Universidade Federal do Paraná

Bioprocess Engineering and Biotechnology Department

Université de Provence (Aix-Marseille I)

Université de La Méditerranée (Aix-Marseille II)

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Production of recombinant α-amylase from *Bacillus subtilis* by *Kluyveromyces lactis*

Author: Bruno Motta Nascimento

Practical Supervisor: Vanete Thomaz Soccol

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Bruno Motta Nascimento

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“Always pass on what you have learned.”

Master Yoda

ABSTRACT

Amylolytic enzymes catalyze the hydrolysis or the modification of starch structure. Many organisms are able to produce these enzymes, but only a few of them exhibit satisfactory characteristics for industrial application. The most common commercial α-amylases with desirable industrial characteristics are produced by bacteria from the genus *Bacillus*. Although the yield of α-amylase produced by these bacterial strains can be increased with culture optimization, the recombinant production of the enzyme could offer some advantages such as easier genetic manipulation/mutation, inducible expression, faster production, higher yields and easier purification steps. The present work proposes the production of a recombinant α-amylase in *Kluyveromyces lactis*. A *Bacillus subtilis* NRRL B-4212 strain provided the α-amylase gene, which was amplified and inserted in a pKLAC2 vector in four different ways: (i) with both the *B. subtilis* signal sequence and *K. lactis* leader sequence; (ii) with only the yeast leader or (iii) only the *B. subtilis* signal, and (iv) without any signal or leader sequence. The final constructs were linearized and the cassettes were used for *K. lactis* CBS 2359 cells transformation. After growth in a selective medium, the positive yeast colonies were assayed for enzymatic activity on agar plates and liquid medium. It was expected that cells with construct (i) and cells with construct (ii) would present extracellular enzymatic activity. On the other way, transformants with constructs (iii) and transformants with construct (iv) should present an intracellular activity. However, only constructs (ii) and (iv) behaved as expected. Construct (i) could not secrete the enzyme, indicating that the presence of both signals might interfere in the secretion machinery, and construct (iii) presented a high extracellular activity (1200 U/L), even though it did not have the yeast leader sequence, indicating that the yeast cell probably can also recognize the bacterial signal peptide. One sample from each construct was selected to determinate the enzyme's optimal temperature and pH, obtaining results between 50 an 55 °C, which is consistent with other observed values for *Bacillus* amylases. Transformant LAC-EXAMY 4 was also cultured in different carbon sources to observe cellular growth and enzyme production. The recombinant yeast was able to quickly grow in the presence of glucose, sucrose and lactose. Even without the induction with lactose or galactose the enzyme was produced with a basal level

(0.44 U/mL). However, the presence of these carbon inducers greatly enhances the production yield, achieving 2.21 U/mL with lactose and 2.55 U/mL with galactose as the carbon source

Keywords: recombinant expression, alpha-amylase, signal sequence, *Kluyveromyces lactis*.

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SYMBOLS AND ABBREVIATIONS

A	Adenine
aa	Amino acid
Arg	Arginine
Asp	Aspartate
BAP	*B. subtilis* α-amylase production broth
C	Cytosine
DNA	Deoxyribonucleic acid
EC	Enzyme Commission number
G	Guanine
Glu	Glutamic acid
GRAS	Generally recognized as safe
INTAMY, INTSIG	Amplified α-amylase genes to be cloned without the yeast secretion leader
EXAMY, EXSIG	Amplified α-amylase genes to be cloned with the yeast secretion leader
kg, g, mg, µg, ng	Kilogram, gram, milligram, microgram, nanogram
K_m	Michaelis-Menten constant
L, mL, µL	Liter, milliliter, microliter
LAC-INTAMY	Cassette integrated in *Kluyveromyces lactis* genome containing only the mature α-amylase gene
LAC-INTSIG	Cassette integrated in *Kluyveromyces lactis* genome containing only the α-amylase with the bacterial signal peptide
LAC-EXAMY	Cassette integrated in *Kluyveromyces lactis* genome containing the mature α-amylase gene and the yeast secretion leader
LAC-EXSIG	Cassette integrated in *Kluyveromyces lactis* genome containing the α-amylase with the bacterial signal peptide and the yeast secretion leader
LB	Luria-Bertani broth
M	Molarity (mol/L)
N	Asparagine

NCM	Nomenclatura comum do Mercosul
ºC	Celsius degree
OD_{600}	Optical density at 600nm
PCR	Polymerase Chain Reaction
pka	Logarithmic acid dissociation constant
pKLAC2	*Kluyveromyces lactis* expression vector
pKLAC-INTAMY	pKLAC2 vector containing the amplified gene INTAMY
pKLAC-INTSIG	pKLAC2 vector containing the amplified gene INTSIG
pKLAC-EXAMY	pKLAC2 vector containing the amplified gene EXAMY
pKLAC-EXSIG	pKLAC2 vector containing the amplified gene EXSIG
R_m	Maximum productivity
S	Serine
s, min, h	Second, minute, hour
sp.	Species
T	Thymine
TGY	Tryptone, glucose, yeast extract broth
Trp	Tryptophan
Tyr	Tyrosine
U	Units of enzyme activity
US$	United States dollar
v_{max}	Michaelis-Menten maximum rate
WT	Wild type (strain *K. lactis* CBS2359)
x g	Relative centrifugal force
YCB	Yeast carbon base broth
YNB	Yeast nitrogen base broth
$Y_{P/S}$	Product yield
YPD	Yeast extract, peptone, dextrose broth
YPL	Yeast extract, peptone, lactose broth
YPLS	Yeast extract, peptone, lactose, starch medium
YPX	Yeast extract and peptone medium, where X could stand for glucose, sucrose, starch, lactose or galactose
$Y_{X/S}$	Biomass yield
α	Alpha

α-MF	α-mating factor
β	Beta
μ_{max}	Maximum specific growth rate

1 INTRODUCTION

Amylolytic enzymes catalyze the hydrolysis or the modification of starch structure. Among them is included the α-amylase, an enzyme responsible for the hydrolysis of α-1,4-glycosidic bonds in starch or its degradation products, acting internally in the polysaccharidic chain. The α-amylase has a broad application in industrial processes. They are vastly used in the textile and paper industries (starch removal from fibers), food processing (for beverages and bakery), detergents formulation (enhancing stain removal efficiency), pharmaceutical studies (diagnostic tests) and ethanol production (GUPTA *et al.*, 2003; SOUZA & MAGALHÃES, 2010).

Many organisms are able to produce this enzyme, but only a few of them exhibit satisfactory characteristics for industrial application, such as optimal range of temperature and pH compatible with those found in industrial processes (GUPTA *et al.*, 2003). The most common commercial α-amylases with desirable biochemical characteristics are produced by bacteria from the genus *Bacillus* (*B. subtilis*, *B. stearothermophilus*, *B. licheniformis* e *B. amyloliquefaciens*) (SATYANARAYANA *et al.*, 2006; PRAKASH & JAISWAL, 2010).

Although the yield of α-amylase produced by these bacterial strains can be increased with culture optimization and strain screening, the recombinant production of the enzyme could offer some advantages such as easier genetic manipulation/mutation, inducible expression, faster production, higher yields and easier purification steps.

Kluyveromyces lactis is a non-conventional yeast used as host for heterologous protein production. It is able to consume a great variety of carbon sources with a high growth rate and without producing ethanol under oxygen-limited conditions (Crabtree-negative). Its genome has already been sequenced and the cells can be easily manipulated for genetic transformation (VAN OOYEN *et al.*, 2006).

Based on these insights, the present work proposes the production of a recombinant α-amylase from *Bacillus subtilis* in *Kluyveromyces lactis* cells for industrial purposes, such as starch degradation for ethanol production.

2 PRACTICAL OBJECTIVES

2.1 GENERAL OBJECTIVE

The present study intends to produce a recombinant α-amylase from *Bacillus subtilis* by transforming *Kluyveromyces lactis* cells with the exogenous gene.

2.2 SPECIFIC OBJECTIVES

- Construct an expression vector of *Kluyveromyces lactis* containing the gene of α-amylase from *Bacillus subtilis* with different signal sequences.
- Obtain recombinant *K. lactis* cells producing intracellular and extracellular α-amylase.
- Perform shake flasks cultivations for the production of the enzyme on bench scale.
- Partially characterize the enzymatic activity of the recombinant enzyme.

3 BIBLIOGRAPHIC REVIEW

3.1 AMYLOLYTIC ENZYMES

Amylolytic enzymes catalyze the hydrolysis or the modification of starch structure. Among them is the α-amylase, which is responsible for the hydrolysis of α-1,4-glycosidic bonds in starch or its degradation products, acting internally in the polysaccharidic chain.

Starch is a common constituent of higher plants, where it plays a role of carbohydrate storage. The metabolites arising from starch degradation are both source of carbon and energy for the plant. Some plants accumulate a great amount of starch granules in their tissues. As an example, the starch content in potato tuber, maize endosperm, sweet potato, and roots of cassava and yam varies between 65% and 90% of total dry weight (BEMILLER & WHISTLER, 2009).

The starch granules are made of two types of polymer: amylose and amylopectin. The ratio between these two components varies depending on the plant species, with an average of 15-25% of amylose and 75-85% of amylopectin (BERTOLDO & ANTRANIKIAN, 2002). The amylose consists of unbranched chains of D-glucose connected by α-1,4 linkages. Amylopectin consists of a chain with α-1,4 linked glucoses branched at every 24-30 residues by α-1,6 linkages (LEHNINGER *et al.*, 2006; BEMILLER & WHISTLER, 2009).

The starch molecule is important in many manufacturing processes, not only in food industry, but also in detergents, textile, paper, fuels and pharmaceutical industries. One of the main modifications performed industrially is the hydrolysis of the polymer to produce syrups, which is mainly accomplished by enzymatic catalysis.

Starch can be hydrolyzed either by chemical treatment with inorganic acids or by enzymatic treatment with hydrolytic enzymes. The second option is favored because it allows better control of hydrolysis reaction, more specific catalysis, more stable products, and milder reaction conditions (with lower temperatures and pH near neutrality). These characteristics reduce the occurrence of undesired reactions, the energetic need for the process and eliminate neutralization steps (SATYANARAYANA *et al.*, 2006).

The first starch degrading enzyme was observed in 1811 by Kirchhoff, but only after 1930 it was suggested the classification in α- and β-amylases, according to the anomeric form of the sugars produced by the enzymatic reaction (GUPTA *et al.*, 2003; SATYANARAYANA *et al.*, 2006).

Starch degrading enzymes can be classified according to their properties, type of action, type of substrate, or similarity of sequences. The classification by the type of bonds they break results in two groups: (i) those which act on α-1,4-glycosidic bonds and (ii) those which act on α-1,6-glicosidic bonds. It is also possible to distinct them by the site of hydrolysis: endo-hydrolases, which act on the interior of the starch polymer, and exo-hydrolases, which act on the substrate from the non-reducing end (Figure 1).

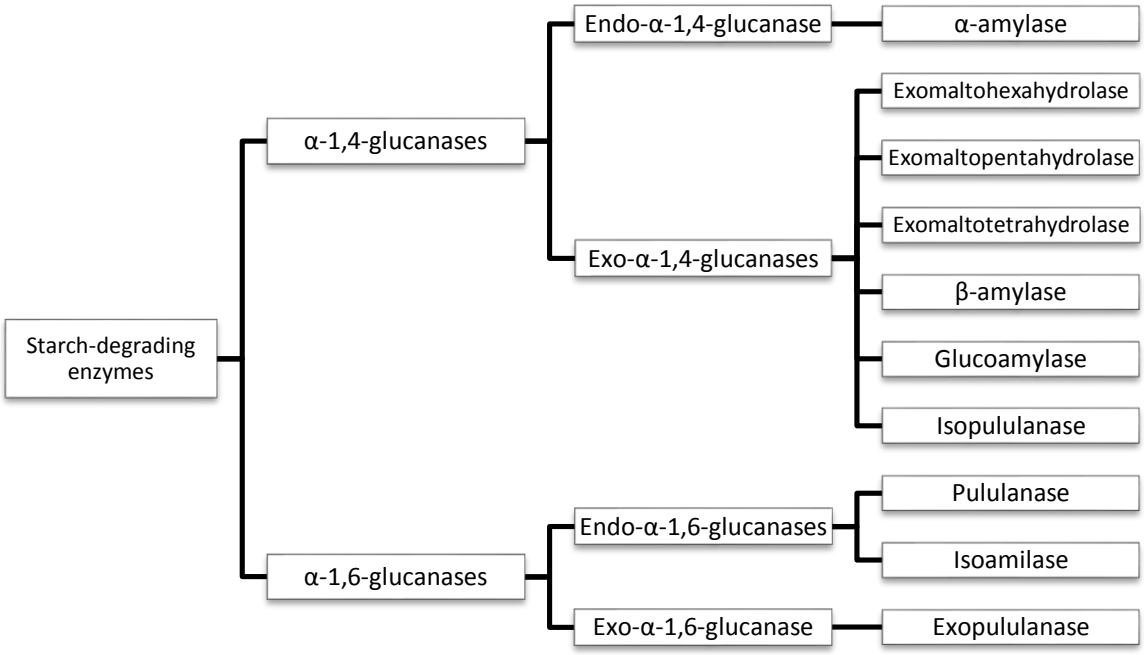


Figure 1 - Starch-degrading enzymes classification based on the type of bond they hydrolyze and site of action. Adapted from NIGAM & SINGH, 1995.

