *Kluyveromyces lactis* NRRL-Y1140 (*MATa*, wild type) and *Saccharomyces cerevisiae* BJ3505 (pep4::HIS3, prb-Δ1.6R HIS3, lys2-208, trp1-Δ101, ura 3-52, gal2, can1). The BJ3505 strain was purchased from Eastman Kodak.

Liquid batch cultures of wild type and transformed cells were grown in Erlenmeyer flasks filled with 20% volume of culture medium at 250 rpm and 30 ºC, unless otherwise stated. *K. lactis* wild type cells were growth in YPL (1% yeast extract, 0.5% bactopeptone, 0.5% lactose) whereas transformed S.

cerevisiae BJ3505 cells were growth in YPHSM (1% yeast extract, 8% bactopetone, 1% dextrose, 3% glycerol, 20mM CaCl2). In the late case, as inocula, a suitable volume of a stationary phase culture in complete medium (CM) (Lowry *et al.* 1983) without the corresponding auxotrophic amino acid was added to obtain an initial OD600 of 0.2. Samples were taken at regular time intervals to measure growth (OD600) and intracellular β-galactosidase activity.

Vectors

The YEpFLAG1-LAC4 (Becerra *et al.* 2001) containing the *LAC4* gene, which codes for *K. lactis* β-galactosidase, inserted between the yeast ADH2 promoter and CYC1 terminator was used. This plasmid also contains the sequence of the FLAG peptide for the immunological detection and affinity purification of the FLAG fusion protein.

Molecular biology procedures

Yeast strains were transformed using the lithium acetate procedure (Ito *et al.* 1983). Plasmid uptake and β-galactosidase production by the transformed strains were identified on plates with the chromogenic substrate X-gal in the corresponding auxotrophic medium.

β-galactosidase activity assays and protein determinations

The method of Guarente (Ito *et al.* 1983) as previously described (Becerra *et al.* 2001) was used. One enzyme unit (E. U) was defined as the quantity of enzyme that catalyzes the liberation of 1 μmol of ortho-nitrophenol from ortho-nitrophenyl-β-D-galactopyranoside per min under assay conditions.

Protein was determined by the method of Bradford (Bradford 1976) using bovine serum albumin (Sigma) as a standard.

Preparation of crude protein extracts

Crude protein extract was prepared as described previously (Becerra *et al.* 1998) from cells cultured in 1 litre of YPL or YPHSM up to an A600nm of 2 (about 3 mg dry wt/ml).

Purification of β-galactosidase

The purification of β-galactosidase from a crude protein extract of the strain of *K. lactis* NRRLY1140 and from a YEpFLAG1-LAC4 transformed S. cerevisiae BJ3505 strain was performed using different chromatographical techniques.

In the first trial, a column with 5 ml agarose-p-aminopheyl-β-D-thiogalactoside (Sigma Chemical, USA) was equilibrated with 50 mM phosphate buffer pH 7, and the enzyme was eluted with 0.1 M sodium borate, pH 10. Fractions of 1 ml were collected at a flow rate of 100 µl/min. The pH of the collected fractions was neutralized to avoid denaturation.

In the second purification method assayed, a column with 0.2 ml of ANTI-FLAG M2 affinity gel (Sigma Chemical, USA), useful for purification of FLAG fusion proteins, was equilibrated with TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4) and the elution of the bound FLAG fusion protein was by competition with a solution containing 100 µg/ml FLAG peptide (Sigma Chemical, USA).

All purification steps were carried out at 4ºC. The β-galactosidase activity was assayed in the eluted fractions obtained from chromatographic steps. Active fractions were pooled and, when required, concentrated by filtration in Amicon ULTRA-4 (Millipore, UFC 803024).

Polyacrylamide gel electrophoresis

This was performed as described in Becerra (Becerra *et al.* 1997).

Protein crystallization

Crystals were grown at several conditions at 20ºC by vapour diffusion in hanging drops containing 1-3 µl of protein solution (9 mg/ml) and 1 µl of reservoir solution.

Experimental design and statistical data analysis

Factorial experimental design was created and data were analyzed with the aid of version 5.1 of the STATGRAPHICS Plus software for Windows (Statistical Graphics Corporation).

The statistical significance of differences between means was determined by Student´s t-test performed with the same software; p values <0.05 were considered significant.

RESULTS AND DISCUSSION

Purification of *K. lactis* β-galactosidase

The success of the crystallization process starts at the protein purification level. In a previous work, the expression and purification of K. lactis β-galactosidase in *E. coli* as a His-tagged recombinant enzyme was tried but left out, due to the formation of insoluble inclusion bodies and the irreversible inhibitory effect of imidazole on the enzyme (Becerra *et al.* 1997). In our study, K. lactis β-galactosidase purification was achieved by two different procedures: affinity chromatography on agarose-p-aminophenyl-β-D-thiogalactoside and immunoaffinity on ANTI-FLAG M2 affinity gel.

The purification of the enzyme from a crude protein extract of the *K. lactis* strain NRRL-Y1140 by affinity chromatography resulted in a purification factor of 2.7 over the crude extract, having an overall yield based on total enzyme units of 17.4%. Values are comparable to those previously reported

for the same enzyme (Becerra *et al.* 1998). The results of this purification process are summarized in Table 1.

Table 1: Summary of the purification procedure of *K. lactis* β-galactosidase by affinity chromatography (A) and *of K. lactis* β-galactosidase fused to the FLAG peptide by immunoaffinity (B).

	Step	Total protein (mg)	Total E.U.	Yield (%)	Specific activity (E. U/mg)	Purification factor
A	Crude extract	87	137 250	100	1 577.59	1
	Affinity and microultrafiltration	1.79	23 882	17.4	4 259.5	2.7
B	Crude extract	133.10	104 610	100	785.95	1
	Immunoaffinity chromatography	1.91	71 980	68.81	37 705.61	47.98

K. lactis β-galactosidase was also purified from a S. cerevisiae BJ3505 strain transformed with YEpFLAG1-LAC4 by affinity purification of the FLAG fusion protein. In this case, a 1.43% protein recovery with a yield of 68.8% and an increase in specific activity of 47.98-fold was obtained (Table 1).

The homogeneity of the isolated β-galactosidases was examined by SDS-PAGE of the purified enzyme (Figure 1), both preparations show a main protein band with the approximate molecular weight of 124 kDa that agreed with the one predicted from the sequence of LAC4, the unique gene coding for β-galactosidase present in the *K. lactis* genome (Poch *et al.* 1992).

These data demonstrate the usefulness of both tested purification procedures for obtaining K.lactis β-galactosidase protein. Although the second procedure gave the highest purification factor and turned out to be more effective.

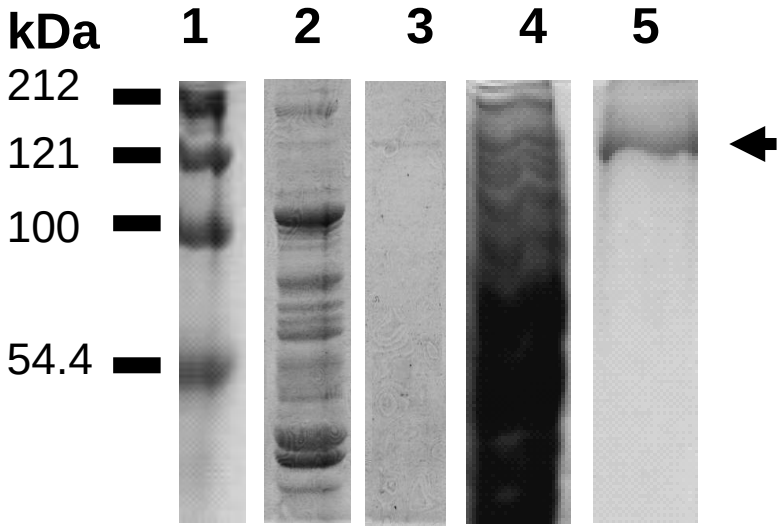

Fig. 1. SDS-PAGE of purified *K. lactis* β-galactosidase. Lane 1, molecular weight markers; lane 2, 60 μg of a crude extract from *K. lactis* NRRL-Y1140; lane 3, 1.5 μg of *K. lactis* β-galactosidase purified by affinity chromatography from *K. lactis* NRRL-Y1140, lane 4, 90 μg of a crude extract of *S. cerevisiae* BJ3505 transformed with YEpFLAG1-LAC4; lane 5, 3.5 μg of *K. lactis* β-galactosidase purified by immunoaffinity. β-Galactosidase is indicated by an arrow.

Initial crystallization screens

Crystallization of macromolecules is usually performed by a somewhat organized trial-and-error procedure using available kits. However, use of these kits locks the experimenter to a relatively narrow set of historical conditions. In our case, the search strategy to get optimal crystallization of *K. lactis* β-galactosidase was based on the use of conditions that have already rendered useful for obtaining crystals of a homologous protein with similar size and function, the β-galactosidase from *E. coli* (Juers *et al.* 2003). Reproducing these conditions, small protein crystals were obtained in presence of 0.1 M Tris pH 8.0 and different concentrations of $(NH_4)_2SO_4$ (0.02 and 0.2 M) and PEG 6000 (5% and 10 %) (Figure 2).

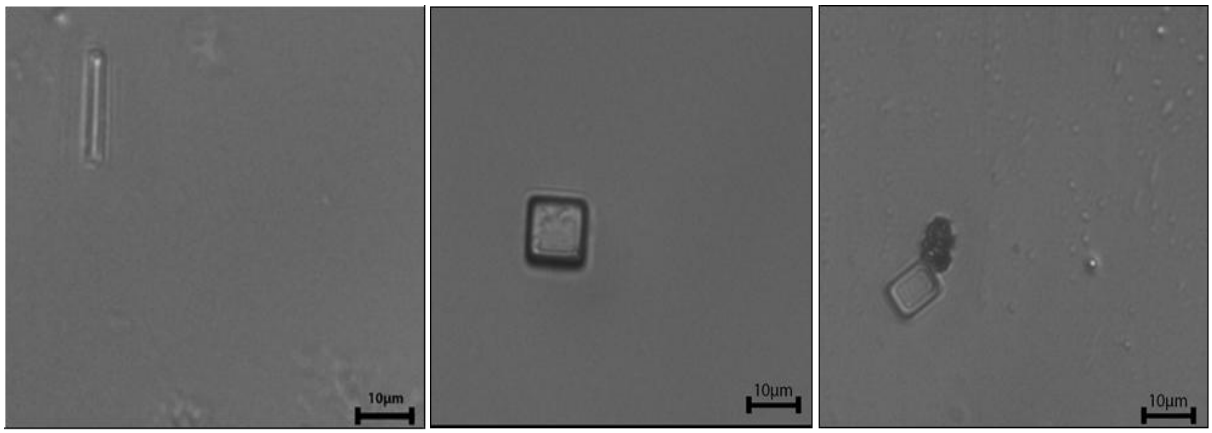

Fig. 2. Photography of one *K. lactis* _-galactosidase crystal obtained in presence of 0.1M Tris, pH 8.0, 0.02M $(NH_4)_2SO_4$ and 5% PEG 6000 (A). Protein crystals obtained using the full-factorial design approach (B and C). Photographs were taken with the objective 40×.

Optimization of the crystallization conditions

The formation of crystals depends on the concentration of macromolecule and precipitant. At higher concentrations of macromolecule, less precipitant is required for crystallization. The function of the different precipitants such as polyethylene glycol and ammonium sulphate in the crystallization drop is to alter the protein-solvent or protein-protein contacts so that the protein molecules precipitate out of solution, preferably as ordered crystals and not as disordered aggregates. In our case, in order to identify optimal conditions for crystal growth, including crystal volume and shape improvements, we studied, by means of a full-factorial design, the influence of three variables (% PEG 6000, $(NH_4)_2SO_4$ and protein concentrations), and their interactions on the response. The range and coding criteria of the variables used are given in Table 2 and Table 3 shows the experimental matrix and the results obtained for the analysed response, the quality of crystals. The quality obtained was quantified taking into account three parameters: morphology, size and amount of crystals. A score of 2 points was given to crystals with regular morphology and 1 point to crystals with irregular morphology. In the center of the experimental domain (0 coded value to the three variables), crystals showed a similar size, and therefore were considered

as average size and scored with 2 points. Bigger crystals than those obtained in the center of domain were scored with 3 points, and smaller crystals with 1 point. Finally, 1 point was given to conditions which showed multiple crystals, and two points were given to conditions with few crystals.

Table 2
Experimental domain and codification of the variables used in the full-factorial design

Natural Values			
Coded values	PEG 6000 (P: %)	$(NH_4)_2SO_4$ (A: M)	Protein concentration (PRO: □ g)
-1	5	0.02	10
0	10	0.1	15
+1	15	0.18	20

Codification: $Vc = (Vn - V0)/DVn$; decodification: $Vn = V0 + (\Delta Vn \times Vc)$ where Vc is the coded value, Vn is the natural value, $V0$ is the natural value in thecenter of the experimental domain and ΔVn is the increase in the natural valuecorresponding to 1U of growth in the coded value.

Table 3: Experimental results of the full-factorial design (2^3) for the study of *K. lactis* β-galactosidase crystals quality obtained taking into account three parameters: morphology, size and amount of crystals. Variables according to Table 2.

	P	**A**	**PRO**	**Quality of Crystals**
1	1	1	1	6
2	-1	1	1	4
3	1	1	-1	0
4	-1	1	-1	5
5	1	-1	1	5
6	-1	-1	1	5
7	1	-1	-1	4
8	-1	-1	-1	0
9	0	0	0	5
10	0	0	0	4
11	0	0	0	4
12	0	0	0	4

Variables are according to Table 2.

The ANOVA table (Table 4A) divides the variability in the response into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 3 effects have *P*-values less than 0.05 (with asterisk in Table 4A), indicating that they are significantly different from zero at the 95.0% confidence level.

After removing the no significant coefficients, the *P*-value for lack-of-fit in the ANOVA table (Table 4B) is greater to 0.05 (0.1149) and the model appears to be adequate for the observed data at the 95.0% confidence level. The lack of fit test is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors.

The R-Squared statistic indicates that the model, as fitted, explains 91.7017% of the variability in the response. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 88.5898 %. The standard error of the estimate shows the standard deviation of the residuals to be 0.64145. The mean absolute error (MAE) of 0.423611 is the average value of the residuals.

The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. Since the DW value is greater than 1.4 (2.05063), there is probably not any serious autocorrelation in the residuals.

The system can be represented by the following codified equation (significance tested by Fisher *F*-test) in which only the concentration of protein, the interaction between the % PEG 6000 and ammonium sulphate

concentration and the interaction between the three variables present influence in the response:

Quality of crystals = 3.83333 + 1.375xPRO - 0.875 x P x A + 1.375 x P x A x PRO

Table 4: Analysis of variance for the response (quality of the crystals) in the full-factorial design (23) studied before (A) and after (B) removing the no significant coefficients and analysis of the significance and adequacy of the proposed model

	Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Ratio	*P*-Value
A	**P:PEG**	0.125	1	0.125	0.50	0.5305
	A:AMMONIUM	0.125	1	0.125	0.50	0.5305
	PRO:PROTEIN	15.125	1	15.1250	60.50	0.0044*
	PxA	6.125	1	6.125	24.50	0.0158*
	PxPRO	1.1250	1	1.125	4.50	0.1240
	AxPRO	0.125	1	0.125	0.50	0.5305
	PxAxPRO	15.1250	1	15.1250	60.50	0.0044*
	Lack-of-fit	1.04167	1	1.04167	4.17	0.1339
	Pure error	0.75	3	0.25		
B	**PRO:PROTEIN**	15.1250	1	15.1250	47.06	0.0002
	PxA	6.1250	1	6.1250	19.06	0.0033
	PxAxPRO	15.1250	1	15.1250	47.06	0.0002
	Lack-of-fit	1.04167	1	1.04167	3.24	0.1149
	Pure Error	2.25	7	0.321429		
	Total (Corr.)	39.6667	11			

(*) Significant coefficients. Variables according to Table 2. R^2 = 91.7017%; R^2 (adjusted for d.f.) = 88.5898%; standard error of est. = 0.64145; mean absolute error = 0.423611; Durbin–Watson statistic = 2.05063 (P = 0.4610).

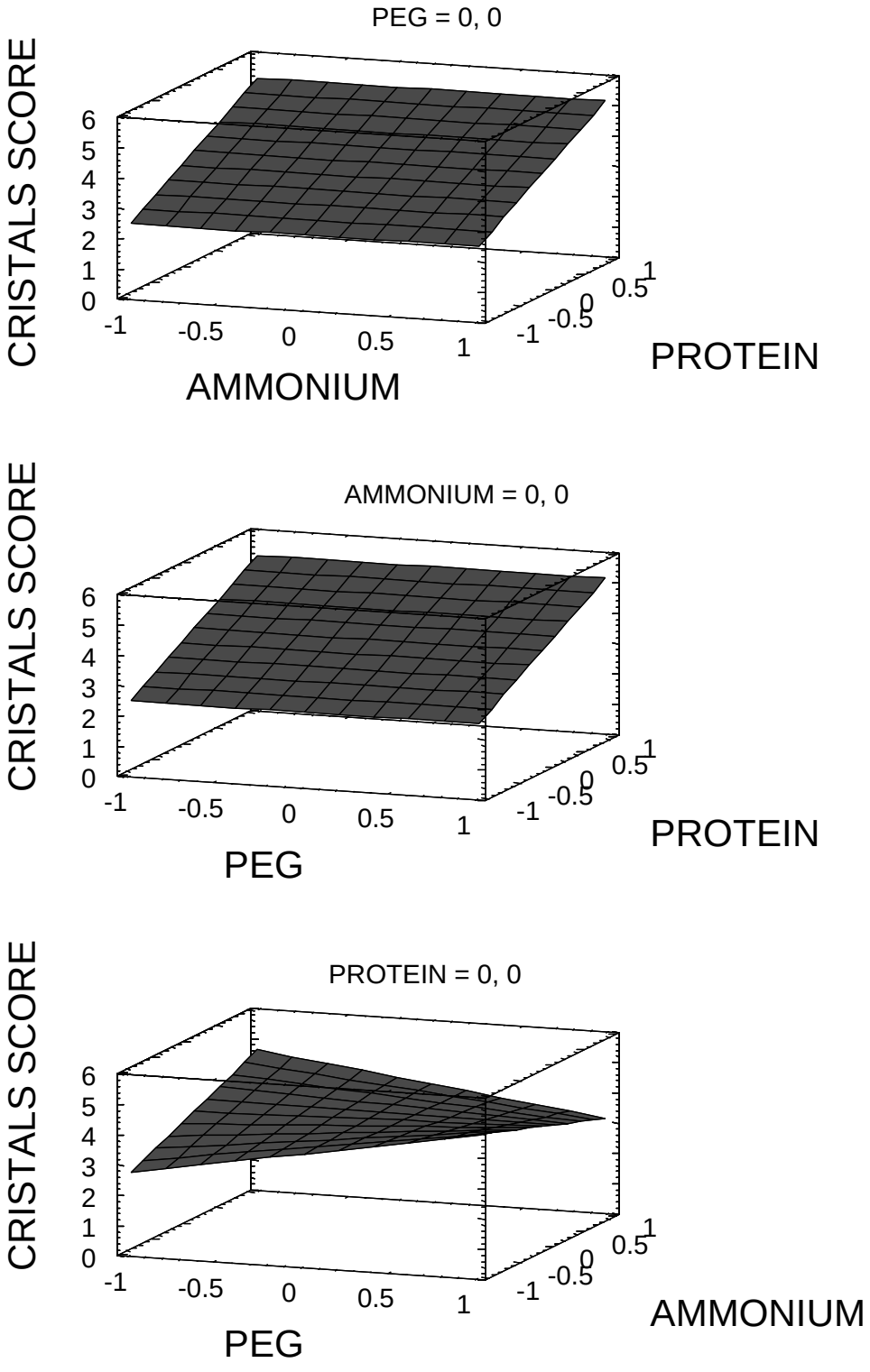

Fig. 3. Response surfaces obtained to identify optimal conditions for *K. lactis* β-galactosidase crystal growth according to the experimental plan defined in Table 2. Crystals score = response (quality of crystals taking into account: morphology, size and amount of crystals). Variable values and nomenclature can be seen in Table 2.

Some of the more representative surface responses corresponding to the mentioned equation are represented in Figure 3. These surfaces are planes defined by pairs of variables having the third variable fixed (values -1, +1). As can be seen in Figure 3, the response increases in each situation of our experimental domain when the concentration of the protein is increased (positive coefficient). The effect of % PEG 6000 and ammonium sulphate concentration, for the same protein concentration, is more complex because response increases in the corners, high % PEG 6000 and small ammonium sulphate concentration and vice versa small % PEG 6000 and high ammonium sulphate concentration. Therefore, the highest values of the response are obtained in the corners: P=+1, A=-1, PRO=+1 or P=-1, A=+1, PRO=+1.

Some of the *K. lactis* β-galactosidase crystals obtained with the optimal conditions obtained by this approach are shown in Figure 2.

CONCLUSIONS

A methodical and efficient approach has been carried out to growth *K. lactis* β-galactosidase crystals. The full-factorial screen with response surface optimization allowed us to find conditions for growing good quality crystals with a small number of experiments to be performed. Optimal crystallization conditions for 20 μg of *K. lactis* β-galactosidase were obtained in the presence of 0.1 M Tris-HCl, pH 8, 15% PEG 6000 and 0.02 M $(NH_4)_2SO_4$. Advantages obtained in this approach include improvements in β-galactosidase crystal volume and shape and also in reproducibility. Similar designs could be of interest to get crystals from other proteins which have special difficulties to solve.

Acknowledgements

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CHAPTER 2

Crystallization and preliminary X-ray crystallographic analysis of β-galactosidase from *Kluyveromyces lactis*

Ángel Pereira-Rodríguez, Rafael Fernández-Leiro, M. Isabel González Siso, M. Esperanza Cerdán, Manuel Becerra and Julia Sanz-Aparicio

Acta Crystallographica Section F Structural Biology and Crystallization Communications (2010) 66: 297-300

SUMMARY

β-galactosidase from *Kluyveromyces lactis* catalyses the hydrolysis of the β-galactosidic linkage in lactose. Due to much industrial applications the biotechnological potential of this enzyme is substantial. This protein has been expressed and purified in yeast for the crystallization trials. However, even optimization of the best crystallization conditions yielded crystals with poor diffraction quality that precluded further structural studies. Finally, thanks to the streak seeding technique, the crystal quality was improved and a complete diffraction data set was collected at 2.8 Å resolution.

INTRODUCTION

The enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) catalyzes the hydrolysis of the disaccharide lactose into glucose and galactose. Enzymes with this activity are present in microorganisms, plants and animals, and have multiple biotechnological applications. β-galactosidases are useful in the treatment of lactose intolerance (Bhatnagar and Aggarwal 2007) and they are frequently used in the food industry in order to increase the sweetening power of the natural saccharides (Gonzalez Siso 1996). In addition, they are also employed in a treatment and transformation of cheeses whey (Gonzalez Siso 1996). β-galactosidase from the yeast *Kluyveromyces lactis* (Kl-β-Gal) is one of the most frequently used β-galactosidases in the biotechnological industry due to its favourable biochemical properties: an optimal neutral pH, and a higher stability than found for β-galactosidases from the other sources (i.e. fungal galactosidases). Furthermore, *K. lactis* is a *GRAS* (Generally Recognized as Safe) organism by the American Food and Drug Administration.

Kl-β-Gal (P00723) is encoded by the gene *LAC4* (Gene ID: 2897170). Although the gene was sequenced in 1992 (Poch *et al.* 1992), the structure of

the protein has not yet been reported. The molecular weight of the Kl-β-Gal monomer is 119 kDa and it was shown that only the dimeric and tetrameric forms of the protein are active, with the tetramer being more active than the dimer (Becerra *et al.* 1998).

On the basis of their sequence, β-galactosidases are classified in CAZy (Cantarel *et al.* 2009) within families 1, 2, 35 and 42 of glycosyl hydrolases. Those from eukaryotic organisms are grouped into family 35 with the only exceptions of *K. lactis* and *K. marxianus* β-galactosidases (99% identitical), which belong to the family 2 together with the prokaryotic β-galactosidases from *Escherichia coli* and *Arthrobacter sp.* Whereas the structures of these last two prokaryotic enzymes have been determined (Juers *et al.* 2000; Skálová *et al.* 2005), none of the yeast β-galactosidases structures has been reported to date. Although their sequence similarity with the prokaryotic enzymes is significant (48% vs. *E. coli* and 47% vs. *Arthrobacter*) there are many differences, particularly some long insertions and deletions, which might play an important role in protein stability and in substrate recognition and specificity. Knowledge of the Kl-β-Gal three-dimensional structure will provide an important insight into understanding of the mechanisms of catalysis and should lead to improvements of its biotechnological applications by rational protein engineering. In this study, we describe expression, purification and preliminary X-ray crystallographic studies of Kl-β-Gal.

MATERIAL AND METHODS

Expression and purification

The gene *LAC4* (Gene ID: 2897170) was amplified by PCR from the pLX8 plasmid, and cloned in the YEpFLAG vector (*Eastman Kodak Company)* as previously reported (Becerra *et al.*, 2001) The construct was used to transform *S. cerevisiae* BJ3505 cells (*Eastman Kodak Company*) by the procedure

of Ito *et al.* (Ito *et al.*, 1983). Cells were incubated at 303 K and 250 r.p.m. during 96 h in a 2 L Erlenmeyer flask containing 1 L of YPHSM modified medium [1% (w/v) Glucose, 3% (v/v) Glycerol, 1% (w/v) Yeast Extract and 8% (w/v) Peptone]; these conditions increased the protein expression. Cells were collected by centrifugation (5000 x g for 10 min at 274 K), resuspended in 0.1 M KH_2PO_4, 1.2 M sorbitol and incubated at 30ºC during 3 h with lyticase (2 mg per g wet weight) in order to obtain the protoplasts (Jigami *et al.* 1986), from which the protein extracts were prepared as described previously (Becerra *et al.* 1998). Kl-β-Gal was purified with *ANTI-FLAG M2* affinity gel (*Sigma Chemical, USA*) and concentrated with *ULTRA-4* (*Millipore, UFC 803024*) as already reported (Rodríguez *et al.* 2008). The purified protein, with the FLAG peptide (SDYKDDDDK) attached to its N- terminus, was concentrated to 7 mg mL^{-1} in 0.05 M Tris-HCl, 0.150 M NaCl and 0.002 M DTT. The homogeneity of the purified protein sample was analyzed (Fig.1) by SDS-PAGE (Laemmli 1970).

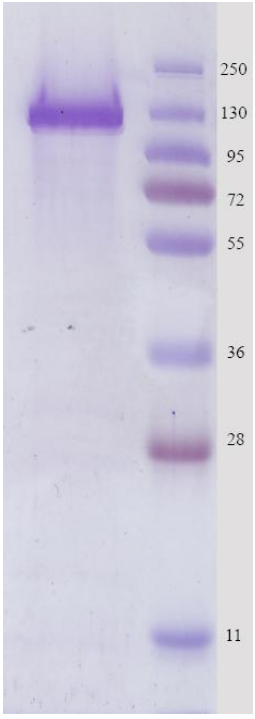

Figure 1: SDS-PAGE analysis of the purified sample of β-galactosidase (119 kDa).

Crystallization

Crystallization conditions were initially explored using high throughput techniques with a NanoDrop robot (*Innovadine Nanodrop I*) and commercially available screens. *Crystal Screen, Crystal Screen II*, Crystal Screen *Lite*, *Salt Rx* and *Index Screen* from *Hampton Research*, *PACT Suite* and *JCSG+ Suite* from *Qiagen* and *Screen Classic* from *Jena Biosciences* were assayed using the sitting drop vapour diffusion method at 291 K. Drops consisting of 0.25 μL of precipitant and 0.25 μL of pure Kl-β-Gal (3.5 mg mL^{-1} in 0.05 M Tris-HCl, 0.150 M NaCl and 0.002 M DTT) were equilibrated against 80 μL of reservoir solution on sitting drop microplates (*Innovaplate SD-2*). Small crystals grew with the *PACT Suite* screen under several conditions when 7% of glycerol (v/v) was added to the protein stock solution prior to the experiment set up. Initial hits were then tested on *Cryschem (Hampton Research)* sitting drop plates by mixing 1 μL of protein with 1 μL of precipitant solution and equilibrating against 500 μL of reservoir solution. Crystallization trials in the presence of agarose were performed adding 0.2 % agarose to the drop using a preheated stock of 0.4 % agarose in the precipitant solution. Crystallization conditions were optimized further and small plate-shaped crystals grew in 23-27% (w/v) Polyethylene Glycol (PEG) 3350, 0.1 M BisTris pH 7.5, 0.2 M Sodium Tartrate. Streak seeding (Stura and Wilson 1991) performed under these conditions gave better quality crystals that were suitable for X-ray diffraction experiments (Fig. 2).

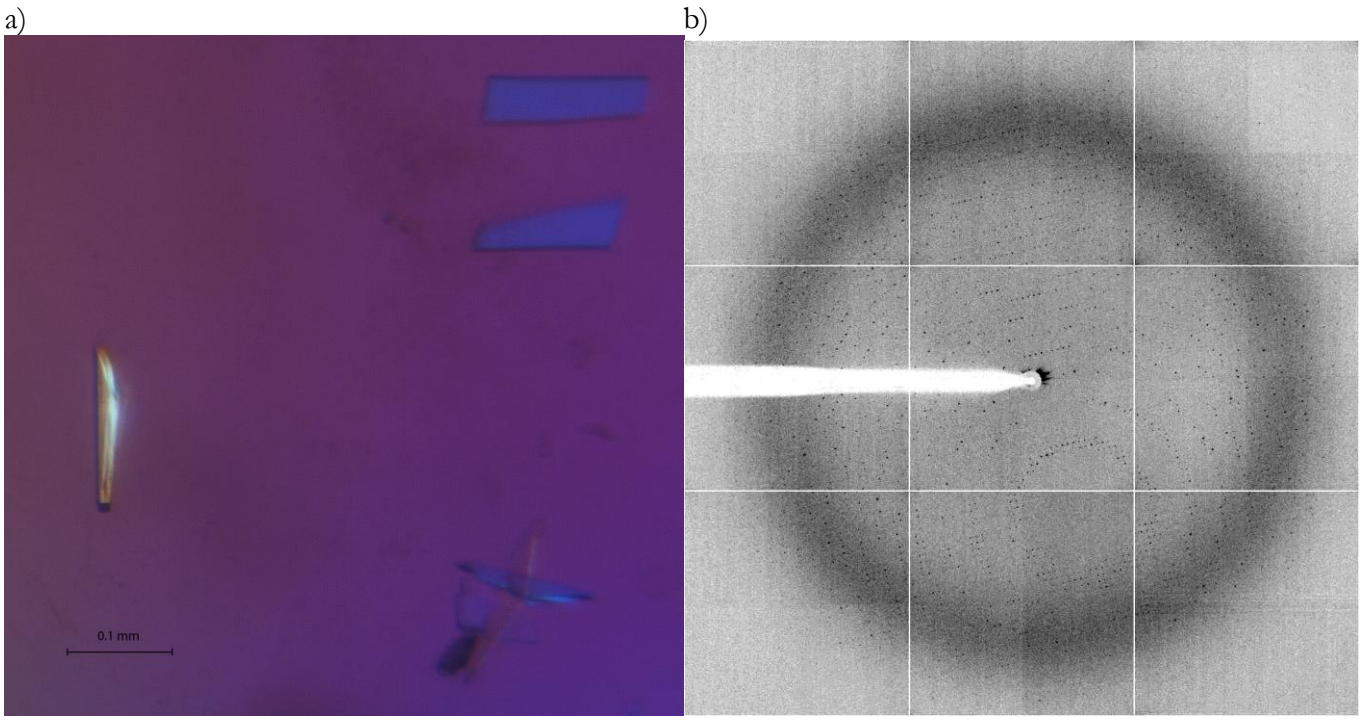

Figure 2: (a) Crystal of β-galactosidase grown in 23-27% (w/v) PEG 3350, 0.1 M BisTris pH 7.5 and 0.2 M Sodium Tartrate by streak-seeding from previous crystals. (b) X-ray diffraction pattern using synchrotron source. Outer resolution shell is 2.8 Å (2.0Å at the edge of the detector).

X-ray data collection and processing

All crystals were cryoprotected before being flash-cooled to 100 K in liquid nitrogen. Mother liquor was substituted by cryoprotectant solution consisting of the crystallization solution containing 20% (v/v) glycerol. Diffraction data were collected using synchrotron radiation at the *European Synchrotron Radiation Facility* (*ESRF*, Grenoble) on ID23-1 beamline using an *ADSC Quantum Q315r* detector fixed at 415.9 mm and a wavelength of 0.979 Å. Exposure time was set to 0.3 seconds and oscillation range to 0.5 degrees per image. Collected diffraction data were processed with *MOSFLM* (Leslie 1992) and scaled using the *CCP4* package (Collaborative Computational Project 1994).

RESULTS AND DISCUSSION

Previous experiments carried out in our laboratory with a full-factorial experimental design (Rodríguez *et al.* 2008) allowed us to grow Kl-β-Gal crystals, but of rather poor diffraction quality. Trials of different modifications in protein preparation led to the improvement of purity of the protein sample. For example, although different methods for the cell disruption have been tried, protein crystals were grown only from the material obtained from the lyticase pre-treated cells. Initial protein concentration in the crystallizations was 7 mg mL^{-1} but, after persisting protein precipitation, 7% (v/v) of glycerol was added to the protein samples and protein concentration was reduced to 3.5 mg mL^{-1} in order to increase its solubility. Repetitions of the some screening tests yielded small plate-like crystals. They grew within two weeks in several PACT Suite screen conditions with PEG 3350 as the main precipitant agent and pH between 6.5 and 8. Subsequently more than a thousand optimization trials were performed, varying pH, PEGs types/concentrations, and different salts. Clusters of medium-size crystals grew in PEG 3350 in the range 23-27% (w/v), 0.1 M BisTris pH 7.5 and 0.2 M Sodium Tartrate. Diffraction of these crystals was too weak hence further improvement of the crystallization conditions was necessary. Neither additives (*Additive Screen* from *Hampton Research*) nor different experiment set up (hanging drop, microbatch and agarose-containing drops) improved the crystal growth, however a crystal streak seeding proved to be crucial for obtaining diffraction quality crystals (Fig. 2).

More than 30 crystals were tested until a full data set from a single Kl-β-Gal crystal was collected to 2.8 Å resolution (Fig. 2). Data processing showed that the crystal belonged to the orthorhombic crystal system with unit cell parameters were a = 139.97 Å, b = 153.40 Å, and c = 216.30 Å. The

analysis of the systematic absences along h00, 0k0 and 00l indicated the space group as $P2_12_12_1$. Data collection statistics are summarized in Table 1.

Analysis of the Matthews coefficient (Matthews 1968), assuming either a dimer or a tetramer in the asymmetric unit, showed a solvent content of 74.91% and 49.83% and the V_m coefficient of 4.90 and 2.45 $Å^3$ Da^{-1}, respectively. The self-rotation functions calculated using *POLARRFN* (Kabsch *et al.* 1976) from the CCP4 package, with Patterson vectors up to a radius of 62 Å and resolution limits within 50-5 Å showed two peaks in the K=180º section of its stereographic projection (Fig. 3), revealing non-crystallographic two-fold symmetry that could be compatible with the tetrameric state of the protein. Structure determination was carried out using the structure of *Arthrobacter sp.* β-galactosidase (PDB code 1YQ2) (Skálová *et al.* 2005) as the template for creating the model for the molecular replacement using the program *Chainsaw* (Stein, 2008) within the CCP4 suite and the *LAC4* sequence (non-conserved residues were pruned to the gamma atom). Sequence homology between these two proteins is 47% (32% identity). Molecular replacement was performed with the program *MOLREP* (Vagin and Teplyakov 1997) using reflections within the resolution range 30 - 2.8 Å and a radius of Patterson vectors of 62 Å. A single solution containing 4 monomers in the asymmetric unit with a final correlation coefficient of 0.27 and an *R* factor of 0.55 was obtained, confirming a tetrameric quaternary structure of this enzyme. Refinement and building of the Kl-β-Gal model, which contains as much as 4000 amino acid residues, is in progress.

Table 1
Data-collection statistics
Values in parentheses are for the outer resolution shell

Crystal data	
Space group	$P2_12_12_1$
Unit cell parameters	
a (Å)	139.97
b (Å)	153.40
c (Å)	216.30
Data collection	
Beamline	ID23.1, ESRF
Temperature (K)	100
Wavelength (Å)	0.979
Resolution (Å)	125.00 - 2.80 (2.95 – 2.80)
Data processing	
Total reflections	643048 (91525)
Unique reflections	115027 (16572)
Redundancy	5.6 (5.5)
Completeness (%)	100.0 (99.9)
I/σ (I)	7.3 (2.3)
Mean I/σ (I)	15.9 (5.2)
R_{merge} [†] (%)	10.7 (35.7)
R_{pim} [††] (%)	4.9 (16.4)
Molecules per ASU	4
Matthews coefficient (Å^3 Da^{-1})	2.45
Solvent content (%)	49.83

[†] $R_{merge} = \sum_{hkl} \sum_i | I_i(hkl) - \langle I(hkl)\rangle | / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the weighted mean of all measurements.
[††] $R_{pim} = \sum_{hkl} [1/(N - 1)]^{1/2} \sum_i | I_i(hkl) - \langle I(hkl)\rangle | / \sum_{hkl} \sum_I$, where N is the redundancy for the hkl reflection.

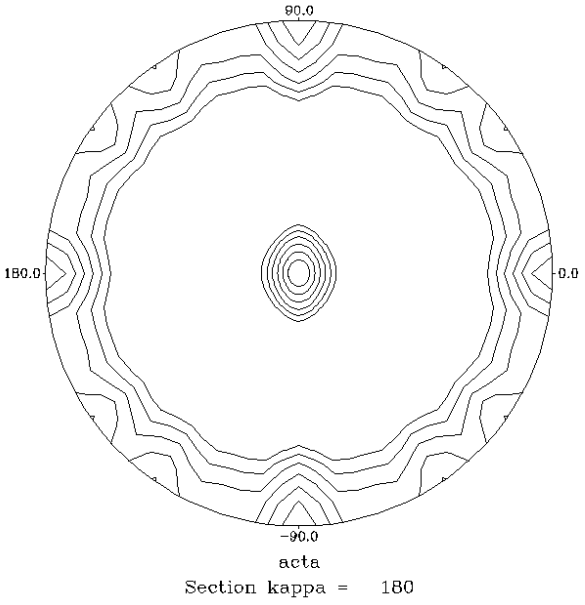

Figure 3: Plot of the self-rotation function of the β-galactosidase Patterson function using data between 50 and 5 Å and a 62 Å radius of integration in the K=180° section. The view is down the *c* axis. ϕ=0˚ and ϕ=90˚ correspond to the *a* and *b* axes, respectively.

Acknowledgements

RFL received a FPU fellowship from Ministerio de Educación y Ciencia. APR received a María Barbeito fellowship from Xunta de Galicia. Research at (1) was supported by grant 07TAL010103PR from Xunta de Galicia co-financed by FEDER (CEE). General support to the lab during 2008-09 was funded by "Programa de axudas para a consolidación e a estruturación de unidades de investigación competitivas da Consellería de Educación e Ordenación Universitaria (Xunta de Galicia). Research at (2) was supported by grant BIO2007-67708-C04-04 from Dirección General de Investigación. This is a product from the project "Factoría de Cristalización", from the program Consolider-Ingenio 2010.

CHAPTER 3

Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase

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SUMMARY

β-Galactosidase or lactase is a very important enzyme in the food industry, being that from the yeast *Kluyveromyces lactis* the most widely used. Here we report its three-dimensional structure both in the free state and complexed with the product galactose. The monomer folds into five domains in a pattern conserved with the prokaryote enzymes of the GH2 family, although two long insertions in domains 2 and 3 are unique and related to oligomerization and specificity. The tetrameric enzyme is a dimer of dimers, with higher dissociation energy for the dimers than for its assembly. Two active centers are located at the interface within each dimer in a narrow channel. The insertion at domain 3 protrudes into this channel and makes putative links with the aglycone moiety of docked lactose. In spite of common structural features related to function, the determinants of the reaction mechanism proposed for *Escherichia coli* β-galactosidase are not found in the active site of the *K. lactis* enzyme. This is the first X-ray crystal structure for a β-galactosidase used in food processing.

INTRODUCTION

β-D-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23), most commonly known as lactase, is one of the most important enzymes used in food processing that catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. Conventionally, its main application has been in the hydrolysis of lactose in milk or derived products, particularly cheese whey. Lactose is a disaccharide formed by glucose and galactose that is found in milk. In humans, lactose intolerance or unabsorbed lactose is a common problem. In fact, it is estimated that lactose intolerance occurs in 70% of the world's adult population, and Eastern Asia has the highest number of lactose malabsorbers with more than 90% of its population (Husain 2010). Lactose maldigestion and intolerance are caused by lactase insufficiency or

non-persistence, which results from a decrease in the activity of the β-galactosidase, in the brush border membrane of the mucosa of the small intestine of adults (Juajun *et al.* 2011). In this case lactose cannot be hydrolyzed, and passes into the large intestine, where it is fermented by colonic microflora causing symptoms such as abdominal pain, gas, nausea and diarrhoea (Husain 2010). The extent of these symptoms is variable and indeed most individuals can tolerate a moderate amount of lactose in their diet (Lifran *et al.* 2000). Nevertheless, there is a considerable market for lactose-free milk and dairy products, which can be obtained by enzymatic hydrolysis using β-galactosidases (Oliveira *et al.* 2011).

Besides lactose maldigestion, crystallization of lactose can be a problem in dairy products such as ice cream and sweetened condensed milk. β-galactosidases derived from food grade organisms can be successfully employed for these problems related to the milk sugar lactose (Juajun *et al.* 2011). The products of lactose hydrolysis, i.e., glucose and galactose, are sweeter and also much more soluble than lactose; hence, sandy defects in dairy products can be avoided (Ganzle and Haase 2008).

Furthermore, disposal of large quantities of the lactose-containing by-products from cheese manufacturing, whey and whey permeates, causes serious environmental problems. It is estimated that approximately 160 million tons of whey are producing worldwide each year (Guimarães *et al.* 2010). Whey's organic load is high (biochemical oxygen demand of 30–50 g/L and chemical oxygen demand of 60–80 g/L), mainly because of the lactose content, which together with the high volumes to which it is generated makes cheese whey a quite concerning environmental issue, and solutions for its valorization are strongly required (Guimarães *et al.* 2010). Whey can be used as a source of cheap, renewable, and fermentable sugars after β-galactosidase-

catalyzed hydrolysis for the production of added-value molecules or bulk commodities by lactose-negative microbes (Oliveira *et al.* 2011).

Apart from lactose hydrolysis, β-galactosidases with transgalactosylation activities are highly attractive for the production of added-value lactose derivatives. In particular, galacto-oligosaccharides (GOS), prebiotics that can stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli, are increasingly finding application in functional foods, namely as low calorie sweeteners in fermented milk products, confectioneries, breads and beverages (Ganzle and Haase 2008; Gosling *et al.* 2010; Park and Oh 2010).

Many organisms naturally synthesize β-galactosidase, including animals, plants, and microorganisms, but an easier manipulation and acceptable productivities and yields from cultivations of the latter have favoured their establishment as a main source for industrial production of β-galactosidases. Although bacteria could offer more versatility, the corroborated GRAS status of yeasts like *Kluyveromyces lactis* and *K. marxianus*, and of fungi like *Aspergillus niger* and *A. oryzae*, still places them among the favourite sources of β-galactosidase for food biotechnology and pharmaceutical industry (Rubio-Texeira 2006).

β-galactosidase sequences can be deduced from various databases, and these can be classified into four different glycoside hydrolase (GH) families 1, 2, 35, and 42, based on functional similarities (Cantarel *et al.* 2009). Those from eukaryotic organisms are grouped into family 35 with the exceptions of *K. lactis* and *K. marxianus* β-galactosidases (99% identity), which belong to the family 2 together with the prokaryotic β-galactosidases from *Escherichia coli* and *Arthrobacter sp.* Whereas the structures of these last two prokaryotic enzymes have been determined (Juers *et al.* 2000; Skálová *et al.* 2005), none of the eukaryotic β-galactosidase structures has been reported. In fact, to date, the X-

ray crystal structures of eight different microbial β-galactosidases are available in the PDB, although none of the enzymes with solved structures is known to be used in food processing.

In this paper, we report the three-dimensional structure at 2.75 Å resolution and the complex structure with galactose at 2.8 Å resolution of the β-galactosidase from *Kluyveromyces lactis*, one of the most important and widely used enzymes of the food industry.

MATERIAL AND METHODS

Cloning, expression and purification

Cloning, expression and purification of *Kluyveromyces lactis* β-galactosidase (KL-β-Gal) was performed as described previously (Pereira-Rodríguez *et al.* 2010).

Crystallization and data collection

Crystallization of KL-β-Gal (3.5 mg mL-1 in 0.05 M Tris-HCl, 0.150 M NaCl and 0.002 M DTT, 7% Glycerol) was performed on Cryschem (Hampton Research) sitting drop plates at 291 K as described previously (Pereira-Rodríguez *et al.* 2010). Small plate-shaped crystals grew in 23-27% (w/v) Polyethylene Glycol (PEG) 3350, 0.1 M BisTris pH 7.5-7.0, 0.2 M Sodium Tartrate. Streak seeding (STURA and WILSON 1991) performed under these conditions gave improved quality crystals that were suitable for X-ray diffraction experiments. Crystals of KL-β-Gal belonged to $P2_12_12_1$ space-group with four molecules in the asymmetric unit and 51% solvent content within the unit cell. For data collection, native crystals were transferred to cryoprotectant solutions consisting of mother liquor plus 20% (v/v) glycerol before being cooled to 100 K in liquid nitrogen. The complex with the

product galactose was obtained by crystal soaking with the substrate lactose (Hassell *et al.* 2007). In order to minimize crystal damage, mother-liquor was substituted by the soaking solution (35% PEG 3350, 0.1 M BisTris pH 7.0, 0.2 M Sodium Tartrate, 2 mM MgCl2) saturated with lactose, incubated for 6 minutes and then cryocooled in liquid nitrogen.

Diffraction data were collected using synchrotron radiation at the European Synchrotron Radiation Facility (ESRF, Grenoble) on ID23.1 and ID14.4 beamlines. Diffraction images were processed with MOSFLM (Leslie 1992) and merged using the CCP4 package (Collaborative Computational Project 1994). A summary of data collection and data reduction statistics is shown in Table 1.

Structure solution and refinement

The structure of KL-β-Gal was solved by molecular replacement using the MOLREP program (Vagin and Teplyakov 1997). The structure of *Arthrobacter sp.* β-galactosidase (PDB code 1YQ2) (Skálová *et al.* 2005) was used to prepare the search model using the program Chainsaw (Stein 2008) and a protein sequence alignment of KL-β-Gal onto *Arthrobacter* β-galactosidase. A single solution containing four molecules in the asymmetric unit was found using reflections within 125 - 3.43 Å resolution range and a Patterson radius of 31 Å, which after rigid body fitting led to an R factor of 51 %. Crystallographic refinement was performed using the program Refmac5 (Murshudov *et al.* 1997) within the CCP4 suite with flat bulk-solvent correction, and using maximum likelihood target features. Tight non-crystallographic symmetry restrictions were applied during first steps of refinement. Loop 246-274, which is ordered in molecules A and C and disordered in molecules B and C (more details in results discussion), and other small regions (as the last portion of the linker between domain 4 and 5), were excluded from the NCS restraints during model building, but best results were

achieved when keeping NCS restrictions for the whole molecule in the last steps of refinement. Free R-factor was calculated using a subset of 5% randomly selected structure-factor amplitudes that were excluded from automated refinement. Several loops in different regions were excluded from the model during the first stages of the refinement since no electron density was observed at the polypeptide chain. After iterative refinement and rebuilding of these regions using the programs O (Jones *et al.* 1991), Buccaneer (Cowtan 2006) and COOT (Emsley and Cowtan 2004), the final 2Fo-Fc map showed continuous density for the whole molecule. As it will be discussed below, some regions in molecules B and C are more disordered than in molecules A and B due to specific interactions in the tetramer. At the latter stages, water molecules, glycerol molecules and metal atoms were included in the model, which, combined with more rounds of restrained refinement, led to a final *R*-factor of 20.7 (R_{free} = 24.4) for all data set up to 2.75 Å resolution. The structure of the complex with galactose was solved by molecular replacement with the native model and refinement was performed as described above. The substrate molecules were manually built into the electron density map, imported to the model and included in the refinement. Refinement with Refmac5 of the galactose-KL-β-Gal complex up to 2.8 Å led to a final *R*-factor of 21.4 (R_{free} = 24.6) at 2.8 Å resolution. Refinement parameters for both structures are reported in Table 1.

Stereochemistry of the models was checked with PROCHECK (Laskowski *et al.* 1993) and MOLPROBITY (Chen *et al.* 2010), while topology assignment by has been analysed by the Protein Families database (PFAM, Finn *et al.* 2010). The figures were generated with PyMOL (DeLano 2002). Analysis of the interfacial surfaces and the oligomer stability was done with the Protein Interfaces, Surfaces and Assemblies service (PISA) at the European Bioinformatics Institute (Krissinel and Henrick 2007). RMS

deviation analysis where made using the program SUPERPOSE within the CCP4 package (Collaborative Computational Project 1994)

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A ultracentrifuge using a Ti50 rotor and six channel centerpieces of Epon-charcoal (optical pathlength 12 mm). Samples of purified KL-β-Gal in the concentration range 0.2 - 0.5 mg ml-1 were equilibrated against 2 mM Tris-HCl pH 7.4, 15 mM NaCl. Samples were centrifuged at 6000, 9000 and 11000 r.p.m. at 293 K. Radial scans at 280 nm were taken at 12, 14 and 16 hours. The three scans were identical (equilibrium conditions were reached). The weight-average molecular mass (M_w) was determined by using the program EQASSOC with the partial specific volume of KL-β-Gal set to 0,73 at 293 K as calculated from its amino acid composition.

PDB accession codes

Model coordinates and structure factors data have been deposited in the Protein Data Bank. Accession codes for the native and complex structures 3OBA and 3OB8 respectively.

Table 1. Crystallographic statistics
Values in parentheses are for the high resolution shell

Crystal data	KL-β-Gal	KL-β-Gal - galactose
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell parameters		
a (Å)	140.030	140.381
b (Å)	153.340	153.454
c (Å)	216.160	217.166
Data collection		
Beamline	ID23.1 (ESRF)	ID14.4 (ESRF)
Temperature (K)	100	100
Wavelength (Å)	0.979	0.939
Resolution (Å)	62.53 - 2.75 (2.90 – 2.75)	49.30 – 2.80 (2.95 – 2.80)
Data processing		
Total reflections	874,614 (123,972)	1,379,068 (193,533)
Unique reflections	121,272 (17,499)	115,849 (16,726)
Multiplicity	7.2 (7.1)	11.9 (11.6)
Completeness (%)	100.0 (100.0)	100.0 (100.0)
I/σ (I)	4.3 (1.4)	7.7 (1.9)
Mean I/σ (I)	10.7 (3.6)	24.5 (6.7)
R_{merge}† (%)	17.2 (53.6)	9.9 (43.1)
R_{pim}†† (%)	6.8 (21.5)	3.0 (13.2)
Molecules per ASU	4	4
Matthews coef. ($Å^3$ Da^{-1})	2.5	2.5
Solvent content (%)	51%	51%
Refinement		
R_{work} / R_{free}††† (%)	20.7 / 24.4	21.4 / 24.6
No. of atoms		
Protein	33300	33300
Carbohydrate	60	48
Refinement	4	24
Water Molecules	1666	1047
Ramachandran (Chen *et al.* 2010)		
Favoured (%)	95.7	95.5
Outliers (%)	0.10	0.00
RMS deviations		
Bonds (Å)	0.008	0.009
Angles (deg.)	1.108	1.131
Protein Data Bank codes	3OBA	3OB8

†R_{merge} = $\sum_{hkl} \sum_i$ | $I_i(hkl)$ – [I(hkl)]| / $\sum_{hkl} \sum_i$ $I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and [I(hkl)] is the weighted mean of all measurements.
††R_{pim} = $\sum_{hkl}$ [1/(N - 1)] 1/2 $\sum_i$ | $I_i(hkl)$ – [I(hkl)]| / $\sum_{hkl} \sum_i$ $I_i(hkl)$, where N is the redundancy for the hkl reflection.
†††R_{work} / R_{free} = $\sum_{hkl}$ | Fo – Fc | / $\sum_{hkl}$ | Fo |, where Fc is the calculated and Fo is the observed structure factor amplitude of reflection hkl for the working / free (5%) set, respectively.

RESULTS AND DISCUSSION

As previously reported (Pereira-Rodríguez *et al.* 2008; Pereira-Rodríguez *et al.* 2010), we have purified and crystallized the *Kluyveromyces lactis* β-galactosidase (KL-β-Gal). The details of crystallization conditions have been given before (Pereira-Rodríguez *et al.* 2008; Pereira-Rodríguez *et al.* 2010). The structure of KL-β-Gal has been determined to 2.75 Å and 2.8 Å resolution, respectively, for the native crystal and its complex with galactose. Experimental and structure determination details are given in Materials and Methods and in Table 1. KL-β-Gal forms a homo-oligomer of four subunits that can be described as a dimer of dimers as it will be discussed below. Each chain (A-B-C-D) consists of 1024 residues with a molecular mass of 119 kDa as calculated from its primary structure. The first nine residues, which correspond to Ser 1 and the eight amino acids from the purification FLAG tag, are missing in the model and probably disordered. The imposition of tight non-crystallographic symmetry during refinement leads to a final model with four identical subunits. However, there are some regions that exhibit poor electron density. This is possibly due to weaker packing interactions in those regions within two of the monomers, which make some loops more exposed to the solvent and consequently more flexible, as it will be discussed. Soaking with the natural substrate lactose was done in an attempt to capture the substrate in the catalytic pocket. However, the high activity that this protein shows at the crystallization pH only allowed us to capture de product galactose. Directed mutagenesis on one of the catalytic residues or the use of substrate analogues should be explored in order to achieve this goal. Nevertheless, some insights into substrate recognition can be done on the basis of comparison with the extensive work made on the *E. coli* β-galactosidase (EC-β-Gal) (Roth and Huber 1996; Roth *et al.* 1998; Juers *et al.* 2000; Juers *et al.* 2001; Huber *et al.* 2003; Juers *et al.* 2003; Juers *et al.* 2009; Dugdale *et al.* 2010; Lo *et al.* 2010).

The fold of the monomer

Topology assignment shows that KL-β-Gal subunit follows the pattern previously described for the two known β-galactosidases, and folds into 5 domains (Figure 1), only one with assigned catalytic function. Domain 1 (residues 32 to 204) presents a jellyroll fold and it is classified as a Glycosyl Hydrolase (GH) family 2 sugar binding domain. Domains 2 (residues 205-332) and 4 (residues 643-720) form two GH family 2 immunoglobulin-like β-sandwich domains. Domain 3 (residues 333-642) folds into a GH family 2 TIM barrel domain harbouring the catalytic pocket and domain 5 (residues 741-1025) is classified as a β-galactosidase small chain. There are two extended regions of the protein that cannot be assigned to any of the domains. One is the N-terminal region (residues 2-31) and the other is a small solvent exposed chain that connects domains 4 and 5 (residues 721-740).

The oligomerization pattern of the tetramer

The *K. lactis* β-galactosidase was found to be tetrameric in the crystal, with the four molecules building up the asymmetric unit. Several studies have reported the presence of two active forms in native electrophoresis analysis of β-galactosidase samples purified from *K.* lactis, which were attributed to the presence of dimers an tetramers (Becerra *et al.* 1998). The fact that the oligomerization pattern observed in the crystal corresponds to a "dimerization of dimers" is consistent with the experimental results. It is significant that the PISA server analysis (Krissinel and Henrick 2007) predicts that the dissociation energy (ΔG^{int}) for this oligomer into two dimers is rather low (6 kcal/mol) when compared with the dissociation energy of the dimers (20 kcal/mol). We have performed preliminary analytical ultracentrifugation analysis and data shows that the average molecular weight corresponds to that of the dimer, under the conditions assayed. Thus, it is feasible that an equilibrium exists between the associated and dissociated dimers, although

more studies need to be carried out to elucidate the conditions that would govern the association equilibrium and its biological implications.

As the model has been refined with tight NCS-restraints, the tetramer is made of four identical subunits A, B, C and D (Fig. 2). Monomers A-C and B-D form two identical dimers. Within each dimer, monomers are related by a NCS twofold axis that brings their catalytic pockets face to face at the interface. Assembling of these dimers occurs essentially through interaction between monomers A and B, although there are also some contacts between monomers A and D, and monomers B and C that help stabilizing the tetramer. Both "dimers" are also related by a NCS twofold symmetry axis. Figure 2 shows the residues that are involved in shaping the different contact surfaces. Surface 1 is identical within monomers A-C and B-D, with a total of 2521 $Å^2$ of surface area buried in each interface. Surface 2 (2438 $Å^2$) is present between monomers A and B making most of the contacts that stabilize the tetramer. There is a third small contact surface (350 $Å^2$) made up by contacts between molecules A-D and B-C that might further stabilize the tetramer. Upon formation of the tetramer, the total surface area is reduced by 11%.

Most of the contacts in the interfaces are non-polar interactions (~75% for surface 1 and ~65% for surfaces 2 and 3). Contact surface 1 is equivalent between monomers A-C and B-D and it involves residues from domains 1, 3 and 5. This surface is responsible for the stabilization of the two identical dimers A-C and B-D. Contact surface 2 is present only in monomers A and C and stabilizes the assembly of the dimers (tetramerization). Contacts in this interface are from domains 1, 2, 4, 5 and one insertion in loop 8 of the catalytic domain (domain 3). The small surface between molecules A-D and B-C is made up from residues from domain 5 in molecules A and B that are making contacts with residues from domain 1 and the insertion in loop 8 of the catalytic domain of molecules D and C. Although surface 1 and 2 are

similar in terms of buried surface area (~2500 Å^2) and in the number of polar links between the residues that build the interfaces, the stability of the assemblies seems to be different. As mentioned above, the dissociation energy calculated for the dimers is ~20 kcal/mol, while for the assembly of dimers is ~6 kcal/mol. The large number of non-polar interactions and the presence of several main chain hydrogen bonds in surface 1 could be accounting for this difference in stability.

The active site

On the basis of sequence alignment, we can identify the catalytic residues in KL-β-Gal as Glu482 and Glu551. These residues are located in a pocket found at one side of the TIM barrel domain, in the centre of each monomer. The catalytic pocket is surrounded by residues from domains 1, 3 and 5 that shape a very narrow cavity about 20 Å deep (see Figure 1c). Moreover, dimerization buries them even more, as both cavities are located face-to-face within the interface (see Figure 2a). This arrangement, together with one insertion in the catalytic domain 3 (residues 420-443) that folds over the entrance in each monomer, make the pockets accessible from the exterior through a narrow slot of no more than 10 Å width. On the other hand, the disposition and the distance between both active sites do not suggest any interaction between them.

Ligand binding

The catalytic pocket was filled with water molecules in the apo-structure whereas in the galactose complex structure, one magnesium and two sodium ions are located at the active site. The magnesium and one of the sodium ions (Na1) were found close to the galactose ring (Figure 3a), resembling the metal binding scheme of EC-β-Gal catalytic site (Juers et al., 2009). A second sodium ion, Na2, also identified in the *Arthrobacter s*tructure (AR-β-Gal), was found filling a gap left by the shorter side chain of residue

Trp190 in KL-β-Gal, which is an arginine in EC-β-Gal. On the other hand, the galactose ring presents orientation and main contacts with surrounding residues conserved through the three structures.

KL-β-Gal presents a manganesium (Figure 3b), not found in the other two structures, coordinated by residues from the insertion (590-605) at loop 8 of the catalytic domain (Asp593) and from one loop from the fifth domain (His975 and Asp978). The strong anomalous signal observed at the wavelength of data collection (0.98, 0.94 Å for the native and the complex), the coordination geometry and the chemical nature of its ligands (two bidentate Asp and one His, completed with a water molecule visible only in two of the monomers) led us to assign this peak to a manganese ion. There is an additional sodium ion, Na3, common to EC-β-Gal, coordinating also to residues from this area. In the case of KL-β-Gal, both ligands may be important for folding stability as these loops are building up part of oligomerization interfaces 2 and 3 and, therefore, are shaping the dimer-dimer interface. This putative structural role in assembling the tetramer may explain the stimulatory effect on KL-β-Gal activity observed in the presentce of Mn^{2+}, previously reported (Pereira-Rodríguez *et al.*, 2006).

Three molecules of glycerol, added as part of the cryoprotectant solution, were found in the apo-crystals and a fourth sodium ion, Na4, was found in the complex structure bound to backbone carbonyls and water molecules. This sodium atom is also in the same position in EC-β-Gal crystals.

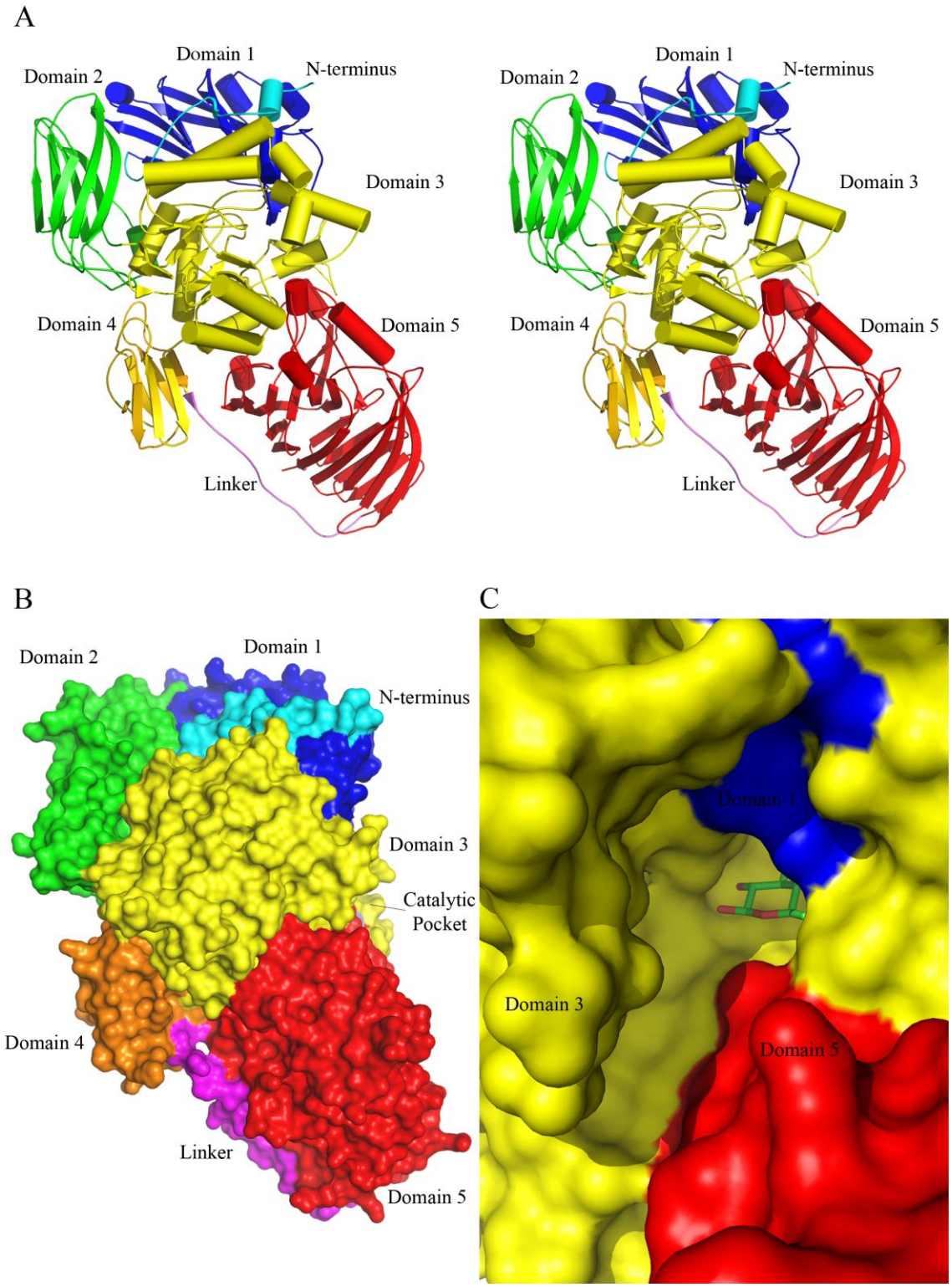

Figure 1. (a) Stereo view of KL-β-Gal monomer in cartoon representation. Domains are represented in different colours. N-terminal region (cyan), domain 1 (blue), domain 2 (green), domain 3 (yellow), domain 4 (orange), linker (magenta) and domain 5 (red). (b) Surface representation of the monomer with coloured domains following the same scheme. (c) Zoomed view of the catalytic pocket entrance. Residues from domains 1, 5 and, mostly, 3 are building up the pocket entrance. A galactose bound to the active site is shown in stick representation.

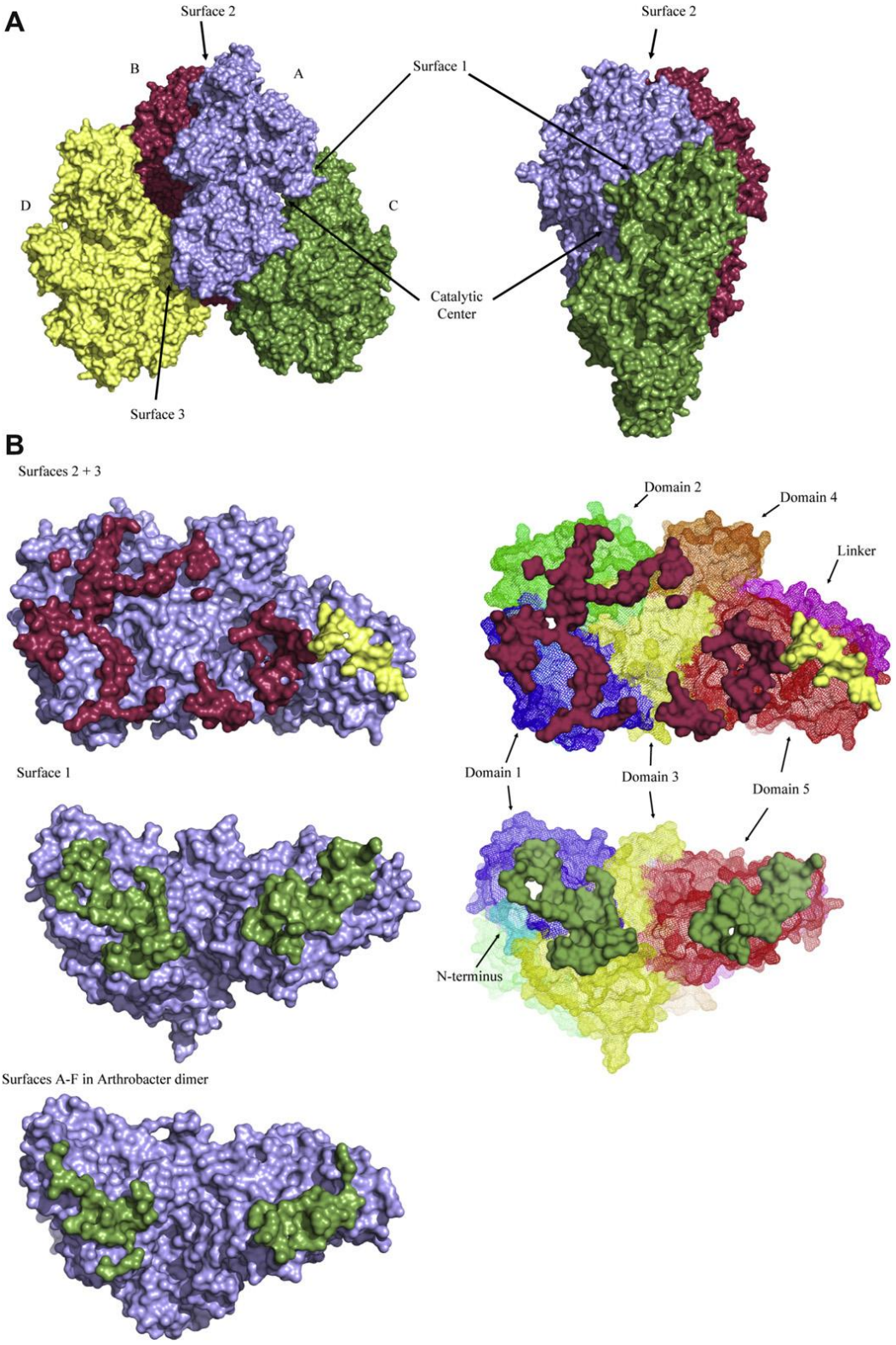

Figure 2. (a) Surface representation of the KL-β-Gal tetramer. Chain A is shown in blue, B in green, C in red and D in yellow. The three different interfaces between monomers are labelled as Surface 1 (A-C), Surface 2 (A-B) and Surface 3 (A-D). (b) Surface representation of KL-β-Gal monomer (upper and medium panels) showing the residues of each interface in the colour of the contiguous molecule following the previous colour scheme (left) and the domains coloured as in Figure 1 (right). Lower panel showing the A-F interface of AR-β-Gal hexamer, similar to surface 1 in KL-β-Gal.

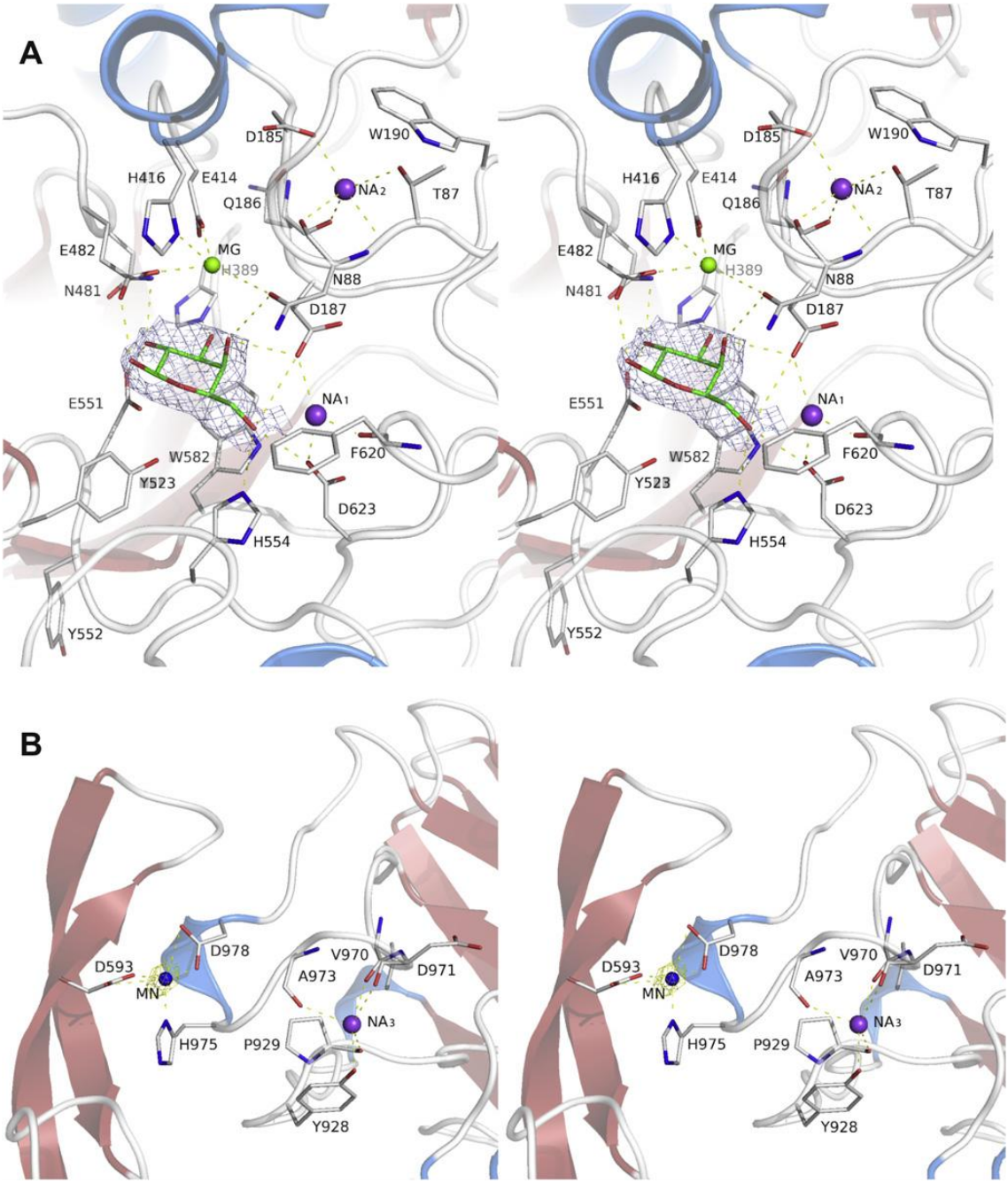

Figure 3. (a) Stereo view of KL-β-Gal catalytic pocket. Residues interacting with the galactose, magnesium (green sphere) and sodium (purple spheres) ions are in stick representation. The 2Fo–Fc electron density map for the galactose residue contoured at 1r is shown. (b) Coordination of the two ions stabilizing the insertion in loop 8 of the catalytic domain (residues 590–605) and the loop 965–985 from domain 5. This region is part of interfaces 2 and 3. The anomalous electron density map shows a strong peak, contoured at 5 s in the figure, that has been assigned to Mn^{2+}.

Structural comparison with *Escherichia coli* and *Arthrobacter sp.* β-galactosidases

Six β-galactosidase structures have been reported to date, all of them classified within clan A in the CAZy database: the *E. coli* (EC-β-Gal) (Juers et al., 2000) and *Arthrobacter sp.* (isoenzyme C.2.2.1, AR-β-Gal) structures (Skalova *et al.* 2005)from GH2, the GH35 structures from *Hypocrea jecorina* (Maksimainen *et al.* 2011), *Penicillium sp.* (Rojas *et al.* 2004) and *Bacteroides thetaiotaomicron* (no reference) and the structure of *Thermus sp.* β-galactosidase (Hidaka *et al.* 2002) from GH42. Only those from GH2 show high levels of homology with KL-β-Gal (48% for EC-β-Gal and 47% for the AR-β-Gal). The other enzymes only show some similarity at the catalytic domain. Interestingly, KL-β-Gal is one of the few eukaryotic β-galactosidases with this folding scheme. In fact, all the other eukaryotic β-galactosidases, including those from other yeast species, are classified within the GH35 family and they share a common overall folding different from that of GH2 structures. This might be suggesting a differential origin for KL-β-Gal and the rest of the eukaryotic enzymes.

The folding pattern of KL-β-Gal is conserved (Figure 4) with that previously reported for EC-β-Gal and AR-β-Gal (Juers *et al.* 2000; Skalova *et al.* 2005). Global RMS deviation between KL-β-Gal and these two structures is 1.9 Å for AR-β-Gal and 3.2 Å for EC-β-Gal (762 and 756 residue alignment respectively). These global RMS deviations are not explained by differences in sequence or folding but by different domain orientations. As it will be discussed below, there are also local differences that must play important roles in oligomerization and function, mostly insertions and deletions in some loops, but these are not taken into account in the RMS calculation. In fact, looking at the RMS deviations by domain (not shown), the differences between KL-β-Gal and the other two structures are smaller. Structure differences between these three enzymes are summarized in the structural

superposition of the three subunits shown in Figure 4a and also in the structure-bases sequence aligment shown in Figure S1 (Appendix A).

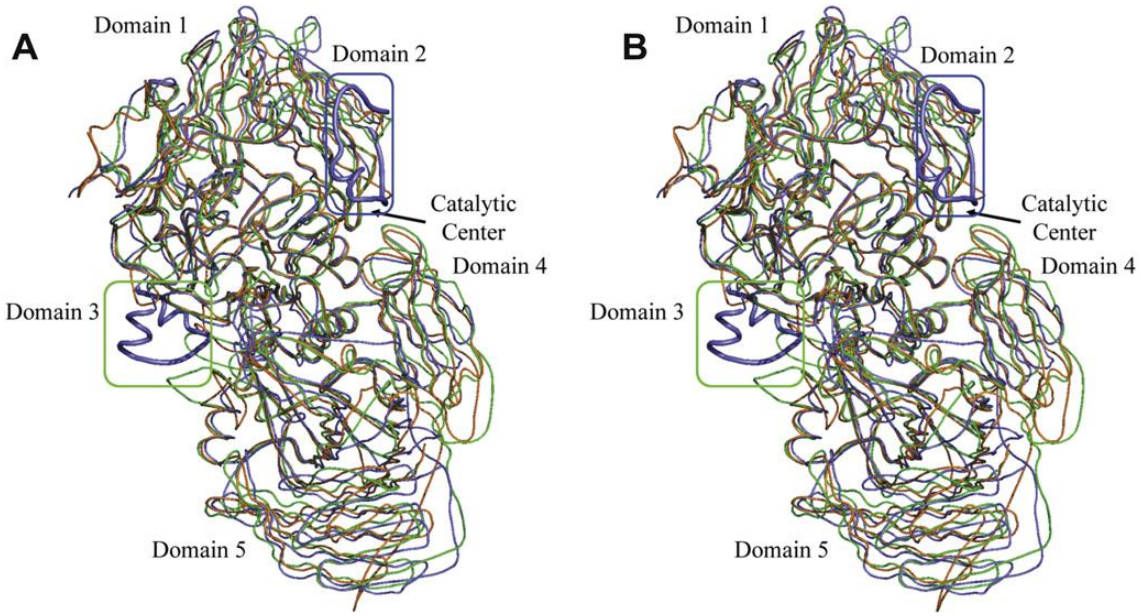

Figure 4. Superimposition of KL-b-Gal (blue), EC-b-Gal (orange) and AR-b-Gal (green) structures. Important insertions in KL-b-Gal are highlighted and domains labeled.

In EC-β-Gal, the N-terminal region is associated with the alpha complementation phenomenon (Juers *et al.* 2000). Such mechanism has not been reported for the KL-β-Gal and, even when we have observed that it is important for protein activity (Becerra *et al.* 2001), no function has been attributed to this region yet. Domain 1 is very similar in all three proteins. Domain 2 differs from the EC-β-Gal domain, where an important insertion (272-288 in EC-β -Gal numbering) emerges from one of the loops and is responsible for some important interactions in the catalytic pocket (Juers *et al.* 2000). This insertion in the prokaryotic enzyme has also been reported to be one of the reasons why this molecule has to be in the form of tetramers to be active (Juers *et al.* 2000). In KL-β-Gal there is one long insertion (246-274, squared in blue in Figure 4) that is contributing to surface 2 (AB) and makes most of the contacts for the assembly of the dimers within the tetramer. This loop is solvent exposed in the other two molecules (C and D), the electron density in that region being quite poor.

Domain 3, the TIM barrel catalytic domain, presents also a long insertion (420-443, squared in green in Figure 4) that fold over the entrance of the catalytic pocket hiding it from the surface. Upon dimerization, this loop makes a channel that makes accessible the catalytic centres of both monomers to the solvent. Moreover, the amino acids in this loop present higher B-factors than the average. Mobility of this region could be one explanation to this high B-factor, and, possibly, this is a requirement to facilitate the binding of substrates to the catalytic pocket. A small insertion also in this domain (599-605) is making interactions with one loop from the fifth domain (965-985). As it was discussed above, the interaction between these two loops is stabilized by a manganesium ion, which is found in both crystals and must be playing a structurally important role. This loop is also part of surface 1 (AC, BD). Domain 4 is clearly smaller in KL-β-Gal when compared to the other two structures, most of the loops and β-sheets being reduced and, also, the long chain that connects domain 4 and 5 shows a different disposition being closer to domain 5. This domain 4 is involved in oligomerization in the EC-β-Gal and AR-β-Gal, while is in the surface in the β-galactosidase tetramer, which may be explaining the smaller size observed in KL-β-Gal. Finally, the fifth domain aligns poorly to both structures (RMS is 1.7 Å and 2 Å for the EC-β-Gal and AR-β-Gal respectively), although it resembles more that of EC-β-Gal.

It is outstanding how proteins with a highly similar folding can have different biochemical characteristics based mostly in a few insertions that modulate oligomerization. The KL-β-Gal assembly of dimers, the EC-β-Gal tetramer and the AR-β-Gal hexamer (described as a dimer of trimers, (Skalova *et al.* 2005) are an interesting example that illustrates this feature). While their overall structure and folding scheme is very similar, small differences in some loops can trigger completely different oligomer arrangements. Monomer interaction surfaces in EC-β-Gal are completely different from those of KL-β-

Gal and AR-β-Gal. On the other hand, contact surface between monomers of different trimers in AR-β-Gal is very similar to Surface 1 in KL-β-Gal (Figure 2), but some differences in other regions lead to completely different oligomers, and, consequently, to a different specificity.

The specificity of KL-β-Gal active site

Although the catalytic pocket of KL-β-Gal (i.e. subsite -1) does not present any substantial change with respect to those of EC-β-Gal and AR-β-Gal, there are some features that define the active site that might be influencing substrate recognition and activity of the enzyme. Catalytic residues are in very similar positions in the three enzymes and the overall shape of the pocket is conserved (Figure 5). Moreover, despite GH35 β-galactosidases showing different overall domain structures, the catalytic domain folds, similarly, into a TIM barrel. This fact reveals common structural features related to function that, nevertheless, are modulated by unique particularities related to specificity.

Many structural studies carried out on the EC-β-Gal have delineated the main features explaining its function, essentially the ability to hydrolyse lactose or allolactose with equal catalytical efficiency, while being only able to produce allolactose by transglycosylation (Juers et al., 2001). This is the natural inducer for the lac operon. Interestingly, the values of K_{cat} for allolactose production are very similar to that for its hydrolysis, this balance being altered by changes in pH and the presence/absence of Mg. Furthermore, through the analysis of different complexes with substrate, intermediate and products, they have proposed a reaction mechanism that involves a movement of the galactosyl moiety from a shallow mode binding (proper of the substrates and the product allolactose) into a deep position (proper of intermediates and the product galactose), in which there is a conformational change in loop 794-804 and in Phe601 position that is stated to be responsible of selecting allolactose

as transglycosylating product. KL-β-Gal, on the contrary, presents a strikingly high hydrolytic activity against lactose but is able to produce 6'galactobiose (Gal-(1,6)-β-D-Gal), allolactose (Gal-(1,6)-β-D-Glc) and the trisaccharide 6'galactosyl-lactose (Gal-(1,6)-β-D-Gal-(1-4)-D-Glc) in high amounts by transglycosylation (Martínez-Villaluenga et al., 2008). The ratio of these products is also altered by temperature and pH changes. This catalytic behavior should be explained on the basis of the KL-β-Gal structural determinants here described.