Endo-hydrolases (such as α-amylase) act in the interior of the starch chain. They are able to hydrolyze in a random way at many positions of the polymeric chain, producing a variety of dextrins and oligomers. Exo-hydrolases (such as β-amylase, glucoamylase and α-glucosidades) attack the chain from the non-reducing end, producing well defined oligosaccharides, such as maltose for β-amylase and glucose for glucoamylase (Figure 2) (GUPTA *et al.*, 2003; BEMILLER & WHISTLER, 2009).

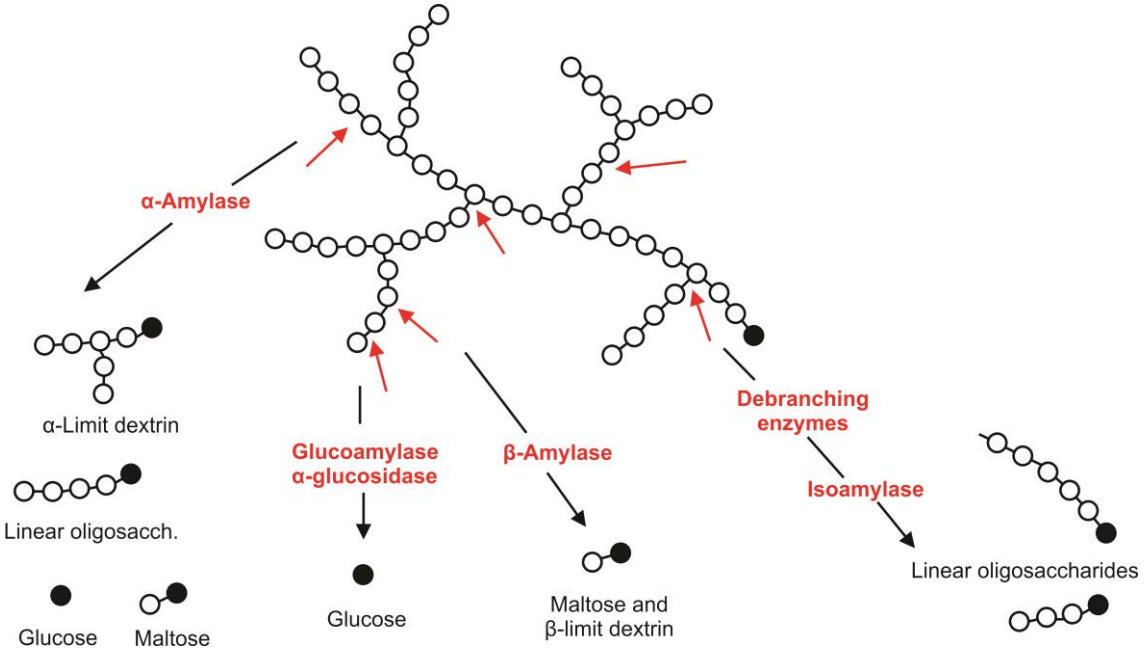


Figure 2 - Enzymatic degradation of starch. The black circle indicates the reducing sugar. Adapted from BERTOLDO & ANTRANIKIAN, 2002.

3.1.1 α-Amylase

The α-amylases are α-1,4-glucan-4-glucohydrolases that catalyze the hydrolysis of α-1,4-glicosydic bonds in starch molecules or its products of degradation. They act internally at random sites of the polysaccharidic chain, producing diverse degradation products. The specificity for the substrate and the range of products formed depends on the source of each α-amylase. They are classified as EC (Enzyme Commission number) 3.2.1.1 based on substrate specificity and the molecular mechanism of catalysis (SATYANARAYANA *et al.*, 2006).

The X-ray analysis of α-amylase structures reveals that it contains three principal domains denominated A, B and C. In Figure 3 it is shown an example of a bacterial α-amylase structure. Domain A consists of a $(\beta/\alpha)_8$ barrel containing the three catalytic residues (Asp, Glu, Asp). Domain C corresponds to the C-terminal portion of the protein and domain B corresponds to a protrusion between the third β-sheet and the third α-helix of the barrel in domain A (HORVÁTHOVÁ *et al.*, 2001; ALIKHAJEH *et al.*, 2007).

The catalytic mechanism of the α-amylase is performed by three main residues: an aspartate (Asp) acting as a nucleophile, a glutamic acid (Glu) acting

as a hydrogen donor in the reaction, and another aspartate (Asp), which is believed to help in the catalysis by doing a hydrogen bond with the substrate and increasing the pKa value of the Glu residue (SATYANARAYANA *et al.*, 2006; PRAKASH & JAISWAL, 2010).

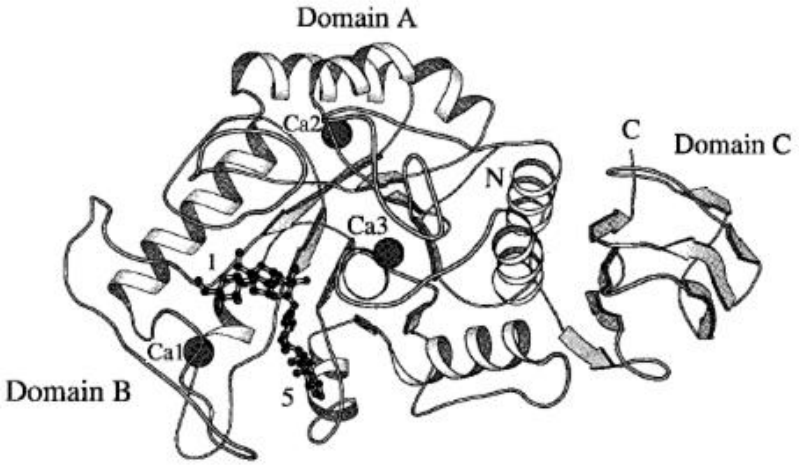


Figure 3 - *Bacillus subtilis* α-amylase structure showing domains A, B and C, three Ca^{2+} ions and the maltopentose used as substrate (molecule numbered from 1 to 5). Adapted from FUJIMOTO *et al.*, 1998.

One of the most advantageous characteristic of α-amylases that permits their industrial application is their thermostability. This term refers to the preservation of the chemical and spatial structure under high temperature conditions. The molecular mechanisms of thermostability depends on the enzyme, but some factors in common are considered to contribute to stability, such as: higher number of hydrogen bonds, ionic interactions and disulfide bonds, higher core hydrophobicity, decreased length of surface loops, presence of metal binding sites, and increased packing density (which leads to a lower level of thermal motion and less flexibility) (PRAKASH & JAISWAL, 2010).

For α-amylases, the most important factor is the presence of calcium ions, substrate or other stabilizing agents. The stabilizing effect of calcium ion on the enzyme's thermostability can be explained by the salting out of hydrophobic residues occurred in the presence of calcium ions, causing the adoption of a compact structure (PRAKASH & JAISWAL, 2010). Most α-amylases are metalloenzymes containing at least one Ca^{2+} ion, and the amount of Ca^{2+} ions

interacting with the protein can vary from 1 to 10 (SATYANARAYANA *et al.*, 2006).

The use of thermostable amylases in an industrial process has some advantages, such as: lower cooling costs, better solubility of the substrate, lower viscosity (allowing a better mixture and pumping of the solution), and reduced risk of microbial contamination (ASGHER *et al.*, 2007).

Nevertheless, many other metallic cations, especially heavy metals ions, sulphydryl group reagents, N-bromosuccinimide, p-hydrohyl mercuribenzoic acid, iodoacetate, BSA, EDTA and EGTA may inhibit the α-amylase (GUPTA *et al.*, 2003). Some animal and *Streptomyces* amylases have inhibition by proteinaceous compounds or substrate analogues. The inhibition by proteinaceous compounds occurs due to the presence of Trp-Arg-Tyr residues in these molecules. Amylases from plants and other microorganisms do not present this kind of inhibition (SUMITANI *et al.*, 1998).

3.2 AMYLASE PRODUCTION

3.2.1 Sources of α-amylases

Amylases are ubiquitous enzymes in distribution, being easily found in plants, animals and microorganisms. However, fungal and bacterial enzymes have more commercial and industrial application due to its easier bulk production, better catalytic properties and stability. Furthermore, microorganisms can be easily manipulated to enhance production yield and protein characteristics (SOUZA & MAGALHÃES, 2010).

The production of microbial α-amylases in bioreactors can occur either by submerged fermentation or solid state fermentation, depending on the characteristics of the producing strain. The physicochemical factors during fermentation will also affect the production of the enzyme, such as medium composition, pH, temperature, aeration, inoculum level and age, carbon/nitrogen ratio, and presence of inducer (PANDEY *et al.*, 2000; SATYANARAYANA *et al.*, 2006).

Industrial amylases typically need little purification during the downstream process, because most of their commercial uses do not need a high degree of purity. Only some pharmaceutical applications and structural studies need highly purified α-amylases (SOUZA & MAGALHÃES, 2010).

The bench scale purification of the protein usually includes an initial separation of the cells from the supernatant, followed by an enzyme concentration by chromatography (ion exchange, gel filtration, hydrophobic interaction, reverse phase), extraction by solvents (ethanol, acetone), precipitation (ammonium sulfate) or ultrafiltration. Some of these steps are expensive, laborious and may result in great loss of product, not being applicable to the industrial environment (PANDEY *et al.*, 2000; GUPTA *et al.*, 2003; SOUZA & MAGALHÃES, 2010).

Many organisms are able to produce these enzymes, but only a few of them exhibit satisfactory characteristics for industrial application, especially the microbial α-amylases. Among the fungal species, the most representative producers of α-amylase are from mesophilic species, such as those in the *Aspergillus* genus (GUPTA *et al.*, 2003). Some yeast also exhibit amylolytic activity, especially *Saccharomyces diastaticus*, *Endomycopsis capsularis* (VERMA *et al.*, 2000), and *Cryptococcus flavus* (GALDINO *et al.*, 2008). Extremophiles belonging to the Bacteria and Archaea domains can produce α-amylases with remarkable industrial characteristics (PRAKASH & JAISWAL, 2010). The most noteworthy producing species are *Pyrococcus furiosus*, *P. woesei*, *Thermococcus profundus* and *T. hydrothermalis* (BERTOLDO & ANTRANIKIAN, 2002).

The most common commercial α-amylases are those produced by bacteria from the genus *Bacillus*, especially *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* (SATYANARAYANA *et al.*, 2006; PRAKASH & JAISWAL, 2010). As this work focus in the production of a bacterial α-amylase, greater details on the subject will be given separately in the next topic.

3.2.1.1 *Bacillus subtilis*

Bacillus subtilis is an α-amylase producer commonly found in soil. It is a gram-positive non-pathogenic eubacterium (Figure 4), which is generally regarded as safe for industrial processes. This species is also known for its usage as a host for production of heterologous proteins, being considered as the gram-positive equivalent of *Escherichia coli* in molecular biology (BOLHUIS *et al.*, 1999; RAJAGOPALAN & KRISHNAN, 2008).