The active site of KL-β-Gal is build up mostly by residues from domain 3, but some residues from domain 1 (Asn88, Val89, Asp187) and from domain 5 (Ala1000, Cys1001) also contribute to the narrow entrance that accesses the binding site (Figures 5). Residue Trp999 in EC-β-Gal is not conserved in AR-β-Gal and KL-β-Gal, where it is replaced by a cysteine (Cys1001 in KL-β-Gal). Mutagenesis analysis in EC-β-Gal has shown that this change is positive for the activity of the enzyme, but tryptophan-stacking interactions are also important for the binding of the glucose as an acceptor molecule in the formation of allolactose. This feature is no longer selected in KL-β-Gal and AR-β-Gal because they do not present the lac operon regulation and that change towards a more effective enzymatic activity is allowed. Apart from lacking Trp999, the most distinguishing feature in KL-β-Gal active site is the insertion at loop 420-443 that shapes the catalytic pocket and makes a narrower cleft when compared to EC-β-Gal (Figure 5). As described above, this loop folds over the entrance of the pocket and buries the binding site. Moreover, when doing a manual docking of a lactose residue into the catalytic centre (Figure 5a), some residues from this loop (Glu431, Tyr440 and Lys436) are within hydrogen bonding distances with the glucose moiety of the substrate, i.e. the aglycone. Consequently, this insertion must be playing essential roles in ligand binding and recognition of the lactose

molecules and, also, in selecting different acceptor molecules during transglycosylation, unique to the eukaryotic enzyme.

In EC-β-Gal, this region is partially occupied by a loop from domain 2 of the neighbour molecule (residues 272 to 283), and it is part of the activating interface of this enzyme (Juers *et al.* 2000). However, none of the residues from this loop is interacting with lactose or allolactose in the complexes of EC-β-Gal and, thus, aglycone binding seems looser in EC-β-Gal as compared to KL-β-Gal. This non-specific binding of the aglycone has been related to the relative promiscuity of the enzyme for various substrates (Juers *et al.* 2001).

This loop and loop 794-804, responsible for the conformational change, are not conserved in the *Arthrobacter* and *K. lactis* enzymes. Moreover, as it can be observed in Figure 5, the position of Phe620, (equivalent to EC-β-Gal Phe601) is intermediate between the deep and shallow stages of the substrate binding process described in EC-β-Gal complexes. Furthermore and contrarily to what is observed in the bacterial enzyme, native and the complex of KL-β-Gal with galactose show no conformational changes in the position of residues at the active site. All these observations point to the conclusion that the reaction mechanism proposed for EC-β-Gal is unique to this enzyme, putatively being common to enzymes being regulated by the *lac* operon.

Finally, it has been shown in EC-β-Gal that a magnesium and a sodium ion are part of the catalytic pocket and their importance for a proper catalysis and substrate binding has been proved (Lo *et al.* 2009).These two ligands are conserved in KL-β-Gal and it is reasonable to think that they will play a similar role in this enzyme.

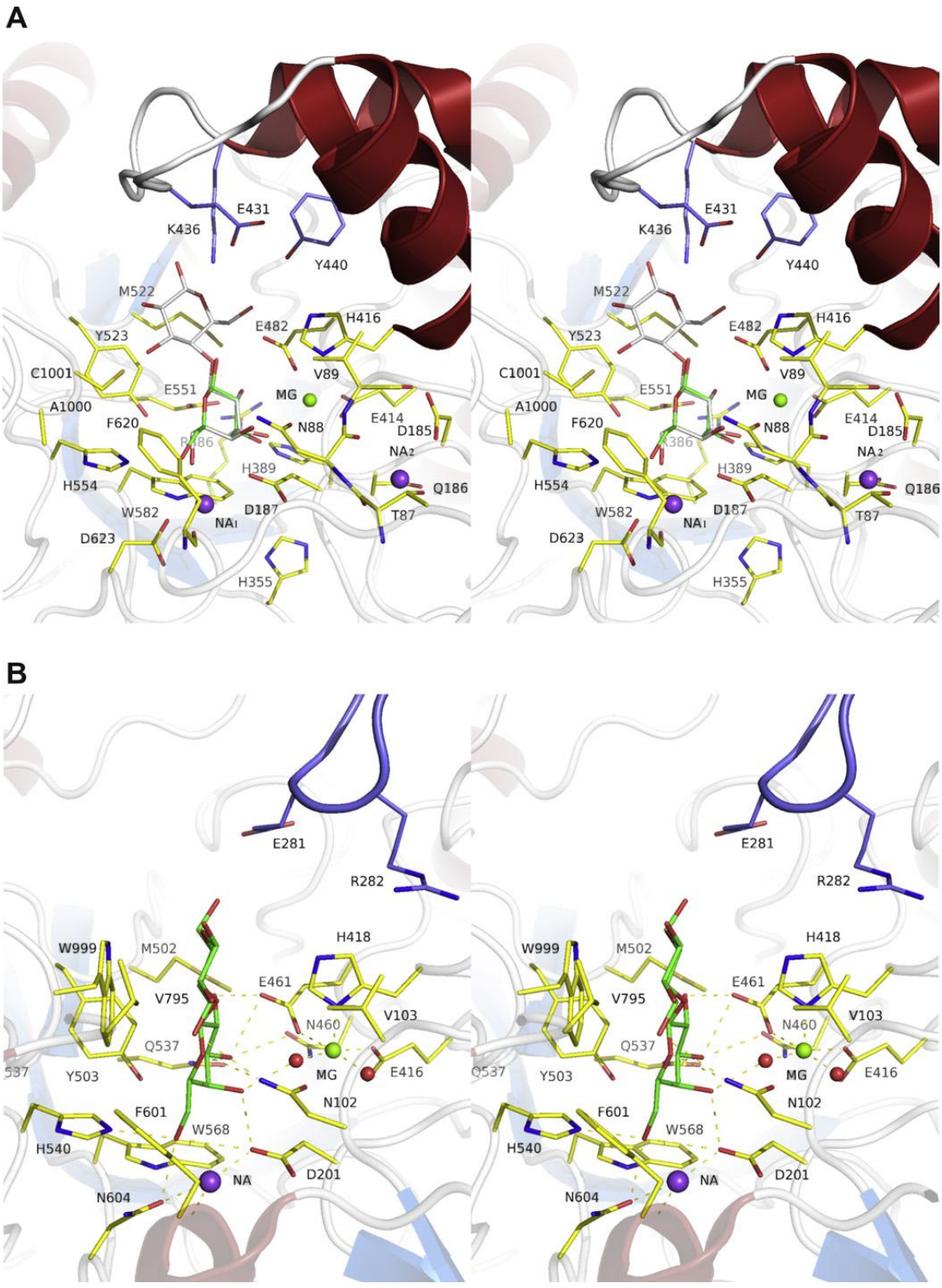

Figure 5. (a) Stereo view of KL-β-Gal catalytic pocket with the bound galactose in green sticks. A putative lactose molecule has been docked by structural superposition of a lactose moiety onto the galactose found in the complex, followed by manual adjustment of the glucose moiety to avoid clashes with the residues at the active site. (b) EC-β-Gal catalytic pocket. Important residues are shown in sticks. Catalytic residues and also Cys1001 (a) and Trp999 (b) are labeled. Loops 272–288 in EC-β-Gal (b) and 420–443 in KL-β-Gal (a) are highlighted in cartoon representation.

CONCLUDING REMARKS

In this study, we have been able to express and purify the β-galactosidase from *Kluyveromyces lactis*, and solved the crystal structures of the free state and its complex with the product galactose at 2.75 and 2.8 Å, respectively. KL-β-Gal subunit folds into five domains in a pattern conserved with other prokaryote enzymes solved for GH2 family, although two long insertions in domains 2 (264-274) and 3 (420-443) are unique and seem related to oligomerization and specificity. The KL-β-Gal tetramer is an assembly of dimers, with higher calculated dissociation energy for the dimers than for its assembly, which can explain that equilibrium exists in solution between the dimeric and tetrameric form of the enzyme. Two active centres are located at the interface within each dimer, in a narrow channel of 10 Å width that makes the catalytic pockets accessible to the solvent. The unique insertion at loop 420-443 protrudes into this channel and makes many putative links with the aglycone moiety of docked lactose, which may account for a high affinity of KL-β-Gal for this substrate and therefore might explain its unusually high hydrolytic activity (Martínez-Villaluenga et al., 2008). None of the structural determinants responsible for the reaction mechanism proposed to the *E. coli* β-galactosidase, which involves transition from a deep to a shallow stage following substrate binding, are envisaged in the KL-β-Gal active site and, consequently, we suggest that this mechanism rules only for GH2 enzymes being regulated by the lac operon. Our results provide key structural determinants of *Kluyveromyces lactis* β-galactosidase activity and specificity, this enzyme being one of the most pursued targets in the food and biotechnological industry.

ACKNOWLEDGEMENTS

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APPENDIX A. SUPPLEMENTARY DATA

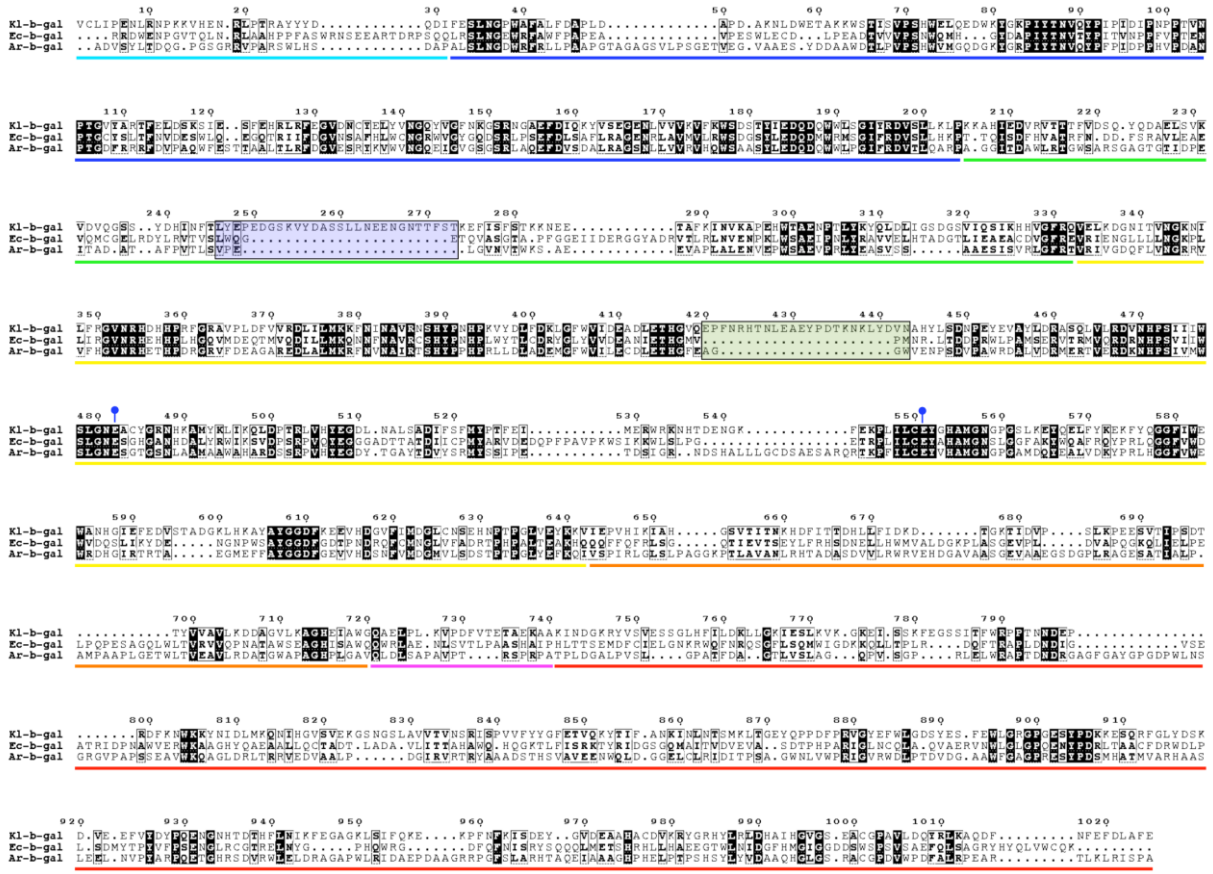

Figure S1.

CHAPTER 4

A hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase

Ángel Pereira Rodríguez

SUBCHAPTER 4.1

Secretion and properties of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase

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Microbial Cell Factories (2006) **5**: 41

SUMMARY

Background: The β-galactosidase from *Kluyveromyces lactis* is a protein of outstanding biotechnological interest in the food industry and milk whey reutilization. However, due to its intracellular nature, its industrial production is limited by the high cost associated to extraction and downstream processing. The yeast-system is an attractive method for producing many heterologous proteins. The addition of a secretory signal in the recombinant protein is the method of choice to sort it out of the cell, although biotechnological success is not guaranteed. The cell wall acting as a molecular sieve to large molecules, culture conditions and structural determinants present in the protein, all have a decisive role in the overall process. Protein engineering, combining domains of related proteins, is an alternative to take into account when the task is difficult. In this work, we have constructed and analyzed two hybrid proteins from the β-galactosidase of *K. lactis*, intracellular, and its *Aspergillus niger* homologue that is extracellular. In both, a heterologous signal peptide for secretion was also included at the N-terminus of the recombinant proteins. One of the hybrid proteins obtained has interesting properties for its biotechnological utilization.

Results: The highest levels of intracellular and extracellular β-galactosidase were obtained when the segment corresponding to the five domain of *K. lactis* β-galactosidase was replaced by the corresponding five domain of the *A. niger* β-galactosidase. Taking into account that this replacement may affect other parameters related to the activity or the stability of the hybrid protein, a thoroughly study was performed. Both pH (6.5) and temperature (40°C) for optimum activity differ from values obtained with the native proteins. The stability was higher than the corresponding to the β-galactosidase of *K. lactis* and, unlike this, the activity of the hybrid protein was

increased by the presence of Ni^{2+}. The affinity for synthetic (ONPG) or natural (lactose) substrates was higher in the hybrid than in the native *K. lactis* β-galactosidase. Finally, a structural-model of the hybrid protein was obtained by homology modelling and the experimentally determined properties of the protein were discussed in relation to it.

Conclusion: A hybrid protein between *K. lactis* and *A. niger* β-galactosidases was constructed that increases the yield of the protein released to the growth medium. Modifications introduced in the construction, besides to improve secretion, conferred to the protein biochemical characteristics of biotechnological interest.

INTRODUCTION

The enzymatic hydrolysis of lactose by β-galactosidase (E.C. 3.2.1.23) is one of the most promising biotechnological processes in development to use the sugar of the milk whey, a by-product of cheese manufacture with high polluting power (Becerra *et al.* 2004). β-galactosidases are widely distributed in nature and are produced by animals, plants and microorganisms (bacteria, fungi and yeast). However, the preparations that are commercially available and rated GRAS come from only a few species of yeast and micro fungi, the most important being *Kluyveromyces lactis* and *K. fragilis*, *Aspergillus niger* and *A. oryzae*. Micro fungi secrete this enzyme extracellularly, however, they produce a lower quantity of enzymatic units than do yeasts and the optimum pH is acid. Micro fungal β-galactosidase utilization for hydrolyzing lactose is restricted to acid wheys (González Siso 1996). In contrast, yeast β-galactosidase optimum pH is near neutral, consequently making it suitable for saccharifying milk and sweet whey. However, the production and industrial use of this intracellular enzyme are problematic due to the high cost associated

with its extraction from the cells and to the low yields obtained as a result of its instability (Becerra *et al.* 2001).

The secretion of β-galactosidase to the culture medium would facilitate remarkably the downstream processing, eliminating the step of extraction from the cells and reducing the risk of degradation by intracellular proteases. In the case of small peptides or proteins, efficient secretion can be achieved simply by fusing a secretory signal sequence 5' to the gene. However, for large oligomeric proteins of cytosolic origin, like the β-galactosidase of *K. lactis*, (Becerra *et al.* 1998) consecution of efficient secretion is not so easy. Protein secretion in yeast heterologous systems is influenced by the composition of the medium, culture conditions, phase of growth and structure of the cell wall (Rossini *et al.* 1993; Henry *et al.* 1997; Wong *et al.* 2002). Protein determinants like size, three-dimensional structure, load, isoelectric point or the glycosylation state are also important (De Nobel and Barnett 1991; Soo-Wan N 1993; Schuster *et al.* 2001), although their influence has not been completely clarified yet.

Recent studies indicate that the most outstanding structural features influencing secretion are, directly or indirectly, related to protein folding: formation of disulphide bridges (Kowalski *et al.* 1998; Bao and Fukuhara 2001), glycosylation (Sagt *et al.* 2000; Lee *et al.* 2003), and union to BiP (Katakura *et al.* 1999) or to ubiquitine (Bao and Fukuhara 2001). Not surprisingly, previous trials of heterologous secretion of β-galactosidase by *S. cerevisiae* rendered levels of 40% of the enzyme in the culture medium in the case of the protein from *A. niger*. This enzyme is extracellular in the micro fungus and therefore suitable structural characteristics for this localization are endogenous. On the contrary, in similar conditions but with the *Escherichia coli* protein, cytosolic in origin, secretion did not surpass 2% in the culture medium (Kumar *et al.* 1992; Pignatelli *et al.* 1998).

In this work, we successfully attempted to convert the intracellular β-galactosidase of *K. lactis* in a protein secreted to the medium. We used engineering techniques based on the construction of hybrid proteins with the extracellular β-galactosidase of *A. niger*. Changes introduced in the hybrid proteins have been evaluated by biochemical methods and discussed to the light of predicted structural models and biotechnological value.

RESULTS AND DISCUSSION

Construction of hybrid enzymes between the intracellular β-galactosidase of *K. lactis* and the extracellular β-galactosidase of *A. niger*

The extracellular β-galactosidase of *Aspergillus niger* presents, along its primary structure, a lower number of charged amino acids (Figure 1) compared to the intracellular *K. lactis* β-galactosidase, showing the *A. niger* β-galactosidase a 50% reduction in histidine and 43% in lysine content. This difference in charged amino acids could facilitate the secretion of the *A. niger* β-galactosidase, since amino acid charge distribution plays an important role in the localization of secreted and membrane proteins (Boyd and Beckwith 1990).

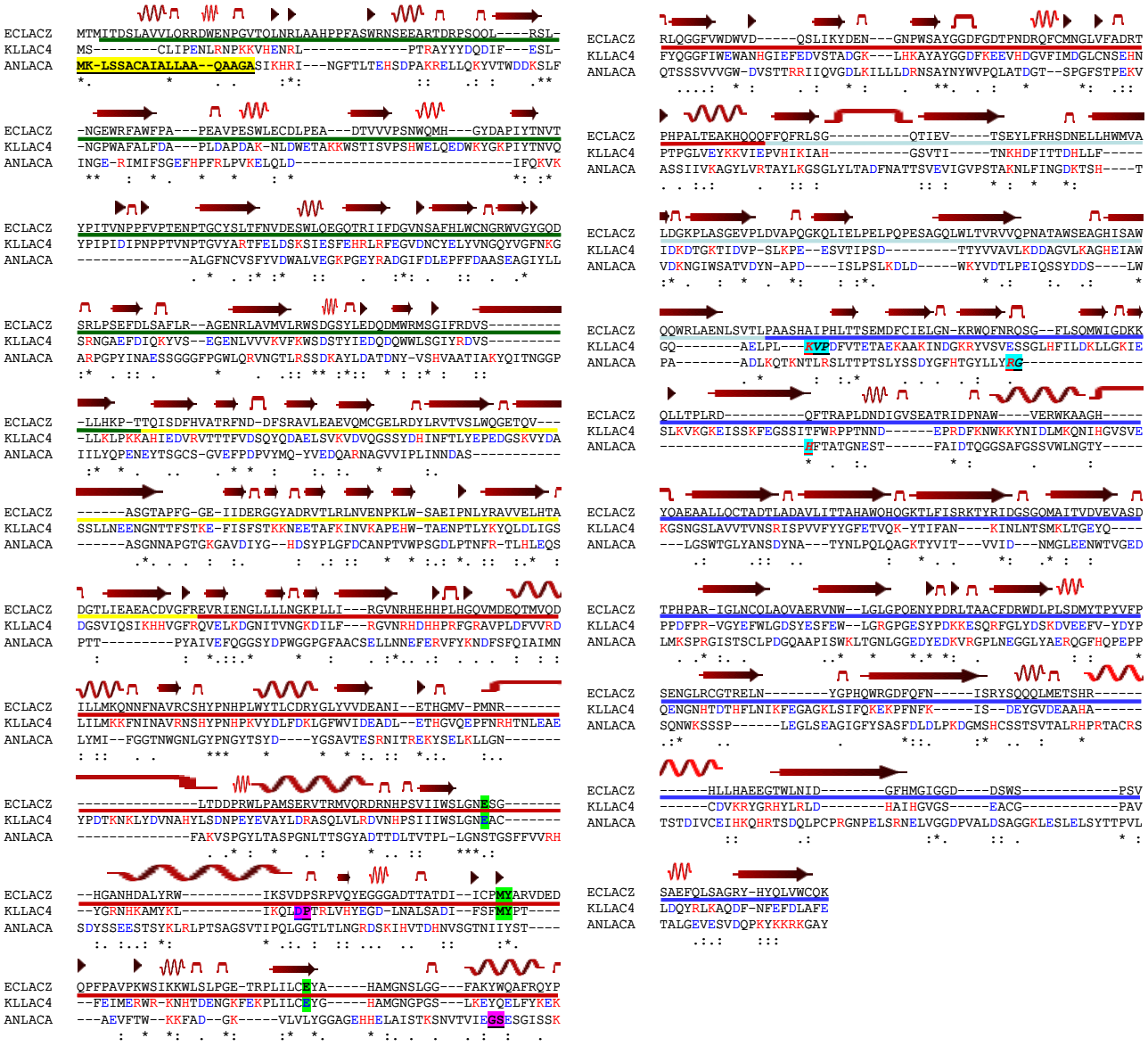

Figure 1. Amino acid sequence alignment of *E. coli* β-galactosidase with the *K. lactis* and *A. niger* β-galactosidase.

Multiple sequence alignment of *Escherichia coli* β-galactosidase (ECLACZ), *Kluyveromyces lactis* β-galactosidase (KLLAC4) and *Aspergillus niger* β-galactosidase (ANLACA). "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed. Acid (blue colour) and basic (red colour) amino acids of *K. lactis* and *A. niger* β-galactosidase are marked. The coloured bar below the *E. coli* β-galactosidase represents the five different domains structurally determined in the protein (Domain 1: green; Domain 2: yellow; Domain 3: red; Domain 4: light blue; Domain 5: dark blue). The secondary structure of *E. coli* β-galactosidase was obtained from the Protein Data Bank (Protein-Data-Bank). The localization of the restriction sites *Bam*HI (residues underlined and pink) and *Kpn*I (residues underlined and blue) are indicated. The conserved residues in *E. coli* β-galactosidase and *K. lactis* β-galactosidase important for catalytic function in *E. coli* β-galactosidase are shown in green. The residues of *A. niger* signal sequence are in yellow and underlined.

The construction of hybrid enzymes can be performed by means of different procedures from which new variants are arising constantly (Nixon *et al.* 1998; Chen 1999; Kikuchi and Harayama 2002; Lutz and Patrick 2004; Bloom *et al.* 2005). In our experimental design, homologous recombination was discarded because the homology between genes was insufficient (Crameri *et al.* 1998; Harayama 1998). Therefore, the corresponding constructions were made by PCR amplification of the selected domains, restriction and ligation. Since previous work had demonstrated that, in *K. lactis*, mutant β-galactosidases with large deletions in the N-terminal region were inactive (Becerra *et al.* 2001) we designed two hybrid proteins between *K. lactis* and *A. niger* β-galactosidases interchanging the C-terminal region. Constructions were made in the pSPGK1 plasmid (Fleer *et al.* 1991) and were called pSPGK1-LAC4-LACA-*Bam*HI and pSPGK1-LAC4-LACA*Kpn*I. Both contain in the N-terminus the secretory signal of the pre-sequence of the *K. lactis* killer toxin that has rendered good levels of secretion in other trials (Becerra *et al.* 2001). In the first construction, the 500 N-terminal amino acids of the *K. lactis* β-galactosidase were fussed in frame to the 478 amino acids of the C-terminal side of the *A. niger* enzyme. In the second, only the segment corresponding to the fifth domain, 297 amino acids positioned at the C-terminus, of the *K. lactis* β-galactosidase was replaced by the corresponding fifth domain, 274 amino acids positioned at the C-terminus, of the *A. niger* enzyme. The prediction of domains in the proteins from *K. lactis* and *A. niger* (Figure 1) was done by multiple alignments and in comparison with the sequence and structure of the *E. coli* β-galactosidase experimentally determined by crystallography (Jacobson *et al.* 1994).

Kinetics of secretion

To examine the kinetics of β-galactosidase secretion, a *K. lactis* β-galactosidase mutant strain, MW190-9B, was transformed with the above described constructions and with the plasmid pSPGK1-LAC4, bearing the gene coding for *K. lactis* β-galactosidase, as a control. Discontinuous cultures were made in liquid medium in Erlenmeyer flasks.

The levels of extracellular and intracellular β-galactosidase produced were different in the three transformants (Figure 2). In all cases extracellular β-galactosidase activity was detected in the media. It is important to remark that values of secreted protein are underestimated in this work if compared to other data in the literature. Usually in the bibliography the term extracellular activity includes also the activity of the periplasmic enzyme that is not effectively released to the medium. We have preferred to use the term extracellular to design uniquely the enzyme available in the medium, out of the cell, because its biotechnological use is easier.

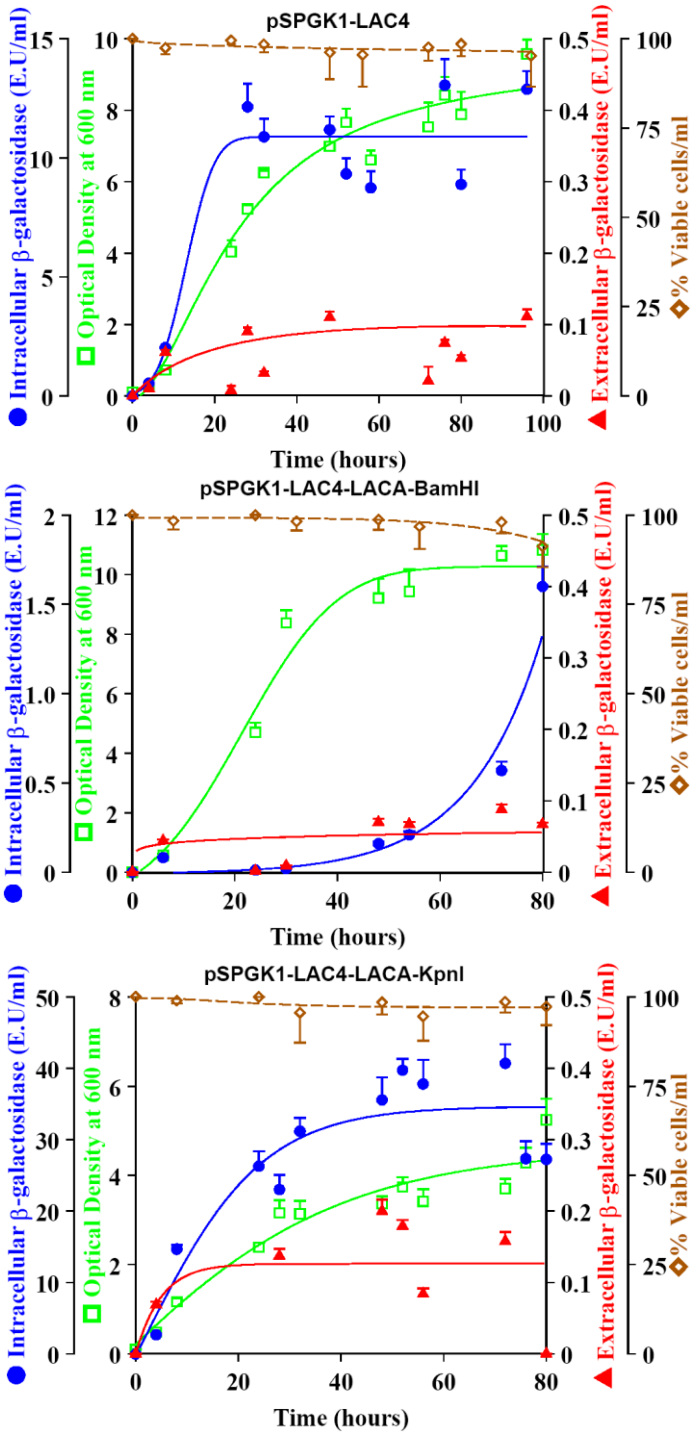

Figure 2. Kinetics of growth and secretion. Growth (Optical Density at 600 nm), percentage of viable cells per ml, extracellular and intracellular β-galactosidase production (E. U. mL^{-1}) by the MW190-9B strain transformed with the corresponding plasmids. Values represent the mean of 5 different cultures.

The strain transformed with the plasmid pSPGK1-LAC4-LACA-*Bam*HI showed a lower intracellular and extracellular β-galactosidase production than the control. This result may be attributed to the fact that a portion of the catalytic site of the *K. lactis* β-galactosidase was replaced by the catalytic site of *A. niger* β-galactosidase. Nevertheless, MW190-9B transformed with pSPGK1-LAC4-LACA-*Kpn*I showed the highest absolute values of intracellular and extracellular β-galactosidase production, almost three times and twice higher, respectively, than those obtained for MW190-9B transformed with pSPGK1-LAC4, although β-galactosidase activity into the culture medium reaches only 2.6% of the intracellular activity. In this case, the catalytic site from the *K. lactis* enzyme remained intact, since only the segment corresponding to the fifth domain was exchanged.

However, the growth rate of MW190-9B transformed with pSPGK1-LAC4-LACA-*Kpn*I diminished to half of the reached by the strain transformed with pSPGK1-LAC4. Cellular lysis was discarded by measuring cellular viability (Figure 2), therefore this slow growth may be attributed to the fact that the cells direct the available energy towards β-galactosidase production rather than division.

Two conclusions are obtained from these results. First, the C-terminal region of *A. niger* β-galactosidase functionally complements the C-terminal region of *K. lactis* β-galactosidase. Similarly, the fifth domain of the *E. coli* β-galactosidase has been related to the ω-fragment and early studies have shown that it folds independently and complements molecules missing this part of the sequence (ω-complementation) (Jacobson *et al.* 1994). Second, the construction pSPGK1-LAC4-LACA-*Kpn*I is of biotechnological value and therefore we decide to further characterize this hybrid protein.

Characterization of the hybrid protein LAC4-LACA-KpnI

Determination of optimum pH and temperature, thermal stability, effects produced by divalent cations upon enzymatic activity and calculation of kinetics constants was performed. To carry out these measures, crude extracts of the strain MW190-9B transformed with pSPGK1-LAC4-LACA-*Kpn*I and with pSPGK1-LAC4 (control) were obtained at the moment of maximum expression of β-galactosidase activity (80 hours).

Determination of the optimum pH

For the determination of the optimum pH, the measurements of enzymatic activity were carried out in buffer Z aliquots modified to obtain pH values from 5 up to 8.5. As seen in Figure 3A, the optimum pH for the β-galactosidase of *K. lactis* is about 7, whereas for the hybrid protein is slightly acid 6.5. The optimum pH values reported for β-galactosidases from *A. niger* are from 2.5 to 4 (Widmer and Leuba 1979) whereas from *K. lactis* are from 7 to 7.5 (Dickson *et al.* 1979; Tello-Solis *et al.* 2005). Therefore, the constructed hybrid protein has characteristics with regard to the pH optimum that differs from its precursors. It was reported that, at pH 6.5, the activity of *K. lactis* β-galactosidase decreased significantly due to local changes in charged residues (Tello-Solis *et al.* 2005). The hybrid protein, with a different composition in charged amino acids, may buffer these local changes and therefore it may be more tolerant to pH changes during culture.

Determination of the optimum temperature

The optimum temperature reported for *A. niger* β-galactosidase is 50°C (Santos *et al.* 1998) whereas for *K. lactis* β-galactosidase is 30°C (Dickson *et al.* 1979). For the determination of the optimum temperature of the hybrid protein and *K. lactis* control, the measurements of enzymatic activity were performed at different temperatures, from 15°C to 50°C (Figure 3B). It was observed that whereas in our conditions the optimum temperature for *K. lactis* β-galactosidase is around 35°C, in the hybrid protein is slightly greater, being near to 40°C. In the same way as for the optimum pH, the constructed hybrid protein presents characteristics that make it more adequate to high temperature during catalysis.

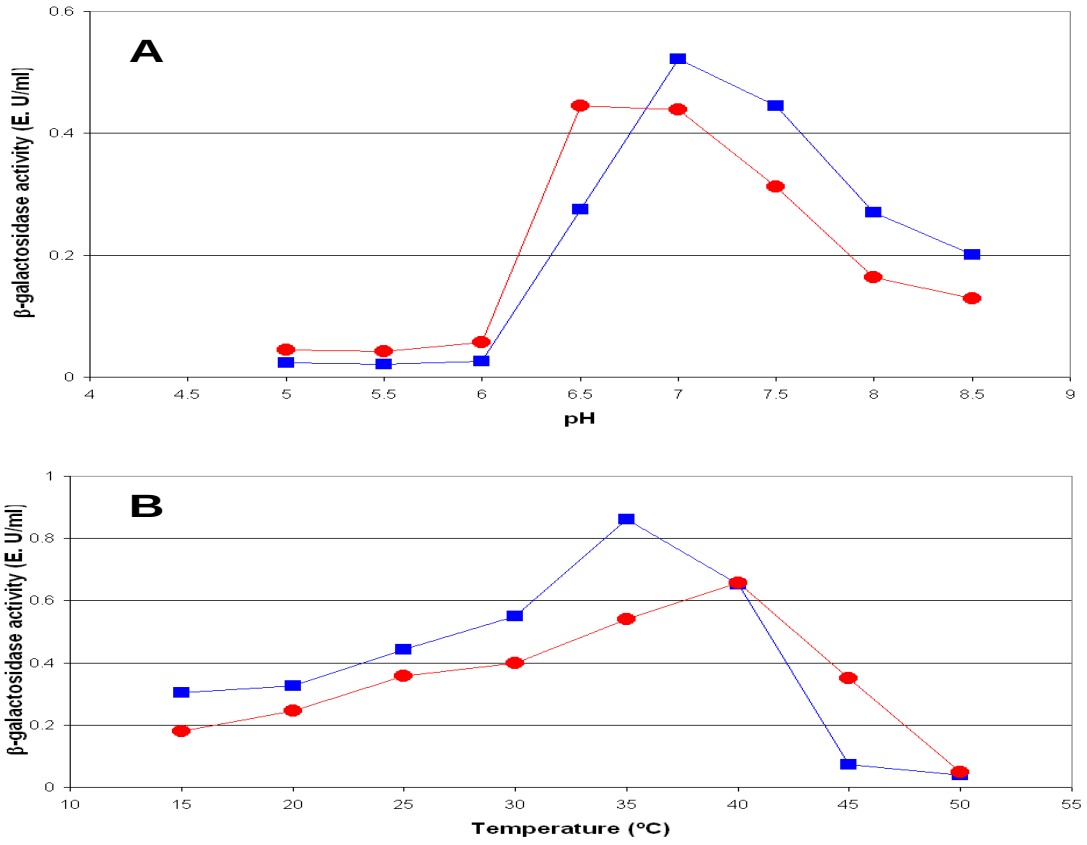

Figure 3. Determination of the pH and temperature optimum. Optimum pH (A) and optimum temperature (B) for the hybrid enzyme between the β-galactosidase of *K. lactis* and *A. niger* (red) and the β-galactosidase of *K. lactis* (blue). Experimental variations are less than 10% of the value of the point. Data are the mean of three independent experiments.

Thermal stability

Thermal stability of the hybrid β-galactosidase was also determined and compared to the native β-galactosidase of *K. lactis*. Before performing the measurement of enzymatic activity, the enzymatic preparation was incubated in buffer Z at different times and temperatures: 30°C, 42°C, 50°C and 60°C. The hybrid β-galactosidase presented a higher stability than the one of *K. lactis* (Figure 4) at all tested temperatures. Almost the 75% of the enzyme kept stable after an hour of incubation at 30°C, the 60% after 15 minutes at 50°C, the 8% after 3 minutes at 60°C (data not show in Figure 4), clearly in advantage to the native *K. lactis* β-galactosidase stability (55%, 40% and 0% respectively). Although biotechnological applications may demand even higher thermal stability of the hybrid β-galactosidase, other procedures exist to improve this factor, i.e. immobilization as previously shown (Makowski *et al.* 2007).

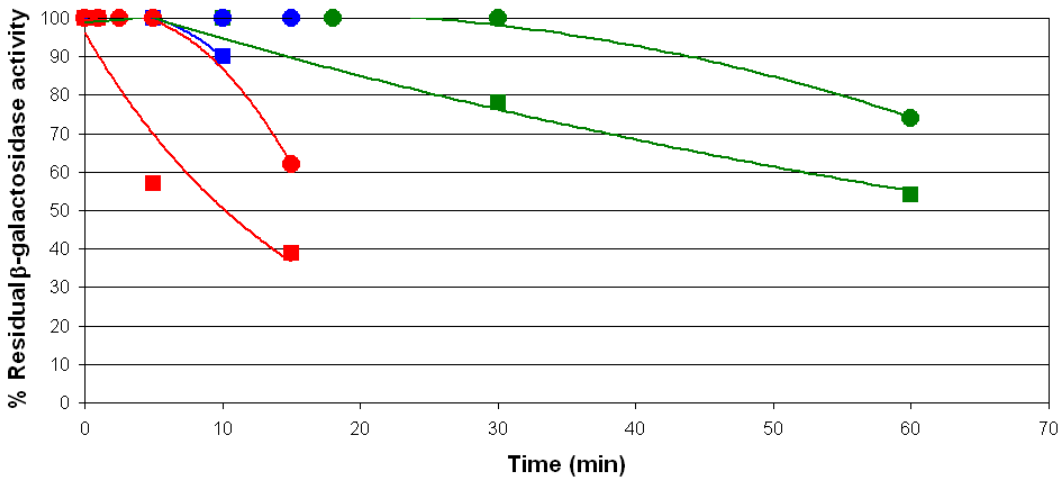

Figure 4. Determination of the thermal stability. Determination of the thermal stability at 30°C (green), 42°C (blue) and 50°C (red) for the hybrid enzyme between the β-galactosidase of *K. lactis* and *A. niger* (circles) and the native β-galactosidase of *K. lactis* (square). Experimental variations are less than 10% of the value of the point. Results are the average of two independent experiments.

Effects of the divalent cations

The activity of *K. lactis* β-galactosidase is stimulated by the presence of some divalent cations, Mg^{2+} or Mn^{2+}, and inhibited by the presence of others, Ca^{2+}, Zn^{2+} and Ni^{2+} (Dickson *et al.* 1979; Kim *et al.* 1997). The effect of Mg^{2+}, Ca^{2+} and Zn^{2+}on the activity of the hybrid protein and the native *K. lactis* β-galactosidase is similar (Figure 5A). Whereas the presence of Ca^{2+} or Zn^{2+} causes a slight inhibition of the activity, Mg^{2+} stimulates it clearly. Although an increase of the β-galactosidase activity has been described in presence of Mn^{2+} (Dickson *et al.* 1979), this stimulatory effect could not be verified in this experiment due to the interference produced by reducing agents present in buffer Z (Figure 5B).

The cation Ni^{2+} exerts different effects in the activity of the native and hybrid proteins (Figure 5B). As previously described by other authors (Kim *et al.* 1997), the cation Ni^{2+} inhibits *K. lactis* β-galactosidase activity but over the hybrid enzyme the effect is activator. Crystallographic studies identified possible divalent cations binding sites in the structure of the *E. coli* β-galactosidase, although no functional significance was ascribed to them (Matthews 2005). Further studies to determine the relationship between structural features, cation binding and activity of β-galactosidase will be required.