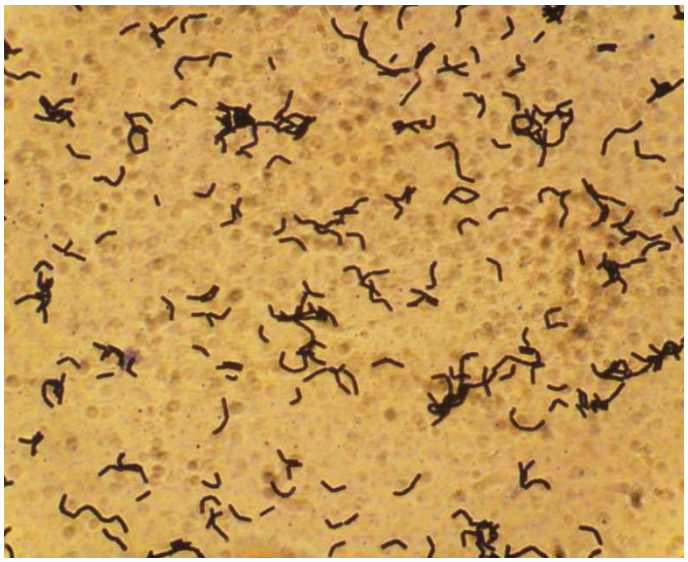


Figure 4 - *Bacillus subtilis* cells after Gram staining at 1000x magnification (Source: the author).

The production of α-amylase by *B. subtilis* is frequently done by submerged fermentation. ROY *et al.* (2012) performed broth optimization for *B. subtilis* AS-S01a cultivation and amylase production in a 5 L bioreactor. They reached a maximum productivity of 799 U (specific activity of 210.2 U/mg) at 72 h in a broth composed of beef extract and starch. The enzyme had a molecular size of 21 kDa and an optimal activity at 55 ºC and pH 9.0. ASGHER *et al.* (2007) produced the enzyme in shake flasks with *B. subtilis* strain JS-2004 and observed that the enzyme synthesis was growth associated. The amylase production peaked at 48 h with approximately 60 U/mL, when the cell population also reached its maximum (4 g/L of cell dry weight). During medium optimization, it was detected that yeast extract and calcium favor growth and expression, while

glucose reduces amylase production. The enzyme had optimal activity at 70 ºC and pH 8.0.

Not only can bacterial cells be cultivated by submerged fermentation, but also by solid state fermentation. BAYSAL *et al.* (2003) isolated a *Bacillus subtilis* strain from hot-spring water and cultivated it in solid state using wheat bran and rice husk as substrates. The first one presented better results, with higher enzyme concentration at 48 h (159,520 U per gram of substrate).

MUKHERJEE *et al.* (2009) isolated the *B. subtilis* strain DM-03 from fermented food and performed a solid state fermentation on trays with different agro-industrial wastes (potato peel, wheat bran, oil cake, rice bran, *Imperata cylindrica* grass, banana leaves and tea leaves). Potato peel and wheat bran had the best enzyme yields (532 U per gram of dry substrate) because they have higher starch content and lower free sugar concentration. Glucose and readily metabolized sugars in the medium have a negative effect on protein production due to possible catabolic repression. ASGHER *et al.* (2007) also observed glucose repression during α-amylase production. However, *B. subtilis* strain KCC103 cultivated by RAJAGOPALAN & KRISHNAN (2008) showed no repression in the presence of glucose, permitting the use of sugarcane bagasse hydrolysate as substrate to produce up to 144.5 U/mL of enzyme. NAJAFI *et al.* (2005) were also able to produce 38 U/mL of α-amylase by strain AX20 without the repression effect in the presence of high amounts of glucose and maltose. Their enzyme had a higher molecular size (139-149 kDa), presented itself as an homodimer, had an optimal activity at pH 6.0 and 55 ºC, and exhibited no inhibition by EDTA or EGTA, suggesting that it does not require metal ions for activity.

The α-amylase from *B. subtilis* can also be immobilized in a solid matrix. Immobilization of enzymes has become a subject of interest because it offers the possibility of enzyme recovering and re-utilization, enhancing stability, simplifying the separation from reaction mixture and facilitating the application to an automated continuous process. ABDEL-NABY *et al.* (1998) tried to immobilize an amylase produced by *Bacillus subtilis* using different methods, such as adsorption on aminoalkaylsilano-alumina, ionic binding onto DEAE-cellulose, covalent binding on chitin, and entrapment in polyacrylamide and calcium-alginate. Though the immobilization of the amylase reduced the enzyme activity

and shifted the optimal pH to lower values, the enzyme became more thermostable, with an increase of optimal temperature from 45 ºC to 60-65 ºC. KONSOULA & LIAKOPOULOU-KYRIAKIDES (2006a) immobilized an α-amylase by calcium alginate entrapment and were able to reuse the capsules up to 20 times. Nevertheless, the entrapped enzyme showed lower hydrolysis rates compared to free enzyme due to the interference of gel matrix in the diffusion of molecules to the active site of the enzyme.

Not only is it possible to immobilize the enzyme, it is also possible to immobilize the *Bacillus* cells. KONSOULA & LIAKOPOULOU-KYRIAKIDES (2006b) accomplished the entrapment of *B. subtilis* cells in calcium-alginate capsules. The immobilized cells were able to sustain 90% of productivity over 5 sequential batches.

Regarding the secretory pathway of amylase production, protein secretion by *B. subtilis* can happen through different ways. One of them is denominated Sec-type secretion (Figure 5), which is mediated by a signal peptide consisting of: a N-region with three positively charged residues of lysine or arginine, a hydrophobic region of approximately 19 residues and a C-region comprising a type I SPase cleavage site with a consensus A-S-A sequence (TJALSMA *et al.*, 2004).

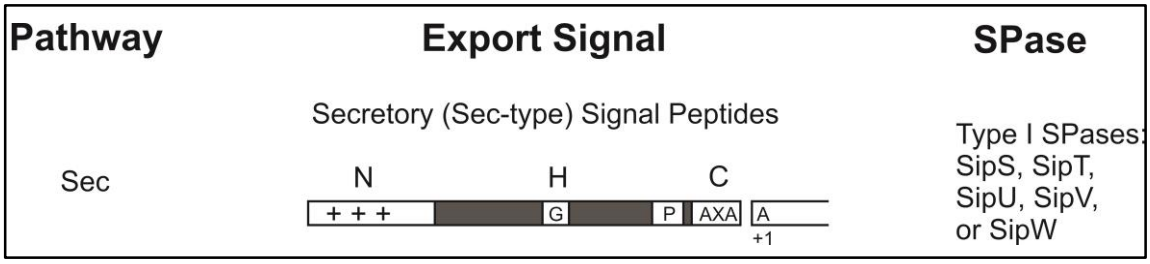


Figure 5 - Consensus sequence for signal peptides of Sec-type secretion. Adapted from TJALSMA *et al.*, 2004.

3.2.2 Heterologous expression of α-amylases

Although the yield of α-amylase produced by natural microbial strains can be increased with culture optimization, the recombinant production of the enzyme could offer some advantages such as easier genetic manipulation/mutation,

inducible expression, faster production, higher yields and easier purification steps.

The development of new genetic engineering tools permitted the introduction of novel genes into an organism and the production of heterologous proteins. These proteins can be expressed in many cell cultures of bacteria, yeasts, fungus, mammals, plants and insects or in transgenic animals and plants. The quality, functionality, rate of production and yield of the final protein depends on the characteristics of each of these hosts (DEMAIN & VAISHNAV, 2009).

One of the most widely used hosts for heterologous expression is the *Escherichia coli*, especially for those proteins which do not need glycosylation. They have as an advantage the rapid growth and expression, and the simplicity in genetic manipulation with many molecular tools available. However, bacterial cells are not able to perform post-translation modifications, such as disulfide bonds and glycosylation (DEMAIN & VAISHNAV, 2009).

Generally the overexpression of recombinant proteins in *E. coli* produces intracellular insoluble aggregates which are denominated inclusion bodies. The resolubilization of such aggregates requires the use of denaturing agents followed by a renaturation of the protein molecular structure. This process may have great loss of activity due to the difficulty of the protein to regain its original conformation (LINDEN *et al.*, 2000). Reports of successful solubilization without using denaturing conditions are scarce, but some of them include heating of insoluble aggregates or glycerol extraction. RASHID *et al.* (2010) were able to resolubilize a recombinant α-amylase produced as aggregates in *E. coli* with a glycerol extraction combined with incubation at 40 ºC.

Other useful and well document bacterial systems are from the *Bacillus* genus, notably the *B. subtilis* and *B. licheniformis* strains. They easily secrete proteins (with no production of intracellular inclusion bodies), are genetically well characterized and are generally recognized as safe (GRAS) by the US FDA (DEMAIN & VAISHNAV, 2009).

The α-amylase was one of the first proteins adopted for molecular biology studies because of the easiness in screening assays, the availability of amylase negative strains and the great knowledge in *B. subtilis* genetics and fermentation technology (SATYANARAYANA *et al.*, 2006). Table 1 shows some examples of α-amylase production in recombinant bacterial hosts.

Table 1 - Examples of recombinant α-amylases produced in bacterial hosts.

Reference	Gene from	Cloned in	Production
AIBA *et al.*, 1983	*B. stearothermophilus*	*B. stearothermophilus*	3.9 U/mL
	B. stearothermophilus	*Bacillus subtilis*	1.6 U/mL
* TAKKINEN *et al.*, 1983	*B. amyloliquecafiens*	*Bacillus subtilis*	-
* YANG *et al.*, 1983	*Bacillus subtilis*	*Escherichia coli*	-
* GRAY *et al.*, 1986	*B. stearothermophilus*	*Escherichia coli*	-
	B. stearothermophilus	*Bacillus subtilis*	-
* GOBIUS & PEMBERTON, 1988	*Aeromonas hydrophila*	*Escherichia coli*	-
* TSUKAMOTO *et al.*, 1988	*Bacillus sp.*	*Escherichia coli*	-
	Bacillus sp.	*Bacillus subtilis*	-
DONG *et al.*, 1997	*Pyrococcus furiosus*	*Escherichia coli*	109 U/mL
JORGENSEN *et al.*, 1997	*Pyrococcus furiosus*	*Escherichia coli*	-
	Pyrococcus furiosus	*Bacillus subtilis*	-
SIDHU *et al.*, 1997	*Bacillus sp.*	*Escherichia coli*	3100 U/mL
	Bacillus sp.	*Bacillus subtilis*	13900 U/mL
KIM *et al.*, 1997	*Streptomyces albus*	*Bacillus subtilis*	136 U/mL
LINDEN *et al.*, 2000	*Pyrococcus woesei*	*Escherichia coli*	13.851 U/mL
ALI *et al.*, 2006	*B. stearothermophilus*	*Escherichia coli*	-
NIU *et al.*, 2009	*B. licheniformis*	*Bacillus licheniformis*	17600 mg/L

* Cited by SATYANARAYANA *et al.*, 2006

3.2.2.1 Yeast as an heterologous host

Whenever a recombinant protein is not produced correctly in a bacterial host because of folding problems or other post-translational processing, yeasts can be used for hosting the heterologous gene. Some yeast strains are genetically well characterized and can easily adapt to the industrial fermentation process. Yeast hosts have as advantages the rapid growth with high cell density, the ability to grow on common substrates and the capacity to perform post-translational modification, such as assisting in folding and performing glycosylation. They are also safe to work because they do not harbor pathogens, viral inclusions or pyrogens (BÖER *et al.*, 2007; DEMAIN & VAISHNAV, 2009).

The glycosylation pattern differs among each cell strain and at each culture condition. Protein glycosylation might enhance its thermostability and protect the enzyme from proteases. Nevertheless, yeasts usually hyperglycosylate N-linked sites, causing the reduction of protein activity, solubility, stability and alter

immunogenicity (DEMAIN & VAISHNAV, 2009). TULL *et al.* (2001) observed that N-glycosylation reduced the thermostability of a recombinant α-amylase from *Bacillus* produced by *Pichia pastoris*. The recombinant enzyme lost 50% of activity at the temperature of 76 ºC, while the natural one produced by the *Bacillus* lost 50% of activity only at 89 ºC. The removal of glycosylation by endoglycosydase H treatment did not enhance the thermostability of the recombinant protein.

The heterologous production in yeasts could be enhanced by optimizing some parameters, such as: culture physical conditions (pH, temperature), culture chemical composition (richer broths usually present higher productivity), the type of gene promoter and signal sequence used (Table 2), the codon bias, and the superexpression of chaperones (WONG *et al.*, 2002). Furthermore, systems biology and proteomics analysis during the recombinant protein expression could help to identify the different types of stresses caused to the cell by the exogenous gene. There is still a reduced amount of information regarding the proteomics of yeast cells during heterologous gene expression. Besides that, lab scale analysis may not correspond to the conditions faced by the microorganism in an industrial plant, where external conditions (temperature, osmolarity and nutrient limitation) can also be stressful (GRAF *et al.*, 2009).

Table 2 - Main promoters used for recombinant gene expression in yeasts. Adapted from PORRO *et al.*, 2005

	Constitutive	**Promoter induced by**					
Species	**Promoter**	**Galactose**	**Lactose**	**Ethanol**	**Starch**	**Xylose**	**Methanol**
S. cerevisiae	GAPDH, PGK, TPI, ENO, α-MP	GAL1-10, GAL7		ADH2			
K. lactis	PGK		LAC4	ADH4			
S. occidentalis	GAM1				AMY1, GAM1		
Y. lipolytica	TEF, RPS7						
Z. rouxii	GAPDH						
Z. bailii	TPI						
P. stipitis						XYL1	
P. pastoris	GAP						AOX1, FLD1
H. polymorpha							MOX
C. boidinii							AOD1
P. methanolica							AUG1

The classical yeast in molecular biology is *Saccharomyces cerevisiae*. The recombinant protein secreted by this microorganism frequently presents a high fidelity in translation, an adequate N-terminal processing and a correct disulfide bond formation. Only 0.5-1% of intracellular proteins leak out of the cell, which simplifies the purification of extracellular proteins. Furthermore, protein secretion to the extracellular medium avoids prolonged exposure to proteases, which may reduce their half-life and stability (WONG *et al.*, 2002).

There are also other yeast strains used in protein production as alternative hosting systems. One of them is *Pichia pastoris*, a methylotrophic yeast which can grow in medium containing methanol as the only carbon source. In a bioreactor it is possible to achieve high cell concentrations (>100 g dry biomass/L; >400 g wet biomass/L; >500 OD_{600}/mL) and high concentrations of secreted proteins (CEREGHINO *et al.*, 2002).

The majority of research projects about the production of recombinant α-amylases in yeasts deal with the production in *Saccharomyces cerevisiae* and *Pichia pastoris*. Some of them are only aiming to produce the heterologous enzyme to perform structural and glycosylation studies, such as JUGE *et al.* (1996), MONTESINO *et al.* (1998), and RYDBERG *et al.* (1999). The present work focuses on the production of a recombinant α-amylase for industrial purposes. Therefore, in this bibliographic review it will be emphasized those articles that give more attention to the industrial application of recombinant yeast expression.

Many works dealing with the production in *Saccharomyces cerevisiae* usually aim the expression of the enzyme and the production of ethanol at the same time. BIROL *et al.* (1998) performed experiments using three different recombinant *S. cerevisiae*: one harboring a fusion gene of *B. subtilis* α-amylase and *A. awamori* glucoamylase; one producing the *B. subtilis* α-amylase and *A. awamori* glucoamylase as separate enzymes; and one producing the *A. awamori* glucoamylase and a mouse α-amylase as separate enzymes. The construct with the mouse gene showed low yields in protein production. The others were able to degrade starch and also produce ethanol. The enzymes expressed separately had higher ethanol productivity than the fused ones, obtaining 43.8 g/L of ethanol. ALTINTAŞ *et al.* (2002) used the same yeast strain harboring the fused gene as the previous work and performed fed-batch experiments by adding starch in

pulses when it dropped to low values. They detected a good plasmid stability (74% of cells retained their plasmids), and high ethanol yield and productivity (0.233 g ethanol/L/h).

SHIBUYA *et al.* (1992) also made a fusion gene of α-amylase and glucoamylase for *S. cerevisiae* transformation, but this time the genes came from *Aspergillus shirousamii*. The fusion protein had almost the same specific activity for soluble starch as those of individual enzymes, with both active sites fully functional without interfering with each other. However, the fused protein was only secreted in small amounts.

The use of SUC2 promoter (which is repressed at high glucose concentration and derepressed at low glucose concentration) in *S. cerevisiae* was attempted by ZHANG *et al.* (2001). The fermentation was performed in two stages: the first stage aimed to achieve high cell density in the presence of both glucose and lactic acid; and the second stage started when most of the glucose was consumed and only yeast extract and lactic acid was fed to the cells (initiating the inducing phase). The feed of a carbon source other than glucose during induction phase provided the energy needed for synthesizing the protein and achieving higher protein concentrations.

GALDINO *et al.* (2008) expressed a *Cryptococcus flavus* in *S. cerevisiae* under the control of the constitutive PGK1 promoter. A maximum of 3.93 U/mL was achieved at 60 h fermentation and the protein production did not impair cell growth.

Barley's α-amylases produced by yeasts have also been much studied because the optimal pH of barley's enzyme is low, which is compatible with the conditions found during alcoholic fermentation (pH 4.0-5.0) (LIAO *et al.*, 2010). WONG *et al.* (2002) produced a barley α-amylase in *S. cerevisiae* under the control of a PGK1 promoter and observed that when glycerol was used as carbon source the enzyme synthesis and secretion was enhanced while the cell growth was suppressed, with a pronounced enhancement in enzymatic activity after 48 h culture.

LIAO *et al.* (2010) produced barley isoenzyme 1 in *S. cerevisiae* either anchored on the cell surface or secreted to the medium. The researchers attempted to anchor the enzyme to improve its stability. However, the secreted form of the enzyme had higher starch consumption rate than the anchored one

and also higher ethanol yield, with 16.7 g/L of ethanol when cultivated for 70 h in 50 g/L starch broth.

In *Pichia pastoris*, the production of barley isoenzymes was attempted by JUGE *et al.* (1996) under the control of a AOX1 promoter. The enzymes could be efficiently secreted to broth by their own signal sequence and they did not present N-glycosylation. However, they appeared in different molecular sizes due to C-terminal sequence processing.

CHOI & PARK (2006) attempted to produce a mouse α-amylase in *P. pastoris*. They optimized some environmental factors, such as temperature, pH and carbon and nitrogen sources and observed that in batch fermentations the combination of methanol and glycerol as carbon sources enhance 1.6-fold the enzyme and biomass yields. In fed-batch experiments, the feeding of a nitrogen mixture (peptone and yeast extract) and a carbon mixture (methanol and glycerol) enabled high biomass concentration (OD_{600}=179) and enzymatic activity (2.4 kU/mL).

KARAKAŞ *et al.* (2010) produced a *B. subtilis* amylase in *P. pastoris* with methanol induction. In shaken flasks fermentations, the maximum activity was achieved at 72 h (44.34 U/mL) and the recombinant enzyme had an optimum activity at 60 ºC and pH 7.0. The presence of calcium ions increased the enzymatic activity in 41%.

LI *et al.* (2011b) produced in *Pichia* a recombinant α-amylase from *Rhizopus oryzae*. Both a constitutive promoter (GAP) and an inducible promoter (AOX1) were tested. The enzyme production with the former was only one fifteenth of that obtained with AOX1 promoter. They also tested the signal peptide for enzyme secretion, observing that the *Rhizopus* native sequence could efficiently direct the secretion of recombinant enzyme to the broth. At last, the best construct obtained (the one induced by methanol and with native signal) was tested in a 7L fed-batch fermentation, achieving 400 mg/L of secreted protein.

Table 3 gives more examples of recombinant α-amylase produced by yeasts, showing the enzymatic production when this data was available.

Table 3 - Examples of recombinant α-amylases produced in yeast.

Reference	Gene from	Cloned in	Production
STRASSER *et al.*, 1989	*Schwanniomyces occidentalis*	*Saccharomyces cerevisiae*	0.02 U/mL
	S. occidentalis	*Kluyveromyces lactis*	0.03 U/mL
	S. occidentalis	*Saccharomyces pombe*	0.01 U/mL
SHIBUYA *et al.*, 1992	*Aspergillus shirousamii*	*Saccharomyces cerevisiae*	-
PAIFER *et al.*, 1994	*Bacteria*	*S. cerevisiae*	0.9 g/L
	Bacteria	*Pichia pastoris*	2.5 g/L
JUGE *et al.*, 1996	*Barley isoenzyme 1*	*Pichia pastoris*	-
	Barley isoenzyme 2	*Pichia pastoris*	-
TOKUNAGA *et al.*, 1997	*Mouse*	*Kluyveromyces lactis*	0.527 U/mL
BIROL *et al.*, 1998	*Bacillus subtilis*	*S. cerevisiae*	4000 U/mL
	Mouse	*S. cerevisiae*	1500 U/mL
RYDBERG *et al.*, 1999	*Human*	*Pichia pastoris*	-
TULL *et al.*, 2001	*Bacillus sp.*	*Pichia pastoris*	311.5 U/mL
KATO *et al.*, 2001	*Mouse*	*Pichia pastoris*	240 mg/L
ALTINTAŞ *et al.*, 2002	*Bacillus subtilis*	*S. cerevisiae*	-
CHOI & PARK, 2006	*Mouse*	*Pichia pastoris*	2400U/mL
NAKANO *et al.*, 2006	*Rice isoenzymes*	*Pichia pastoris*	173 mg/L
ARRUDA, 2008	*Bacillus subtilis*	*Pichia pastoris*	250 U/mL
GALDINO *et al.*, 2008	*Cryptococcus flavus*	*S. cerevisiae*	3.93 U/mL
KARAKAŞ *et al.*, 2010	*Bacillus subtilis*	*Pichia pastoris*	44.34 U/mL
LIAO *et al.*, 2010	*Barley isoenzyme 1*	*S. cerevisiae*	-
MONTAÑO, 2010	*Bacillus subtilis*	*Pichia pastoris*	218 U/mL
LI *et al.*, 2011a	*Rhizopus oryzae*	*Kluyveromyces lactis*	22.4 U/mL
LI *et al.*, 2011b	*Rhizopus oryzae*	*Pichia pastoris*	450 U/mL

3.2.2.2 *Kluyveromyces lactis*

Kluyveromyces lactis (Figure 6) is a non-conventional yeast used as host for heterologous protein production. It has also been used for a long time in the food industry for the production of β-galactosidase (lactase), being considered a GRAS (generally recognized as safe) microorganism for this application. Thus, its culture in large scale is well known and studied. Their ability to grow in cheap substrates such as lactose and cheese whey makes them interesting hosts for industrial production (VAN OOYEN *et al.*, 2006).

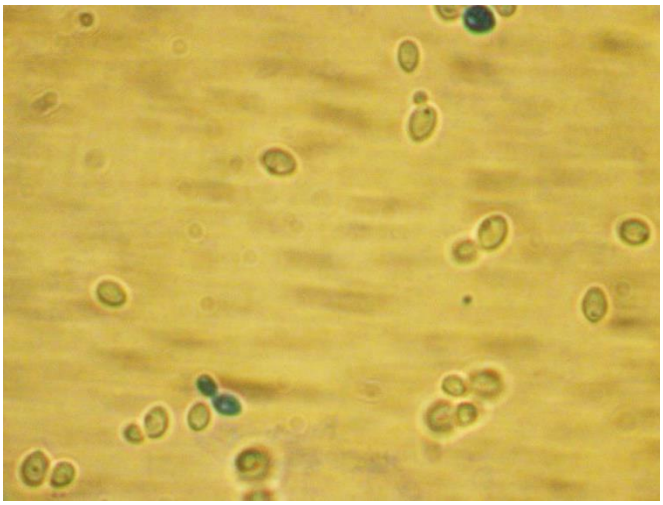


Figure 6 - *Kluyveromyces lactis* cells after methylene blue staining (dead cells appear as dark blue) at 1000x magnification (Source: the author).

An interesting characteristic of this yeast for the heterologous production is the absence of a Crabtree effect. Crabtree-positive yeasts such as *Saccharomyces cerevisiae* produce ethanol under oxygen limitation, reducing yield in ATP and biomass production. Therefore, with Crabtree-negative strains such as *K. lactis* it is possible to achieve high biomass concentration without metabolic deviations to other secondary products other than the desired protein (SCHAFFRATH & BREUNIG, 2000).