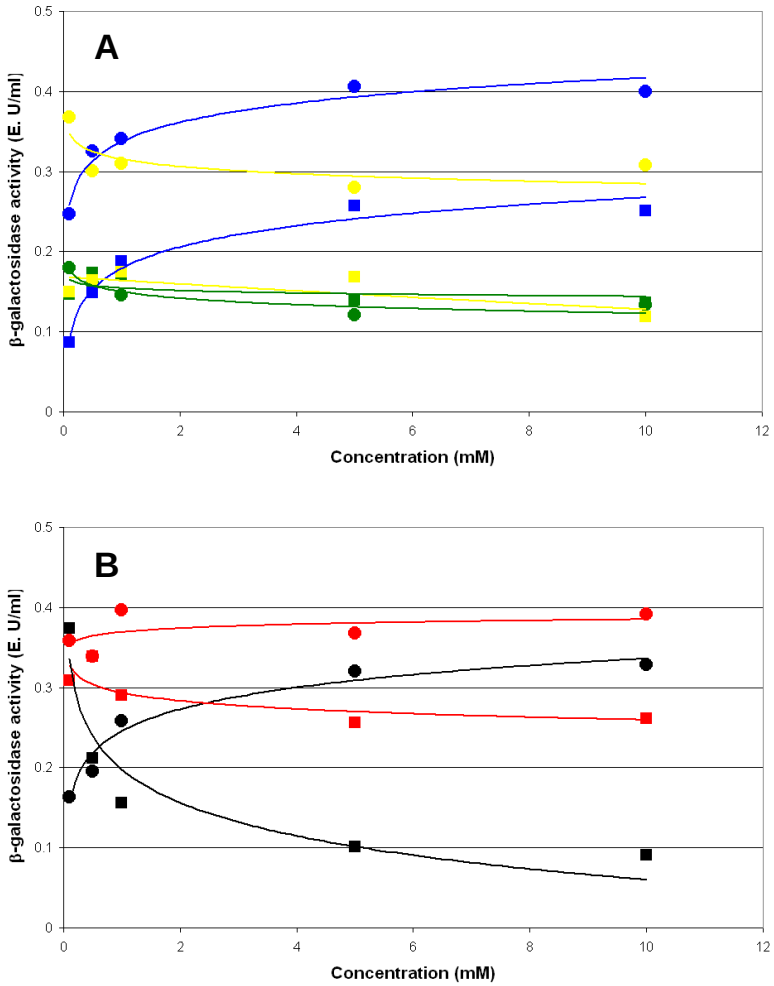

Figure 5. Determination of the effects of the divalent cations. Determination of the effects of the divalent cations Mg^{2+} (blue), Ca^{2+} (green) and Zn^{2+} (yellow) (A) and Mn^{2+} (red) and the Ni^{2+} (black) (B) on the enzymatic activity of the β-galactosidase hybrid between *K. lactis* and *A. niger* (circles) and the native β-galactosidase of *K. lactis* (square). Experimental variations are less than 10% of the value of the point. Results are the average of two independent experiments.

Determination of the kinetic constants

The values of kinetic constants for the hybrid and native β-galactosidases were obtained from double-reciprocal plots (Figure 6). Hybrid β-galactosidase presents a greater affinity both for ONPG (Km 0.8 mM) and lactose (Km 8.7 mM) than *K. lactis* β-galactosidase (1.5 mM and 21 mM, respectively). This striking increase in affinity aimed us to look for a structural explanation of the change.

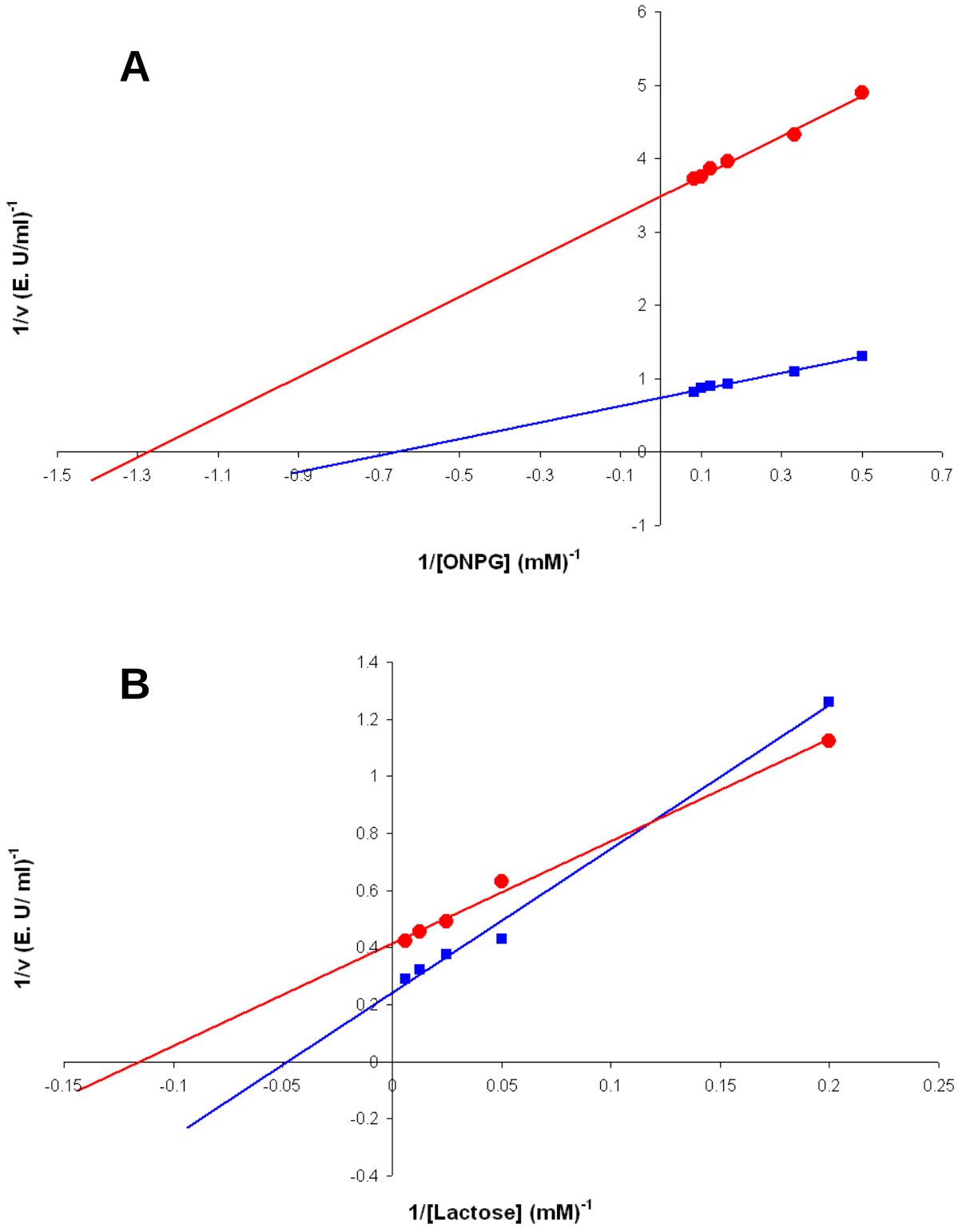

Figure 6. Lineweaver-Burk plots. Lineweaver-Burk plot of the reaction catalyzed by the β-galactosidase hybrid between *K. lactis* and *A. niger* (red circles) and the native β-galactosidase of *K. lactis* (square blue) in the presence of the synthetic substrate ONPG (A) or the natural substrate lactose (B). Experimental variations are less than 10% of the value of the point. Results are the average of two independent experiments.

Prediction of the tertiary structure of the β-galactosidase of *K. lactis* and the hybrid protein

Three-dimensional protein structures are important for a detailed understanding of the molecular basis of protein function. In absence of direct experimental data, a computational approach by homology modelling is a reliably method to generate a three-dimensional model for a protein. In order to understand the differences between the hybrid and native *K. lactis* β-galactosidases, a prediction of the tertiary structure of these proteins and the *A. niger* β-galactosidase was made. The server for automated comparative modeling (Swiss-Model) was used. The amino acids E461, M502, Y503 and E537, considered important residues for the catalytic activity of the *E. coli* β-galactosidase (Jacobson *et al.* 1994; Matthews 2005) and which form the active-site pocket, are highly conserved in the *K. lactis* β-galactosidase (residues E482, M522, Y523 and E551) (Poch *et al.* 1992). As depicted in Figure 7A, a part of the active site is formed by a deep pit that intrudes well into the core of the TIM barrel at the third domain. In addition, there are loops coming from the first and fifth domain. In the case of the hybrid protein (Figure 7C), the fifth domain of the *K. lactis* β-galactosidase was replaced by the corresponding domain of the *A. niger* enzyme (Figure 7B). Structurally, as predicted by the model, this causes a slight opening of the third domain. This could favour the accessibility of the substrate and could explain the change in the kinetic constants.

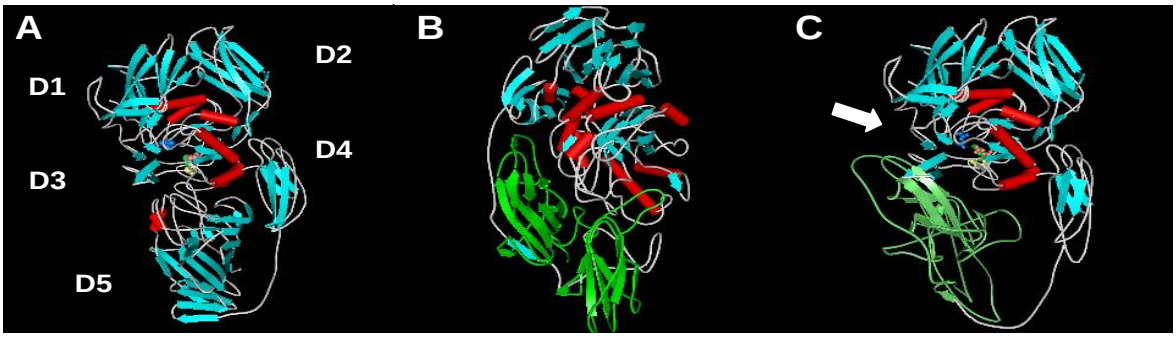

Figure 7. Ribbon representations. Ribbon diagram corresponding to the prediction of the tertiary structure of *K. lactis* β-galactosidase (A), *A. niger* β-galactosidase (B) and hybrid β-galactosidase (C) using the Swiss-Model program. The residues mentioned in Figure 1 have been drawn as spheres of colours (E482 blue, M522 green, Y523 yellow, E551 red). D1–D5 identify the five domains of *K. lactis* β-galactosidase (A) predicted by alignment in comparison with the sequence of the *E. coli* β-galactosidase (Figure 1). The fifth domain of the *A. niger* β-galactosidase is coloured in green (B and C). The white arrow (C) shows the slight opening of the third domain in the hybrid β-galactosidase.

CONCLUSION

The cellular wall represents in yeasts an additional barrier for the excretion of proteins to the culture. The secretory signal directs the proteins across the secretion route up to the periplasmic space but this does not imply that the protein could cross the cell wall. The hybrid protein obtained in this work, by replacing the fifth domain of the β-galactosidase of *K. lactis* by the one of *A. niger*, is active, reaches the culture medium and presents, in addition, greater stability at high temperatures and more convenient kinetics parameters for its biotechnological utilization. Some of these features may be explained to the light of structural changes predicted by homology modelling.

METHODS

Strains and culture conditions

The *Kluyveromyces lactis* MW 190-9B strain (*MATa lac4-8 uraA Rag*+) was used. Liquid batch cultures of transformed cells were grown in Erlenmeyer flasks filled with 20% volume of culture medium at 250 rpm, unless otherwise stated. As inocula, a suitable volume of a stationary phase culture in complete medium (Zitomer and Hall 1976) without the amino acid corresponding to

the strain auxotrophy was added to obtain an initial OD_{600} of 0.2. The same medium was also used as culture media. Samples were taken at regular time intervals to measure growth (OD_{600}), percentage of viable cells, intracellular and extracellular β-galactosidase activity.

Vectors and DNA constructions

The pSPGK1-LAC4 (Becerra *et al.* 2001), a derivative of pSPGK1 plasmid (Fleer *et al.* 1991) containing the secretory signal that corresponds to the pre-sequence (16 amino acids) of the *K. lactis* killer toxin (α-subunit) and the PCR-amplified *LAC4* gene (which codes for *K. lactis* β-galactosidase) inserted between the constitutive promoter and the terminator of the *S. cerevisiae* phosphoglycerate-kinase (*PGK*) gene, was used for building new vectors. Vectors were constructed as follows:

-pSPGK1-LAC4-LACA-BamHI: plasmid pSPGK1-LAC4 was digested with *Bam*HI. The *Bam*HI-*Bam*HI fragment that contains the C-terminal segment of the *K. lactis* β-galactosidase was removed and replaced by the C-terminal segment of the *Aspergillus niger* β-galactosidase amplified from pVK1.1 (Kumar *et al.* 1992) with the following oligonucleotides creating *Bam*HI sites on the ends of the PCR product:
GAAGGATCCTGAGTCTGGCATCTCG,
CCACACCCGTCCTGTGGATCC.

*-pSPGK1-LAC4-LACA-Kpn*I: plasmid pSPGK1-LAC4 was digested with *Kpn*I and ligated to the segment corresponding to the five domain of the *A. niger* β-galactosidase amplified from pVK1.1 with the following oligonucleotides generating *Kpn*I sites on the ends of the PCR product:
GCGGTACCCCGCGGACACTTCACCGC,
GCGGTACCGCCATCTCCTTGCATGC.

PCR conditions

A 20 ng amount of template DNA was incubated with 30 pmol of primer-1 and 30 pmol of primer-2 in the presence of 0.25 mM dNTPs, *Taq* or *Pwo* polymerase buffer and 2 U of the corresponding polymerase. Initial denaturation was done at 94°C for 2 min, followed by 30 cycles of 1 min at 95°C, 2 min at 50–57°C and 1.5–2.5 min at 72°C. There was a final incubation at 72°C for 10 min to fill-in ends.

Molecular biology procedures

Escherichia coli DH5a strain (*supE44 DlacU169 f80lacZDM15 hsd*R17 *recA1 endA1 gyrA96 thi-1 relA1*) was used for the construction of the plasmids and propagation by means of the usual DNA recombinant techniques according to Ausubel *et al.* (Ausubel FM and K 1995). Yeast strains were transformed using the lithium acetate procedure (Ito *et al.* 1983). Plasmid uptake and β-galactosidase production by the transformed strains were identified on plates with the chromogenic substrate X-gal in the corresponding auxotrophic medium.

Percentage of viable cells

The methylene blue solution, which contained 0.01% Methylene Blue (Sigma-Aldrich, M9140) and 2% (w/v) tri-sodium citrate dihydrate in phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4), was mixed with an equal volume of yeast suspension for 10 min. Unstained cells were assumed to be viable. The stained cells in the mixture were quantified under an optical microscope (Nikon Eclipse 50i). The viability of 100 cells, from five replicates of each sample, was assessed and expressed as the mean percentage of viable cells.

β-galactosidase activity assays

The method of Guarente (Guarente 1983) as previously described (Becerra *et al.* 2001) was used. One enzyme unit (E. U) was defined as the quantity of enzyme that catalyzes the liberation of 1 μmol of ortho-nitrophenol from ortho-nitrophenyl-β-D-galactopyranoside per min under assay conditions. E.U. are expressed per mL of culture medium.

Throughout this paper and unless otherwise specified, the term extracellular β-galactosidase is used to mean the enzyme in the culture medium and the term intracellular β-galactosidase is used to mean the cell-associated enzyme, both periplasmic and cytoplasmic.

Preparation of crude protein extracts

For the preparation of crude protein extracts, the cells were harvested by centrifugation at 7000 rpm for 5 min at 4°C and washed once with distilled water. They were suspended in 20 mM Tris-HCl, pH 7.8, 300 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol buffer with 0.1 mM PMSF, 4 mM Pepstatin, 4 mM Leupeptin and 2 μM β- mercaptoethanol and broken using a sonicator at 16 μm for a total of 20 min at 4°C making four exposures of 5 min, with 5 min intervals after each. Cell debris was removed by centrifugation at 40000 rpm for 90 min at 4°C. The supernatant constituted the cell-free extract.

Protein determinations

Protein was determined by the method of Bradford (Bradford 1976) using bovine serum albumin (Sigma) as a standard.

Characterization of the hybrid enzyme

The characterization was carried out from a crude extract of the strain of *K. lactis* MW190-9B/pSPGK1-LAC4-LACA*Kpn*I obtained at the moment of maximum expression of β-galactosidase activity (80 hours). As a control, in all the essays performed, the same quantity of protein of a crude extract of the strain of *K. lactis* MW190-9B/pSPGK1-LAC4, obtained at the moment of maximum expression of β-galactosidase activity, was taken.

In order to calculate the optimum pH, 60 μg of the crude yeast protein extract were incubated in buffer Z (100 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.6 mM $MgSO_4$ and 2.7 mL of β-mercaptoethanol for litre of dissolution) adjusted respectively to pH 5; 5.5; 6; 6.5; 7; 7.5 and 8.

For optimum temperature determination, the enzymatic activity of 60 μg of the crude protein extract was measured at different temperatures: 15, 20, 25, 30, 35, 40, 45 and 50°C.

In thermal stability experiments, 30 μg of the yeast crude protein extract were incubated during different periods of time to different temperatures: 30, 42, 50 and 60°C.

For the determination of the effects of divalent cations on the enzymatic activity, 100 μg of the crude protein extract and several increasing concentrations (0.1, 0.5, 1.5 and 10 mM) of $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$ or $NiCl_2$ were added to the buffer Z and the enzymatic activity was measured as previously described.

Kinetic studies

The β-galactosidase activity was tested with the artificial substratum ONPG and the natural substratum lactose. The determination of the β-galactosidase activity in 30 μg of the crude extract was made as above

explained, but in presence of different concentrations of ONPG: 2, 3, 6, 8 and 12 mM. Alternatively, 60 μg of the crude extract were incubated with different concentrations of lactose: 5, 20, 40, 80 and 160 mM. To determine lactose hydrolysis, a commercial kit was used (*Boehringer-Mannheim*) following the supplier instructions. The method is based on the oxidation of the product D-galactose by the β-galactose dehydrogenase. The amount of NADH formed in this last reaction is stoichiometric to the amount of lactose and D-galactose. The NADH production was measured following the absorbance increase at 340 nm.

Homology modelling

The models of the *K. lactis* and *A. niger* β-galactosidases and the hybrid protein were made with the fully automated protein structure homology-modelling server (Swiss-Model).

Acknowledgements

We are grateful for kindly providing materials to Dr. Wésolowski-Louvel (Université Claude Bernard, France) for the MW190-9B strain and to Dr. Fukuhara (Institute Curie, France) for the pSPGK1 plasmid. This research was supported by PGIDIT03BTF10302PR grant from the Xunta de Galicia (Spain). RFL received a fellowship from the Universidade da Coruña (Spain), APR and MCT were supported by the PGIDT01PX110303PN grant from Xunta de Galicia (Spain). Part of the results presented here has been communicated at the 4th Recombinant Protein Production Meeting (Barcelona, 2006).

SUBCHAPTER 4.2

Production of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase in a continuous immobilized culture

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INTRODUCTION

At present the importance of the enzymatic processes applied to the food or pharmaceutical industry is so big, that some of the products could not be made by other way. The industrial enzyme production is a business which in the beginning of the present XXI century involves around 1.6 billion of dollars per year (Cherry and Fidantsef 2003). One of the most interesting types of enzymes in the industrial production is the group of the β-galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23) which hydrolyze the o-glycosidic linkages between β-D-galactose terminal and non-reducing residues. They are known as lactases due to their catalysis of the lactose (milk's sugar) into its constituent sugars: glucose and galactose.

The β-galactosidases are obtained principally by a small amount of microbial sources as for example *Kluyveromyces* genre yeasts and *Aspergillus* genre fungus, which are considered as GRAS (Generally Regarded As Safe) by the FDA. Therefore, they can be used in the food or pharmaceutical industry.

Currently β-galactosidase preparations, obtained from *K. lactis* and *K. fragilis*, are used to saccharified milk and sweet whey, but due to the β-galactosidase intracellular nature and its poor stability, the extraction and purification of the enzyme involves high costs (Cherry and Fidantsef 2003).

The *Aspergillus niger* β-galactosidase is naturally secreted to the medium, but it has an optimum pH too much acid to be used in sweet cheese whey (the most important variety of cheese whey in Galicia, and in Spain too), therefore it can be only utilized in acid wheys which have less biotechnological possibilities because of its acid taste and because of its high content in salts (Rubio-Texeira 2006). Other advantage in the *A. niger* β-galactosidase (furthermore of the extracellular nature) is that it has got a good

thermostability, and could be used at high temperatures like for example 50ºC (Panesar *et al.*, 2006).

A hybrid β-galactosidase between *K. lactis* and *A. niger* was constructed, and it duplicated the secretion percentages with respect to the wild β-galactosidase from yeast with the same secretion signal (Rodriguez *et al.* 2006). This hybrid β-galactosidase showed an optimal pH around the neutrality and it had a better stability at high temperatures compared with the wild protein from yeast.

In this work different culture mediums were assayed to make a first approximation to scale-up the production of this biotechnological value hybrid β-galactosidase. The use of spent grains (a by-product of brewery industry) as an immobilizing material (yeasts colonize the surface of the carrier by spontaneous adhesion or attachment of the yeast to the surface of the nonporous cellulose-based carrier material prepared from spent grains), was checked too.

MATERIAL AND METHODS

The general material and methods to develop this work are summarized in (Rodriguez *et al.* 2006).

Strain

The *K. lactis* MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I strain (Rodriguez *et al.* 2006) secreting a hybrid protein between *K. lactis* and *A. niger* β-galactosidases was used.

Media

The following media were used:

YPD: complex medium which contains 1% yeast extract, 2% peptone and 2% glucose.

YPL: complex medium which contains 1% yeast extract, 2% peptone and 2% lactose.

Minimum medium (MM): minimum medium which contains 2% glucose or lactose and YNB 1X (6.7 gL^{-1} Yeast Nitrogen Base from BD without amino acids).

SS-Lactose (5%): minimum medium which contains 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.2% Yeast Extract and 5% Lactose.

Cheese Whey Powder provided by a Portuguese dairy (Lactogal - Porto).

Microtiter Assay of β-galactosidase activity

Intracellular

Grown cells in flasks or culture tubes were span in Eppendorf tubes in a centrifuge for 5 minutes at 5000 rpm, until achieve a pellet of 100-150 μL. Then the biomass was washed in the Eppendorf tube with 9 volumes of PE buffer (Table 1), centrifuged and the supernatant was discarded.

The biomass was resuspended in 3 volumes of PE buffer, and 4 volumes of glass beads were added to the tube. Cycles of 30 seconds of vortex and 1 minute on ice, for 9 minutes were perfomed.

Then 3 volumes of Z buffer (Table 2) were added and vortex gently. After that, a centrifugation for 15 minutes at 13200 rpm and 4ºC was made to separate the cell debris against the supernatant.

Finally, the supernatant was collected in a new Eppendorf tube and maintained on ice. The quantity of total protein was measured using the

Bradford method (Bradford 1976) To determinate the β-galactosidase activity at least three dilutions were measured and the following formula was used:

Intracellular β-galactosidase activity (nmoL/min/mL)= $S^{1}/6{,}1366^{2}/(V^{3}*1000)*250/200*1000000$

[1] -> Slope (in the kinetic measure)

[2] -> Extinction molar coefficient (empirically measured using buffer Z and pNP disolved in buffer Z. Nine different concentrations of pNP were measured using the same protocol as followed in the samples)

[3]-> Sample volume used in mL

Table 1. PE Buffer (200mL) [1, 2]

Na_2HPO_4	**1.708g**
$NaH_2PO_4.H_2O$	**1.1g**
KCl	**0.15g**
$MgSO_4.7H_2O$	**0.05g**
EDTA $Na_2.2H_2O$	**0.0744g**
$(NH_4)_2SO_4$	**7.92g**
Glycerol	**10mL**
Milli-Q Water	**up to 200 mL final volume**

[*1] It is necessary to adjust the pH to the optimum of the protein activity, and autoclave the solution.

[*2] It is necessary to add: 20 µL/mL of Protease Inhibition Cocktail (Roche) stock solution 25X, 10 µL/mL of PMSF (100mM) stock solution 100X, 1 µL/mL of DTT (1M) stock solution 1000X and 2.8 µL/mL of 2-Mercaptoethanol

Table 2. Buffer (500mL)*

$Na_2HPO_4.7H_2O$	**8.05g**
$NaH_2PO_4.H_2O$	**2.75g**
KCl	**0.375g**
$MgSO_4.7H_2O$	**0.123g**
2-Mercaptoethanol	**1.35mL**
Milli-Q Water	**up to 500 mL final volume**

*It is necessary to adjust the pH to the optimum of the protein activity.

To measure the β-galactosidase activity, 200 µL of extract or diluted extract was added into a microtiter plate, and 50 µL of pNPG (p-Nitrophenyl-beta-D-galactopyranoside at 4 mg/mL) were added to initialize the reaction. The kinetic reaction was measured at 405 nm and 30ºC, for 15-30 minutes.

Extracellular

50 µL of fermentation supernatant were mixed with 150 µL of Buffer Z (Table 2). Then, 50 µL of pNPG (p-Nitrophenyl-beta-D-galactopyranoside at 4 mg/mL) were added and the kinetic reaction was measured as in the intracellular protocol. To determinate the extracellular β-galactosidase activity the following formula was used:

Extracellular β-galactosidase activity (nmoL/min/mg)=$S^1/6{,}1366^2/(V^3*1000)*250/200*1000000$

[1] -> Slope (in the kinetic measure)

[2] -> Extinction molar coefficient (empirically measured using Z buffer and pNP dissolved in Z buffer)

[3]-> Protein sample concentration used in mg/mL

Microtiter assay of the Bradford method

It is based on the Bradford protocol (Bradford 1976). Coomassie Plus Protein Assay Reagent (Pierce) was used. As standard a stock of 2 mg/ mL of Bovine Serum Albumin (BSA): 100 mg of BSA dissolved in 50 mL of 0.9% NaCl was used. 1 mL aliquots of BSA stock were stored at -20 ºC. The working range used was between 100 – 1500 µg/mL.

Standard	BSA (µL)	MilliQ water (µL)	[BSA] (µg/mL)
A	**375 *(Stock)***	**125**	**1500**
B	**325 *(Stock)***	**325**	**1000**
C	**175 *(Std A)***	**175**	**750**
D	**325 *(Std B)***	**325**	**500**
E	**325 *(Std D)***	**325**	**250**
F	**325 *(Std E)***	**325**	**125**
G	**100 *(Std F)***	**400**	**25**

Procedure

Firstly, 10 µL of each standard or sample (diluted with water if it was necessary) were added into the plate wells and 10 µL of the diluent (water) were used for the blank wells. Then, 300 µL of the Coomassie Plus Protein Assay Reagent were added to each well, and mixed on a plate shaker for 30 seconds.

Finally, measure the absorbance at 595 nm (A595).

NOTES:

- *The average A595 for the blanks were subtracted from the standard or sample readings.*
- *If higher A595 readings were required, 15 µL of standard or sample and 300 µL of reagent per well were used.*
- *Always two different dilutions of each sample were assayed, to check for possible interferences.*

Fermenters conditions

A Plexiglas airlift was used to carry out the fermentations. The total capacity is 10 liters, and the working volume is 6 liters. In the first 24 hours the inocula grew in "batch" conditions, because the entry and the out of the medium were closed, to achieve enough amounts of cells to immobilize. The following days different flows and medium were used. Temperature, pH and airflow were controlled at 30ºC, 5 and 0.5 vvm respectively. Samples to measure the absorbance at 600nm, dry weight, HPLC analysis (lactose, glucose, glycerol and ethanol), and immobilized biomass were taken during the fermentation.

Immobilization

The immobilization was carried out following the Branyik protocol with small modifications (Branyik *et al.* 2002).

Carrier preparation

Dry spent grains (100 g), obtained after dry roller milling of malt and wort separation, were mixed in 1500 mL of 3 vol % HCl solution at 60°C for 2.5 h to hydrolyze the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was cooled, washed with water and dried. The remaining solids (approximately 30 g), mainly the husks of the barley grain, were partially delignified by shaking (120 rpm) in 500 mL of 2% (wt/vol) NaOH solution at 30°C for 24 h. After being washed several times with water (until neutral pH) and dried, the carrier (approximately 10 g) was ready to be used. The preparation procedure gave a 10% yield (on a weight basis) from dry spent grains. The drying steps applied in the preparation procedure were necessary only to quantify the yields.

Airlift System Preparation

The Plexiglas airlift reactor (ALR) was sterilized with sodium hypochlorite solution (2% active chlorine) at least 2 days prior to fermentation. After draining the reactor, the sterile air supply was started and the reactor was filled with the sterilized slurry consisting of pretreated spent grains (90 g dry state) in Milli-Q water (1 L). Prior to inoculation, the reactor containing fresh carrier was washed with 50 L of sterile water. Subsequently, the reactor was charged with concentrated medium to obtain the desired concentration of the medium and then inoculated with 1 L of yeast cell suspension grown by using a rotary shaker. At the end of a 24 h batch growth, fresh sterilized medium was added and the continuous system was considered to be in steady state conditions after a period of 5 residence times.

Immobilized biomass determination

A sample of approximately 100 mL was taken from the reactor in an Erlenmeyer flask (previously dried at 105°C for 12 h and weighted). The bulk liquid was removed by decantation in a sink and the carrier was washed twice with 200 mL of distilled water each one. Then the flask with the carrier was dried at 105°C for 12 h and afterwards was weighted. After that the carrier was incubated with 100 mL of 3% (wt /vol) NaOH solution and shaken at 150 rpm for 24 h. During this time the attached biomass was completely removed from the carrier and this was verified using microscopy. The biomass free carrier was weighted again after being washed several times with 400 mL of distilled water (until the NaOH completely disappeared) and dried at 105°C for 12 h. The amount of yeast biofilm was determined from the weight difference before and after the treatment with caustic. Corrections of the biomass weight for the losses of carrier itself (approximately 6% wt) during the washing procedures were carried out with blank experiments with clean carrier.

Dry weight

Samples of 25 mL were harvested in Falcon tubes (previously weighted and dried at 105ºC overnight), and centrifugated at 5000 rpm during 10 minutes to separate the cells from the medium. Then, they were weighted and dried again at 105ºC overnight. The amount of yeast was determined from the weight difference before and after the sample acquirement.

Sugars and alcohol measures

Lactose, glucose, ethanol and glycerol were analyzed by HPLC (CHROMPACK Jasco), using a Varian MetaCarb 87H column eluted at 60ºC with 0.005M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector (Jasco 830-RI).

Plasmid Stability

Cells were grown during almost 149 hours in complex medium, and cells dilutions were grown in selective and non-selective media (YPD, YPL and MM), where cfu (colony forming units) were countered. The % of stability was calculated dividing the number of cfus in selective medium against the number of cfus in complex medium.

RESULTS AND DISCUSSION

Flask's cultures

Firstly, cultures were done in the same complex medium (YP) but with different carbon source (glucose or lactose) to check the growth, the carbon source consumption of the strain producing the hybrid protein (Figure 1 and 2), and the stability of the plasmid. The stability of the plasmid in YPD was 100% and in YPL was 96,43%, after almost 149 hours (more than 50 generations).

The strain MW190-9B/pSPGK1-LAC4-LACA-KpnI shows a similar growth in both assayed media (Figure 1 and 2) and the consumption of all the sugar (glucose or lactose respectively) finishes before 24 hours, for this reason the following experiments were focused in the first 30 hours of culture.

In order to analyse the intracellular and extracellular β-galactosidase activity of the transformed strain new culture were made in both media (Figure 3). Both intra and extracellular β-galactosidase activities are higher in YPL than in YPD.

To determinate the production of the β-galactosidase and to check the ability to grow in 5% of lactose (average concentration of lactose in cheese whey) new cultures were done at this sugar concentration (Figures 4 and 5).

An improvement in both intra and extracellular β-galactosidase activity is observed when the transformed strain is grown in YP-lactose (5%) (Figure 4) compared with the same strain growing in YP-lactose (2%) (Figure 3). At 26 hours of culture with YPL (2% lactose) only 2050 nanomol/min*mg and 135 nanomol/min*mL, intra and extracellular activity respectively were measured. However with YPL (5% lactose) at the same time, 7000 nanomol/min*mg and 340 nanomol/min*mL, intra and extracellular activity respectively were measured, being 3.4 and 2.5 times higher (intra and extracellular β-galactosidase activity respectively) compared with the condition with less sugar per liter.

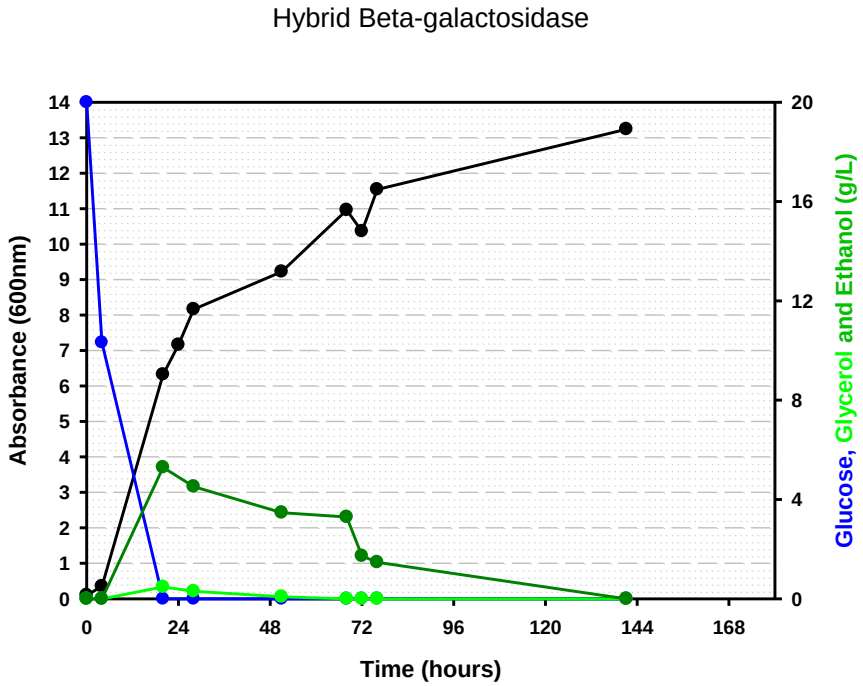

Figure 1. Growth (absorbance at 600 nm), glucose consumption, glycerol and ethanol production in YPD-glucose (2%) of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I.

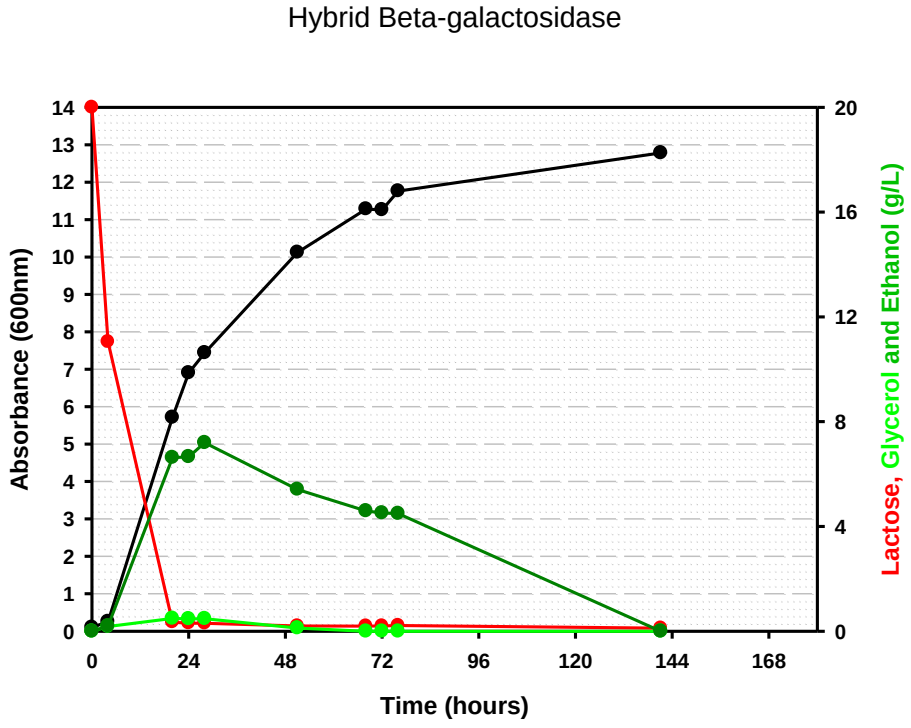

Figure 2. Growth (absorbance at 600 nm), lactose consumption, glycerol and ethanol production in YPD-lactose (2%) of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I.

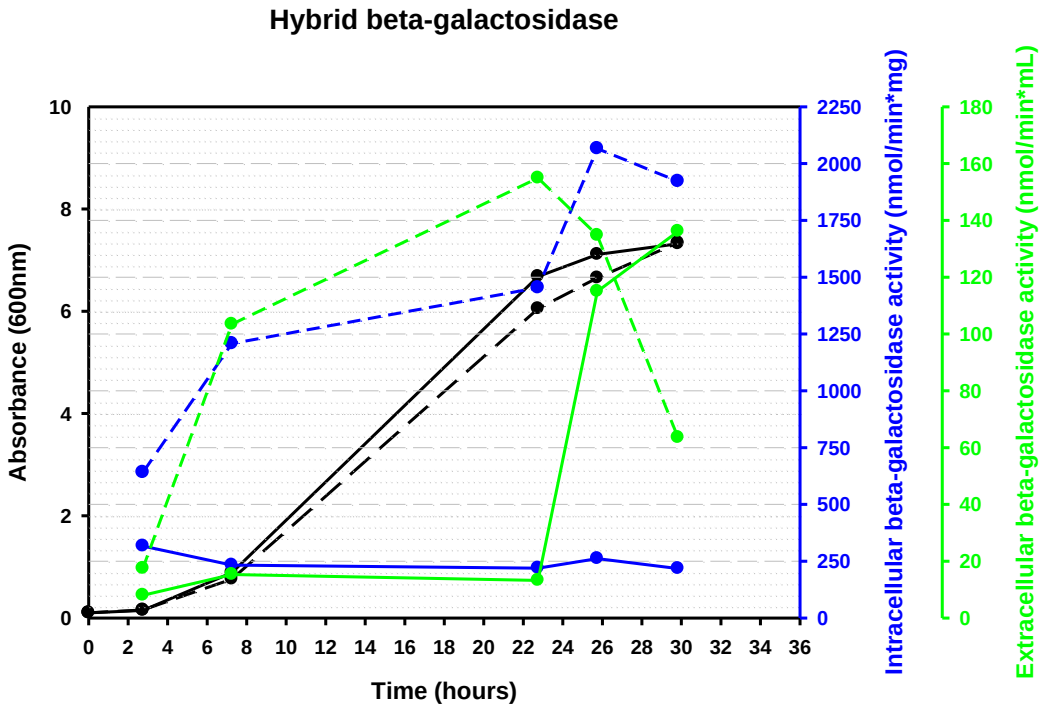

Figure 3. Growth (absorbance at 600 nm), intracellular and extracellular β-galactosidase activity of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I in YPD-glucose (2%) (full line) and in YP-Lactose (2%) (dots).

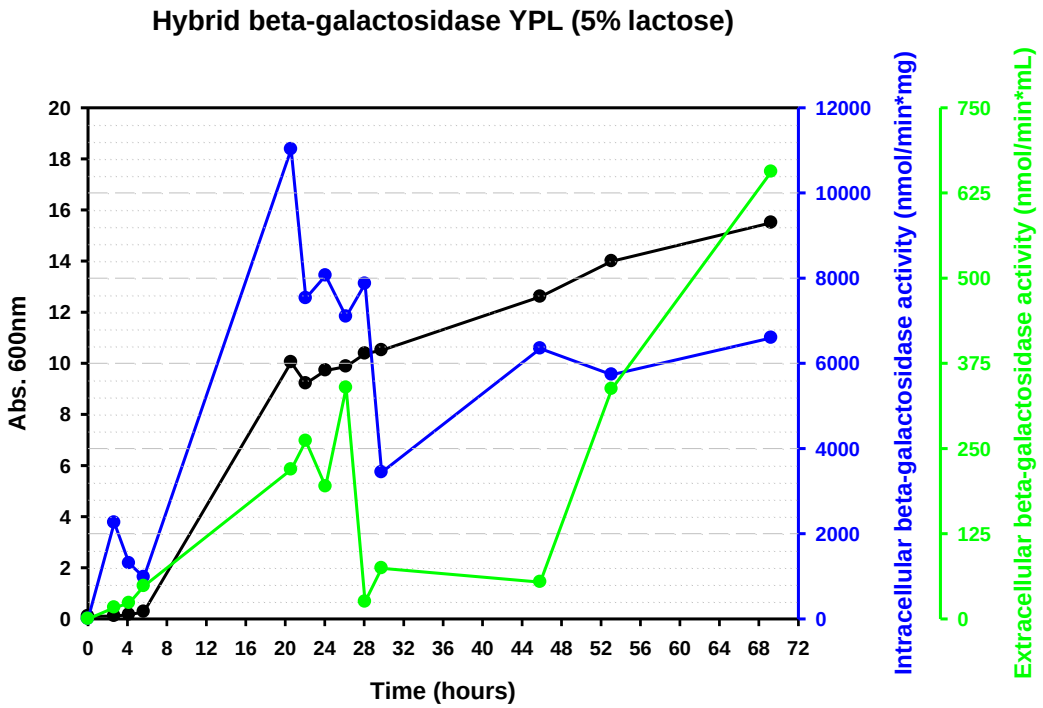

Figure 4. Growth (absorbance at 600 nm) of the yeast strain MW190-9B /pSPGK1-LAC4-LACA-*Kpn*I in YP-Lactose (5%); and intracellular (activity per mg of protein) and extracellular (activity per mL of medium) β-galactosidase activities during 72 hours.

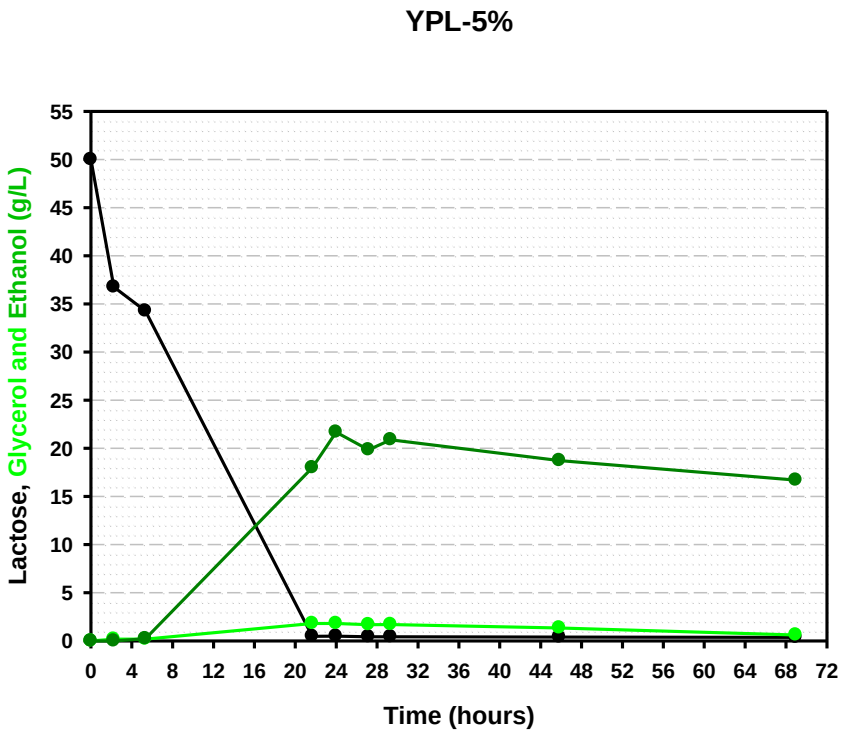

Figure 5. Lactose consumption, and glycerol and ethanol production of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I growing in YP-Lactose (5%).

As it is well known, the utilization of peptone and yeast extract in industrial processes is avoidable because they are very expensive products and make the economic balance to fall down (Johansson *et al.* 2001; Michael J. Waites 2001). Therefore, other experiments were done in SS-5% lactose medium, due to its benefits in the industrial production, measuring only the extracellular β-galactosidase activity (Figure 6).

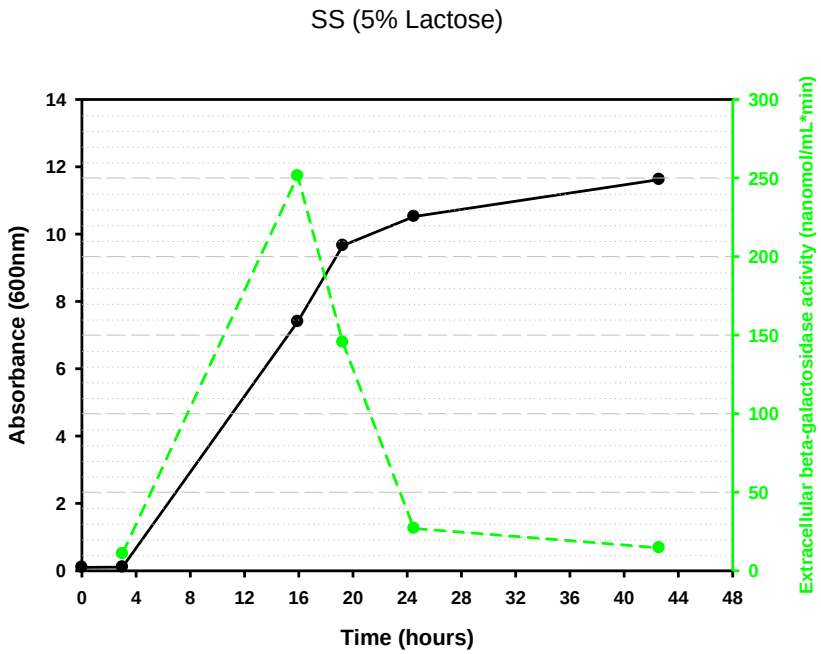

Figure 6. Growth (absorbance at 600 nm) of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I in SS-Lactose (5%), and extracellular β-galactosidase activity.

The extracellular β-galactosidase activity was improved in the SS-Lactose (5%) at 16 hours of culture with regard to the YP-Lactose (5%) medium, because the absorbance was similar in both cultures (7.5 and 7 respectively) and the extracellular β-galactosidase activity was 250 and 160 nanomol/min*mL respectively in both media.

As the SS-Lactose it is a cheaper medium (in the scale-up the economy influence the decisions of which medium is possibly profitable or not), and better production results were obtained, SS-Lactose (5%) medium was used in the following experiments.

Immobilization

Some previous experiments with *Saccharomyces* genre strains in spent grains as a support material have been done in the past, and good results were obtained (Branyik *et al.* 2002). However, to our knowledge, it is the first time that experiments with spent grains and *Kluyveromyces lactis* are made.

The procedure to prepare the spent grains and to immobilize the cells is explained in: Branyik *et al.*, 2002. Two grams of spent grains and 100 mL of culture medium inoculated to reach an initial optical density of 0.3 was maintained at 30ºC and 160 rpm during 96 hours.

0,1491 gbio/gdrech were achieved, which means that the 24,47% of the cells have been immobilized, and taking into account that the immobilization yield is usually bigger in airlifts, these results were a good start point.

Continuous Fermenters

A first fermenter was made in an airlift with 10 liters of capacity and 6 liters of working volume. The culture medium was SS-Lactose (5%), and the inoculation of the yeast reached an initial optical density of 0,602. Temperature, pH and air flow were maintained at 30ºC, 5 and 0.5 vvm respectively. Bioreactor was maintained in batch operation for 19.25 h before switching to continuous feeding. Results obtained are summarized in figures 7 and 8.

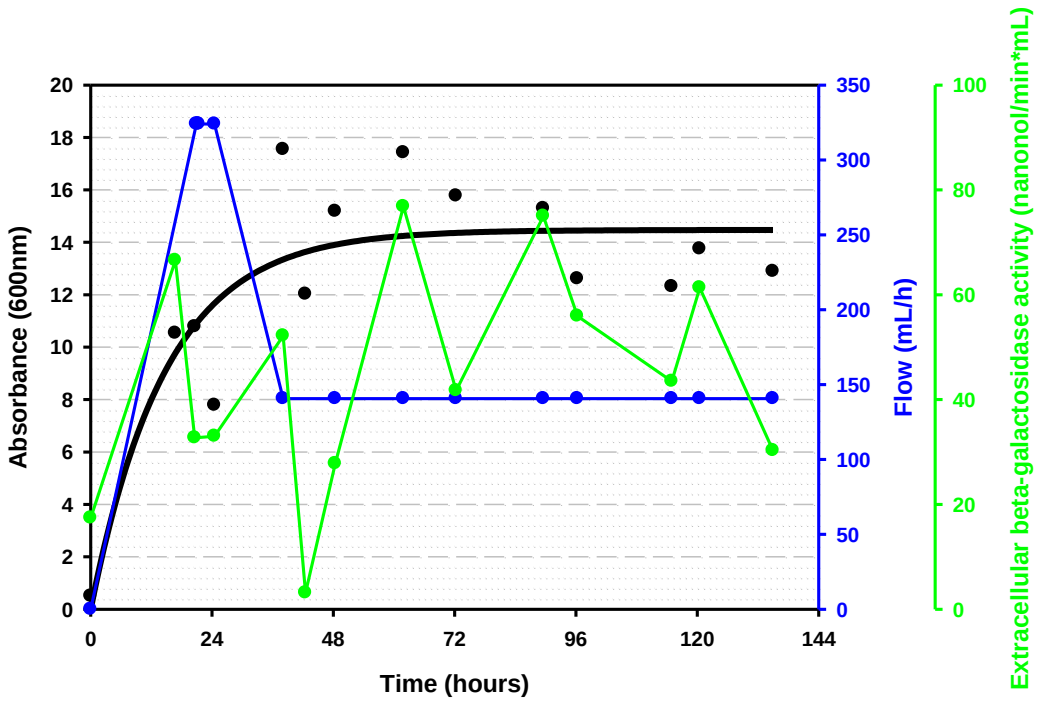

Figure 7. Growth, flow and extracellular beta-galactosidase activiy of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I in the SS-Lactose (5%) continuous airlift fermenter

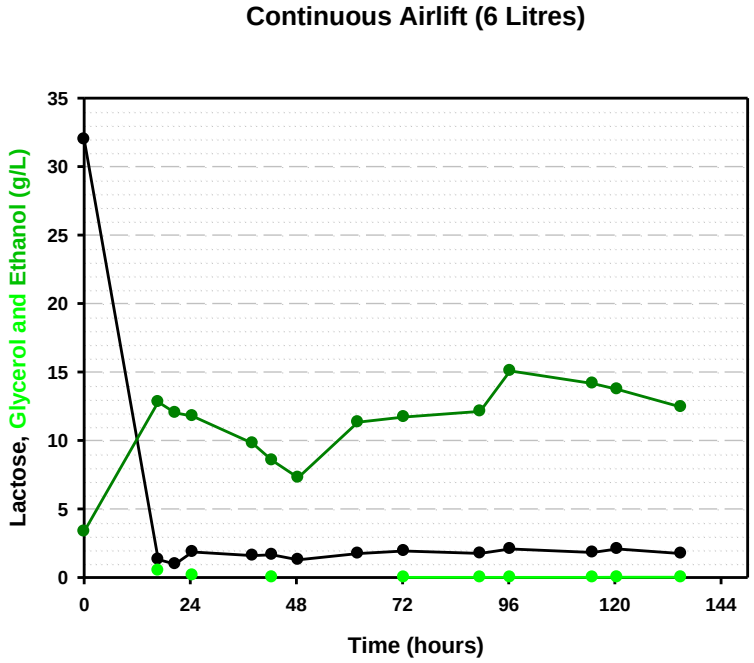

Figure 8. Lactose consumption and glycerol and ethanol production of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I growing in the SS-Lactose (5%) continuous airlift fermenter.

In this airlift the carbon source was finished before 24 hours, and the maximum of extracellular β-galactosidase activity was reached after this.

The dilution rate was not enough because the lactose concentration after the batch phase was always under 2-3 g/L, therefore the cells did not grow properly and the results obtained were poor.

A second fermenter was done in the same airlift, but using this time SS-lactose (5%) in the first 250 hours, and cheese whey powder (diluted in water to reach a concentration of 5% lactose) afterwards (Figure 9).

In this case, different dilution rates and two different feed mediums were analyzed. Dry weight was calculated to obtain a measure of "productivity", which reflects the extracellular β-galactosidase activity per gram of cells, and should give us an idea of which dilution rate is better. Table 3 shows a summary of the results of this last continuous airlift.

In general, the maximum of extracellular β-galactosidase activity corresponds with the ingestion of lactose by the yeast, and is correlated with the dilution rate and the growth of the cells (Figure 9).

Ethanol is produced during the use of the lactose, and is consumed when the lactose was not present.

Moreover cells are washed when the dilution rate is close to their exponential growth rate.

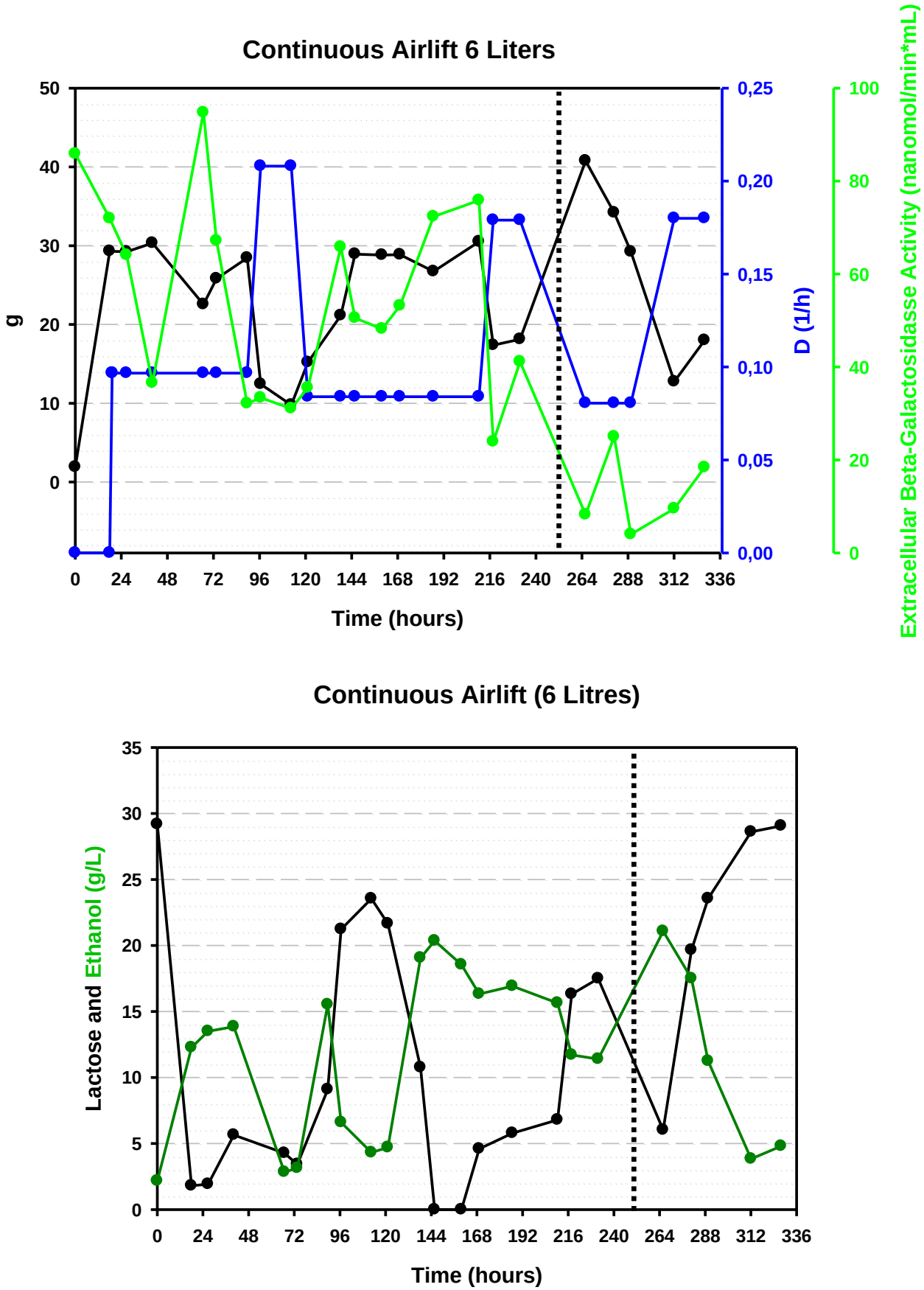

Figure 9. On the top, growth (g cells), extracellular β-galactosidase activity and dilution rate; at the bottom, lactose consumption, and ethanol production of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I growing in continuous airlift fermenter. The dots line means the change of the culture medium (from SS-lactose to cheese whey).

Table 3. Time, dilution rate, extracellular β-galactosidase activity**,** total β-galactosidase activity, g cells / g spent grains, total immobilized biomass and productivity (in terms of liberation of 1 nanomol of ortho-nitrophenol from ortho-nitrophenyl-β-D-galactopyranoside /min*g of cells) of the yeast strain MW190-9B/pSPGK1-LAC4-LACA-*Kpn*I growing in continuous airlift fermenter.

Time	D (1/h)	Extracellular β-galactosidase activity (nanomol/min*mL)	Total Extracellular β-galactosidase activity (activity in the whole reactor)	g cells/g spent grains	Total immobilized biomass g	"Productivity" (nanomol/min per g)
24-72 hours	0.097	68	410564	0.23	1.853	**221616**
73-120 hours	0.208	44	262660	0.28	1.543	**170259**
121-210 hours	0.084	51	304957	0.24	3.197	**95377**
211 - 234 hours	0.179	32	194252	0.30	2.034	**95504**
235 -289 hours	0.081	4	25405	0.37	7.547	**3366**
290 - 328 hours	0.18	4	25030	0.45	2.678	**9346**

A minimum 0.23 g dry cell g^{-1} dry carrier was obtained in the first measure (after 24 hours of continuous mode) (Table 3) which is almost the minimum immobilized biomass that was obtained in previous works (Branyik *et al.* 2002), but a maximum of 0.45 g dry cell g^{-1} dry carrier was obtained in the last measure (after 13 days). Therefore, it seems that the spent grains are a good immobilizing substrate for this recombinant yeast strain.

In the case of cheese whey the immobilization was almost the double than in the SS-lactose, so a big improvement was done using a cheaper and more pollutant culture medium.

The optimum dilution rate in the whole process was close to 0.1 h-1, which was the half of the dilution rate expected, because generally in this type of productions the optimum is reached near to the exponential growth rate, which was previously calculated as 0.213 h-1 (data not shown).

The production of extracellular β-galactosidase from cheese whey was not probably good due to the preparation of the medium (although it was tried to avoid the protein fraction of the cheese whey, it was not separated mechanically. These proteins probably affect to the mix) in the fermenter. Doing an effective protein separation before using it, will allow the use of cheese whey as medium culture.

Although good results were carried out with regard to the immobilization and production of the hybrid β-galactosidase, more experiments have to be carried out to scale-up the process, and this is probably the first step in order to produce the hybrid β-galactosidase from cheese whey with K. lactis strains immobilized in spent grains.

Acknowledgements

A.P.R. was supported by a journey grant from María Barbeito Program from Xunta de Galicia (Spain) during his stay at the Department of Biological Engineering in the University of Minho (Portugal). We want to appreciate the invitation and help of the Professor Jose Antonio Couto Teixeira, the assistant professor Lucília Maria Alves Ribeiro Domingues and the rest of the components of the research group.

CHAPTER 5

Heterologous expression and directed evolution of *Aspergillus niger* β-galactosidase

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Manuscript in preparation

SUMMARY

Saccharomyces cerevisiae strains expressing under the control of the yeast *ADH2* promoter the *Aspergillus niger* β-galactosidase gene fused in frame to the yeast α-factor secretion signal were able to secrete up to 94% of the total β-galactosidase activity into the growth medium and were able to grow in lactose-containing media. Moreover, approaches to rationally engineer the pH activity profiles of the acid *A. niger* β-galactosidase were done. Replacement of three acid residues of the surface of the enzyme: Glu439, Asp469 and Asp476 with His, Gly and Ala, respectively, led to decreases in activity and a shift in the pH profile of the enzyme to the neutral range. The most striking success in this study was in proving the feasibility of shifting the pH profile of this important enzyme, *A. niger* β-galactosidase, to enhance its catalytic efficiency in milk and sweet cheese-whey.

INTRODUCTION

The use of β-galactosidase (EC 3.2.1.23) in the hydrolysis of lactose in milk and milk products is one of the most promising applications of enzymes in the food industry (Husain 2010). The enzyme has been isolated and purified from a wide range of microorganisms but most widely used β-galactosidases in industry are derived from a few species of yeast (the most important being *Kluyveromyces lactis*) and fungal (*Aspergillus niger*) sources (Panesar *et al.*, 2006), because these could be readily obtained with acceptable productivities and yields from cultivations of these microorganisms (Oliveira *et al.* 2011). Additionally, products obtained from these organisms are generally recognized as safe (GRAS status) for human consumption, which is critical for food related applications (Panesar *et al.*, 2006). *A. niger* β-

galactosidase is secreted to the extracellular medium, which is an additional advantage in industrial applications, whereas *K. lactis* β-galactosidase is intracellular. Fungal enzyme has a pH optimum in the acidic range (2.5-5.4) and a high temperature optimum that allows their use at temperatures up to 50 °C (Panesar *et al.*, 2006). Their main application is in the hydrolysis of acid whey, which derives from the production of fresh or soft cheeses (Yang and Silva 1995). Acid whey has, however, lower interest for nutrition purposes because of its undesirable acidic flavour and high saline contents (Rubio-Texeira 2006).

For most of the applications, therefore, the pH optimum of *A. niger* β-galactosidase are too acidic. It largely limits the applications of the enzyme in milk and the neutral sweet cheese-whey (derived from hard cheese manufacturing). Generating an *A. niger* β-galactosidase more active and tolerant in neutral conditions would help to increase its applications.

The main objective of this work was the heterologous expression of the *A. niger* β-galactosidase by *Saccharomyces cerevisae* strains in order to make experiments to shift the pH-profile of the enzyme to match the sweet cheese-whey pH by rational protein engineering. Moreover, it was tested the ability of this recombinant yeast strain to grow on lactose media.

MATERIAL AND METHODS

Yeast and bacterial strains, plasmids and culture conditions

To maintain and propagate the plasmids by means of the usual DNA recombinant techniques (Ausubel *et al.*, 1995) was used the strain of *Escherichia coli*: XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsd*R17 *supE44 relA1 lac* [F'*proAB lacI*^{q}*ZD M15 Tn10* (Tet^{r})]) (Stratagene Cloning Systems).

The following vectors were used: YEpFLAG (*Eastman Kodak Company*), allows the expression in *S. cerevisiae* of the cloned gene in frame to the yeast α factor secretion signal and expressed under the control of the yeast *ADH2* promoter and *CYC1* terminator. This plasmid also contains the sequence of the FLAG peptide for the immunological detection of the secreted protein. The plasmid pVK1.1 (Kumar *et al.* 1992) which contains the *LACA* gene (which codes for *Aspergillus niger* β-galactosidase) was also used.

The following yeast strain from *S. cerevisiae: BJ3505 (pep4::HIS3, prb-Δ1.6R HIS3, lys2-208, trp1-Δ101, ura3-52, gal2, can1) (Eastman Kodak Company)* was used.

Yeast cells were transformed by the procedure of Ito *et al.* (Ito *et al.* 1983).

Liquid batch cultures of transformed strains were performed (a) in Erlenmeyer flasks filled with 20% volume of culture medium at 30ºC and 250 r.p.m. Samples were taken at regular time intervals to measure growth (absorbance at 600 nm), intra- and extracellular β-galactosidase activity, pH and ethanol; (b) in Biostat-MD (Braun-Biotech) 2 l vessel chemostat. The working volume of the culture was 1 l and the temperature was maintained at 30ºC. The air flow was 2 l/min sparged through the culture with an agitation speed of 250 r.p.m. Samples and measurements were as described above.

YPHSM modified (1% D-Glucose, 3% Glycerol, 1% Yeast Extract, 8% Bactopeptone), YP-2,5% Lactose (2.5% Lactose, 0.5% Bactotriptone, 1% Yeast Extract), YPD (0.5% Bactotriptone, 1% Yeast Extract, 0.5% D-Glucose) or sweet cheese-whey permeate were used as culture media. Sweet cheese-whey permeate from a Galician (Spain) local dairy industry (Queizuar, S. L.) was obtained by ultrafiltration; the lactose concentration was approximately 5%. The cheese-whey permeate was further concentrated by

nanofiltration and a product with approximately 15% lactose was obtained and tested.

Bacterial strain was grown in LBA (1% NaCl, 1% Bactotriptone, 0.5% Yeast Extract, 0.1% D-Glucose and 80 mg/ml of ampicillin).

PCR conditions

A 20 ng amount of template DNA was incubated with 30 pmol of primer-1 and 30 pmol of primer-2 in the presence of 0.25 mM dNTPs, *Taq* or *Pwo* polymerase buffer and 2 U of the corresponding polymerase. Initial denaturation was done at 94°C for 2 min, followed by 30 cycles of 1 min at 95°C, 2 min at 50–57°C and 1.5–2.5 min at 72°C. There was a final incubation at 72°C for 10 min to fill-in ends.

β-Galactosidase activity assays

Intra- and extracellular β-galactosidase activity was measured by the method of Guarente (Guarente 1983) with modifications. For extracellular activity, a suitable volume of the culture medium was used instead of the permeabilized cellular suspension. β-Galactosidase activity was assayed by using ortho-nitrophenyl-β-D-galactopyranoside as the substrate. The reaction mixture contained 0.8 ml of 1.7 mM ortho-nitrophenyl-β-D-galactopyranoside in 0.075 M sodium acetate buffer (pH 4.5) and 0.2 ml of suitably diluted enzyme. The reaction was allowed to proceed for 10 min at 45°C and was stopped by adding 1 ml of 0.1 M Na_2CO_3. The absorbance was measured at 420 nm. One enzyme unit (E. U) was defined as the quantity of enzyme that catalyzes the liberation of 1 nanomol of ortho-nitrophenol from ortho-nitrophenyl-β-D-galactopyranoside per min under assay conditions. E.U. are expressed per mL of culture medium.

In order to calculate the optimum pH, the reaction mixture was incubated in sodium acetate buffer adjusted respectively to pH 2; 3; 4; 5; 6; 7 and 8.

Lactose and ethanol concentration determination

Lactose and ethanol concentration were determinated using the Lactose/D-Galactose kit (Boehringer Mannheim/R-Biopharm) and the Ethanol kit (Boehringer Mannheim/R-Biopharm), respectively, following the supplier specifications, or by HPLC. In this case, two HPLC systems were used:

1. Waters HPLC, using a Shodex SC-1011 column eluted at 70ºC with Milli-Q water at a flow rate of 0.5 mL/min, and a refractive-index detector (Waters 410).

2. Waters HPLC (Waters Breeze I), using a Waters Sugar-Pak column eluted at 90ºC with Milli-Q water at a flow rate of 0.5 mL/min, and a refractive-index detector (Waters 2414).

Directed Evolution

The server for automated comparative modeling, Swiss-Model (Arnold *et al.* 2006) was used to generate a three-dimensional model of *A. niger* β-galactosidase.

The Rosetta Design Server (Liu and Kuhlman 2006; Kaufmann *et al.* 2010) was used to determine how the mutations affected the global energy of the protein.

The program GETAREA (Fraczkiewicz and Braun, 1998) was used to compute the surface exposure of each amino acid in the homology modeling of *A. niger* β-galactosidase structure.

The *QuikChange® XL Site-Directed Mutagenesis Kit* (Stratagene) was used according to manufacturer's recommendations to make the site-directed mutagenesis.

RESULTS AND DISCUSSION

Design of the YEpFLAG-LACA constructions

LACA gene, which codifies for *Aspergillus niger* β-galactosidase, was amplified by the polymerase chain reaction with the following primers using the pVK1.1 plasmid (Kumar *et al.* 1992) as template:

LACACI

5'- GAC TAC AAG GAT GAC GAT GAC AAG GAA TTC AAG CTT TCC TCC GCT TGT GC- 3'

LACACII

5'- CCG CGG GTC GAC GGG CCC GGA TCC ATC GAT CTA GTA TGC ACC CTT CCG CT- 3'

LACASS

5'- GAC TAC AAG GAT GAC GAT GAC AAG GAA TTC TCC ATT AAG CAT CGA ATC AA- 3'

LACA

5'- CTA TAT CGT AAT ACA CCA AGC TCG ACC TCG ATG AAG CTT TCC TCC GCT TG -3'

Primers present a fragment of *LACA* sequence and carried 30 nucleotides of the specific sequence required for homologous recombination with the vector YEpFLAG.

LACACI and LACACII were designed to amplify *LACA* gene by PCR and to clone into YEpFLAG vector maintaining the two secretion signal (the yeast α-factor secretion signal from the vector and the endogenous signal sequence of the *LACA*). The construction was called Y2SSLACA.

LACACII and LACASS were designed to amplify *LACA* gene by PCR and to clone into YEpFLAG maintaining only the yeast α-factor secretion signal from the vector. The construction was called YPSSLACA.

LACACII and LACA were designed to amplify *LACA* gene by PCR and to clone into YEpFLAG maintaining only the endogenous signal sequence of the *LACA* gene. The construction was called YOSSLACA.

To make the constructions Y2SSLACA and YPSSLACA, YEpFLAG was previously digested with *XhoI* and *BglII*, and in the case of YOSSLACA, YEpFLAG was digested with *NruI* and *BglII*. PCR products and the digested YEpFLAG were cotransformed in the *Saccharomyces cerevisiae* strain BJ3505. The three constructions were obtained by homologous recombination.

Kinetics of growth and secretion

Discontinuous cultures were performed for 170 hours (≈ 7 days) in 120 ml of YPHSM in *Erlenmeyer* flasks to study the kinetics of growth and secretion of *A. niger* β-galactosidase by the transformed yeasts with the different constructions (Figure 1). YPHSM media is a high stability expression media that allowed maintain the pH between 6 and 7.5. Synthesis of the enzyme is accelerated in all cases after 24 h of culture, when glucose is exhausted, since the *ADH2* promoter is glucose-repressed. β-galactosidase activity in the culture medium increased with increasing cell concentration, reaching its maximum when cell growth approached the stationary phase.

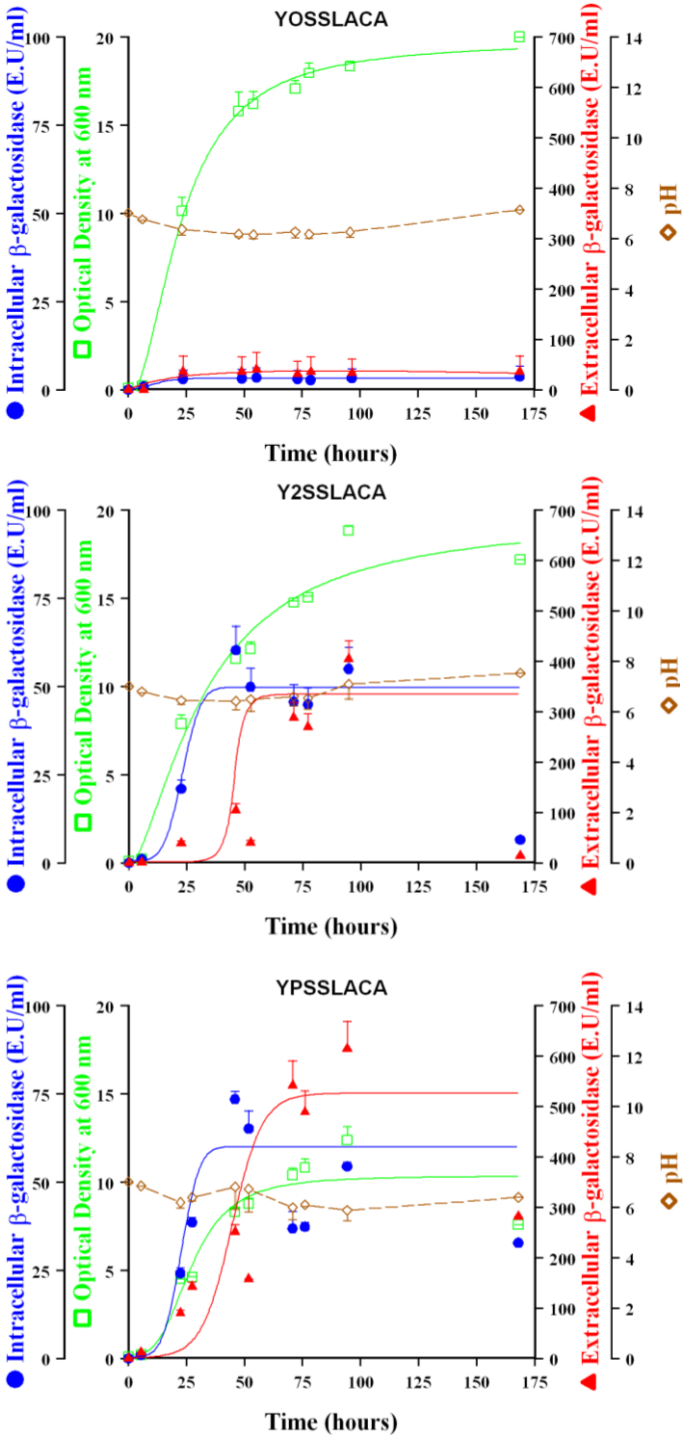

Figure 1. Growth (Optical Density at 600 nm), pH, extracellular and intracellular β-galactosidase production (E. U. mL^{-1}) by the BJ3505 strain transformed with the corresponding plasmids. Values represent the mean of 2 different cultures.

The strain transformed with the plasmid YOSSLACA showed the lowest intracellular and extracellular β-galactosidase activity (Figure 1). This result may be attributed to the fact that the endogenous signal sequence of the *LACA* gene is not operating correctly in the *S. cerevisiae* strain BJ3505.

The strain transformed with the plasmid YPSSLACA, with the signal sequence of the yeast α-factor, showed the highest levels of extracellular and intracellular β-galactosidase activity (Figure 1). This recombinant strain reached around 10-fold increase of extracellular β-galactosidase compared with the recombinant strain transformed with the plasmid YOSSLACA and a 1.7-fold increase compared with the recombinant strain transformed with the plasmid Y2SSLACA. Moreover, the strain transformed with the plasmid YPSSLACA was able to secrete up to 94% of the total β-galactosidase activity into the growth medium. Previous trials of heterologous secretion of *A. niger* β-galactosidase by *S. cerevisiae* rendered levels of 40% (Kumar *et al.* 1992) and 61% (Ramakrishnan and Hartley 1993) of the enzyme in the culture medium.

To the best of our knowledge, there have been no reports on the expression of the *LACA* gene fused in frame to the yeast α-factor secretion signal by recombinant *S. cerevisiae* cells.

Determination of the optimum pH

The supernatant of the liquid cultures and the permeabilized cells obtained at 62 hours of culture of the three recombinant *S. cerevisiae* strains were assayed to determinate the optimum pH of the different recombinant *A. niger* β-galactosidase. Measurements of the enzymatic activity were performed in sodium acetate buffer adjusted to different pH values from 2 to 8. Both intracellular and extracellular protein of the three different recombinant *A. niger* β-galactosidase showed an optimum pH of 3 (Figure 2). These results are similar to previous studies which indicate that the optimum pH values for β-galactosidase from *A. niger* are from 2.5 to 4 (Widmer and Leuba 1979).

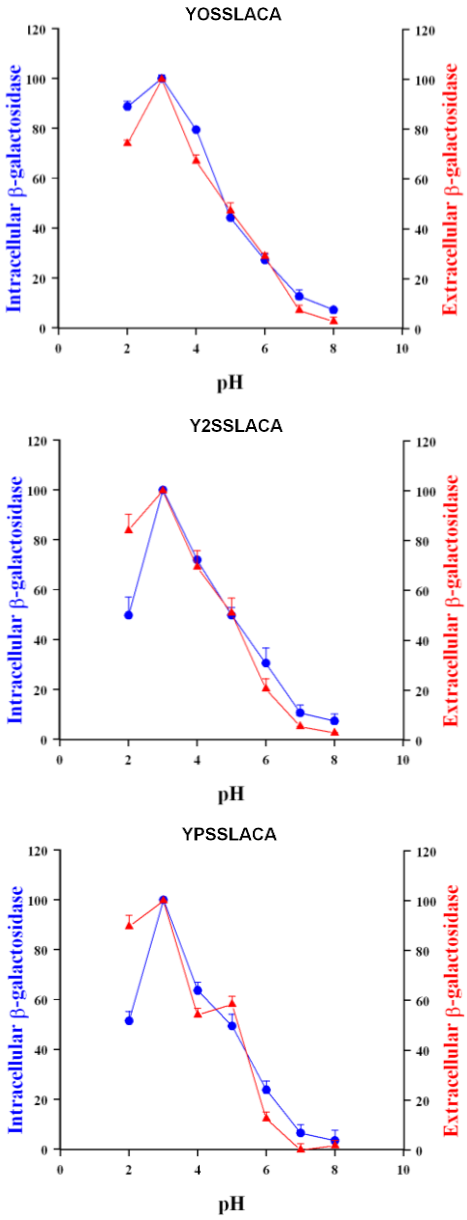

Figure 2. Determination of the optimum pH for the three different constructions. Data are the mean of two independent experiments.

Kinetics of secretion and ethanol production in lactose media.

Saccharomyces cerevisiae is the organism of choice in most commercial biotechnological processes. However, it cannot ferment lactose since it lacks both β-galactosidase and a lactose-permease system to transport the disaccharide into the cytoplasm. This inability to ferment lactose prevents this yeast from using milk whey. Milk whey could be a cheap substrate for the production of ethanol, yeast biomass or associated metabolites and, at the same time, the disposal problem would be alleviated. The development of *S.*

cerevisiae strains with the capability of metabolizing lactose is an important biotechnological objective (Becerra *et al.* 2003; Becerra *et al.* 2004).

One of the attempts to construct a *S. cerevisiae* strain than could utilize lactose included the use of the vector pVK1.1 (Kumar *et al.* 1992) to engineer different recombinant *S. cerevisiae* strains with the capacity to produce and secrete *A. niger* β-galactosidase (for a review see: (Oliveira *et al.* 2011)). Although, the main objective of this work was the heterologous expression of *A. niger* extracellular β-galactosidase in order to make experiments to shift the pH-profile of the enzyme to match the sweet cheese-whey pH, we decided to test the ability of the strain transformed with the plasmid YPSSLACA to grow in lactose media. Growth, ethanol formation and β-galactosidase production by the recombinant strain were measured both in a synthetic medium with 2.5% of lactose (Figure 3) and ultrafiltration-permeate of cheese-whey three times concentrated (Figure 4).

The recombinant strain transformed with the plasmid YPSSLACA was able to grow efficiently in lactose media without the diauxic growth typical of wild-type *S. cerevisae* in pre-hydrolyzed lactose (Porro *et al.* 1992). A 1.1-fold and 1.8-fold increase in extracellular and intracellular β-galactosidase production, respectively, were attained in a synthetic medium with 2.5% of lactose (Figure 3) comparing with the previous fermentation conducted in glucose medium (Figure 1). Domingues (Domingues *et al.* 2002) described that the β-galactosidase activity of a flocculent *S. cerevisiae* strain secreting *A. niger* β-galactosidase increased linearly with increasing lactose concentrations, between 0.5 and 15% (w/v). However, when an ultrafiltration-permeate of cheese-whey three times concentrated (around 15% w/v) was used as culture medium the maximum extracellular β-galactosidase detected was around 150 E.U/ml (Figure 4). This may be attributed to the presence of a β-galactosidase activity inhibitor in the whey permeate. Examples of *A. niger* β-galactosidase

inhibition by whey have been reported (Richmond *et al.*, 1981). Trace amounts of divalent cations in the whey were pointed out as a possible cause of this inhibitory effect.

Low ethanol productions were obtained. The reduced ethanol levels produced are in agreement with results obtained by other authors using different approaches to construct *S. cerevisiae* strains with the capability of metabolizing lactose (Porro *et al.* 1992; Rubio-Texeira *et al.* 1998), with the exception of the flocculent strain reported by Domingues *et al.* (Domingues *et al.* 1999). This non-fermentative behaviour of our recombinant strain may have interesting biotechnological advantages because they favour an efficient transformation of lactose into biomass and biomass-associated products.

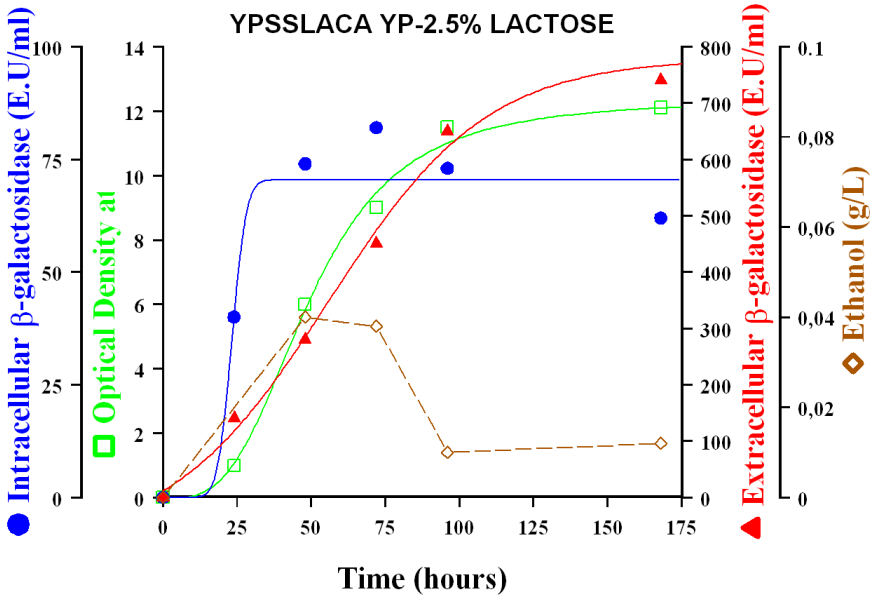

Figure 3. Growth (absorbance at 600 nm), ethanol production, extracellular and intracellular β-galactosidase activity (E. U. mL^{-1}) by the recombinant BJ3505 strain transformed with the plasmid YPSSLACA growing in YP-2.5% Lactose.

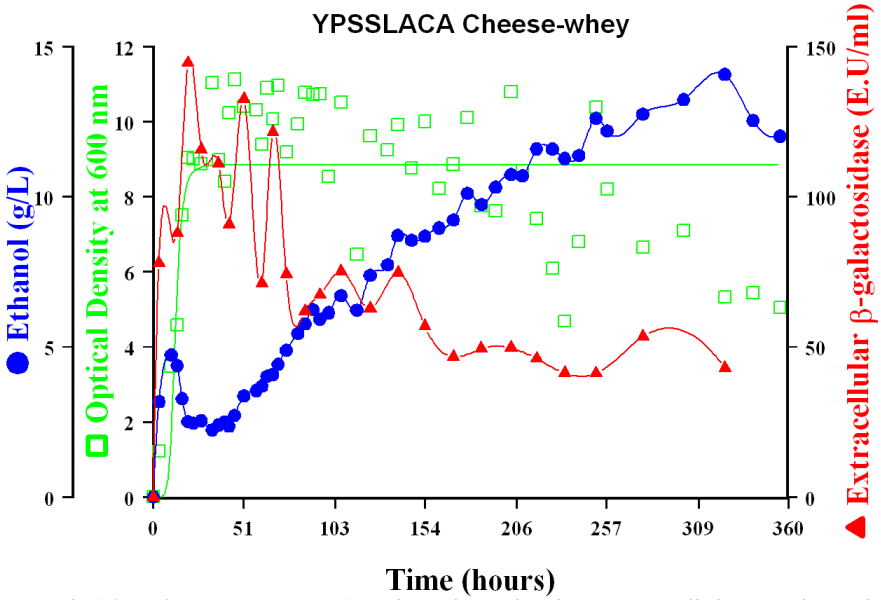

Figure 4. Growth (absorbance at 600 nm), ethanol production, extracellular β-galactosidase activity (E. U. mL^{-1}) by the recombinant BJ3505 strain transformed with the plasmid YPSSLACA growing in permeated cheese-whey three times concentrated.