A great variety of *K. lactis* strains have been deposited in culture collections. One of the most used in research is CBS 2359, which is well characterized and whose genome has already been sequenced (VAN OOYEN *et al.*, 2006).

Both episomal and integrative vectors are available for *K. lactis* cell transformation. Episomal vectors (e. g., pKD1) may be present in many copies inside the yeast cell. However, they might be very unstable without the presence of a selecting agent. On the other hand, integrative vectors (e. g., pKLAC) are genetically more stable, but may be present in a low number of copies (VAN OOYEN *et al.*, 2006).

A few promoters are used in *K. lactis* expression vectors. The most characterized is the LAC4 gene promoter, which is induced 100-fold in the presence of lactose or galactose and is usually not repressed by glucose. Other

promoters from *S. cerevisiae* are recognized by *K. lactis* and can also be used for the expression of foreign genes, such as PGK1 and PHO5 promoters (VAN OOYEN *et al.*, 2006).

There are few reports regarding the production of recombinant α-amylase in *Kluyveromyces lactis*. One of the first attempts was performed by STRASSER *et al.* (1989), when they compared the productivity of a *Schwanniomyces occidentalis* α-amylase produced by *Saccharomyces cerevisiae*, *S. pombe* and *K. lactis*. The *K. lactis* cells had the highest extracellular production, achieving an enzymatic activity of 30.5 U/L.

TOKUNAGA *et al.* (1997) transformed *K. lactis* cells to produce α-amylase from mice. They tested gene constructs containing different promoters (PHO5 and PGK1) and observed that both had similar efficiency in inducing protein production. The yeast secreted both the glycosylated and the non-glycosylated forms of the protein. By non-denaturing PAGE electrophoresis and iodine staining they were able prove that both forms of the protein are enzymatically active. The recombinant cells efficiently secreted the enzyme when cultivated in glucose, lactose or galactose as a carbon source, with the highest productivity of 0.527 U/mL. The production of the amylolytic enzyme also enabled the yeast to grow on starch as the only carbon source.

The α-amylase gene has also been used as a reporter gene in *K. lactis* basic research. BARTKEVICIUTE & SASNAUSKAS (2003) wanted to screen for *K. lactis* mutants with super-secretion phenotype after UV mutagenesis. They used a *Bacillus amyloliquefaciens* α-amylase as a reporter gene for protein secretion because they could be easily detected by iodine staining.

LI *et al.* (2011a) attempted to produce an α-amylase from *Rhizopus oryzae* in *Kluyveromyces lactis* using the LAC4 gene promoter to induce protein expression. A number of carbon sources were tested for the expression of the enzyme, and the culture in galactose medium had a slightly higher productivity, achieving 22.4 U/mL. However, in lactose broth the secretion of the enzyme was not induced as expected, having similar yield to those cultures in glucose, glycerol, starch and maltose medium. The strain was also able to grow on starch as the only carbon source, with a yield of 12.25 g/L in biomass and 11 U/mL in secreted enzyme (present both in a glycosylated and non-glycosylated form).

3.3 APPLICATIONS OF α-AMYLASES

Amylolytic enzymes have a broad application in industrial processes, including in the textile, paper, food, detergent and pharmaceutical fields.

Currently, almost 90% of liquid detergents contain α-amylase. The use of enzymes during the washing procedure enhances the ability of the detergent to remove stains, making the product environmentally friendly and avoiding the use of other aggressive chemical compounds which might damage the laundry and dishware (GUPTA *et al.*, 2003; SOUZA & MAGALHÃES, 2010). The α-amylases used for this application need to have a good activity and stability at the physicochemical conditions achieved during the washing procedure, such as alkaline pH, the presence of metal ion biding agents (polyphosphates, nitrilotriacetics acid and zeolites), anionic surfactants, and bleaching agents. As an example, MUKHERJEE *et al.* (2009) produced an α-amylase from *B. subtilis* and tested its compatibility with compounds present in detergents formulation. After simulating a washing process at 37 and 45 ºC, they observed that the enzyme was able to maintain a good activity. ROY *et al.*, (2012) also tested their α-amylase for this application, and discovered that it was stable in the presence of laundry detergents components and improved stain removal.

At the food processing industry this enzyme is used for the production of bread, cakes and beverages. In bakery, α-amylase is used to break starch into smaller dextrins, accelerating the fermentation performed by the yeast and reducing the viscosity of the dough. The application of the enzyme enhances the texture and volume of the final bread and helps in developing the flavor and color. In beverages production, amylolytic enzymes are mainly used for beer and juice clarification (GUPTA *et al.*, 2003; SOUZA & MAGALHÃES, 2010).

Another application is the treatment of animal feed with α-amylase to enhance digestibility and nutrient absorption (SOUZA & MAGALHÃES, 2010). Exogenous enzymes complement the enzymes insufficiently produced by the animal, improving their digestive capacity and stabilizing the gut flora. As an example, the product AVIZYME 1500 produced by Danisco comprises a mixture of xylanase, amylase and protease. The amylase acts in the initial part of the animal's gastrointestinal tract, enhancing the incomplete digestion of starch present in the endosperm of cereals used as feed (POLITZER & BON, 2006).

Starch is used during weaving in the textile manufacturing process to increase fiber's resistance. Afterwards it is removed from the fabric with the application of amylolytic enzymes. They degrade starch into smaller dextrins more soluble in water that can be washed away (GUPTA *et al.*, 2003; SOUZA & MAGALHÃES, 2010).

Although in Brazil the main raw material used for ethanol production is sugarcane, in other countries starch is predominantly used for this purpose. In the United States most of their corn crops are destined to the production of alcohol for fuel (Figure 7). The starch from maize must be treated with α-amylases and glucoamylases to produce a mixture of fermentable sugars which can be consumed by yeasts (*Saccharomyces cerevisiae*) to produce ethanol. On average, 9.5 L of ethanol are produced from 14.5 kg of starch (BEMILLER & WHISTLER, 2009).

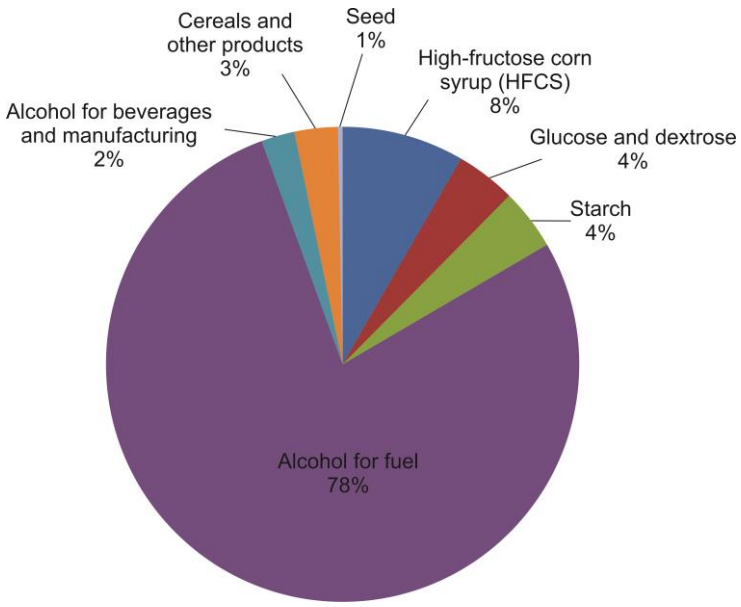


Figure 7 - Industrial uses of corn produced in the United States between September/2010 and August/2011 according to United States Department of Agriculture (USDA).

The hydrolysis of starch initially needs a cooking step to gelatinize the starch granules. This procedure is called gelatinization and is achieved by heating up the granules in an aqueous solution (pH 5.8-6.5) to 105-110 ºC for 5 minutes. If the gelatinization temperature is kept under 105 ºC there will be only a partial solubilization of the material, which may cause pumping and filtration problems

in the subsequent steps. Subsequently the solution is cooled down to 95 ºC to perform the starch liquefaction: a partial hydrolysis with α-amylases for 2 to 3 hours. During this step occurs the breakdown of starch polysaccharidic chains in dextrins, which causes the reduction of solution viscosity. Afterwards the solution is cooled to 55-60 ºC and the pH is adjusted to 4.2-4.5 to perform the saccharification of the material. This step comprises the final enzymatic hydrolysis with glucoamylase for 24 to 72 h to produce fermentable sugars such as glucose and maltose (VIEILLE & ZEIKUS, 2001; PRAKASH & JAISWAL, 2010).

As it can observed, this process involves many heating, cooling and pH adjusting steps, creating massive costs with energy and reagents (VIEILLE & ZEIKUS, 2001). The use of enzymes with optimal pH and temperatures near each other would assist the decrease in costs. Moreover, the use of raw starch degrading enzymes could cut out the gelatinization cooking step, having a simplification of the process and reducing the energy consumption (SUN *et al.*, 2010).

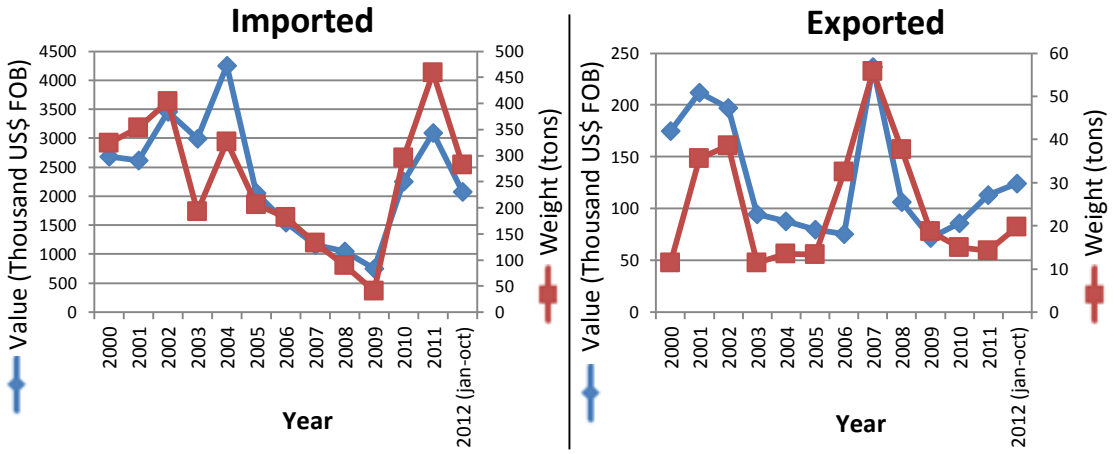
3.4 AMYLOLYTIC ENZYMES MARKET

The need for new sustainable and environmentally friendly processes to substitute chemical processes based in non-renewable sources has increased the requirement for new biotechnology tools. Processes involving the use of enzymatic technology are included in these cleaner biotechnologies, having a huge demand in the worldwide market. The global industrial enzyme market is expected to reach $4.4 billion by 2015 (SARROUH *et al.*, 2012). Amylolytic enzymes corresponded in 2005 to 13% of all the enzymes exported in Brazil, and to 14% of all imported enzymes. Some of the main producers of industrial amylolytic enzymes in Brazil are Novozymes Latin America Ltda., Danisco (now acquired by DuPont), and DSM (POLITZER & BON, 2006).

According to the Brazilian Ministry of Development, Industry and International Trade (Ministério do Desenvolvimento, Indústria e Comércio Exterior - http://aliceweb2.mdic.gov.br/), the amylase's data are divided in two groups of NCM (nomenclatura comum do Mercosul): NCM 3507.90.11 for α-amylase (*Aspergillus oryzae*) and NCM 3507.90.19 for other amylases and their

concentrate. In 2011 Brazil imported 459 tons of α-amylase (*Aspergillus oryzae*) and exported 14 tons. During the same period, the importations accounted for US$ 3 million in expenses, while the income with exportations was only of US$ 113 thousand. For other amylases and their concentrate, data show that 848 tons were imported in 2011 and 60 tons were exported. This indicates a technological disadvantage in Brazil in terms of production and use of these catalysts. The mean price of the imported α-amylase (*A. oryzae*) was US$ 6.72/kg and for other amylases and their concentrate US$ 7.98/kg. Analyzing throughout a period of 12 years, it can be observed that importation prevailed most of the time for both α-amylase (*A. oryzae*) and other amylases (Figure 8). Because of the present need and tendency of the market it is expected a significant increase in enzyme consumption in the next years.

(a) Alpha-amylase (*Aspergillus oryzae*) (NCM 3507.90.11)



(b) Other amylases and their concentrate (NCM 3507.90.19)

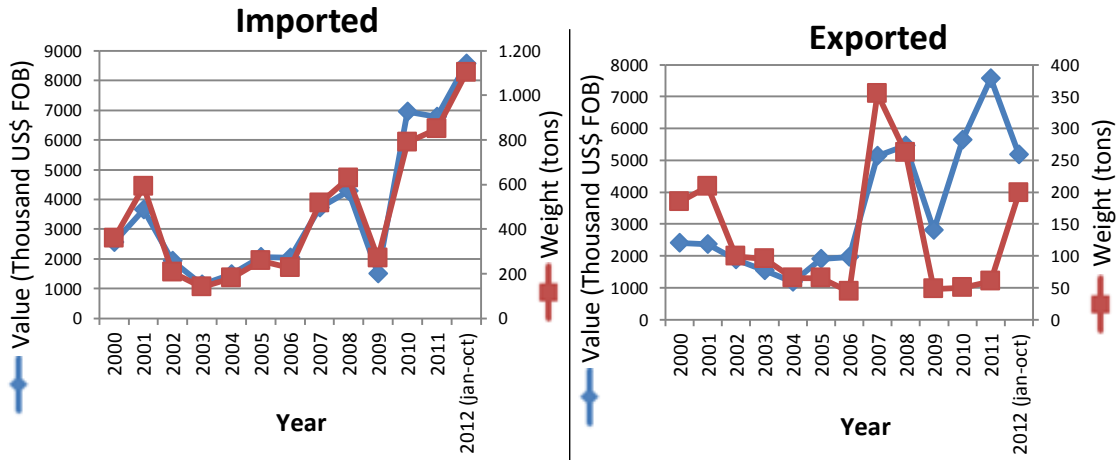


Figure 8 - Annual value and weight of imported and exported amylases in Brazil according to Ministério do Desenvolvimento, Indústria e Comércio Exterior.

In starch's hydrolysis process, the costs with enzymes are US$ 17.00 per ton of starch, and the total cost for the production of hydrolyzed syrup is from US$165.00 to US$250.00. Therefore, the enzymes account for 11 to 7% in the total cost for starch hydrolysis (POLITZER & BON, 2006). For that reason, it is crucial to develop more economic processes for producing the enzyme and reducing the final product price, which will eventually strengthen even more the use of enzymes in the industry.

4 MATERIALS AND METHODS

4.1 MICROORGANISM STRAINS

The *Bacillus subtilis* subspecies *subtilis* NRRL B-4212 strain supplied by ARS Culture Collection was used as a donor of the α-amylase gene. The *Escherichia coli* XLI-Blue strain was used during the vector construction and cloning steps in bacteria. The *Kluyveromyces lactis* CBS 2359 strain was used for yeast transformation and protein expression.

4.2 CULTURE MEDIA

- Luria-Bertani (LB) was used for strain propagation and preservation of *Escherichia coli* cells during molecular biology procedures (ZIMBRO *et al.*, 2009). It consisted in 1 g/L of glucose, 10 g/L of tryptone, 5 g/L of yeast extract, 5 g/L of NaCl, and, in the case of solid medium, it was added 18 g/L of agar. Whenever the cells where transformed with the plasmids, ampicillin was added to the cooled medium to a final concentration of 100 mg/L for cell selection.
- *Tryptone, Glucose, Yeast Extract* (TGY) was used for *Bacillus subtilis* cultivation. It consists of 5 g/L tryptone, 2.5 g/L yeast extract, 1 g/L glucose and 15 g/L agar.
- *B. subtilis production broth* (BAP) was used for the production of α-amylase by *B. subtilis*. It consists of 5 g/L yeast extract, 10 g/L soluble starch, 20 g/L tryptone, 25 g/L NaCl, 0.02 g/L $MgSO_4.7H_2O$, 0.026 g/L $CaCl_2.2H_2O$, pH 7.0.
- *Yeast carbon base* (YCB) with acetamide 5 mM (New England Biolabs® #B9017S) was used to select recombinant yeast cells right after transformation, because only those cells with proper cassette integration are able to metabolize acetamide as the only nitrogen source.
- *Yeast extract, peptone, dextrose* (YPD) was used for *K. lactis* cultivation (ZIMBRO *et al.*, 2009). It consists of 20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose.

- *Yeast extract, peptone, lactose, starch* (YPLS) was used for the screening of the strains producing the recombinant enzyme in Petri dishes. It has the same composition of YPD, except for the replacement of glucose by lactose (20 g/L) and the addition of starch (10 g/L) and agarose (15 g/L).
- *Yeast extract, peptone, lactose* (YPL) was used for the production of the recombinant enzyme by *K. lactis* containing the cassette induced by lactose. It has the same composition of YPD, except that glucose was replaced by lactose.
- *Yeast extract, peptone, carbon source* (YPX) was used for the production of the recombinant enzyme by *K. lactis* in different carbon sources. It consists of 20 g/L peptone, 10 g/L yeast extract and 20 g/L of the carbon source (X = glucose, sucrose, starch, lactose or galactose).

4.3 CASSETTE CONSTRUCTION

4.3.1 Basic molecular biology procedures

Bacterial competent cells of *E. coli* XLI-Blue were prepared and transformed according to SAMBROOK & RUSSELL (2001). Plasmid extraction (miniprep) was performed using the commercial kit Invisorb® Spin Plasmid Mini Two from Invitek. DNA digestion with restriction enzymes and DNA ligation procedures were done according to the manufacturer recommendations (Invitrogen, New England Biolabs). Horizontal DNA electrophoresis in agarose gel was performed according to SAMBROOK & RUSSELL (2001). Yeast genomic DNA was extracted according to AUSUBEL *et al.* (2003).