On comparison with previously published heterologous *A. niger* β-galactosidase expression levels, it was found that the maximum of extracellular β-galactosidase activity reached by our recombinant strain (740 E.U./ml) was noteworthy in relation to initial attempts of expression of this enzyme by recombinant *S. cerevisiae* strains (Kumar *et al.* 1992; Ramakrishnan and Hartley 1993; Domingues *et al.* 2000). Although, subsequent works have demonstrated that increased levels of extracellular *A. niger* β-galactosidase were reached after a further optimisation (Domingues *et al.* 2002; Domingues *et al.* 2004; Domingues *et al.* 2005; Oliveira *et al.* 2007). Optimization of bioreactor operation together with culture conditions (lactose and yeast extract concentration) were described (Domingues *et al.* 2010) to led to a 21-fold increase in the extracellular *A. niger* β-galactosidase activity produced by a recombinant *S. cerevisiae* strain when compared with preliminary shake-flask fermentations. Therefore, further studies about optimisation of the medium composition and growth conditions should be done to achieve increased production and secretion of β-galactosidase activity by our recombinant *S. cerevisiae* cells. Moreover, scope also exists for industrial-scale optimisation

work with our recombinant strain, which could result in further increases in expression levels.

Even though our main goal was to produce heterologously *A. niger* β-galactosidase, we have observed that this recombinant *S. cerevisiae* strain was able to grow in lactose media. The production system hereby presented seems an interesting one for cheese-whey treatment. While reducing the organic load by hydrolyzing lactose and metabolizing the resulting monosaccharides, the recombinant strain produces *A. niger* β-galactosidase.

Modification of the optimum pH of the *Aspergillus niger* β-galactosidase

As previously mentioned, *A. niger* β-galactosidase have optimal pH within the acidic range and this restrict the use of the enzyme to acid whey hydrolysis with lower interest for nutrition purposes. Generating an *A. niger* β-galactosidase more active and tolerant in neutral conditions would help to use this enzyme for saccharifying milk and sweet cheese-whey.

Application of enzymes in processes with a specified pH condition necessitates protein engineering to change pH performance. Rational design and site-directed mutagenesis were employed to improve the activity of phytases at lower pH (Tomschy *et al.* 2002) and to alter the pH optimum of xylanases (Turunen *et al.* 2002), α-amylases (Hidaka *et al.* 2002; Bessler *et al.* 2003; Rubin-Pitel and Zhao 2006) and cellulases (Heinzelman *et al.* 2009; Cockburn and Clarke 2011).

Different strategies can be chosen to modify the pH activity profile of an enzyme (Tomschy *et al.* 2002; Cockburn and Clarke 2011). The first is the replacement of ionisable groups that are directly involved in substrate or product binding and/or catalysis by non ionizable ones or by amino acids

with different charge or pK values (Tanner *et al.* 1999; Kim *et al.* 2006). The second is the replacement of residues that are in direct contact with the binding/catalytic residues by forming hydrogen bonds and/or salt bridges. Substitution of such residues may disturb the hydrogen-bonding network in the active site or alter the electronic environment of binding/catalytic residues (Fang and Ford 1998; Wang *et al.* 2005). The effects on the pH activity profile caused by this type of mutations are particularly difficult to predict. The third is the alteration of longer-range (indirect) charge-charge interactions by modification of the surface charge of the enzyme. This can be achieved by either (non selective) chemical modification of surface residues (Rashid and Siddiqui 1998) or by selective, site-directed modification of surface charge (Loewenthal *et al.* 1993; Cockburn and Clarke 2011).

In general, it has been found that while introduction of charges near or in catalytic residues can have the most dramatic effect in altering the pKa values, this will often result in an inactive enzyme (Tynan-Connolly and Nielsen 2007). Therefore, for a first approximation to shift the optimum pH of *A. niger* β-galactosidase we have preferred focus on the introduction or removal of charged groups on the surface of the protein.

The three-dimensional structure of *A. niger* β-galactosidase has not been solved but there are several homologs that do have known structures. The server for automated comparative modeling, Swiss-Model (Arnold *et al.* 2006) was used to generate a three-dimensional model of *A. niger* β-galactosidase to facilitate the analysis of which residues would be suitable candidates for replacement (Figure 5). The homology modeling structure obtained from Swiss-Model of *A. niger* β-galactosidase was based on the structure of *Penicillium sp.* β-galactosidase (1tg7A.pdb) (Rojas *et al.* 2004). Both enzymes are members of the GH family 35. The significant sequence identity between β-galactosidase from *A. niger* and β-galactosidase from *Penicillium sp.* (69%)

enables us to affirm that both proteins should share significant structural homology.

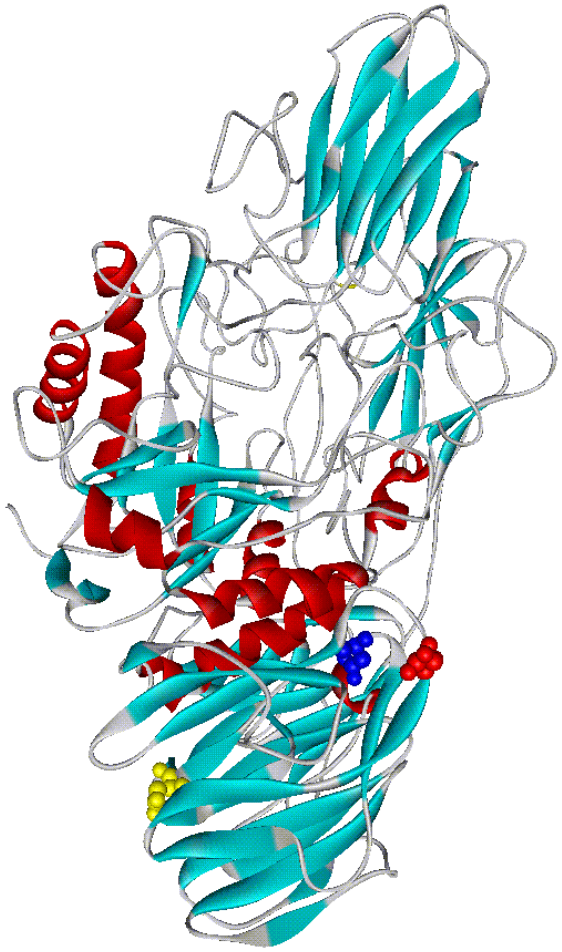

Figure 5. Ribbon diagram corresponding to the prediction of the tertiary structure of *A. niger* β-galactosidase using the Swiss-Model program. The residues selected to perform site-directed mutagenesis have been drawn as spheres of colors (Glu439 red, Asp429 blue and Asp436 yellow).

Two criteria were used for the selection of residues for replacement: first, the charged residue would be solvent exposed, which would increase the likelihood of the enzyme being able to tolerate the introduction of removal of charged residues and, secondly, the residue would pass the test of the RosettaDesign Server (Liu and Kuhlman 2006; Kaufmann *et al.* 2010). TheRosettaDesign server identifies low energy amino acid sequences for target protein structures (http://rosettadesign.med.unc.edu). The client provides the backbone coordinates of the target structure and specifies which

residues to design. The server returns to the client the sequences, coordinates and energies of the designed proteins. RosettaDesign has been experimentally validated and has been used previously to stabilize naturally occurring proteins and design a novel protein structure(Liu and Kuhlman 2006; Kaufmann *et al.* 2010). With respect to the first criteria, the program GETAREA (Fraczkiewicz and Braun, 1998; http://curie.utmb.edu/getarea.html) was used to compute the surface exposure of each amino acid as a ratio of its exposed surface area in the homology modeling of *A. niger* β-galactosidase structure to the area exposed when it is in the peptide Gly-X-Gly. Residues were scored as 'surface exposed' if this ratio was ≥0.30. 38 acid amino acids and 21 basic amino acids were considered to be surface exposed in the homology modeling of *A. niger* β-galactosidase structure.

Only mutations of three residues matched the two criteria: Glu439His (charge reversal), Asp469Gly (charge removal) and Asp476Ala (charge removal) (Figure 5). These mutations and all the possible combinations of these three mutations were created (except for the combination of the three mutants that for unknown reasons it was impossible to create it). Site-directed mutagenesis was performed using the *QuikChange® XL Site-Directed Mutagenesis Kit* (Stratagene) using the construction YPSSLACA as template and the appropriate primers (Table 1) to make the PCR.

To obtain the double mutations, Glu439His mutant was used as template to generate Glu439His/Asp469Gly mutant and Glu439His/Asp476Ala mutant, whereas Asp469Gly mutant was used as template to generate Asp469Gly/Asp476Ala mutant.

Table 1. List of primers and mutations performed.

Mutant	Forward	Reverse
Glu439His	CTACAGCAGCGAAcAcTCAACATCATACAA	TCCGAATGTCTGACCACGAAGAAAGAG
Asp469Gly	CACTTAATGGACGCGgTTCAAAGATACACG	TGAGTGTACCACCAAGCTGAGGGATA
Asp476Ala	AAGATACACGTGACCGcTCACAATGTCTC	TGAATCGCGTCCATTAAGTGTGAGTGTACC
Glu439His/Asp469Gly	CACTTAATGGACGCGgTTCAAAGATACACG	TGAGTGTACCACCAAGCTGAGGGATA
Glu439His/Asp476Ala	AAGATACACGTGACCGcTCACAATGTCTC	TGAATCGCGTCCATTAAGTGTGAGTGTACC
Asp469Gly/Asp476Ala	AAGATACACGTGACCGcTCACAATGTCTC	TGAATCGCGTCCATTAAGTGTGAGTGTACC

After polymerase chain reaction, the product was subjected to *DpnI* (Stratagene) digestion to remove template DNA and then transformed into *E. coli* XL1-Blue cells. The mutations were confirmed by nucleotide sequencing and the plasmids were transformed into *S. cerevisiae* BJ3505 strain for expression trials.

The β-galactosidase activities of the various mutant enzymes were screened at pH 3, 4, 5, 6 and 7 to determine the effect, if any, of the amino acid replacements on the pH-activity profile of *A. niger* β-galactosidase (Figure 6). The introduction of mutations at these positions with basic and neutral replacements resulted in dramatic losses of activity. Whereas the Glu439His mutant maintained only 0.4% of wild-type activity, the Asp469Gly mutant had 3.1% of wild-type activity and the Asp476Ala mutant retained 1.5% of wild-type activity. However, while the activity was decreased, there were a shift in the pH profile to the neutral range in the mutant Glu439His and the mutant Asp476Ala (Figure 6). More than 54% and 48% of the residual activity of the Glu439His mutant was retained at pH 6.0 and pH 7.0, respectively, which

revealed that mutant Glu439His was most neutral-tolerant among the three mutants and wild-type *A. niger* β-galactosidase.

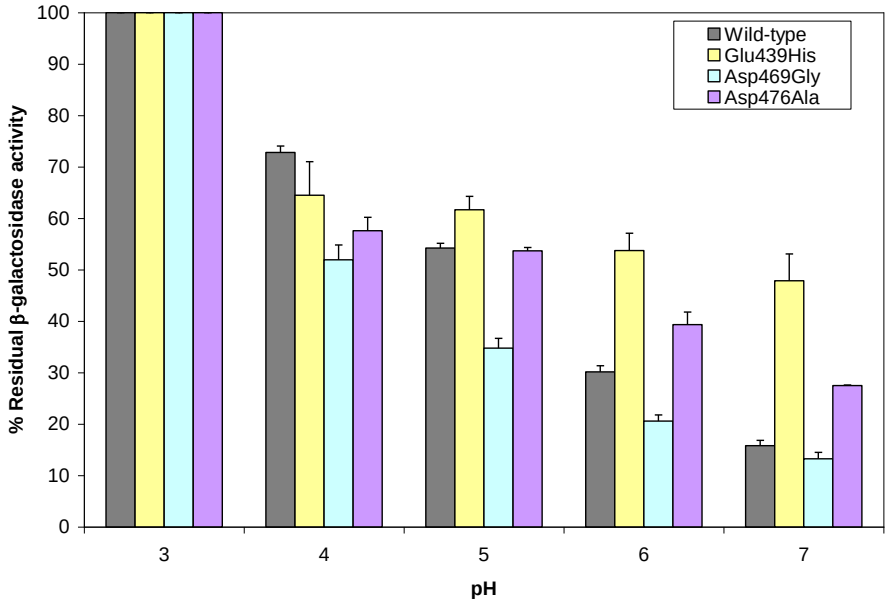

Figure 6. pH-activity profiles of the *A. niger* β-galactosidase wild-type and the three mutants generated. Activities at optimal pH were defined as 100%. Error bars represent the standard deviation from four separate experiments.

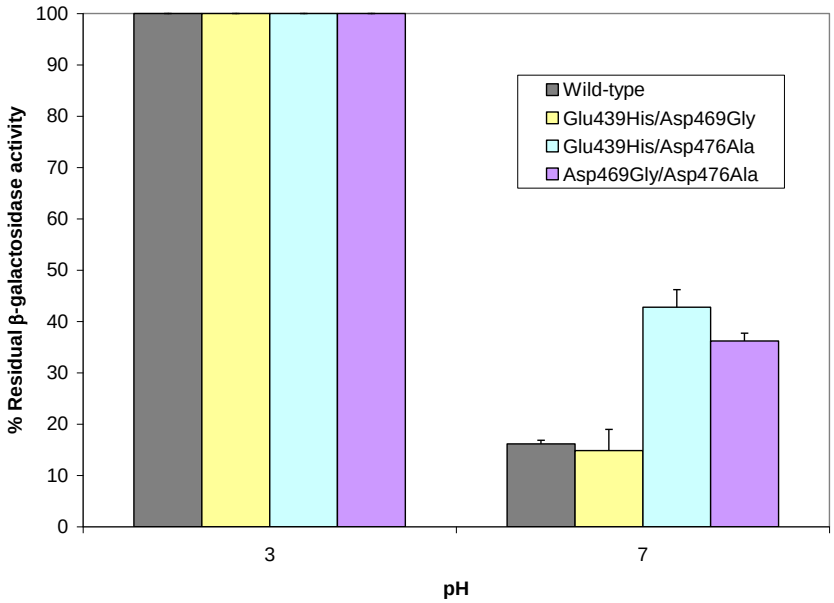

Figure 7. pH-activity at 3 and 7 of the *A. niger* β-galactosidase wild-type and the three doubled mutants generated. Activities at optimal pH were defined as 100%. Error bars represent the standard deviation from four separate experiments.

The double mutants also led to decreases in activity. The double mutant Glu439His/Asp469Gly retained 1.7% of wild-type activity, the Glu439His/Asp476Ala mutant had 0.95% of wild-type activity and the Asp469Gly/Asp476Ala retained 1.0 % of wild-type activity. In this case, the double mutants Glu439His/Asp476Ala and Asp469Gly/Asp476Ala showed a shift in the pH profile to the more neutral side (Figure 7).

Although the effect of the single and double mutants, far of the hypothetical catalytic residues: Glu200 and Glu298 (http://www.uniprot.org/uniprot/P29853), over the pH-activity profile of *A. niger* β-galactosidase is difficult to explain, it has been described that the structural bases of the alkalophilic character of the alkaline endoglucanases produced by bacteria and alkaline proteases involved an increase in the number of Arg, His, and Gln residues, and a decrease in Asp and Lys residues (Wang *et al.* 2005). In our case, the decrease in Asp residues and/or the increase in His residue could explain the effect of the single mutants Glu439His, Asp476Ala and the double mutants Glu439His/Asp476Ala and Asp469Gly/Asp476Ala over the pH profile of *A. niger* β-galactosidase. Nevertheless, the precise mechanisms for the decrease of activity and over the pH-activity profile by these mutations are difficult to predict and structural analysis are necessary to further characterize the *A. niger* β-galactosidase and these mutations.

In conclusion, although our data are preliminary, our study shows that it is feasible to improve the function of *A. niger* β-galactosidase under neutral pH conditions by rational protein engineering.

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Concluding remarks

The concluding remarks of this thesis are the following:

1. A methodical and efficient approach has been carried out to growth *Kluyveromyces lactis* β-galactosidase crystals. The full-factorial screen with response surface optimization allowed us to find conditions for growing good quality crystals with a small number of experiments to be performed. Optimal crystallization conditions for 20 μg of *K. lactis* β-galactosidase were obtained in the presence of 0.1 M Tris-HCl, pH 8, 15% PEG 6000 and 0.02 M $(NH_4)_2SO_4$. Advantages obtained in this approach include improvements in β-galactosidase crystal volume and shape and also in reproducibility.
2. Crystallization of *K. lactis* β-galactosidase (3.5 mg mL-1 in 0.05 M Tris-HCl, 0.150 M NaCl and 0.002 M DTT, 7% Glycerol) was performed using the sitting drop method. Small plate-shaped crystals grew in 23-27% (w/v) Polyethylene Glycol (PEG) 3350, 0.1 M BisTris pH 7.5-7.0, 0.2 M Sodium Tartrate. Streak seeding performed under these conditions gave improved quality crystals that were suitable for X-ray diffraction experiments.
3. *K. lactis* β-galactosidase subunit folds into 5 domains in a pattern conserved with other prokaryote enzymes solved for GH2 family, although two long insertions in domains 2 (264-274) and 3 (420-443) are unique and seem related to oligomerization and specificity.
4. *K. lactis* β-galactosidase tetramer is an assembly of dimers, with higher calculated dissociation energy for the dimers than for its assembly, which can explain that equilibrium exists in solution between the dimeric and tetrameric form of the enzyme.
5. Two active centres are located at the interface within each dimer, in a narrow channel of 10 Å width that makes the catalytic pockets accessible to the solvent. The unique insertion at loop 420-443 protrudes into this channel and makes many putative links with the aglycone moiety of docked lactose, which may account for a high affinity of *K. lactis* β-galactosidase for this substrate and therefore might explain its unusually high hydrolytic activity.
6. None of the structural determinants responsible for the reaction mechanism proposed to the *Escherichia coli* β-galactosidase, which involves transition from a deep to a shallow stage following substrate binding, are envisaged in the *K. lactis* β-galactosidase active site and, consequently, we suggest that this mechanism rules only for GH2 enzymes being regulated by the lac operon.

7. A hybrid protein obtained by replacing the fifth domain of the β-galactosidase of *K. lactis* by the one of *Aspergillus niger*, is active, reaches the culture medium and presents, in addition, greater stability at high temperatures and more convenient kinetics parameters for its biotechnological utilization.
8. The *K. lactis* strain secreting the hybrid protein between *K. lactis* and *A. niger* β-galactosidases was immobilized in spent grains to produce the hybrid protein using lactose as carbon source. Spent grains were a good carrier to immobilize the recombinant strain.
9. *Saccharomyces cerevisiae* strains expressing under the control of the yeast *ADH2* promoter the *A. niger* β-galactosidase gene fused in frame to the yeast α-factor secretion signal were able to secrete up to 94% of the total β-galactosidase activity into the growth medium and were able to grow in lactose-containing media.
10. Approaches to rationally engineer the pH activity profiles of the acid *A. niger* β-galactosidase were done. Replacement of three acid residues of the surface of the enzyme: Glu439, Asp469 and Asp476 with His, Gly and Ala, respectively, led to decreases in activity and a shift in the pH profile of the enzyme to the neutral range.

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Appendix I – Material and Methods

1. BIOLOGICAL MATERIAL

1.1 Bacterial strains

All the *Escherichia coli* strains used in this work to amplify the plasmids and to make the constructions are shown in the following table 1.

Table 1. List of the *Escherichia coli* strains used.

STRAIN	GENOTYPE	REFERENCE
DH5α	*supE44 DlacU169 f80lacZDM15 hsd*R17 *recA1 endA1 gyrA96 thi-1 relA1*	GibcoBRL
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsd*R17 *supE44 relA1 lac [F´proAB lacI^q ZΔM15 Tn10 (Tet^r)]^c*	Stratagene Cloning Systems

The bacterial strains were usually conserved in LB plates at 4ºC, after their growth at 37ºC overnight and periodically recultured each two months. The same happened with the strains transformed with plasmids with ampicillin resistance, but making them grow in LB plates supplemented with ampicillin.

To maintain the strains during a long time, bacteria were inoculated in the same culture mediums but in liquid state. They grew with stirring overnight at 37ºC, and afterwards they were added into a vial (volume=2mL) with glycerol at 20%. Then, samples were frozen at -80ºC.

1.2 Yeast strains

Saccharomyces cerevisiae (table 2) and *Kluyveromyces lactis* (table 3) yeast strains were conserved in YPD or CM at 2% glucose plates (see yeast culture mediums) at 4ºC, after having grown at 30ºC during two or three days. They were recultured periodically each two or three months. To keep them during

long time, they were inoculated in YPD or CM, grown with stirring at 30ºC, and afterwards they were added into a vial (volume=2mL) with glycerol at 50%, and finally were frozen at -80ºC.

Table 2. List of the *Saccharomyces cerevisiae* strains used.

***Saccharomyces cerevisiae* STRAINS**		
BJ3505	*pep4::HIS3, prb-⊿1.6R HIS3, lys2-208, trp1-⊿101, ura 3-52, gal2, can1*	Eastman Kodak

Table 3. List of the *Kluyveromyces lactis* strains used.

***Kluyveromyces lactis* STRAINS**		
Y1140	*MATa wt*	Provided by R. Zitomer, University at Albany, USA
MW190-9B	*MATa lac4-8 uraA Rag*$^+$	Provided by Micheline Wesolowski-Louvel. *Université Claude Bernard.* Lyon. France.

2. CULTURE MEDIA

1.5 % of bacto agar was added to the liquid mediums to prepare the semi-solid mediums. Solutions were sterilized in an autoclave at 121ºC and 2Ba during 20 minutes.

2.1 Bacteria culture media

LB (Luria-Bertani)

It is a general medium for bacteria growth, and its composition is:

- 1% Bactotryptone
- 0.5% Yeast Extract
- 0.5% NaCl
- 0.1% Glucose

LBA (Luria-Bertani supplemented with ampicillin)

A Luria-Bertani medium supplemented with ampicillin (6-[D(-)-alpha-Aminophenylacetamide]-penicillanic acid sodium salt from Sigma Aldrich) for the growth of bacteria transformed with plasmids which give the bacteria the ampicillin resistance

The ampicillin solution is prepared at a final concentration of 70 mg/mL with distilled water and is conserved at -20ºC. The final concentration in the LBA medium is 70 μg/mL.

The gene which confers the ampicillin resistance codifies for an enzyme which is secreted to the periplasmic space, where catalyze the hydrolysis of the antibiotic β-lactamic ring.

LBA/X-Gal/IPTG

LBA plates supplemented with X-Gal (Eppendorf) and IPTG (Roche Diagnostics Corporation). After the sterilization of the LBA medium, 2 mL of 20 mg/mL of X-Gal in N,N-dimethylformamide and 400 μL of a IPTG solution 0.23 mg/mL in sterile distilled water were added to the medium.

These plates were used to distinguish which colonies had or not β-galactosidase. This happens due to the X-Gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside) which is hydrolyzed by the β-galactosidase giving a specific blue color. IPTG (Isopropyl-D-1Thiogalactopyranoside) is the inductor of the β -galactosidase in bacteria. It is a chemical analogue of allolactose which is non-hydrolyzable by the β-galactosidase, so works as inductor of the *E. coli* operon lac.

SOC

This liquid medium is used to obtain competent cells. Its composition is:

- 2% Bactotryptone
- 0.5% Yeast Extract
- 0.0584% NaCl
- 0.0186% ClK

2.2 Yeast culture media

YPD

It is a general medium for yeast growth. Its composition:

- 1% Yeast Extract
- 0.5% Bacto peptone
- 2% Dextrose

YPL

It is the same medium as YPD, but the carbon source is changed from glucose to lactose.

CM

Also known as SC, Synthetic Complete (Zitomer & Hall, 1976). It is a synthetic medium prepared in different way depending on the selection that it is needed. For example, a CM-Ura is a complete synthetic medium but

without uracil. Usually the carbon source used is glucose at 2%, but can be changed if It is necessary (for example: lactose, glycerol, galactose, etc).

Its composition is (to make 1L):

Glucose	**20 g**
Amino acids mix (200X)	**5 mL**
YNB*	**67 mL**
Histidine, Leucine, Uracil, Adenine, Tyrosine**	**40 mg each**
Trytophan	**30 mg**

* Added in sterile conditions after the sterilisation of the rest of the components, when the medium reaches 60ºC.

** The auxotrophic marker was not added.

Amino acids mix (200X) composition (to make 1L):

Arginine, Methionine, Threonine	**2 g each**
Isoleucine, Phenylalanine	**12 g each**
Lysine	**8 g**

YNB (Yeast Nitrogen Base) composition (to make 1L):

Vitamins Mix 300X	**50 mL**
Trace Salts 150X	**100 mL**
Ammonium sulphate*	**75 g**
KH_2PO_4	**15 g**
$MgSO_4$	**7.5 g**
NaCl	**1.5 g**
$CaCl_2$	**1.5 g**

*Added when the rest of the components are dissolved, because of its insolubility.

Vitamins Mix 300X (to make 1L)	mg	Trace Salts 150X (to make 1L)	mg
Biotin	0.6	Boric acid	75
Calcium pantothenate	120	Cupric sulphate	6
Folic cid	0.6	Potassic Iodure	15
Inositol	600	Ferric chloride	30
Niacin	120	Manganese sulfate	60
p-Aminobenzoic acid	60	Sodium molybdate	30
Pyridoxine	120	Zinc sulfate	60
Riboflavin	60		
Thiamine	120		

YPHSM modified

General medium for yeast growth, recommended by Eastman Kodak for the production of recombinant proteins with YEpFLAG.

Its composition is:

- 1% Yeast Extract
- 8% Bacto peptone
- 1% Dextrose
- 3% Glycerol

SS-Lactose

Minimum medium which contains:

- 0.5% KH_2PO_4
- 0.2% $(NH_4)_2SO_4$
- 0.04% $MgSO_4 \cdot 7H_2O$
- 0.2% Yeast Extract

- 5% Lactose.

Cheese Whey

Initially cheese whey obtained from the local dairy plant Queizuar S.L. (Bama, A Coruña, Spain) was used to do the cultures without any modifications. Afterwards, ultrafiltrated permeate of cheese whey concentrated (3X) was used in the fermentations. If a protein precipitation was observed after autoclave sterilization at 121ºC for 15 min, then it was removed by centrifugation (15 min at 10000 r.p.m.) under sterile conditions.

3. CLONING VECTORS AND MOLECULAR MARKERS

3.1 Cloning vectors

- **T-vector** (*pMBL*)**:** contains the bacterium replication origin, the α-peptide, a Multiple Clonig Site (MCS), the *lac* promoter, the pUC replication origin and the ampicillin resistance gene.

- **YEpFLAG** (*Eastman Kodak Company*)**:** contains *ADH2* promoter, which is repressed with glucose, yeast α factor secretion signal, twenty-four base pairs that codifies the FLAG peptide for the inmunological detection, 2 *micron* to use it in *S. cerevisiae*, the ampicillin resistance gene, the selection marker *TRP1* and the bacterial replication origin.

- **pSPGK1** (X. J. Chen. Institut Curie. Orsay. France)**:** contains the secretory signal that corresponds to the pre-sequence (16 amino acids) of the *K. lactis* killer toxin (α-subunit) and the constitutive promoter and the terminator of the *S. cerevisiae* phosphoglycerate-kinase (*PGK*) gene.

- **pLX8** (Das and Hollenberg 1982)**:** contains the gene *LAC4* which codes for *Kluyveromyces lactis* β-galactosidase, and the *LAC12* gene which codes for *K. lactis* lactose permease.

- **pVK1.1** (Kumar *et al.* 1992)**:** contains the gene *LACA* which codes for *Aspergillus niger* β-galactosidase.

3.2 Molecular Markers

To calculate the molecular weight of the PCR fragments and the constructions, two different molecular markers were used:

- DNA lambda phage digested by the restriction enzyme *BstEII* from BioLabs™ (Daniels *et al.* 1983) (Figure 1).

- GeneRuler™ 1 kb DNA Ladder, 250-10,000 bp. The ladder is a mixture of chromatography-purified individual DNA fragments. (Figure 1)

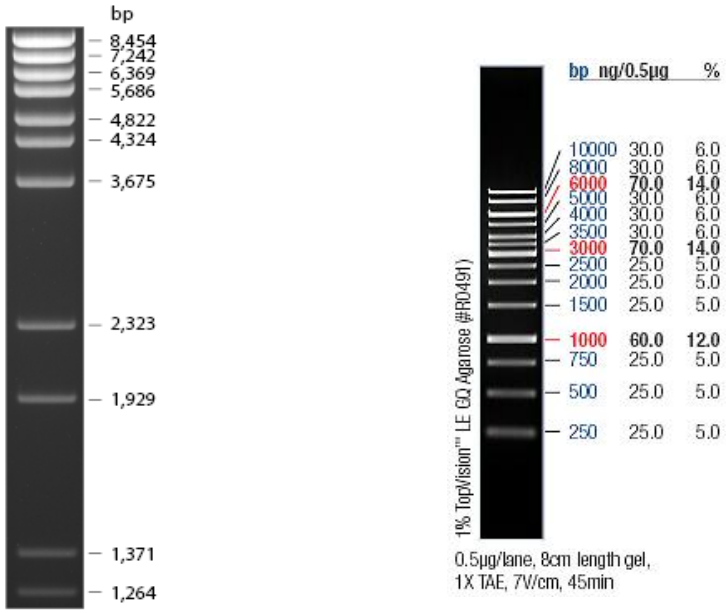

Figure 1. On the left, DNA lambda phage digested by the restriction enzyme BstEII. On the right, GeneRuler™ 1 kb DNA Ladder, 250-10,000 bp.

In Southern Blot experiments the DIG Labeled DNA Molecular Weight Marker VII (Roche) was used (Figure 2).

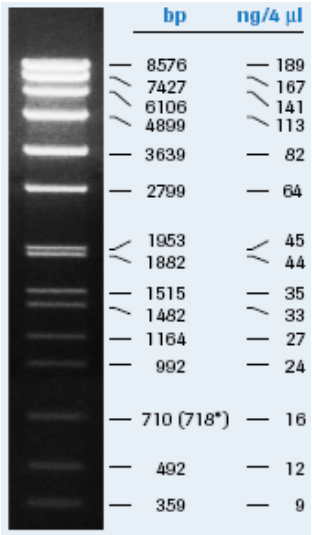

Figure 2. DIG Labeled DNA Molecular Weight Marker VII.

To calculate the molecular weight of the proteins, two different molecular markers were used (Figure 3):

- SDS- Broad Range Marker (New England Biolabs)
- PageRuler™ Plus Prestained Protein **Ladder** (Fermentas)

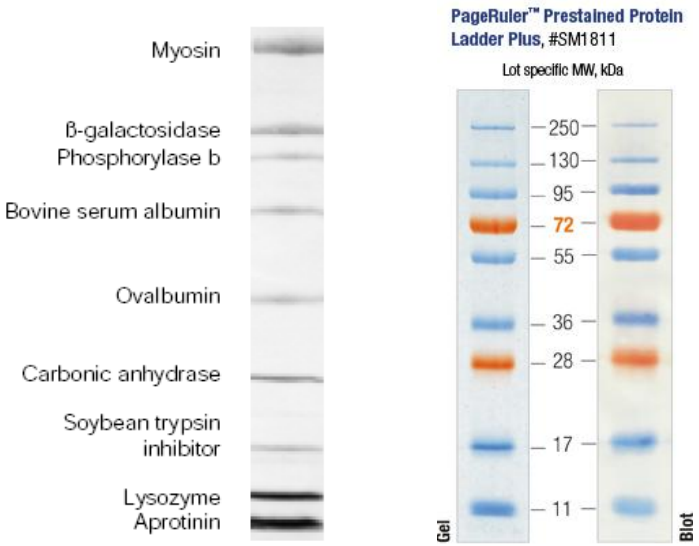

Figure 3. On the left, SDS- Broad Range Marker. On the right, PageRuler™ Plus Prestained Protein Ladder.

4. Molecular Biology Techniques

4.1 DNA extraction from bacteria

4.1.1 Plasmid DNA extraction from *E. coli* (Sambrock *et al.*, 1989; Ausubel *et al.*, 1995). Alkaline Lysis Protocol.

To obtain plasmid DNA in small and large scale (miniprep or maxiprep), the same procedure was used, changing only the initial volume (1 mL or 25 mL respectively). The volume of the solutions was adapted to the scale of the extraction.

25 mL:

Cells were inoculated in 25 mL of adequate media (with the appropriate antibiotic), and grew overnight at 37ºC with stirring. The following day, cells were precipitated at 8000 r.p.m. during 5 minutes. Supernatant was discarded, and pellet was resuspended in 1.5 mL of Solution I using vortex. Then an incubation of 5 minutes at room temperature was done, with the tube opened. Afterwards, 3 mL of Solution II were mixed thoroughly in the tube by inversion 4–6 times, and incubated during 5 minutes in ice. Then 2.5 mL of solution III were added, and mixed again inverting the tube 4–6 times. After 5 minutes of incubation in ice, the mix was centrifuged at 12000 r.p.m. during 10 minutes. The supernatant was transferred to a new clean tube. 5 mL of PCIA (mix of phenol, chloroform and isoamyl alcohol) were then added, mixed by vortex, and centrifuged 5 minutes at 8000 r.p.m. Then the aqueous phase was collected and was added to a new clean tube, with 10 mL of ethanol at 95% to precipitate the DNA. A new vortex was made, and the samples were incubated during 10 minutes at -10ºC. Afterwards the samples were centrifuged at 13000 r.p.m. 15 minutes. Pellet was washed with 10 mL of

ethanol at 70%, and centrifuged again at 13000 r.p.m. 15 minutes. Finally, pellet was dried at vacuum and resuspended in 100-250 μL of Milli-Q water.

- **Solutions:**

Solution I

Glucose	**50 mM**
EDTA	**10 mM**
Tris-HCl pH 8	**25 mM**

It was sterilised in the autoclave and kept at 4ºC in the fridge.

Solution II

NaOH	**0.2 M**
SDS	**1%**

It was prepared once it was needed from two sterile solutions of NaOH 2M and SDS at 20%.

Solution III

Potassium Acetate 5M	**60 mL**
Glacial acetic acid	**11.5 mL**
Milli-Q Water	**28.5 mL**

It was sterilised in the autoclave and kept at 4ºC in the fridge.

PCIA

To prepare, liquid phenol was mixed in a proportion of 1:1 with chloroform, and was equilibrated with 1xTE in a proportion of 3:1 shaking vigorously during several minutes. After that, the mix was incubated at room temperature in the darkness, until the aqueous phase was completely separated. Then the aqueous phase was aspired, repeating this procedure at least 2 or 3 times. Finally a proportion of 1/25 isoamyl alcohol was added and the mix was kept at 4ºC in the darkness.

Treatment with RNAse

Using the last protocol, although a great amount of DNA is purified, a considerable amount of RNA is also extracted, and for some procedures it was necessary to eliminate it. In these cases, 0.1 μg of RNAse (Roche and Fermentas) were added for each 1 mL of DNA, and incubated during 15 minutes at 37ºC.

4.1.2 Plasmid DNA extraction from *E. coli* using QIAprep® or similar commercial kits

Cells were inoculated in LBA plates and were grown overnight at 37ºC. Then, cells were collected using a toothpick, and added into an *Eppendorf* tube with 250 μL of buffer 1 (Resuspension buffer). After a vigorous vortex during 20 seconds, 250 μL of buffer 2 (Lysis buffer) were added, and the samples were mixed thoroughly by inverting the tube 4–6 times. Afterwards 350 μL of buffer 3 (DNA Binding Buffer) were added and mixed thoroughly by inverting the tube 4–6 times again.

Then, the samples were centrifuged at 13000 r.p.m. 10 minutes. Supernatant was applied to a spin column and centrifuged at 13000 r.p.m. 1 minute. The flow-through was discarded, and the column was washed with

600 μL of buffer 4 (Column Wash Buffer), and centrifuged at 13000 r.p.m. 1 minute.

Finally, the spin column was placed in a new 1.5 mL *Eppendorf* tube, and DNA was eluted adding 50 μL of Milli-Q water and centrifuging the tube at 13000 r.p.m. 1 minute.

4.2 DNA extraction from yeast

4.2.1 Plasmid Extraction using the miniprep solutions

A modified miniprep protocol was used to extract the plasmids from yeast.

Yeast cells were grown in selective media during 24-48 hours (until the culture was saturated). Cells were centrifuged at 5000 r.p.m. 5 minutes and supernatant was discarded. 200 μL of Solution I (minipreps) and glass beads (Ø=425-600 μm) just until the top of the liquid were added to the pellet. The mix was vigorously stirred by vortex during 3 or 4 minutes, putting the cells on ice every 30 seconds. Then, mix was centrifuged at 13000 r.p.m. 2 minutes, and supernatant was taken.

200 μL of solution II were added and mixed inverting the tube several times. Afterwards 150 μL of solution III were added and mixed inverting the tube several times again. Then they were incubated 5 minutes on ice, and centrifugated at 12000 rpm during 5 minutes. The pellet was discarded, and the supernatant was put in a new clean tube.

1 volume of PCIA was added and vortex was applied gently during 5 seconds, and centrifugated at 13000 rpm during 2 minutes. The aqueous phase was taken to another clean tube, and two volumes of ethanol (95%) and 0.1 volumes of NaAcOH (3M) were added. The tube was incubated on ice (or

at -20ºC) during 30 minutes, and centrifugated at 13000 rpm during 15 minutes. Then the pellet was washed with 1 mL of ethanol (70%) and centrifugated at 13000 rpm during 15 minutes.

Finally, the samples were dried in vacuum and the samples weer resuspended in Milli Q water.

4.2.2 Plasmid Extraction using the plasmid miniprep kit solutions

A modified plasmid miniprep protocol was used to extract the plasmids from yeast.

Firstly grow culture to saturation in 4 ml selective media and spin1.5 mL for 10 min in Eppendorf tube, and remove supernatant. Add another 1.5mL of culture to pellet, spin, and remove supernatant (optional). Then resuspend the cells in 500 μL sterile Milli-Q water. Spin cells 5 minutes at 5000 rpm, remove supernatant, and resuspend pellet in 250 μL of buffer 1 (Resuspension buffer containing RNAse A).

Afterwards add 250 μL buffer 2 (Lysis buffer) and 250 μL acid washed glass beads, vortex for 2 min and let sit 5 min at 4ºC. Then add 350 μL chilled buffer 3 (DNA Binding Buffer) and mix by inverting, and incubate on ice for 5 min.

Spin at 13000 rpm during 10 min and place a spin column in 1.5 mL Eppendorf tube and apply supernatant. Afterwards spin 60 sec at 13000 rpm, drain tube and wash column with 0.75 mL of buffer 4 (Column Wash Buffer). Spin 60 sec at 13000 rpm, drain tube and spin again.

Finally place column in a clean 1.5 mL Eppendorf tube and elute DNA with 50 μL Milli-Q water and spin 30 seconds.

4.2.3 Genomic DNA extraction

4.2.3.1 mi-Yeast Genomic DNA Isolation Kit (Metabion)

For the yeast genomic DNA extraction the mi-Yeast Genomic DNA Isolation Kit (Metabion) was used.

Firstly add 1 mL cell resuspension (e.g. overnight culture containing approximately 1-2x10^8 cells) to a 1.5 mL tube on ice, and spin at 13,000 rpm for 1 min and remove the supernatant. Then add 300 µL of 50 mM EDTA (pH 8.0) to the cell pellet and gently pipet up and down until cells are suspended. Add 7.5 µL of Lyticase (stock solution 20 mg/mL) and invert the tube 25 times to mix.

Incubate at 37°C for 30 min on shaker to digest the cell walls. Invert the sample occasionally during the incubation and spin at 13,000 rpm for 1 min to pellet the cells. Remove the supernatant, add 400 µL of Cell Lysis Buffer and mix thoroughly. Add 10 µL of Proteinase K (stock solution 10 mg/mL) to the 1.5 mL tube and mix thoroughly. Incubate the sample at 65°C for 15 - 30 min.

Then cool the sample to room temperature and add then 3 µL of RNase A (stock solution 10mg/mL) to the 1.5 mL tube and incubate at 37°C for 15 - 30 min. Afterwards add 100 µL of PPT Buffer and vortex for 20 sec. Incubate the sample on ice for 5 min. The spin at 13,000 rpm (12,000 x g) for 1 min at room temperature and transfer the supernatant into a new 1.5 mL tube. Add 600 µL of Column Binding Buffer without wetting the rim and mix by vortexing. Set one spin column into a collection tube and transfer 650 µL of the sample (prepared previously) to the spin column.

Then spin at 13,000 rpm for 1 min and discard the flow through. The liquid will flow through the spin column membrane leaving the genomic

DNA bound to the filter membrane. (Repeat these steps until all supernatant has passed through the column.)

Remove the spin column from the collection tube and discard the flow through. Place the spin column in the same collection tube and add 650 µL of Column Wash Buffer without wetting the rim. Spin at 13,000 rpm for 1 min and remove the spin column from the collection tube and discard the flow through.

Replace the spin column in the same collection tube, add 350 µL of Column Wash Buffer without wetting the rim and spin at 13,000 rpm for 1 min.

Spin again at full speed (13,000 rpm) for 1 min, place the spin column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate.