4.3.2 Primers design, PCR amplification and vector construction

Primers were designed based on the *amyE* gene from *Bacillus subtilis* OI1085 (GenBank: FJ643607.1). Observing the N-terminal portion of the protein coded by this sequence, it can be noted that it probably contains a signal peptide of the Sec-type (Figure 9).

```
   ++ +         G            P   ASA
MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASAETANKSNELTAPSIKSGTILHAWN
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Figure 9 - N-terminal of the protein coded by the *amyE* gene, highlighting in gray the probable signal peptide.

Therefore, five primers were designed for gene amplification (Figure 10): *EXSIG For* and *INTSIG For*, which are the forward primers that anneal at the beginning of the signal sequence; *EXAMY For* and *INTAMY For*, which anneal right after the signal sequence; and *AMY Rev*, which is the reverse primer used in combination with all the forward primers. This procedure was carried out to compare if the presence of this bacterial signal sequence would interfere in protein production at the yeast host.

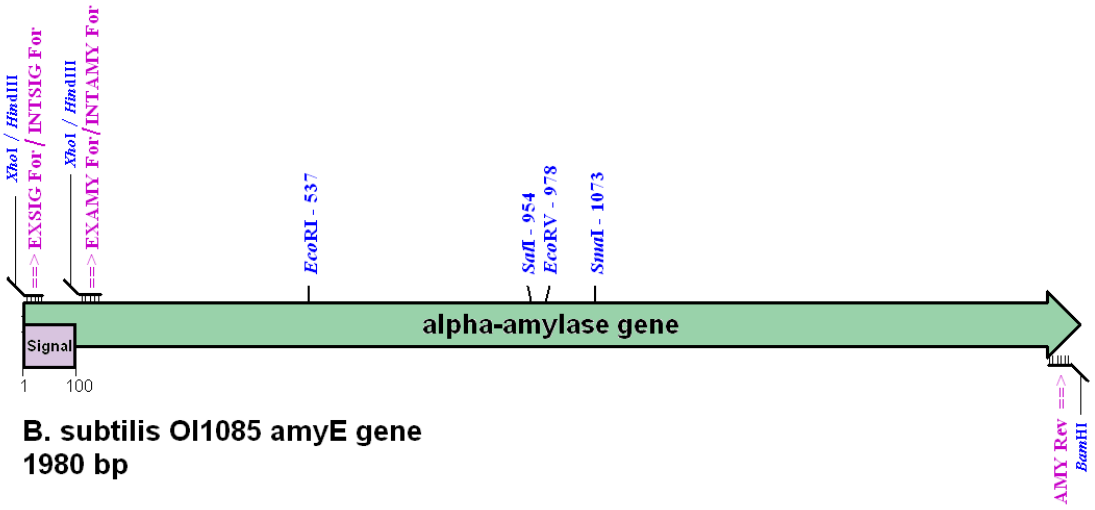


Figure 10 - Sequence scheme of the *amyE* gene (green arrow), highlighting the signal sequence (gray box), the restriction sites (in blue) and the designed primers (in pink).

The amplified gene must be inserted in the pKLAC2 vector (

Figure 11). Therefore, it was necessary to add to the amplification product restriction sites compatible with those present at the vector. This plasmid permits the expression of proteins in *K. lactis* extracellularly or intracellularly. The protein secretion occurs when the desired gene is placed in frame with the *α-mating factor* (α-MF) present in the plasmid, which directs the protein to the secretory

pathway and is removed by the presence of a Kex protease cleaving site. Thus, primers *EXSIG For* and *EXAMY For* contain a *XhoI* site (located right after the α-MF in pKLAC2) and a coding sequence recognizable by Kex protease (Table 4). For the intracellular production in yeast, primers *INTSIG For* e *INTAMY For* were used. They contain a *HindIII* site, which is located in pKLAC2 right after the promoter and before the α-MF. The reverse primer (AMY Rev) anneals at the end of the gene and contains a *BamHI* site, which is also present in the pKLAC2 polylinker.

PLAC4-PBI3'	3' end of LAC4 gene promoter
a-MF	Leader sequence of *alpha-mating factor* from *K. lactis*
TTLAC4	Transcription terminator of LAC4 gene
PADH1	Alcohol dehydrogenase promoter from *S. cerevisiae*
amdS	Acetamidase gene for acetamide transformant selection
PLAC4-PBI5'	5'end of LAC4 gene promoter
ori	Replication origin of *pMB1*
Ap R	*bla* gene conferring ampicillin resistance in *E. coli*

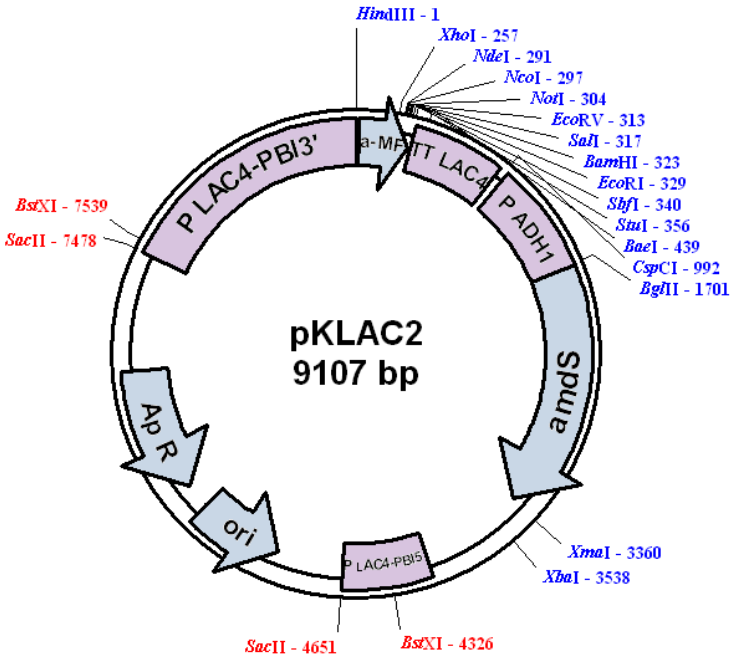


Figure 11 - Plasmidial vector pKLAC2 used for *K. lactis* cassette construction.

Table 4 - Primer sequences indicating the position of the restriction sites and the codons coding for Kex protease site.

Primer	Sequence
EXSIG For	5'-AGACTCGAGAAAAGAATGTTTGCAAAACGATTCAAA *XhoI* Kex↑
INTSIG For	5'-ACGGCAAAGCTTATGTTTGCAAAACGATTCAAA *HindIII*
EXAMY For	5'-GCAGACTCGAGAAAAGAGAAACGGCGAACAAATCGAAT *XhoI* Kex↑
INTAMY For	5'-CAGGCAAAGCTTATGGAAACGGCGAACAAATCGAAT *HindIII*
AMY Rev	5'-ACTGAGGATCCTCAATGGGGAAGAGAACCGCT *BamHI*

Bacillus subtilis genomic DNA was extracted with the method described by SHARMA & SINGH (2005) and the sample was used as template for the PCR reaction. The amplification reaction consisted of 100 ng of *Bacillus* genomic DNA, 3 mM of $MgCl_2$, 150 µM of each dNTP, 0.5 µM of forward primer, 0.5 µM of reverse primer, Taq DNA polymerase buffer and 0.05 U/µL of Taq DNA polymerase (Invitrogen), completing the final volume to 100 µL with ultrapure water. The cycling parameters were: 6 min at 95 ºC (hot start); followed by 35 cycles of denaturation (30 s at 95 ºC), annealing (45 s at 65 ºC), and extension (60 s at 72 ºC); and a final extension for 10 min at 72ºC.

The four amplification products obtained with the primers were digested with the appropriate enzymes and inserted in the pKLAC2 vector (

Figure 11), generating four constructs: (i) pKLAC-EXSIG; (ii) pKLAC-EXAMY; (iii) pKLAC-INTSIG; and (iv) pKLAC-INTAMY. Prior to yeast transformation, these vectors were linearized with *SacII*, creating the final cassettes presented in Figure 12.

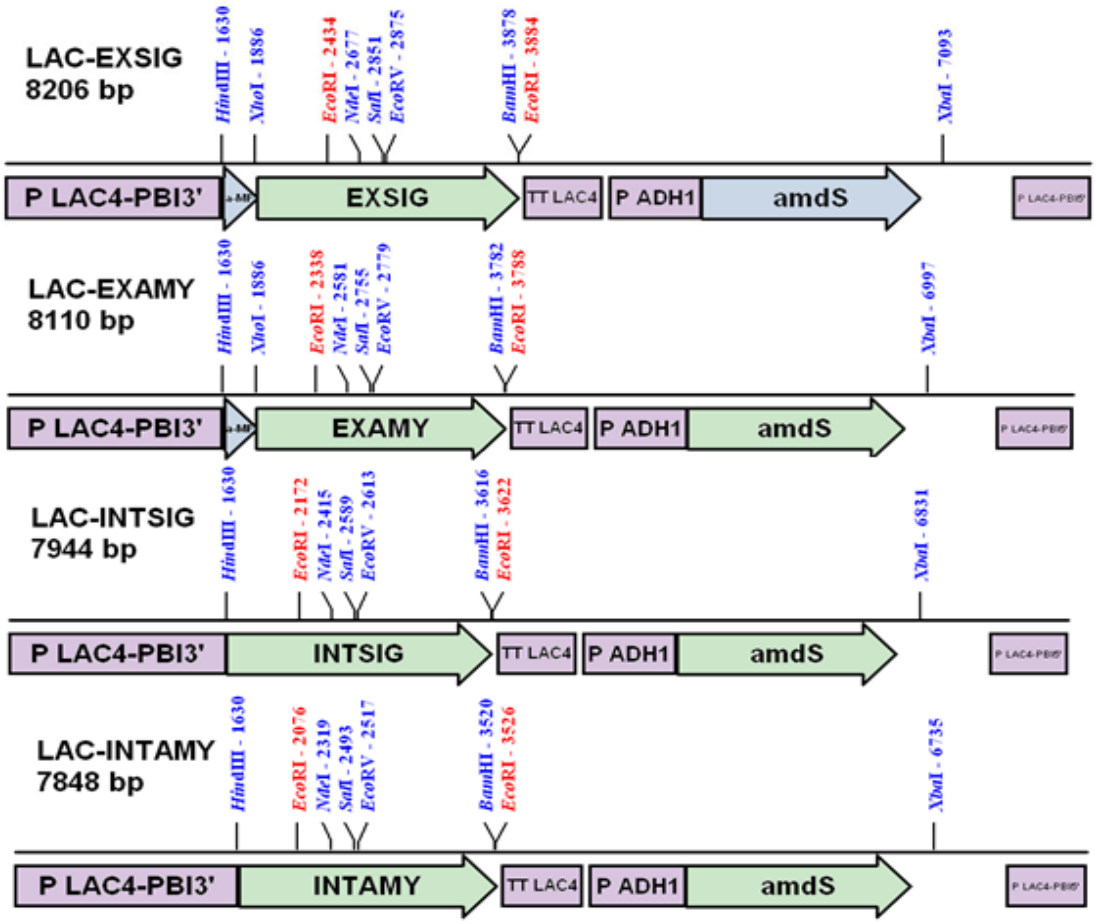


Figure 12 - Final cassettes used for yeast transformation.

These four constructs were also sequenced to confirm its codon sequence. The α-amylase gene was divided in fragments and inserted in an appropriate vector for amplification and labeling with BigDye™ reagent. The samples were purified, resuspended in 10 µL of Hi-Di formamide and denatured at 95 ºC for 5 min. Applied Biosystems 3130 Genetic Analyzer was used for separation and detection of the labeled DNA fragments.

4.3.3 Yeast transformation and transformant screening

K. lactis CBS2359 cells were transformed by the lithium acetate method described by ITO *et al.* (1983). After transformation cells were plated in YCB with acetamide for selection of the transformed colonies able to grow with acetamide as the only nitrogen source. The positive ones were isolated and had the integration of the cassette checked by PCR.

For screening the cells based on their enzymatic activity on agar plates, each positive transformant was resuspended in sterile medium to achieve the same cell concentration and was plated on a YPLS medium (which contains 1% soluble starch) and grown for 2 days at 30 ºC. After incubation, these plates were stained with an iodine solution (5 mM I_2 and 50 mM KI), which interacts with the starch molecule giving a purple color. Those strains capable of secreting the α-amylase may present a colorless halo around the colony, indicating enzymatic activity.

4.4 FLASK CULTIVATION OF CELLS AND ENZYME PRODUCTION

4.4.1 Shake flask cultivation

The cells which presented amylolytic activity in the YPLS test were selected and further tested in Erlenmeyer liquid cultivations. A pre-inoculum was prepared by inoculating each yeast strain in a sterile vial containing 3 mL of YPL that was incubated at 30 ºC and 150 rpm overnight. On the following day, the optical density of each vial was measured at 600 nm and they were used to inoculate a 50 mL YPL broth in a 125 mL Erlenmeyer flask. The appropriate volume of the pre-inoculum was used to achieve an initial optical density of 0.200

in the Erlenmeyer flasks, which were then incubated at 30 ºC and 150 rpm for 48 h. Cell cultivation was performed in duplicate and the following analysis of biomass, sugar consumption, protein production and enzymatic activity were performed in triplicate.

4.4.2 Determination of biomass production

Yeast growth was followed by measuring optical density at 600 nm and dry cell mass determination. Samples from the culture supernatant were appropriately diluted to achieve OD_{600} lower than 0.600 and measured in a spectrophotometer. For dry mass determination, a known volume of the culture was filtered and the retained biomass was washed and dried until constant weight for measurement in an analytical balance. After biomass determination samples were centrifuged at 4000 xg for 10 min. The supernatant and the pellet were separated for further analysis.

4.4.3 Determination of carbohydrates consumption and proteins production

Reducing sugars in the supernatant were measured by the dinitrosalicylic acid (DNS) assay (MILLER, 1959). Total sugars were measured by the phenol-sulfuric acid method for microplate (MASUKO *et al.*, 2005).

Protein quantification was carried out by the Bradford method (BURGESS & DEUTSCHER, 2009). The protein content was measured in the supernatant and in the lysed cells. To perform cell lysis, the pellet obtained after culture centrifugation was resuspended in 100 mM pH 7 phosphate buffer and 5% protease inhibitor cocktail (nº P8215, Sigma-Aldrich). Glass beads (425-600 µm) were added and the mixture was vortexed for 5 periods of 1 minute, intercalating between each period a 1 min incubation on ice. The cellular debris were separated by centrifugation at 4000 xg for 10 min and this supernatant corresponded to the intracellular fraction.

4.4.4 Enzymatic activity determination

The dextrinizing activity of the α-amylase was assayed by the starch-iodine method. It was originally described by FUWA (1954) and has been adapted by many authors. In the present work, the modified protocol from XIAO *et al.* (2006) was used combined with the one from MANONMANI & KUNHI (1999). The combination of both protocols was necessary because in initial trials a loss of iodine color was observed due to interference of thiol-compounds, which are present in peptones and yeast extracts (both used as substrates in the experiments on this dissertation). The addition of copper sulfate and hydrogen peroxide suggested by MANONMANI & KUNHI (1999) protects the starch-iodine complex and prevents the loss of color in the presence of thiol-compounds.