Finally, elute by adding 50 - 100 µL of Milli-Q water and incubate the spin column with TE buffer or distilled water at room temperature for 1 min. Spin at 13,000 rpm for 1 min and the Yeast Genomic DNA is now ready to use.

4.2.3.2 Fast Chromosomal Yeast DNA extraction protocol

For the yeast genomic DNA extraction the Fast Chromosomal Yeast DNA extraction protocol (Ausubel FM and K 1995) was used.

Firstly prepare 10 mL of yeast culture and let grow overnight. Spin at 4000 rpm for 5 minutes, and discard supernatant. Resuspend the pellet with the micropipette in 500 µL of Milli-Q water and transfer into an Eppendorf tube. Then spin at 13000 rpm for 10 seconds, discard the supernatant and resuspend the pellet in 200 µL of Breaking Buffer (Table 4) with 2 µL of

RNAse (25mg/mL) plus 0.3 g of glass beads (approx. 200 µL of volume) plus 200 µL of PCIA. Vortex at 15-20 Hz for 3 minutes.

Then add 200 µL of buffer 1xTE and vortex again briefly (approx. 4 seconds), spin at 13000 rpm at 5 minutes and room temperature, and transfer the supernatant by decantation into a new clean tube.

Afterwards add 1 mL of ethanol (100%) and mix inverting the tube. Let the tube 5-10 minutes at room temperature. Then spin at 13000 rpm for 5 minutes and eliminate the supernatant by decantation.

Finally dry the tube with vacuum, and resuspend the DNA with 40 µL of 1xTE. Leave the samples at 4ºC (fridge).

Table 4. Breaking Buffer (10 mL)

Triton 100X	**200 µL**
SDS 20%	**500 µL**
NaCl 2.5 M	**400 µL**
Tris ClH pH=8.0 1M	**100 µL**
EDTA pH=8 0.5M	**20 µL**
Milli-Q Water	**8.78 mL**

4.3 DNA purification from agarose gels

For the DNA purification from agarose gels the mi-Gel Extraction Kit (Metabion) was used.

Firstly cut the desired DNA band with a clean sharp scalpel from the TAE agarose gel. Place the gel piece in a 1.5 mL microcentrifuge tube and add 3 volumes of Gel Extraction Buffer to the 1.5 mL microcentrifuge tube.

Then incubate for 5 - 10 min at 65°C (mix thoroughly for 10 sec and invert the tube every 2-3 min to melt the gel). Insert the spin column into the collection tube and add the solution into the spin column.

Centrifuge the spin column at 13,000 rpm for 1 min at room temperature and discard the flow through liquid and replace spin column in collection tube. Add 500 μL of Column Wash Buffer to the spin column and centrifuge at 13,000 rpm for 1 min at room temperature.

Afterwards discard the flow through liquid and repeat the previous steps once more. To eliminate any possibility of Column Wash Buffer carryover, spin at 13,000 rpm for 1 min at room temperature and replace the spin column into a clean microcentrifuge tube.

Finally elute the DNA by adding 10 μL of Milli-Q water directly onto the centre of the white spin column membrane and spin at 13,000 rpm. The DNA is now ready to use.

4.4 DNA enzymatic manipulation

4.4.1 Enzymatic digestion

Genomic and plasmid DNA were digested using restriction endonucleases (New England Biolabs, Roche, Takara and Fermentas) following the manufacturer recommendations with regard to the buffer, optimal temperature, enzyme concentration and digestion time.

4.4.2 Ligation reaction

4.4.2.1 T4 DNA ligase protocol

T4 DNA ligase (Roche and Fermentas) and the specific supplied buffer were used using a final volume of 10 μL. Several proportions of vector/insert were assayed, but normally the relationship employed was 1/5 or 1/10. Different times and temperatures were assayed too, but normally the reaction was incubated during 2-4 hours at room temperature, or overnight at 4ºC or 14ºC (incubator).

4.4.2.2 T-Vector protocol

It is a commercial kit to clone pcr products. Figure 4 is a representation of the pMBL vector

Protocol:

1. Mix in a vial:
    a. 1 μL T_4 DNA ligase 10X buffer
    b. 1 μL pMBL T-vector (50 ng/μL)
    c. x μL DNA insert
    d. 1 μL T_4 DNA ligase (5U)
    e. H_2O up to 10 μL
2. Incubate the vial at room temperature (20-22ºC during an hour)
3. Transform 50 mL of competent cells with 5 μL of ligation mix.

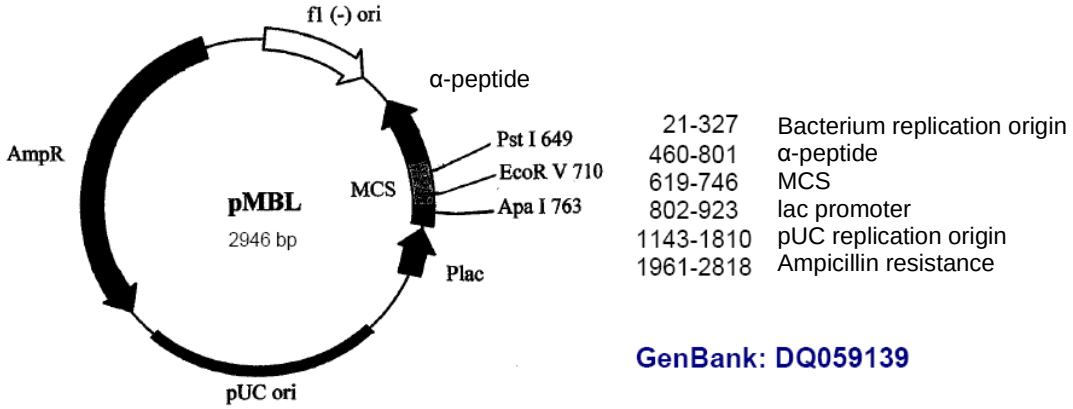

Figure 4. pMBL vector.

4.4.2.3 GAP-repair technique (Zaragoza, 2003)

It is based on the yeast DNA recombination using the nick repair mechanisms. Firstly a nick is made in the plasmid (where we want to insert the gene) using restriction enzymes, and afterwards the insert is amplified by PCR using primers with thirty nucleotides tails (homologues of the region of the plasmid where we made the nick).

Then the yeast is cotransformed with the PCR and the linear plasmid, and the yeast makes the recombination, creating a circular plasmid with the insert in the chosen place.

4.4.3 Amplification of DNA using the PCR (polymerase chain reaction)

It is based on the property of the DNA polymerases to synthesize a complement strand, using a primer and a template. The most used polymerase is the Taq polymerase which is an enzyme obtained from *Thermus aquaticus* (a thermophile bacterium). Sometimes a more accurate replication is needed and other polymerases as for example Pfu or Pwo were used. In the case of very long templates, the Taq Long (New England Biolabs) was used. Finally to

make the site directed mutagenesis, the Quikchange or the Quikchange XL were used (Stratagene).

Typical mix to make the pcr:

Products	Volume (µL)
Template	**0.2 (depending on the sample)**
dNTPs	**4**
Each primer	**2.5**
Specific Buffer	**5**
Polymerase	**0.5**
Milli-Q Water	**35**

The programs used in the thermocyclers only have differences depending on the annealing temperature of the primers, and the length of the fragment of DNA to amplify (for Taq for example, aprox. 1 minute per 1000pb).

PCR cycles:

Number of cycles	Temperature (ºC)	Time (seconds)	
1	**95**	**180**	Initialization
30	**95**	**30**	Denaturation
	Depend on the primers	**45**	Annealing
	72	**Depend on the fragment to amplify**	Extension / Elongation
1	**72**	**600**	Final Elongation
1	**4-8**	∞	Final Hold

4.4.4 Purification of the DNA amplified by PCR

For the purification of the DNA amplified by PCR, the commercial kit mi-PCR Purification Kit (Metabion) was used.

Firstly add 5 volumes of DNA Binding Buffer to the PCR reaction and mix well by pipetting. Place a spin column into a 2 mL collection Tube. Transfer the PCR/DNA Binding Buffer mixture to a spin column and spin at 13,000 rpm for 1 min at room temperature.

Then remove the spin column and discard the liquid flow-through from the collection tube by decanting and replace the spin column in the same decanted collection tube.

Add 750 μL of Column Wash Buffer to the spin column, spin at 13,000 rpm for 1 min at room temperature, add 250 μL of Column Wash Buffer to the spin column, spin at 13,000 rpm for 1 min at room temperature again and remove the spin column discarding the liquid flow-through by decanting. Afterwards replace spin column back into the same collection tube.

To eliminate any possiblity of Column Wash Buffer carryover spin at 13,000 rpm for 1 min at room temperature. Transfer the spin column to a clean microcentrifuge tube and elute the DNA by adding 50 μL of Milli-Q water

Finally spin at 13,000 rpm for 1 min at room temperature and discard the spin column. The purified DNA is now in the microcentrifuge tube and ready to use.

After the protocol the DNA is free of all reaction components, such as primers, linkers, enzymes, salt, and dNTPs.

4.5 Bacteria Transformation Protocols

4.5.1 Rubidium chloride method (Kushner, 1978)

Competent cells preparation

SOC medium was inoculated in a proportion of 1mL of preinoculum per 100 mL of SOC, and was incubated at 37ºC with stirring (300rpm) until the culture reached 0.6-0.8 DO_{600nm}. Then the culture was put on ice during 10 minutes to stop the cell division. Afterwards cells were taken to sterile tubes and were centrifugated at 5000 rpm during 7 minutes in a refrigerated centrifugue. Once the cells are in ice, be aware that in every step of the protocol they should be on ice.

Then the supernatant was discarded, and a small amount of TBF-1 solution (table 5) was added to gently resuspend the cells. After they were resusended, the rest of the volume up to 40 mL was added, and the cells were incubated during 5 minutes to let the TBF-1 solution work properly.

After this time of incubation, cells were precipitated again with a centrifugation of 5000 rpm during 7 minutes. Supernatant was discarded, and the solution TBF-2 (15 mL) (table 6) was added in the same way as the TBF-1, using the solution to gently resuspend the cells.

Finally cells were poured in small aliquots in *Eppendorf* tubes, and conserved at -80ºC

To prepare 80 mL of TBF-1:

Table 5. TBF-1

Cl_2Rb	0.964 g
Cl_2Mn	0.788 g
CH_3COOK	0.232 g (pH 5,8)
Cl_2Ca	0.116 g
Glycerol	12 mL

To prepare 15 mL of TBF-2:

Table 6. TBF-2

Cl_2Rb	**0.02 g**
Cl_2Ca	**0.2 g**
Glycerol	**2.28 mL**

Add Milli-Q water to achieve 80 mL or 15 mL (respectively) of final volume, and filter the solution.

Cells transformation

The transformation was carried out using aliquots of 100 µL of fresh defrosted cells (during 20 minutes on ice) per transformation. Then the necessary amount of DNA was added to the cells, and they were incubated on ice for 15 minutes.

Afterwards, cells were forced to a thermal shock at 42ºC during 45 seconds (depending on the strain could be less or more time). Cells were incubated again on ice during 3 minutes, and after that, 1 mL of LB was added to the mix of cells and DNA, and was incubated at 37ºC during 1 hour.

Finally, cells were centrifuged at 2500 rpm during 3 minutes, and they were inoculated with 100 µL of LB in LBA plates.

4.6 Yeast Transformation Protocols

4.6.1 Litium acetate modified method (Ito *et al.*, 1983)

It is a transformation method for yeasts.

Firstly inoculate 50 mL YPD with a fresh colony, and leave growing at 30ºC and 150 rpm overnight. Then inoculate 50 mL of fresh YPD with the preinoculum grown overnight (calculate to leave the inoculums at approx. 0.4 (OD600). Monitor the growth until the OD600 is around 0.6-0.8, and then spin down the cells (5000 rpm for 5 minutes).

Afterwards resuspend cells in 1 mL of lithium acetate buffer-1XTE (prepared using 900 μL of Lithium Acetate 1M and 100 μL of 10XTE), and spin cells again at 5000 rpm for 5 minutes.Turn on a thermal blockand set for 42ºC.

Then resuspend cells in 100 μL of lithium acetate buffer 1XTE (for each transformation) and mix them in an Eppendorf tube with 5 μL of boiled sheared salmon sperm DNA (5 mg/mL), and with 0.5-10 μg of DNA. Invert the tube to mix them, and add 300 μL of PEG 4000 (50%).

Incubate 30 min at 30ºC and 150 rpm on an orbital shaker, add 88 μL of DMSO, and incubate 7 min 42°C. Then spin down cells (5000 rpm for 5 minutes), and wash cells with 1 mL of Milli-Q water. (Optional if it is a very difficult transformation) Resuspend cells in 1 mL of fresh YPD and incubate at 30ºC for 2 hours, and spin down cells (5000 rpm for 5 minutes).

Finally resuspend cells in 100 μL of Milli-Q water and grow them in adequate plate.

To prepare 100 mL of 10XTE:

1 M Tris pH 7.5	10 mL
0.5 M EDTA pH 8	2 mL
Milli-Q water	88 mL

4.6.2 Electroporation Modified Method (Kooistra *et al.* (2004))

It is a transformation method with more efficiency than the Lithium acetate.

Protocol:

Put a yeast preinoculum growing overnight in 10 mL YPD. The following day use 200 µL of this preinoculum in fresh YPD, until the cells reach an O.D. 600nm of approx. 0.6-0.8 (exponential phase). Meanwhile, put the electroporation cuvettes (2mm path, Cell Projects LTD) and the cuvette holder in ice during at least 15 minutes. Spin the cells during 5 minutes at 5000 rpm, discard the supernatant and wash the cells in 1 mL of Milli-Q water and centrifugated again during 5 minutes at 5000 rpm. Discard the supernatant, and incubate the cells in 1 mL of pre-treatment buffer (see composition in table 7) during 30 minutes at 30ºC.

Centrifugate the cells at 5000 rpm during 5 minutes, discard the supernatant and add 1 mL of electroporation buffer (see composition in table 8). Mixed the cells with the DNA, and incubate other 15 minutes on ice. Then put 50 µL of the suspension in each electroporation cuvette, and place them to the electroporator (BioRad Gene Pulser II). Voltage 1000 V, capacitance 25µF and a resistance 400 ohm have to been fixed. Then make a pulse, and

afterwards add 1 mL of YPD and transfer the suspension in an Eppendorf tube in ice during 15 minutes.

Finally incubate at 30ºC during 1 hour, and plate on appropriate media.

Table 7. Pre-treatment Buffer.

Volume: 100 mL
YPD
95 mL
DTT 1M (dithiothreitol)
20 mL
HEPES 1M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
30 mL

Table 8. Electroporation Buffer.

Volume:100 mL
Tris-HCl 1M, pH 7.5
1 mL
Sacarose 1M
27 mL
Lithium acetate 1M
100 µL
Milli-Q Water
71,9 mL

4.7 Electrophoresis techniques

4.7.1 Agarose gels

To observe the PCR products, plasmid digestions and in general the DNA used in the constructions, agarose (Sigma) gels were made with a concentration of agarose between 0.7-1% in 1xTAE Buffer (Table 9).

The voltage used was between 60-100 V and the migration time depended in the size and the concentration of the gel, and in the size of the DNA fragments.

Before loading the samples in the gel, 1/10 of final volume of blue loading buffer (Table 10) was added.

Table 9. 1xTAE Buffer.

Tris-Acetate 0.04M
EDTA 0.1M
Acetic Acid 30mM

Table 10. Blue Loading Buffer

Glycerol 50%
Bromophenol Blue 0.25%
Xylene Cyanol 0.25%

4.7.2 Agarose gels staining

4.7.2.1 Ethidium Bromide

To observe the bands of DNA in the agarose gels, gels were stained with Ethidium Bromide in a concentration of 5 drops of diluted ethidium bromide stock (1/10 of a stock of ethidium bromide at 10 mg/mL) per 100 mL of TAE. Afterwards gels were watched in an UV transiluminator Bio-Rad Universal Hood II.

The photographs were taken using the software Quantity One 4.5.0 (Build 46).

4.7.2.2 SYBR® Safe DNA Gel Stain

To observe the bands of DNA in the agarose gels, SYBR® Safe DNA Gel Stain was added to all of the samples (following the distributor instructions), before loading the gel. Gels were watched in an UV transiluminator Bio-Rad Molecular Imager Gel Doc XR+ System.

The photographs were taken using the software Image Lab.

4.7.3 Polyacrylamide gels (SDS-PAGE)

To observe the proteins, polyacrylamide gels were made at a fixed concentration of 10% following the Laemmli protocol(Laemmli 1970). The electrophoretic cell used was a Bio-Rad Mini Protean.

Separating and Stacking gels were prepared as follows:

Solution	*Separating Gel*	*Stacking Gel*
Acrilamide 45%	2,2 mL	0,55 mL
Tris 1.5 M pH 8.8 (6.8)	2,5 mL	(0,63 mL)
SDS 10%	0,1 mL	0,05 mL
APS 10%	0,1 mL	0,05 mL
TEMED	0,004 mL	0,005 mL
Milli-Q H_2O	5,1 mL	3,68 mL

All the components of the separating gel were mixed adding the APS and the TEMED at the end, and the solution was introduced between the crystals. Distilled water was added in the top of the solution to avoid the air contact with the solution which makes the polymerization slower.

After 20-30 minutes (when the polymerization finished), the water was discarded and the components of the stacking gel were introduced between the crystals, and finally the comb was introduced. All the system was flooded in Tris-ClH-Glycine Buffer (table 11).

The protein samples were loaded after being mixed with 50% of 2X Laemmli loading buffer (table 12), and incubated at 95ºC for 5 minutes.

Electrophoresis was made at 100 V meanwhile the sample migrates into the stacking gel, and at 120-150 V when the sample migrates into the separating gel. Once the bromophenol blue dye went out, the electrophoresis was stopped.

Table 11. Tris-ClH-Glycine Buffer.

Tris-HCl 25 mM pH 8.3
Glycine 192 mM
SDS 0,1%

Table 12. 2X Laemmli Loading Buffer (1mL).

SDS 10%	**400 µL**
Glycerol	**200 µL**
Tris-HCl 1M pH 6.8	**120 µL**
Bromophenol Blue 0.1%	**20 µL**
Milli-Q Water	**160 µL**
B-mercaptoethanol	**100 µL**

4.7.4 Polyacrylamide gels (SDS-PAGE) Staining

Three different protocols of staining were assayed:

4.7.4.1 Coomasie Brilliant Blue method

To stain the proteins in the polyacrilamide gels the protocol used was as described by (Volker et al. 1985). Gels were incubated in Coomassie brilliant blue (CBB) G-250 with slow stirring for two or three hours (or even overnight), and were washed with distilled water to eliminate the excess of dye. The sensibility is 0.05-0.1 μg each band.

4.7.4.2 Two solutions with methanol

To stain the proteins in the polyacrilamide gels two solutions were used. The first was named as "Solution I or Staining Solution" (Table 13) and the second was named as "Solution II or Fade Solution" (Table 14).

Firstly the polyacrylamide gel was incubated with slow stirring in Solution I for 1 hour. Then the solution I was discarded and the gel was incubated in the same conditions as solution I but with solution II for 30 minutes. Finally the dehydrated gel was incubated in distilled water for 10 minutes.

Table 13. Solution I or Staining Solution (500mL).

Coomasie Brilliant Blue 0.1%	**0.5 g**
Methanol 40%	**200 mL**
Acetic Acid 10%	**50 mL**
Milli-Q Water	**250 mL**

Table 14. Solution II or Fade Solution (100mL).

Methanol 40%	**40 mL**
Acetic Acid 10%	**10 mL**
Milli-Q Water	**50 mL**

4.7.4.3 Microwave protocol

To stain the proteins in the polyacrilamide gels two solutions were used. The first was named as "Solution I or Staining Solution" (Table 15) and the second was named as "Solution II or Fade Solution" (Table 16).

Firstly the polyacrylamide gel was heated in the microwave at maximum potency (up to 90ºC) with Solution I for 15 seconds. Then it was incubated for 10 minutes with stirring. Afterwards the solution I was substituted by solution II and the gel was heated in the microwave at maximum potency (up to 90ºC) again for 15 seconds. Finally the gel was incubated at room temperature for at least 2 hours with stirring in this solution II.

Table 15. Solution I or Staining Solution (100mL).

Coomasie Brilliant Blue	**25 mg**
Acetic Acid	**10 mL**
Milli-Q Water	**90 mL**

Table 16. Solution II or Fade Solution (100mL).

Acetic Acid	**10 mL**
Milli-Q Water	**90 mL**

4.8 Crude Protein Extracts

To obtain crude protein extracts a 1L culture growing for at least 48-72 hours absorbance at 600 nm of 3) was centrifugated at 7000 rpm for 10 minutes at 4ºC (all the protocol has to be made at 4ºC and with ice). Discarded the supernatant, the cells were washed once in 5 mL cool distilled water and once in 5 mL Buffer A (Table 17), and centrifugated after the washes at 6000 rpm during 5 minutes.

Then cells were suspended in 3 mL of Buffer A with 0.5 volumes of glass beads (Ø=425-600 μm). Afterwards, 3 μL of pepstatin (4mM final concentration), leupeptin (4mM), aprotinin (2 μg/mL), beta-mercaptoethanol (2 μM) and PMSF were added.

Five or six cycles of 20 seconds at maximum vortex and 20 seconds on ice were made to disrupt the cells. Then the extract was centrifugated at 8000 rpm for 15 minutes.

Finally discarded the pellet, the supernatant was centrifugated again in a new clean tube at 13000 rpm for 60 minutes. The supernatant of this last step was used as the crude protein extract, and depending on the following experiments it was stored at 4ºC or at -20ºC or -80ºC.

Table 17. Buffer A.

Tris HCl 20 mM pH 7.8
$(NH_4)_2SO_4$ 300 mM
$MgCl_2$ 10 mM
EDTA 1 mM
Glycerol 10%

5.Analytical techniques

5.1 β-galactosidase activity

5.1.1 Eppendorf Assay

5.1.1.1 Intracellular

Grown cells in flasks or culture tubes were span in 10 mL tubes in a centrifugue for 5 minutes at 5000 rpm. Supernatant was discarded and then cells were washed with 2 mL of Buffer Z (Table 18), and span again for 5 minutes at 5000 rpm. Cells were resuspended in 2 mL of Buffer, and 110 µL of chloroform and 175 µL of SDS (Sodium Dodecyl Sulphate) 0.1% were added to the tube.

Afterwards, a vortex of 20 seconds was made to break the cells and the extract was incubated for 10 minutes at 30ºC. Once the incubation finished, 440 µL of O-nitrophenyl-β-D-galactoside (4 mg/mL) were added and the reaction was neutralized adding 0.5 mL of Na_2CO_3 1M. The absorbance at 420 nm after cells centrifugation and the initial reaction, middle reaction and

finish reaction time were measured, and were used with the following formulas to calculate the β-galactosidase activity.

Formulas:

1. (Abs 420nm t2 –Abs 420nm t1)/(ε*(t2-t1))
2. Abs 420nm t1/(ε*(t1-t0))
3. Abs 420nm t2/(ε*(t2-t0))

An average of the three formulas was done.
The volume of the reaction and the volume of cells were taken into account.
ε is the molar extinction coefficient : 4500 M^{-1} cm^{-1}.

Table 18. Buffer Z.

Volume:500 mL*

$Na_2HPO_4.7H_2O$	**8.05g**
NaH_2PO_4. H_2O	**2.75g**
KCl	**0.375g**
$MgSO_4.7H_2O$	**0.123g**
2-Mercaptoethanol	**1.35mL**
Milli-Q Water	**up to 500 mL final volume**

*It is necessary to adjust the pH.

5.1.1.2 Extracellular

Grown cells in flasks or culture tubes were span in 10 mL tubes in a centrifugue for 5 minutes at 5000 rpm, and 100-300 μL of supernatant were collected and added into a tube with 2 mL of Buffer Z (Table 18).

Then the tube was incubated for 10 minutes at 30ºC. Once the incubation finished, 440 μL of O-nitrophenyl-β-D-galactoside (4 mg/mL) were added and the reaction was neutralized adding 0.5 mL of Na2CO3 1M. The absorbance at 420 nm and the initial reaction, middle reaction and finish reaction time were measured in minutes, and were used with the following formulas to calculate the β-galactosidase activity.

Formulas:

1. (Abs 420nm t2 –Abs 420nm t1)/(ε*(t2-t1))
2. Abs 420nm t1/(ε*(t1-t0))
3. Abs 420nm t2/(ε*(t2-t0))

An average of the three formulas was done.
The volume of the reaction and the volume of extract were taken into account.
ε is the molar extinction coefficient : 4500 M^{-1} cm^{-1}

5.1.2 Microtiter Assay

5.1.2.1 Intracellular

Grown cells in flasks or culture tubes were span in Eppendorf tubes in a centrifugue for 5 minutes at 5000 rpm, until achieve a pellet of 100-150 μL. Then the biomass was washed in the Eppendorf tube with 9 volumes of PE buffer (Table 19), and the supernatant was discarded.

The biomass was resuspended in 3 volumes of PE buffer, and 4 volumes of glass beads were added to the tube. A vortex was made with cycles of 30 seconds of vortex and 1 minute on ice, for a total time of 9 minutes.

Then 3 volumes of buffer Z were added and vortex gently. Then a centrifugation for 15 minutes at 13200 rpm and 4ºC was made to separate the cell debris against the supernatant.

Finally, the supernatant was collected in a new Eppendorf tube and maintained on ice. The quantity of total protein was measured using the Bradford method. To determinate the β-galactosidase activity the following formula was used:

Formula:

A Δ405*D/ ε/ Prot

A Δ405*D is an average of the Δ405 multiplied by the respective dilution.
ε is the molar extinction coefficient : 6136'6 M^{-1} cm^{-1}
Prot is the quantity of protein measured by the Bradford method

Volume:200 mL*[1*2]

Table 19. PE Buffer.

Na_2HPO_4	1.708g
NaH_2PO_4. H_2O	1.1g
KCl	0.15g
$MgSO_4.7H_2O$	0.05g
EDTA $Na_2.2H_2O$	0.0744g
$(NH_4)_2SO_4$	7.92g
Glycerol	10mL
Milli-Q Water	up to 200 mL final volume

***1** It is necessary to adjust the pH, and autoclave the solution.

*2 It is necessary to add:

- 20 µL/mL of Protease Inhibition Cocktail (Roche) stock solution 25X.
- 10 µL/mL of PMSF (100mM) stock solution 100X.
- 1 µL/mL of DTT (1M) stock solution 1000X.
- 2.8 µL/mL of. 2-Mercaptoethanol

To measure the β-galactosidase activity, 200 µL of extract or diluted extract was added into a microtiter plate, and 50 µL of pNPG (p-Nitrophenyl-beta-D-galactopyranoside at 4 mg/mL) were added to initialize the reaction. The kinetic reaction was measured at 405 nm and 30ºC, for 15-30 minutes.

5.1.2.2 Extracellular

Mix 50 µL of fermentation supernatant with 150 µL of Buffer Z. Add 50 µL of pNPG (p-Nitrophenyl-beta-D-galactopyranoside at 4 mg/mL) and measure the kinetic reaction as in the intracellular protocol.

Formula:

A **Δ**405*D/ ε/ Vol

A Δ405*D is an average of the Δ405 multiplied by the respective dilution.
ε is the molar extinction coefficient : 6136'6 M^{-1} cm^{-1}
Vol is the volume of extract used.

5.2 Protein Quantification

5.2.1 Bradford method

All the following protocols are based on Bradford protocol (Bradford 1976).

5.2.1.1 Eppendorf Assay

At first, it is necessary to prepare dilutions of known protein concentrations, to calculate a linear regression between absorbance and protein concentration, as in the example with BSA 1mg/mL (Bovine Serum Albumin):

1. 0.5 μL + 999.5 μL Milli-Q water
2. 1 μL + 999 μL Milli-Q water
3. 2 μL + 998 μL Milli-Q water
4. 4 μL + 996 μL Milli-Q water
5. 8 μL + 992 μL Milli-Q water
6. 16 μL + 984 μL Milli-Q water

Then, prepare your samples and dilute them if it is necessary. After that take 800 μL of every sample and pattern, and mix in a new Eppendorf with 200 μL of Bradford Reactive (BIORAD, Pierce, etc), mix gently and incubate at room temperature for 10 minutes. Remind to make a blank with 200 μL of Bradford Reactive and 800 μL of Milli-Q water.

Then measure the absorbance at 595nm, against the blank, and use the linear regression to estimate the protein concentration.

5.2.1.2 Microtiter Assay

Coomassie Plus Protein Assay Reagent (Pierce) was used.

STANDARDS

Working Range: 100 – 1500 µg/mL

BSA Stock: 2 mg/mL (100 mg of BSA dissolved in 50 mL of 0.9% NaCl)

Store 1 mL aliquots at -20 ºC.

Standard	BSA (µL)	MilliQ water (µL)	**[BSA] (µg/mL)**
A	375 *(Stock)*	125	**1500**
B	325 *(Stock)*	325	**1000**
C	175 *(Std A)*	175	**750**
D	325 *(Std B)*	325	**500**
E	325 *(Std D)*	325	**250**
F	325 *(Std E)*	325	**125**
G	100 *(Std F)*	400	**25**

Procedure

Add 10 µL of each standard or sample (dilute with water if necessary) into the plate wells. Use 10 µL of the diluent (water) for the blank wells.

Add 300 µL of the Reagent to each well, and mix on a plate shaker for 30 seconds.

Measure the absorbance at 595 nm (A595).

NOTES:

- *Subtract the average A595 for the blanks from the standard or sample readings.*
- *If higher A595 readings are required, use 15 μL of standard or sample and 300 μL of reagent per well.*
- *Always assay two different dilutions of each sample, to check for possible interferences.*

5.2.2 Nanophotometer

1-2 μL of sample were taken to the nanophotometer cuvette, and measured following the manufacturer information.

5.3 DNA quantification

5.3.1 Spectrophotometric Assay

5 μL of sample were diluted with Milli-Q water in a final volume of 1 mL. This dilution was chosen to make easy the following calculations, because with this dilution the absorbance measured was multiplied by 10 to express the concentration in μg/μL. Measures were done in a *Cecil CE 2041* using 1 mL quartz cuvettes. Sample absorbances were measured between two wavelengths, 260 and 280 nm, and the real concentration was calculated assuming that one unit of A_{260} is equivalent to a concentration of 50 μg/mL of double stranded DNA.

The relationship A_{260}/A_{280} is an index of the purity grade of the sample. The ideal value is 1.8 (Sambrook *et al.*, 1989).

5.3.2 Nanophotometer

1-2 μL of sample were taken to the nanophotometer cuvette, and measured following the manufacturer information.

5.4 High Performance Liquid Chromatography (HPLC)

Three different HPLC systems were used to measure the sugars and alcohols in the fermentations.

5.4.1 Waters HPLC (I)

Initially a Waters HPLC was used. Lactose, Glucose, Galactose, Glycerol and Ethanol were analyzed by HPLC (Waters), using a Shodex SC-1011 column eluted at 70ºC with Milli-Q water at a flow rate of 0.5 mL/min, and a refractive-index detector (Waters 410).

5.4.2 Waters HPLC (II)

A Waters HPLC was used. Lactose, Glucose, Galactose, Glycerol and Ethanol were analyzed by HPLC (Waters Breeze I), using a Waters Sugar-Pak column eluted at 90ºC with Milli-Q water at a flow rate of 0.5 mL/min, and a refractive-index detector (Waters 2414).

5.4.3 JASCO HPLC

In Braga, lactose, glucose, ethanol and glycerol were analyzed by HPLC (CHROMPACK Jasco), using a Varian MetaCarb 87H column eluted at 60ºC with 0.005M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector (Jasco 830-RI).

5.5 Ethanol and Lactose Kits

Sometimes, lactose and ethanol were measured with specific Lactose and Ethanol kits from Boehringer Mannheim and Megazyme, following strictly the distributor protocol.

5.6 Protein Purification

5.6.1 Afinity Chromatography purification

Biologic LP from Bio-Rad (a chromatographic system) was used with a 5 mL of volumen agarose column refilled with p-Aminephenyl-1-thio-β-D-galactopiranoside (Sigma) which was maintained by temperature control (4ºC) using a refrigerated bath (model Multitemp III from Pharmacia Biotech).

The column was equilibrated with phosphate buffer 50mM pH 7 during 1 hour. Then 7.5 mL of sample (approx. 1.5 mg of total protein) was loaded into the column, and the buffer loading was fixed at 0.4 mL/min during 100 minutes (40 mL).

Afterwards, the elution was made changing the phosphate buffer into sodium borate buffer 0.1 M pH 10.1 during approx. 100 minutes at 0.4 mL/min again. The fractions were collected using a fraction collector (model 2110 from Bio-Rad), neutralizing the pH to avoid the denaturalization of the protein.

β-galactosidase activity was measured, and those samples which have the maximum activity were concentrated and desalinized using the microultrafiltration systems (Amicon ULTRA-4, 30000 MWCO).

5.6.2 Immunopurification

Firstly prepare TBS (50 mM Tris HCl, with 150 mM NaCl, pH 7.4) buffer which is the optimum buffer for protein and resin.

Then suspend the resin by gentle inversion and make sure the bottle of ANTI-FLAG M2 affinity gel is a uniform suspension of gel beads. Remove 0.250 mL aliquot for use and immediately transfer the suspension to an Eppendorf tube. Centrifuge the resin at 5000 G (maximum) in a refrigerated centrifuge, and discard the glycerol buffer with a pipette.

Then place 500 μL of TBS into the Eppendorf and wash the gel by loading five sequential aliquots (500 μL each one) of TBS using the pipette to mix the beads with the TBS. Between the loads, centrifugue again at 5000 G (maximum) in a refrigerated centrifuge, and discard always the liquid. The meaning of these sequential washes is to replace the glycerol with TBS and therefore equilibrate the resin for use.

Once, the resin is equilibrated, load it with the TBS and the protein solution (crude extract), and incubate overnight in a Petri plate with gently stirring.

The following day, the resin and the protein solution are applied to an empty chromatography column, and let the crude extract leaves the column by gravity. The elution is performed by adding FLAG peptide in a concentration of 150ng/μL to compete with beta-galactosidase.

Finally the purified protein is concentrated around 3-8 mgr/ml by filtration in AMICON ULTRA-4 (Millipore, UFC 803024).

6. Cell fractionation

To make the cell fractionation, put an inoculum at 0.3-0.4 of absorbance at 600 nm, and wait until it reaches 0.6-0.8. Then centrifugate cells at 3000 rpm for 5 minutes at 4ºC. The supernatant has to be collected and named as S1*. On the other hand, cells have to be suspended in 25 mL of Milli-Q water, and afterwards centrifugated in a weighted tube at 4000 rpm and 4ºC for 5 minutes.

Once the centrifugation finishes, weight the tube again, and calculate the cells wet weight. Then use Tris-SO_4 pH 9.4 100 mM to resuspend the cells (2 mL per gram of cells). Add DDT 1M to a reach a final concentration of 10 mM, and incubate cells at 30ºC with stirring.

Afterwards, centrifugate the cells at 4000 rpm and 4ºC for 5 minutes, and resuspend them in Lyticase Buffer (Table 20) at a final concentration of 0.15 g/mL. Add 2-3 mg of lyticase per gram of wet weight, and incubate at 30ºC with soft stirring for 20-40 minutes (measuring every 10 minutes the absorbance to check the protoplasts formation).

Then centrifugate again at 4000 rpm and 4ºC for 5 minutes. Collect the supernatant and name it as S2*, and resuspend the pellet in 1-2 mL of Milli-Q water. Centrifugate the resuspended pellet at 4000 rpm and 4ºC for 5 minutes. Collect the supernatant and name it as S3*. Resuspend the pellet in 1-2 mL of Milli-Q water and centrifugate again at 4000 rpm and 4ºC for 5 minutes. Discard the supernatant, and finally resuspend the pellet in 1 mL of Milli-Q water. This final fraction is named as P1*.

Table 20. Lyticase Buffer

Sorbitol 1.2 M
KH_2PO_4 10 mM pH 6.8

* S1-> Extracellular fraction (culture medium)

S2-> Periplasmic fraction

S3-> Membrane fraction

P1-> Intracellular fraction

7.Statistical Methods

A factorial approach to experimental design, permits the assay of a large number of crystallization conditions with as few experiments as possible. This is accomplished by varying more than one factor at a time in a given experiment; this saves material, and from the analysis of the results, it is possible to readily determine the factors that are critical for crystallization (Fisher 1942; Carter and Carter 1979).

In this work it was studied the influence of three variables (Amonium sulphate oncentration, Protein concentration and PEG6000 %), and their interactions in the response. Once the variables were chosen, the experimental patterns (domains) were established and codified their edge values as +1 and -1, an experimental matix was constructed.

In that matrix:

1.1 The number of elements of each column is equal to the number of experiments (2H, being H the number of variables)

1.2 Each variable should take only the edge values (+1 and -1) and, in each column, the number of +1 and -1 values should be the same (means that each variable should present the same number of values in its upper and lower levels).

1.3 It should be orthogonality condition: the scalar product of all column vectors must be equal to zero.

Furthermore of the experiments of this matrix, three replicates of the central point (all the variables with value 0) were made, to determine the intrinsic variability of the system. The obtained results were used for the determination of the system equation and the analysis of the significance using the experimental design module of the software Statgraphics Plus:

Design with two variables:

Coded Value	**V1**	**V2**
-1	V_{1n}-dV_{10}	V_{2n}-dV_{20}
0	V_{1n}	V_{2n}
+1	V_{1n}+dV_{10}	V_{2n}+dV_{20}

Encoding: $Vc = (Vn-Vo)/ dVn$

Decoding: $Vn = Vo + (dVn \times Vc)$

Vc : Coded value

Vn : Natural Value

Vo : Natural Value in the center of the experimental domain

dVn : Increase of the natural value according to a unit increase in the coded value

The response is visualized presenting the surface defined by the equation (surface response) through their projections over defined plans for each pair of variables.

8. Crystallization Techniques and Structural Resolution

8.1 Crystallization

Several crystallization techniques have been use: hanging drop, sitting drop, and streaking.

8.1.1 Hanging Drop Technique

In this technique, a small volume of protein (1-5 μL) is mixed with the precipitant solution maintaining a ratio normally of 1:1. The drop is loaded in a silanized cover glass, and it is stuck in the crystallization plate, being the drop hanging by gravity in a hermetically closed chamber. In the bottom of the plate, there is a reservoir with 250-1000 μL of precipitant solution.

Due to the highest concentration of the reservoir with regard to the drop, there is a steam diffusion net flux from the drop to the reservoir until the equilibrium is achieved. As a consequence of that water flux, the protein and reservoir concentration in the drop increase slowly until the solution is oversaturated.

Along the way this happens, there are nucleation events, and if the conditions are appropriated, the protein precipitates orderly and the crystals appear.

8.1.2 Sitting Drop Technique

It is based in the same phenomenon as the Hanging Drop Technique, with the difference that the drop is not loaded in a cover glass. In this case the drop is "sit" it in a small "chair", which is surrounded by the precipitant reservoir. The chamber is hermetically closed sealing the crystallization plate with a film.

There were made lots of experiments and test of crystallization modifying the several variables (protein concentration, concentration and type of precipitant, additives, drop and reservoir volumes, temperature, pH, dimensions, characteristics of the crystallization plates, etc).

Initially the first experiments were carried out in the laboratory with multiwell plates (similar to the cellular cultures ones), in which a thin layer of silicone (Type B Silicone from Panreac) was added around 12 of the 24 wells per plate. For that purpose, a syringe (10 mL of volume) was use (the syringe was loaded previously with the appropriate silicone).

The hanging drop technique was used and the cover glasses were home-made silanized. To do that, the cover glasses were immersed in a solution with Dichloromethylsylane at 2% in 1,1,1 Threechloromethane. Then the cover glasses were dried.

The drops were loaded (1-3 μL of final volume and 500 μL of reservoir) in the cover glasses manually with micropipettes (, and the drops were incubated at room temperature and at 4ºC. The drops were checked every day in the first weeks, and after that more periodically, in a Nikon Eclipse 50i microscope. Photos were taken with the Nikon Digital Sight DS-SM camera and with the software NIS-Elements F. Version 2.10.

Later, the crystallization conditions were tested using high yield techniques using a crystallization robot (Innovadine Nanodrop I) and with several different commercial crystallization kits (Crystal Screen, Crystal Screen II, Crystal Screen Lite, Salt Rx e Index Screen (Hampton Research), y Screen Classic (Jena Biosciences).

In this case the drops were loaded by the robot with 0,25 μL of protein solution and 0,25 μL of reservoir (0,5 μL final volume). These experiments were carried out in controlled temperature (18ºC and 4ºC)

Once the optimal conditions were checked (in where crystals grew), these conditions were replicated in bigger supports (Cryschem, Hampton Research) and volumes manually (2 μL of final volume and 0,5 mL of reservoir). Some experiments were made also with against diffusion using the Granada Crystallization Box (Triana Tech) and with Streak seeding (Stura and Wilson 1991) performed under these conditions gave improved quality crystals that were suitable for X-ray diffraction experiments

To obtain data from the diffraction, the crystals were transferred in cryoprotectant solutions (crystallization solution + 20% of glycerol) before the freezing at -173,5 ºC in liquid nitrogen.

The galactose-protein complexes were obtained with the lactose substrate applying the soaking technique (Hassell *et al.* 2007).

8.2 DIFFRACTION

X-Ray diffraction experiments were carried out with the Instituto de Química-Física Rocasolano (IQFR, Madrid) equipment and with the European Synchrotron Radiation Facility (ESRF, Grenoble) radiation sources (specifically lines ID23.1 and ID14.4).

8.3. STRUCTURAL RESOLUTION

Diffraction images were processed with the MOSFLM software (Leslie 1990), with which the unit cell parameters were characterized, and an index was made to give a hkl index (Miller indexes) to each reflection. With the same software an estimation of the intensities of each maximum of diffraction and the determination of the spacial group was made.

Scale-up of these intensities and the calculation of the modules of the structure factors were carried out using a group of several programs that are included in the CCP4 (Collaborative Computational Project 1994).

The *K. lactis* β-galactosidase structure was solved by molecular replacement using the MOLREP software (Vagin and Teplyakov 1997) and using the *Arthrobacter sp.* β-galactosidase structure (PDB code: 1YQ2) (Skalova et al. 2005) to prepare the seeking model using the CHAINSAW software (Stein 2008), and the alignment of both protein sequences.

Crystallographic refinement was made using the REFMAC5 software (Murshudov *et al.* 1997) as part of the CCP4 software. After several refinement and reconstruction manual cycles using the O (Jones *et al.* 1991) and COOT (Emsley and Cowtan 2004) the final map showed the continuous density of the whole molecule.

The galactose-protein complex was solved using the native structure and the refinement was made using the previously procedure.

The models stereochemistry was checked using PROCHECK (Laskowski *et al.* 1993) and MOLPROBITY (Chen *et al.* 2010). The figures were made using PyMOL (DeLano 2002). For the interaction surfaces analysis and the oligomer stability the PISA software (Krissinel and Henrick 2007), which is hosted in the European Bioinformatics Institute, EBI, http://www.ebi.ac.uk/, was used.

9. Software

Furthermore to the Microsoft Office (from XP versión to Professional Plus 2010) and the previous mentioned software in the other sections, the following software and internet webpages were used:

pDRAW 32 (www.acaclone.com)

For restriction analysis and vector images.

OLIGO version 6

Oligonucleotides design, to order their synthesis (Roche Applied Science), and use them as primers in sequencing.

CLUSTAL V

For sequence alignment (Higgins *et al.* 1992)

NCBI

Internet webpage for papers search, and sequence alignment (BLAST software)

http://www4.ncbi.nlm.nih.gov/entrez/query.fcgi

SGD (Saccharomyces Genome Database)

Saccharomyces cerevisiae strain database for gene searching, and sequence analysis.

http://genome-www.stanford.edu/Saccharomyces/

GENOSCOPE

Genome database of several microorganisms (yeasts included).

http://www.genoscope.cns.fr/

EUROSCARF

It is the EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis. It is a huge database for strains and plasmids.

ExPASy *(Expert Protein Analysis System).*

Webpage with a variety of bioinformatic software (for example Swiss-Model).

http://www.expasy.ch/

SWISS-MODEL

This homology modeling server was used to obtain the structural model of the *K. lactis* and *A. niger* β-galactosidase, and also the hybrid protein from the *K. lactis* and *A. niger* β-galactosidase.

http://swissmodel.expasy.org/

PDB (Protein Data Bank)

Protein data bak with programs to analyze the secondary structure of a problem protein, and give you the three dimensional structure of the protein with more homology, which has been crystallized and included in its database.

http://www.rcsb.org/pdb

STATGRAPHICS Plus

Statistical analysis of the factorial design.

Kodak Digital Science 1 D o Quantity One version 4.5.0

DNA agarose gel analysis and quantification.

Breeze I (Waters)

Analysis of the data obtained in the Waters HPLC.

Workstation Toolbar Version 6.30

Analysis of the data obtained in the JASCO HPLC

Sigmaplot 10.0 (Systac Software Inc)

Easy and complete graphing software

Transform 0.10.0

Free software very useful and flexible to format DNA, RNA or protein sequences.

Endnote X-X4

Software for bibliography organization.

PyMOL (DeLano Scientific)

For the protein images.

CaZY Database

It is the Carbohydrate-Active enZYmes Database.

http://www.cazy.org/

Rosetta Design Server

To emulate modifications in the amino acids of the protein, and guess if it is possible or not in the nature.

http://rosettadesign.med.unc.edu/

Net Primer Biosoft

To design the primers on-line. Give information about Molecular Weight, Melting Temperature, GC % content, ΔH, hairpins, dimmers, palindromes, etc.

http://www.premierbiosoft.com/netprimer/index.html

Appendix II - Resumen

Resumen

Las β-galactosidasas son las enzimas encargadas de la hidrólisis de los β-galactósidos en sus monosacáridos correspondientes.

Las β-galactosidasas están ampliamente representadas desde organismos procariotas hasta organismos eucariotas (como p.ej. los humanos).

La primera β-galactosidasa secuenciada fue la de *Escherichia coli* en 1970 (Fowler and Zabin 1970) la cual contiene 1024 aminoácidos. Este pequeño paso, abrió el camino hacia varias líneas de investigación destinadas a mejorar el conocimiento de este tipo de enzimas así como a la producción y evolución de las mismas. Hubo que esperar 20 años hasta que su estructura tridimensional fue descubierta (Jacobson *et al.* 1994).

En el caso de la β-galactosidasa de *Kluyveromyces lactis* la información sobre su secuencia fue publicada en 1992 (Poch *et al.* 1992), pero su estructura tridimensional, a pesar de numerosos intentos de resolución usando técnicas como por ejemplo el Dicroismo Circular (Tello-Solis *et al.* 2005) no ha sido resuelta.

Existe una elevada demanda de aplicaciones cuyo uso implica la actividad de las β-galactosidasas, las cuales se pueden resumir en los tres siguientes campos:

1. Valorización del suero de leche.
   En este caso existen varias soluciones para este subproducto de la elaboración del queso entre las cuales pueden destacar las siguientes: SCP, producción de biosurfactantes, producción de bacteriocinas, producción de proteínas recombinantes, producción de biopolímeros, producción de biogás, producción de bioetanol y producción de ácido láctico.
2. Aplicaciones farmacéuticas/médicas.
   Destacan entre ellas el uso de la β-galactosidasa como sistema de comprobación de que un kit ha funcionado correctamente ("screening") y su uso para el tratamiento de la intolerancia a la lactosa (desde pastillas de β-galactosidasa al uso de la propia enzima en la producción de alimentos sin lactosa)
3. Otros usos habituales en la industria de la alimentación.

Un uso habitual de las β-galactosidasas en la industria alimentaria va destinado a incrementar el poder edulcorante de la lactosa en algunos alimentos como por ejemplo en helados, tartas, etc.

Debido a esta elevada demanda, se hace necesario el comprender más profundamente cómo funcionan estas enzimas. Por ello uno de los objetivos en esta tesis es el de la expresión, purificación, cristalización, determinación de la estructura tridimensional y análisis de la β-galactosidasa de *Kluyveromyces lactis*.

Otro objetivo de la presente tesis es la construcción y producción de una β-galactosidasa híbrida entre la β-galactosidasa de *Kluyveromyces lactis* y la β-galactosidasa de *Aspergillus niger*.

El último de los objetivos el cuál también está relacionado con los dos anteriores, trata de la construcción, producción y modificación por evolución dirigida de una cepa de *Saccharomyces cerevisiae* que secrete la β-galactosidasa de *Aspergillus niger*.

Las técnicas usadas para la elaboración de la tesis, están explicadas en la sección "Material and Methods".

La β-galactosidasa de *Kluyveromyces lactis*

La β-galactosidasa de *K. lactis* es la enzima responsable de la hidrólisis de lactosa a sus dos monosacáridos correspondientes (glucosa y galactosa).

Es una proteína de unos 120 kDa (Becerra *et al.* 1998), compuesta por un tetrámero, y cuya secuencia fue liberada 1992 (Poch *et al.* 1992).

Tiene una temperatura y pH óptimos de 30ºC y 7 respectivamente (Rodriguez *et al.* 2006), lo que la convierte en una enzima muy apta para su uso biotecnológico e industrial.

Aunque se han realizado diversos estudios para descubrir la estructura tridimensional de la enzima, como por ejemplo el del grupo de Tello-Solis (Tello-Solis *et al.* 2005), a día de hoy no se ha resuelto su estructura terciaria ni cuaternaria.

En la presente tesis, hemos realizado una nueva construcción (Becerra *et al.* 2001), en la cual se ha insertado la β-galactosidasa de *K. lactis* en el vector de expresión de proteínas YEpFLAG (Eastman Kodak) entre el promotor

inducible de levaduras *ADH2* y el terminador *CYC1* (el vector cuenta además con un péptido FLAG para su detección inmunológica).

Este vector se transformó en la cepa de *Saccharomyces cerevisiae* recomendada por el *kit*, cuya denominación es BJ3505.

Una vez transformada la levadura, se realizaron diversos estudios de expresión para conseguir optimizar la producción de la β-galactosidasa, y una vez finalizados se comenzó con la etapa de purificación.

Inicialmente se realizaron varias purificaciones por exclusión e intercambio iónico, pero la solución se halló realizando la purificación usando una columna con resina anti-FLAG (Sigma-Aldrich), y siguiendo las instrucciones del fabricante.

Una vez optimizadas las purificaciones y comprobadas en gel tanto en cantidad como en calidad (ya que ambos factores son limitantes a la hora de cristalizar), se realizaron unos test usando como herramienta para la optimización de las condiciones de cristalización un diseño de plan factorial completo.

El uso de los planes factoriales para la cristalización de proteínas se demostró en el trabajo de Carter y Carter (Carter and Carter 1979), permitiendo este método el ensayo de un elevado número de condiciones de cristalización con un número reducido de experimentos.

Se realiza variando más de un factor a la vez en cada experimento, de manera que se ahorran ensayos, y además analizando los resultados es posible determinar cuáles son los factores críticos en la cristalización.

Usando este método y condiciones similares a las que ya se habían obtenido cristales en la β-galactosidasa de *E. coli* (Juers *et al.* 2003) mediante la técnica de la gota colgante, se obtuvieron los primeros cristales, cuyo tamaño (unas 10 μm aprox) y forma no fueron suficientes para difractar, pero que nos dieron una información muy valiosa sobre los factores y compuestos que más afectan a la cristalización de la β-galactosidasa de *K. lactis* (Rodríguez *et al.* 2008).

El siguiente paso para la mejora de los cristales pasó por usar técnicas de alto rendimiento con el robot NanoDrop (Innovadyne Nanodrop) y varios kits de "screening" (Crystal Screen, Crystal Screen II, Crystal Screen Lite, SaltRx y Index Screen de Hampton Research, PACT Suite y JCSG+ Suite de Qiagen y JB Screen Classic de Jena Biosciences) para acotar más las condiciones

óptimas para el crecimiento de los cristales, usando de nuevo la técnica de cristalización de la gota colgante.

La mejora de los rendimientos y pureza de las purificaciones, así como la gran cantidad y variedad de kits usados, sirvió para mejorar los cristales tanto en tamaño como en capacidad de difracción. Se testaron más de 30 cristales, para finalmente conseguir un set completo de datos a 2.8 Å (Pereira-Rodriguez *et al.* 2010).

Finalmente mediante la técnica de "streak seeding", que se basa en romper los cristales con un pelo de gato o similar y con el mismo extenderlo sobre una nueva gota, de manera que la estructura tridimensional se reproduce si las condiciones son similares, y crecen los cristales en torno a una línea común.

Se consiguieron así cristales con un mayor poder de difracción, logrando un set completo de datos a 2.75 Å y 2.80 Å, consiguiendo descifrar tanto la estructura tridimensional del cristal por separado, como la estructura de la enzima con galactosa en su interior (tras la adicción de lactosa sobre el cristal) respectivamente.

El análisis de la estructura se obtuvo a través de reemplazamiento molecular con el programa MOLREP. La enzima tiene un peso molecular de 119kDa, contiene 1024 aminoácidos, y su forma activa comprende una estructura tetramérica que comprende la unión de dos dímeros entre sí (es la primera β-galactosidasa conocida que tiene esta estructura) y no de cuatro monómeros individuales como por ejemplo ocurre en el caso de la β-galactosidasa de *E. coli*.

Cada monómero está constituído por 5 dominios: Dominio 1 (residuos 32 a 204) presenta una estructura "jelly-roll" y es un dominio de unión a azúcar clasificado dentro de la familia 2 de las Glicosil Hidrolasas (GH), el Dominio 2 (residuos 205 a 332) que forma una estructura "β-sandwich" similar al de la inmunoglobulina y que pertenece a la familia 2 de las GH, el Dominio 3 (residuos 333 a 642) forma una estructura "TIM Barrel" perteneciente también a la familia 2 de las GH y que además es el centro catalítico, el Dominio 4 (residuos 643 a 720) que forma también una estructura "β-sandwich" similar al de la inmunoglobulina y que pertenece a la familia 2 de las GH, y finalmente el Dominio 5 (residuos 741 a 1025) que está clasificado como una pequeña cadena de β-galactosidasa. Existen dos regiones en la proteína que no pueden ser asignadas a ninguno de los dominios; una es la

región N-terminal (residuos 2 a 31) y la otra es una cadena pequeña expuesta al solvente que conecta los dominios 4 y 5 (residuos 721 a 740).

La unión de los monómeros para formar los dímeros de dímero, se establece entre agrupaciones de monómeros 2 a 2, uniéndose los dímeros mediante el contacto de dos de los monómeros principalmente, aunque se observan interacciones entre todos los monómeros para dar lugar a la estructura tetramérica.

El centro catalítico está constituido por un par de residuos catalíticos que son dos ácidos glutámicos posicionados en las posiciones 482 y 551. Ambos residuos se encuentran en las profundidades del "TIM Barrel" dentro del dominio 3. El bolsillo catalítico abarca unos 20 Å de profundidad y está rodeado por residuos de los 4 monómeros constituyentes.

En cuanto a la información obtenida en el cristal con el ligando, se ha encontrado la galactosa dispuesta de una manera muy parecida a su disposición en las β-galactosidasas de *E. coli* y *Arthrobacter*, y dos iones sodios y un ión magnesio. Uno de esos sodios y el ión magnesio están dispuestos en la misma posición que lo hacen en *E. coli*, y el otro sodio se encuentra en una posición similar a como lo hace en *Arthrobacter*.

Se ha podido localizar un ión manganeso, que no aparece en ninguna de las estructuras descritas previamente coordinado por residuos del loop 8 del dominio catalítico (Asp593) y de un loop del 5º dominio (His975 y Asp978). También aparece un ión sodio estabilizando el loop del 5º dominio, el cual está presente también en *E. coli*. Ambos ligandos pueden tener un rol importante en la oligomerización de la enzima en sus interfaces 2 y 3.

Construcción, expresión y producción de una β-galactosidasa híbrida entre la β-galactosidasa *Kluyveromyces lactis* y la β-galactosidasa de *Aspergillus niger*

La β-galactosidasa de *K. lactis* tiene un interés muy elevado en biotecnología debido a la gran cantidad de usos que se le atribuyen. Sin embargo, el hecho de ser una enzima intracelular provoca que aumenten las dificultades en cuanto a su recuperación una vez producida, es decir, al ser una enzima intracelular, es necesario extraerla de las células y separarla de las mismas. Esto además de ser difícil, incrementa el presupuesto necesario para su uso en la industria.

Es por ello que se ha construido una enzima híbrida entre la β-galactosidasa de *K. lactis* (intracelular) y la β-galactosidasa de *A. niger* (extracelular), para conseguir una proteína que se secrete al exterior.

Para ello se sustituyó el dominio 5 de la β-galactosidasa de *K. lactis* por el dominio 5 de la β-galactosidasa de *A. niger* (Rodriguez *et al.* 2006). Para predecir los dominios se usó como base la estructura cristalográfica de *E. coli*. Las técnicas usadas para la construcción de la enzima híbrida fueron la amplificación por PCR, la digestión por restricción y el ligamiento, ya que la recombinación homóloga tuvo que ser descartada por la poca homología existente entre ambas enzimas.

Una vez construida la enzima, se estudió su expresión y producción en matraces, siendo los resultados un incremento en 2 y 3 veces la producción de la β-galactosidasa extra e intracelular respectivamente.

La conclusión de estos resultados es que la región C-terminal de la β-galactosidasa de *A. niger* complementa perfectamente la región C-terminal de la β-galactosidasa de *K. lactis*.

Debido a la presencia de actividad enzimática, se decidió estudiar tanto el pH óptimo como la Tª óptima de la enzima, con miras a su uso en la industria.

Los resultados indican que la proteína híbrida adquirió unas propiedades bioquímicas híbridas entre ambas β-galactosidasas, ya que el pH óptimo desciende de 7-7,5 (Dickson *et al.* 1979; Tello-Solis *et al.* 2005) en *K. lactis* a 6,5 (el pH óptimo de *A. niger* está entre 2,5-4 (Widmer and Leuba 1979)) y la temperatura óptima aumentó de 30-35ºC en *K. lactis* (Dickson *et al.* 1979) a 40ºC (la Tª óptima de *A. niger* es de 50ºC (Santos *et al.* 1998)).

Posteriormente se estudió su estabilidad térmica así como el efecto de los cationes en la enzima. Los resultados presentan una mayor estabilidad térmica de la enzima híbrida respecto a la silvestre tanto a 30ºC, como a 42 y 50ºC. En cuanto a los cationes, su efecto fue similar en el caso de Mg^{2+}, Ca^{2+}, Zn^{2+}; pero en el caso del Ni^{2+} su efecto fue contrario a *K. lactis*, ya que este catión provocó un aumento de la actividad en la enzima híbrida, y no una inhibición como ocurre en el caso de la enzima de *K. lactis*.

Se analizaron finalmente las K_M del ONPG y la lactosa, que son el sustrato artificial y natural, respectivamente; siendo mayor la afinidad por ambas moléculas en el caso de la enzima híbrida.

También se realizó un modelado por homología de la enzima híbrida, en función de la estructura cristalográfica de *E. coli*.

Visto que la enzima híbrida tiene unas propiedades muy interesantes para su producción a gran escala, se realizó un estudio de su producción inmovilizando la cepa en "spent grains" (un subproducto de la elaboración de la cerveza) y haciéndola crecer en un medio rico en lactosa en un fermentador tipo "air-lift".

Este tipo de fermentadores es especialmente indicado para cultivos en aerobiosis, ya que la función de agitación se realiza por la entrada en la parte interior del mismo un flujo de aire, lo que mejora la oxigenación del medio y además es una manera menos agresiva de agitar las células.

Para la inmovilización de las células se siguió el protocolo de Branyik (Branyik *et al.* 2002).

Inicialmente se realizaron pruebas de crecimiento, consumo de lactosa, etanol, glicerol y actividad β-galactosidasa intra y extracelular en matraces.

Una vez determinadas las actividades, se realizó el cultivo en un fermentador de 6 litros de volumen de trabajo, en SS-Lactosa (5%) y luego en suero de leche (LACTOGAL), modificando los tiempos de residencia para calcular la mejor tasa de dilución para la producción de la β-galactosidasa híbrida.

La tasa de dilución óptima fue de 0.1, aproximadamente la mitad de la tasa de crecimiento exponencial (0.213), lo cual es sorprendente debido a que mayoritariamente la mejor tasa de dilución suele coincidir con la fase de mayor crecimiento celular.

Se consiguió además una tasa máxima de inmovilización de 0,45g cél/g de agente inmovilizador, lo cual demuestra que las "spent grains" son un substrato apropiado para inmovilizar *K. lactis*.

El que se pueda usar el suero de leche como medio de cultivo, facilita si cabe aún más su aprovechamiento, ya que evita el problema medioambiental que lleva asociado.

De todas maneras deberían realizarse más estudios para validar su uso a nivel industrial.

Construcción de cepas de *Saccharomyces cerevisiae* que secretan la β-galactosidasa de *Aspegillus niger* y evolución dirigida

La construcción de cepas capaces de crecer en lactosa es un objetivo muy importante en el campo de biotecnología (Becerra *et al.* 2002; Becerra *et al.* 2003).

Se han construido tres cepas diferentes capaces de secretar la β-galactosidasa de *A. niger*: una con la señal de secreción de la propia β-galactosidasa, otra con las dos señales de secreción (la del plásmido y la de la β-galactosidasa) y una última con la señal de secreción del plásmido únicamente.

Las tres construcciones se consiguieron mediante recombinación homóloga, usando el plásmido YEpFLAG (Eastman Kodak) y transformándose en BJ3505 ya que era la cepa de *Saccharomyces cerevisiae* que recomendaba el kit.

Se analizaron las actividades β-galactosidasa, y de las tres construcciones, la que más actividad β-galactosidasa extracelular producía era la construcción con la señal de secreción del propio plásmido. Esta cepa fue capaz de secretar más del 90% de la actividad β-galactosidasa total. El pH óptimo de las proteínas también fue calculado y resultado obtenido fue 3.

Para terminar debido a que la enzima β-galactosidasa de *A. niger* tiene un pH óptimo muy ácido (pH=3) se estudió la estructura obtenida mediante modelado por homología y se analizaron varias mutaciones en el servidor Rosseta (Liu and Kuhlman 2006; Kaufmann *et al.* 2010), que analiza la energía global de la proteína y en función de ello, se puede prever si la mutación es posible o no en la naturaleza (si es estable), para tratar de modificar el pH óptimo usando la evolución dirigida.

La técnica usada fue el kit *QuikChange® XL Site-Directed Mutagenesis* (Stratagene) con el que se construyeron los 3 mutantes simples así como las diferentes combinaciones, de manera que finalmente se obtuvieron 6 mutantes de la β-galactosidasa de *A. niger*. El reemplazamiento de tres residuos ácidos de la superficie de la enzima: Glu439, Asp469 y Asp476 por His, Gly y Ala, respectivamente, aunque disminuyó la actividad enzimática presentó un cambio en el perfil de pH de la enzima hacia una enzima más neutral.

Conclusiones

Las principales conclusiones del presente trabajo son las siguientes:

1. Se ha realizado una aproximación métodica y eficiente para el crecimiento de cristales de la β-galactosidasa de *Kluyveromyces lactis*. El plan factorial completo empleado permitió encontrar condiciones para el crecimiento de cristales proteicos de buena calidad realizando un bajo número de experimentos. Las condiciones óptimas de cristalización de 20 μg de β-galactosidasa de *K. lactis* se obtuvieron en presencia de 0,1 M de Tris-HCl, pH 8, 15% PEG 6000 y 0,02 M de $(NH_4)_2SO_4$. Las ventajas obtenidas con esta aproximación incluyen mejoras en la forma y el volumen de los cristales de la β-galactosidasa de *K. lactis* así como en la reproducibilidad.

2. La cristalización de la β-galactosidasa de K. lactis (3,5 mg ml^{-1} en 0,05 M de Tris-HCl, 0,150 M de NaCl, 0,002 M de DTT y 7% de Glicerol) se realizó usando el método de la gota sentada. Pequeños cristales en forma de plato crecieron en presencia de PEG 3350 al 23-27% (p/v), 0,1 M de BisTris pH 7,5-7,0 y 0,2 M de Tartrato Sódico. La micro-siembra de los cristales realizada empleando estas condiciones, mejoró la calidad de los cristales obtenidos e hizo posible que fuesen adecuados para los experimentos de difracción mediante Rayos-X.

3. El monómero de la β-galactosidasa de *K. lactis* se pliega en cinco dominios en un patrón conservado con otras enzimas procarióticas resueltas pertenecientes a la familia GH2, aunque dos inserciones largas en el dominio 2 (264-274) y el 3 (420-443) son únicas y parecen estar implicadas en la oligomerización y la especificidad.

4. El tetrámero de la β-galactosidasa de *K. lactis* es un ensamblado de dímeros, con una energía de disociación superior para los dímeros que para el tetrámero, lo que podría explicar que existiese un equilibrio en solución entre la forma dimérica y tetramérica de la enzima.

5. Dos centros activos se localizan en la interfase dentro de cada dímero, en un canal estrecho de 10 Å de ancho que hace los bolsillos catalíticos accesibles al solvente. La inserción única en el bucle 420-443 se introduce en este canal y presenta muchos enlaces posibles con la fracción aglicona de la lactosa, lo que

podría explicar la alta afinidad de la β-galactosidasa de *K. lactis* por este sustrato y por tanto su inusual elevada actividad hidrolítica.

6. Ninguno de los determinantes estructurales responsables del mecanismo de reacción propuesto para la β-galactosidasa de *Escherichia coli*, que implica una transición de un estado profundo a uno superficial tras la unión del sustrato, están presentes en el sitio activo de la β-galactosidasa de *K. lactis* y, consecuentemente, se sugiere que este mecanismo se aplica sólo para las enzimas pertenecientes a la familia GH2 reguladas por el operon lac.

7. Una proteína híbrida obtenida tras el reemplazamiento del dominio quinto de la β-galactosidasa de *K. lactis* por el de *Aspergillus niger*, fue activa, alcanzó el medio de cultivo y presentó además una estabilidad superior a temperaturas elevadas y unos parámetros cinéticos más adecuados para su utilización biotecnológica.

8. Una cepa de *K. lactis* secretando la proteína híbrida entre la β-galactosidasa de *K. lactis* y la de *A. niger* fue inmovilizada en afrecho (*spent grains*) para producir la proteína híbrida usando lactosa como fuente de carbono, alcanzando un máximo de 0,45 gramos de peso seco de células por gramo de peso seco de afrecho.

9. Una cepa recombinante de *Saccharomyces cerevisiae* expresando bajo el control del promotor *ADH2* de levaduras la β-galactosidasa de *A. niger* fusionada a la señal de secreción del factor α de levaduras presentó hasta el 94% de la actividad total β-galactosidasa en el medio de cultivo y fue capaz de crecer en medios con lactosa.

10. Mediante experimentos de evolución dirigida de la β-galactosidasa de *A. niger* se intentó modificar su pH óptimo. El reemplazamiento de tres residuos ácidos de la superficie de la enzima: Glu439, Asp469 y Asp476 por His, Gly y Ala, respectivamente, aunque disminuyó la actividad enzimática presentó un cambio en el perfil de pH de la enzima hacia una enzima más neutral.

Appendix III – *Curriculum vitae*

Publications

2012

"Heterologous expression and directed evolution of *Aspergillus niger* β-galactosidase" Ángel Pereira Rodríguez *et al.* Manuscript in preparation.

"Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase" Angel Pereira-Rodríguez; Rafael Fernández-Leiro; M Isabel González-Siso; M Esperanza Cerdán; Manuel Becerra; Julia Sanz-Aparicio. ***Journal of Structural Biology.*** 2012 Feb;177(2):392-401. DOI: 10.1016/j.jsb.2011.11.031

2010

"Structural Analysis of *Saccharomyces cerevisiae* α-Galactosidase and Its Complexes with Natural Substrates Reveals New Insights into Substrate Specificity of GH27 Glycosidases" Fernández-Leiro R, Pereira-Rodríguez A, Cerdán ME, Becerra M, Sanz-Aparicio J.***Journal of Biological Chemistry.*** 2010,285:28020-28033. DOI: 10.1074/jbc.M110.144584

"Crystallization and preliminary X-ray crystallographic analysis of β-galactosidase from *Kluyveromyces lactis*" Ángel Pereira-Rodríguez, Rafael Fernández-Leiro, M. Isabel González Siso,M. Esperanza Cerdán, Manuel Becerra and Julia Sanz-Aparicio. ***Acta Crystallographica section F: Structural Biology and Crystallization Communications*** (2010). 66, 297–300. DOI: 10.1107/S1744309109054931

"**Crystallization and preliminary X-ray diffraction data of alpha-galactosidase from *Saccharomyces cerevisiae*"** Fernández-Leiro R, Pereira-Rodríguez A, Cerdán ME, Becerra M, Sanz-Aparicio J.
Acta Crystallogr Sect F Struct Biol Cryst Commun. 2010 Jan 1;66(Pt 1):44-7. DOI: 10.1107/S1744309109047794

2008

"Production of recombinant proteins from cheese whey". Book: Advances in Cheese Whey Utilization. Editorial Research Signpost-Transworld research network. M. Isabel González-Siso, Angel Pereira Rodríguez, Rafael Fernández Leiro, M. Esperanza Cerdán & Manuel Becerra. ISBN:978-81-7895-359-5.

"*Kluyveromyces lactis* β-galactosidase crystallization using full-factorial experimental design" Ángel Pereira Rodríguez, Rafael Fernández Leiro, M. Esperanza Cerdán, M. Isabel González Siso, Manuel Becerra Fernández. ***Journal of Molecular Catalysis B: Enzymatic*** 52–53 (2008) 178–182. DOI: 10.1016/j.molcatb.2007.11.013.

2006

"Secretion and properties of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase" Ángel Pereira Rodríguez, Rafael Fernández Leiro, M. Cristina Trillo, M. Esperanza Cerdán, M. Isabel González Siso, Manuel Becerra. ***Microbial Cell Factories*** 5: 41. DOI: 10.1186/1475-2859-5-41.

Patents

2011

Two new biotechnology patents are being processed.

2010

EP20100761222 - "Strains of *S.cerevisiae*, capable of growing in media with melibiose, stachyose and raffinose" Rafael Fernández Leiro, Ángel Pereira Rodríguez, Manuel Becerra Fernández, Mª Isabel González Siso, Mª Esperanza Cerdán Villanueva.

2009

ES20090000961 - **"Cepas de *Saccharomyces cerevisiae* capaces de crecer en medios con melibiosa, estaquiosa y rafinosa".** Rafael Fernández Leiro, Ángel Pereira Rodríguez, Manuel Becerra Fernández, Mª Isabel González Siso, Mª Esperanza Cerdán Villanueva. Material Transfer Agreement with BIO4-Soluçoes Biotecnológicas LTDA.

2007

ES20070001946 - "Cepas de levadura capaces de secretar beta-galactosidasa al medio y su uso para la producción de biomasa, etanol, beta-galactosidasa y proteínas de interés". Ángel Pereira Rodríguez, Rafael Fernández Leiro, Manuel Becerra Fernández, Mª Isabel González Siso, Mª Esperanza Cerdán Villanueva. Material Transfer Agreement with Kerry Bio-Science.

Conference communications

2011

"Structure of the β-galactosidase from *Kluyveromyces lactis*". Poster communication to the XXII International Union of Crystallography Congress and General Assembly. Fernández-Leiro Rafael, Ángel Pereira Rodríguez, M. Esperanza Cerdán Villanueva, Manuel Becerra Fernández, Juliana Sanz Aparicio. (22-29/08/2011), (Madrid, Spain).

"Production of Angiotensin Converting Enzyme (ACE) Inhibitors by proteolysis of cheese whey and preliminary identification of active peptides using proteomic approaches". Poster communication to the 2nd International Congress on Analytical Proteomics – ICAP. Estévez, N., Rodrigues, A. C., Fuciños, C., Míguez, M., Pereira, A., Fuciños, P. (18_20/07/2011), (Ourense, Spain).

2010

"Improvement of ethanol production from cheese whey using full-factorial experimental design". Poster communication to the Yeast, an evergreen model – Tribute to P. Slonimski. A. Pereira Rodríguez, S. Pedraza de la Cuesta, R. Fernández Leiro, M.E. Cerdán Villanueva, M.I. González Siso, M. Becerra Fernández. (23_25/09/2010), (Rome, Italy).

"Aplicación de métodos estadísticos para mejorar la producción de etanol a partir de suero de leche". Poster communication to the XXXIII Congreso Sociedad Española de Bioquímica y Biología Molecular. A. Pereira Rodríguez, S. Pedraza de la Cuesta, R. Fernández Leiro, M.E. Cerdán Villanueva, M.I. González Siso, M. Becerra Fernández. (14_17/09/2010), (Cordoba, Spain).

"The Structure of alpha-galactosidase from *Saccharomyces cerevisiae*". Poster communication to the VI Reunión de la Red Temática Nacional de Estructura y Función de Proteínas. Fernández-Leiro Rafael, Ángel Pereira Rodríguez, M. Esperanza Cerdán Villanueva, Manuel Becerra Fernández, Juliana Sanz Aparicio. (21-23/04/2010), (Madrid, Spain).

2009

"Cristalización de la beta-galactosidasa de *Kluyveromyces lactis*". Poster communication to the XXXII Congreso Sociedad Española de

Bioquímica y Biología Molecular. A. Pereira Rodríguez, R. Fernández Leiro, M.I. González Siso, J. Sanz Aparicio & M. Becerra Fernández. (23_26/09/2009), (Oviedo, Spain).

"Caracterización estructural de la alfa-galactosidasa de Saccharomyces cerevisiae". Poster communication to the XXXII Congreso Sociedad Española de Bioquímica y Biología Molecular. R. Fernández Leiro, A. Pereira Rodríguez, M. E. Cerdán Villanueva, J. Sanz Aparicio & M. Becerra Fernández. (23_26/09/2009), (Oviedo, Spain).

"*Kluyveromyces lactis* β-galactosidase crystallization using incomplete factorial experiments". Poster communication to the Hands-on Course in Proteins and Proteomics. A. Pereira Rodríguez, R. Fernández Leiro, M. E. Cerdán, M. I. González Siso, M. Becerra Fernández. (07_18/09/2009), (Costa da Caparica, Portugal). Awarded best poster prize.

"Ethanol production from cheese whey using respiratory mutants of *Kluyveromyces lactis*". Poster communication to the 27th ISSY – International Specialized Symposium on Yeasts – Institut Pasteur. Pereira Rodríguez, A., Fernández Leiro, R., Cerdán Villanueva, Mª E., González Siso, M.I. & Becerra Fernández, M. (26_29/08/2009), (Paris, France).

"Analysis of respire-fermentative metabolism, oxidative stress and sensitivity to cis-platinum in four mutants of *K. lactis* defective in transcriptional regulatory factors". Poster communication to the 27th ISSY – International Specialized Symposium on Yeasts – Institut Pasteur. Ángel Pereira-Rodríguez, Ana García-Leiro, Esther Rodríguez-Belmonte, Mónica Lamas Maceiras, Manuel Becerra Fernández, M. Isabel González Siso y M. Esperanza Cerdán Villanueva. (26_29/08/2009), (Paris, France).

"The *alpha-galactosidase* from *Saccharomyces cerevisiae*. Crystallization and preliminary X-ray diffraction data". Poster communication to the 27th ISSY – International Specialized Symposium on Yeasts – Institut Pasteur. Fernández Leiro, R., Pereira Rodríguez, A., Sanz Aparicio, J., Cerdán Villanueva, Mª E., & Becerra Fernández, M. (26_29/08/2009), (Paris, France).

"Producción de bioetanol a partir de suero de leche". Conference presentation in the "Biotecnología e industrias lácteas: valorización de residuos" seminar (organised by the UIMP). Ángel Pereira Rodríguez. (15/07/2009), (A Coruña, Spain).

"Crystallization of a highly glycosylated protein. The alpha-galactosidase from *Saccharomyces cerevisiae*". Poster communication to the International School on Biological Crystallization. Fernández Leiro, R., Pereira Rodríguez, A., Cerdán Villanueva, Mª E., Becerra Fernández, M. & Juliana Sanz Aparicio. (18_22/05/2009), (Granada, Spain). Awarded best poster prize.

2008

"Production of bioethanol in cheese whey using *Saccharomyces cerevisiae* expressing the *Aspergillus niger* β-galactosidase". Poster communication to the 5th EFB Meeting on Recombinant Protein Production. Pereira-Rodríguez, A., Fernández-Leiro, R., Cerdán-Villanueva, Mª. E., González-Siso, Mª. I. & Becerra-Fernández, M. (23_28/09/2008), (Sardinia, Italy).

"Expression, purification and characterization of an α-galactosidase from *Saccharomyces cerevisiae*". Poster communication to the 5th EFB Meeting on Recombinant Protein Production. Fernández-Leiro, R., Pereira-Rodríguez, A., Cerdán-Villanueva, Mª. E., González-Siso, Mª. I. & Becerra-Fernández, M. (23_28/09/2008), (Sardinia, Italy).

"Mutagénesis dirigida del centro catalítico de la α-galactosidasa de *Saccharomyces cerevisiae*". Poster communication to the XXXI Congreso Sociedad Española de Bioquímica y Biología Molecular. Fernández Leiro, R., Pereira Rodríguez, A., Cerdán Villanueva, Mª E. & Becerra Fernández, M. (10_14/09/2008), (Bilbao, Spain).

"Producción de bioetanol a partir de suero de leche". Poster communication to the XXXI Congreso Sociedad Española de Bioquímica y Biología Molecular. Pereira Rodríguez, A., Fernández Leiro, R., González Siso, Mª. I. & Becerra Fernández, M. (10_14/09/2008), (Bilbao, Spain).

2007

"Heterologous *Aspergillus niger* β-galactosidase secretion by *Saccharomyces cerevisiae*". Poster communication to the 13th European Congress on Biotechnology. "Symbiosis:Science, Industry & Society" Pereira Rodríguez, A., Fernández Leiro, R., Cerdán M. E., González Siso, M.I., Becerra Fernández, M. (16_19/09/2007), (Barcelona, Spain).

"Secreción heteróloga de la β-galactosidasa de *Aspergillus niger* en *Kluyveromyces lactis*". Poster communication to the XXX Congreso Sociedad Española de Bioquímica y Biología Molecular. Fernández Leiro, R., Pereira Rodríguez, A., Cerdán M. E., Becerra Fernández, M Pereira Rodríguez, A., Fernández Leiro, R., González Siso, M.I., Becerra Fernández, M. (12_15/09/2007), (Málaga, Spain).

"Aproximación a la cristalización de la α-galactosidasa de *S. cerevisiae*". Poster communication to the XXX Congreso Sociedad Española de Bioquímica y Biología Molecular. Fernández Leiro, R., Pereira Rodríguez, A., Cerdán M. E., Becerra Fernández, M. (12_15/09/2007), (Málaga, Spain).

"*Kluyveromyces lactis* β-galactosidase crystallization using incomplete factorial experiments". Poster communication to the 8th International Symposium on Biocatalysis and Biotransformations "BIOTRANS 2007". Pereira Rodríguez, A., Fernández Leiro, R., Cerdán M. E., González Siso, M.I., Becerra Fernández, M. (08_13/07/2007), (Oviedo, Spain).

2006

"Secretion of a hybrid *K. lactis-A. niger* β-galactosidase". Poster communication to the 4th Recombinant Protein Production Meeting. Pereira Ángel, Fernández Rafael, Cerdán María Esperanza, González Siso María Isabel, Becerra Manuel. (21_ 23/09/2006), (Barcelona, Spain).

"Properties of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase". Poster communication to the XIXth International Meeting on the "Biology of *Kluyveromyces*". Pereira-Rodríguez Ángel, Fernández-Leiro Rafael, Cerdán María Esperanza, González-Siso María Isabel, Becerra Manuel. (16_17/09/2006), (Parma, Italy).

"Construcción de enzimas híbridas entre la β-galactosidasa intracelular de *Kluyveromyces lactis* y la β-galactosidasa extracelular de *Aspergillus niger*". Poster communication to the XXIX Congreso de la Sociedad Española de Bioquímica y Biología Molecular. Pereira-Rodríguez Ángel, Fernández-Leiro Rafael, González-Siso María Isabel, Becerra Manuel. (07_10/09/2006), (Elche, Spain).

"Cambio de especificidad de sustrato de una α-galactosidasa de *Saccharomyces cerevisiae* a una β-galactosidasa". Poster communication to the XXIX Congreso de la Sociedad Española de Bioquímica y Biología

Molecular. Fernández-Leiro Rafael, Pereira-Rodríguez Ángel, González-Siso María Isabel, Becerra Manuel. (07_10/09/2006), (Elche, Spain).

2005

"Aproximación de la cristalización de la β-galactosidasa de *K. lactis* y estudio de variantes obtenidas mediante la evolución molecular dirigida con actividad enzimática modificada". Poster communication to the XXVIII Congreso de la Sociedad Española de Bioquímica y Biología Molecular. A.Pereira, M.I. González, M. Becerra. (12_15/09/2005), (Zaragoza, Spain).

Research Fellowships/Contracts

01/01/2009 - 14/10/2010	"María Barbeito" Contract (Xunta de Galicia Predoctoral Programme).
01/10/2009 – 31/12/2009	Visitor Researcher Fellowship (Xunta de Galicia) in the Departamento de Engenharia Biológica (Universidade do Minho, Portugal).
01/01/2008 - 31/12/2008	UDC Predoctoral Contract.
01/01/2007 - 31/12/2007	UDC Predoctoral Fellowship.

Participation in Research Grants/Projects

2009-2010

Regional Project: "Valorización dos soros de queixería mediante a producción de bioetanol". Funded by the Xunta de Galicia. Consellería de Economía e Industria 09REM001E (DOG 24-06-2009). In collaboration with Queizuar SL - Queixerías Bama (Traditional Cheese Factory – Touro (A Coruña)).

2008-2011

Program "Consolidación y estructuración de unidades de investigación competitivas del sistema Universitario de Galicia" Funded by the Xunta de Galicia, Conselleria de Educación y Ordenación Universitaria (DOG 3-12-2008). Biochemistry area. Department of Cellular and Molecular Biology,

University of A Coruña (Spain). 2008 – 2011. Principal Investigator: María Esperanza Cerdán Villanueva.

2007-2008

Regional Project: "Valorización de los sueros de quesería mediante la producción de ingredientes alimenticios funcionales y producción de bioetanol". Funded by the Xunta de Galicia 07TAL006E (DOG 21-11-2007). In collaboration with Universidade de Vigo and Queizuar SL - Queixerías Bama (Traditional Cheese Factory – Touro (A Coruña)).

2007

Program: "Consolidación y estructuración de unidades de investigación competitivas del sistema gallego de I+D+I". Funded by the Xunta de Galicia - Consellería de Innovación e Industria. 1INCITE07PXI103013ES (DOG 21-12-2007).

2004

Regional Project: "Evolución dirigida y estudios cristalográficos de la β-galactosidasa de *Kluyveromyces lactis*". Funded by the Xunta de Galicia PGIDIT03BTF10302PR (DOG 20-10-2003).