One enzymatic unit (U) was defined as the amount of enzyme necessary to hydrolyze 0.1 mg of soluble starch per minute at the reaction conditions (50 ºC, 0.1 M phosphate buffer pH 7.0, 2% soluble starch).

It is difficult to compare results from different references because each author may adopt different quantification methods, reaction conditions, and enzymatic unit definition. Furthermore, the type of starch used in the assay and its origin (maize, potato, cassava) may influence the results due to different proportion of amylose and amylopectin.

4.4.5 Enzyme production with different carbon sources

One of the strains (*K. lactis* LAC-EXAMY 4) was selected to be cultivated in different carbons sources to observe the protein induction. The growth media YPX consisted on 10 g/L of yeast extract, 20 g/L of bacteriological peptone and 20 g/L of a selected carbon source (glucose, sucrose, starch, lactose or galactose). The strain was cultivated in 200 mL of the media in a 500 mL Erlenmeyer flask and incubated at 150 rpm and 30 ºC. The inoculum was standardized to achieve an initial OD_{600}=0.200. Samples were taken throughout time until 70 h for measurement of: 1) biomass formation; 2) carbohydrate consumption; 3) pH variation; 4) protein production; and 5) enzymatic activity.

4.5 PARTIAL CHARACTERIZATION OF THE ENZYME

4.5.1 Determination of optimal pH and temperature

The supernatant of *K. lactis* LAC-EXAMY 4 and LAC-INTSIG 24 cultures in YPL, and the cellular lysate of LAC-EXSIG 2 and LAC-INTAMY 1 containing the produced α-amylases were also used to determine an optimal pH and temperature range. It was done by a rotatable central composite design 2^2 with 4 axial points and 4 central points (Table 5). This analysis was employed because it offers an adequate amount of information with the points tested. It also allows to observe the interaction between pH and temperature variation at the same time. This kind of information is important for process instrumentation and control, making it possible to establish a range where pH and temperature can vary without significant loss of activity (RODRIGUES & IEMMA, 2005).

The pH was adjusted in each experiment with the addition of glycine-HCl 0.1 M buffer for pH 2.75 and 4; phosphate 0.1 M buffer for pH 7; and glycine 0.1 M buffer for pH 10 and 11.24.

Table 5 - Experimental design used in the determination of the optimal temperature and pH.

Experiment	Temperature		pH	
	Level	ºC	Level	Value
1	-1	30	-1	4
2	-1	30	+1	10
3	+1	80	-1	4
4	+1	80	+1	10
5	-1.41421	19.6	0	7
6	+1.41421	90.4	0	7
7	0	55	-1.41421	2.75
8	0	55	+1.41421	11.24
9 (C)	0	55	0	7
10 (C)	0	55	0	7
11 (C)	0	55	0	7
12 (C)	0	55	0	7

(C): Central point

4.6 STATISTICAL ANALYSIS

All the statistical data was analyzed by the software STATISTICA 10 (StatSoft, Inc.) according to the recommendations from CALADO & MONTGOMERY (2003) and RODRIGUES & IEMMA (2005).

5 RESULTS AND DISCUSSION

5.1 GENE AMPLIFICATION

The genomic DNA of *Bacillus subtilis* was extracted and used as template for amplification with each of the designed primers. The gel electrophoresis of the amplification products (Figure 13) corresponds to the expected fragment sizes, with the constructs containing the native signal (EXSIG and INTSIG) having a slightly higher molecular size than the others without it (EXAMY and INTAMY). The expected sizes are: 2004 bp for EXSIG, 2003 bp for INTSIG, 1909 bp for EXAMY, and 1907 bp for INTAMY.

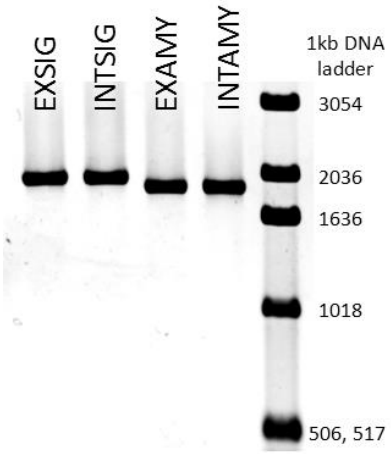


Figure 13 - Agarose gel electrophoresis of *B. subtilis* genomic DNA amplification with the designed primers EXSIG, INTSIG, EXAMY and INTAMY.

5.2 CASSETTE CONSTRUCTION

Each amplified fragment contains restrictions sites at both ends to permit its insertion in a pKLAC2 vector. The PCR products and the pKLAC2 were double-digested with *XhoI-BamHI* or *HindIII-BamHI* and the digested DNA was purified. The DNA fragments were ligated, transformed in competent *E. coli* and the colonies capable of growing in selective media had their plasmidial DNA extracted. To confirm the proper insertion of the amplified gene, the plasmids were checked by digestion with different restriction enzymes. Figure 14 shows an example of a restriction analysis of construct pKLAC-INTAMY. All the four genes

were correctly inserted in a pKLAC2 vector and presented the expected fragment sizes in agarose electrophoresis

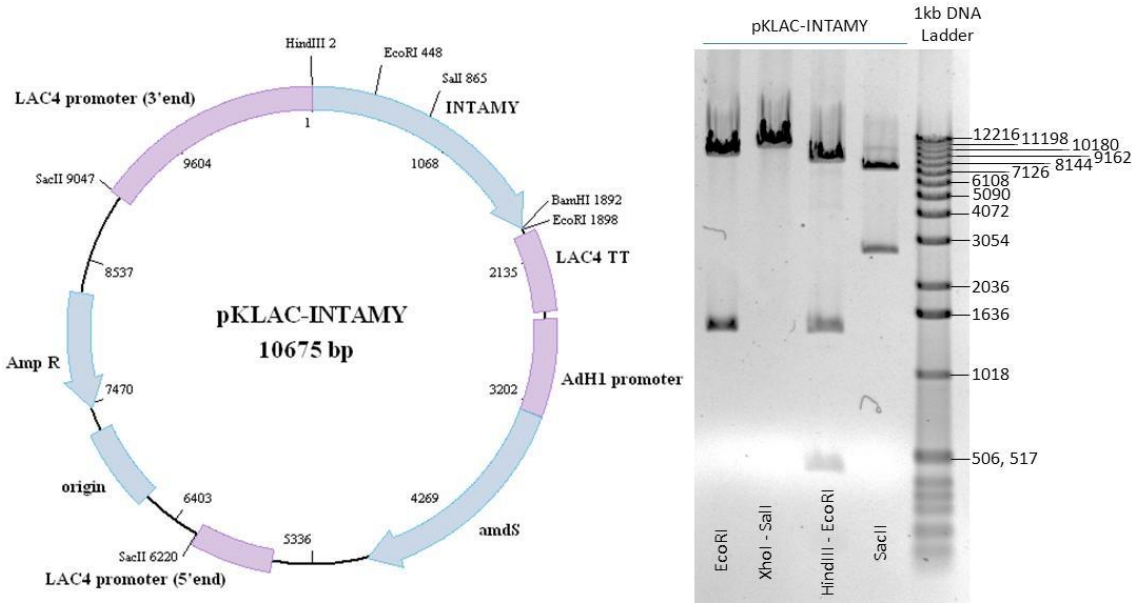


Figure 14 - Agarose electrophoresis of pKLAC-INTAMY digested with different restriction enzymes to confirm the correct insertion of the gene.

5.3 GENE SEQUENCING

The confirmation of the constructs was also performed by DNA sequencing. The sequences obtained after electrophoretograms analysis and reading alignments are shown at Appendix 1. The DNA sequencing of the constructs revealed that the EXAMY and EXSIG genes have the same sequence as the *amyE* gene from *Bacillus subtilis* OI1085 (GenBank: FJ643607.1). However, the INTSIG gene presented two silent point mutations (without changes in the amino acid sequence): one at position 165 (replacing an A for a G) and one at position 450 (replacing a G for an A). The INTAMY gene exhibited a non-synonymous point mutation at position 458, replacing an A for G. This resulted in a change of the amino acid, replacing the uncharged polar asparagine (N) for an uncharged polar serine (S). However, this mutation is not near the active site and the $(\beta/\alpha)_8$ barrel structure. These mutations might have occurred due to an error of the Taq polymerase used during the initial amplification or during cloning steps in *E. coli*. The protein sequence alignment is shown at Appendix 2.

5.4 YEAST TRANSFORMATION

The pKLAC-EXAMY, pKLAC-INTAMY, pKLAC-EXSIG and pKLAC-INTSIG vectors obtained were linearized with *SacII* and used for *Kluyveromyces lactis* transformation by the lithium acetate method. The transformed cells were plated in YCB medium containing acetamide as the only nitrogen source and after 3 to 4 days incubation at 30ºC the colonies started to appear. Those colonies were isolated in a new YCB with acetamide and had their genomic DNA extracted for confirmation of proper integration of the cassette. The extracted yeast DNA was used as template on a PCR reaction with the designed primers on Table 4 and the product was run on agarose gel.

Many colonies appeared in YCB medium, but only some of them contained the cassette properly integrated, presenting the expected fragment size on the agarose gel. Figure 15 shows an example of the PCR results for *K. lactis* transformants with LAC-INTSIG. As it can be seen, only colonies 25, 37 and 40 contain the INTSIG gene (2003 bp). As a negative control, genomic DNA of K. lactis CBS2359 (denominated WT – wild type) was used.

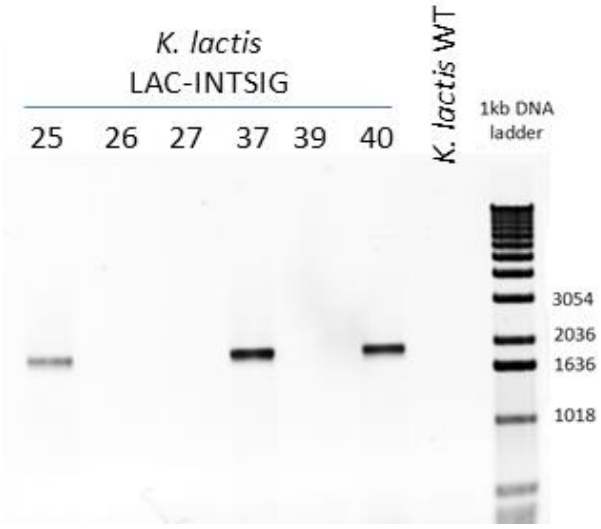


Figure 15 - Example of PCR performed with genomic DNA extracted from transformed cells and primers *INTSIG For* and *AMY Rev*. Colonies 25, 26, 27, 37, 39, 40 are from transformed *K. lactis* LAC-INTSIG and *K. lactis* WT is the negative control (strain CBS 2359).

5.5 SCREENING OF TRANSFORMED CELLS

Some of the colonies on the PCR reaction were selected for a screening based on the enzymatic activity. Each yeast liquid culture had its optical density corrected to 0.200 and 5µL of this cell suspension was inoculated on an YPLS plate. After 48h growth at 30ºC, the plates were stained with iodine solution and photographed. Clear zones around the colonies indicate the consumption of starch and, therefore, the production of the enzyme. The *K. lactis* CBS2359 (WT) was also inoculated as a negative control.

Based on the predicted protein configuration of the constructs (Figure 16), it is expected that the LAC-EXSIG and LAC-EXAMY strains, which contain the α-mating factor leader sequence recognizable by the yeast cell, would secrete the α-amylase; while the LAC-INTSIG and LAC-INTAMY strains, which do not possess the secretion leader, would retain the protein intracellularly.

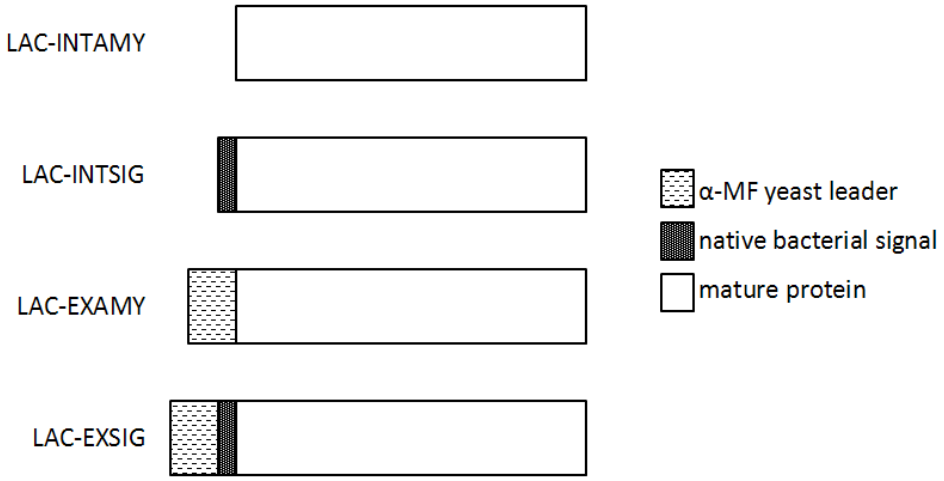
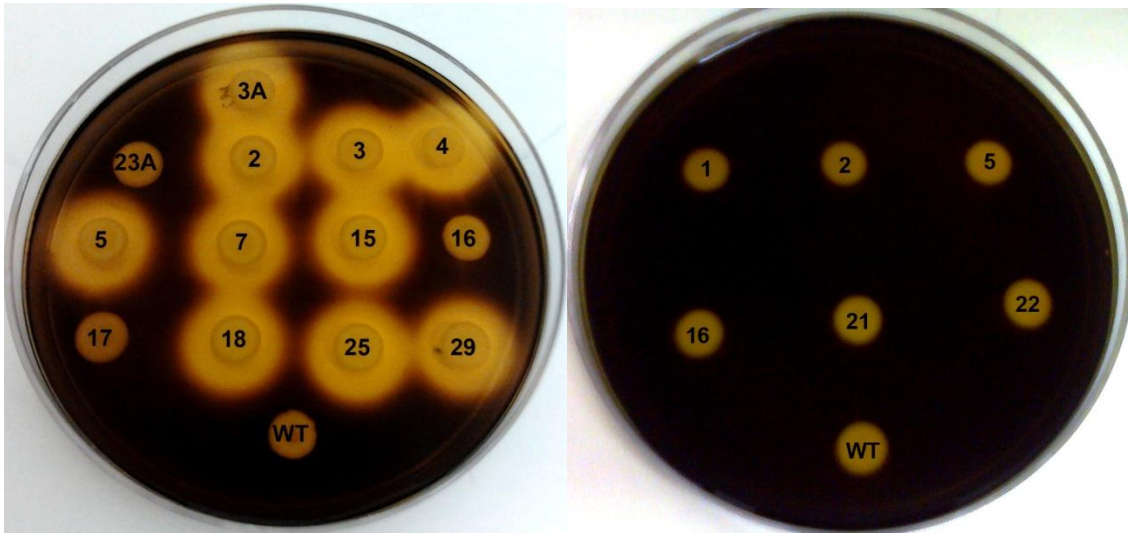


Figure 16 – Schematic representation of the expected proteins to be expressed with each construct.

The *K. lactis* LAC-EXAMY cells presented clear halos around some colonies after iodine staining, indicating the production of the enzyme (Figure 17 A). As expected, the WT strain used as a negative control did not present a halo. Even though colonies 23A, 16 and 17 were able to grow in acetamide, they were not positive on the PCR reaction. They also did not present a halo of enzymatic activity on the agar plate assay.

(A) (B)



(C) (D)

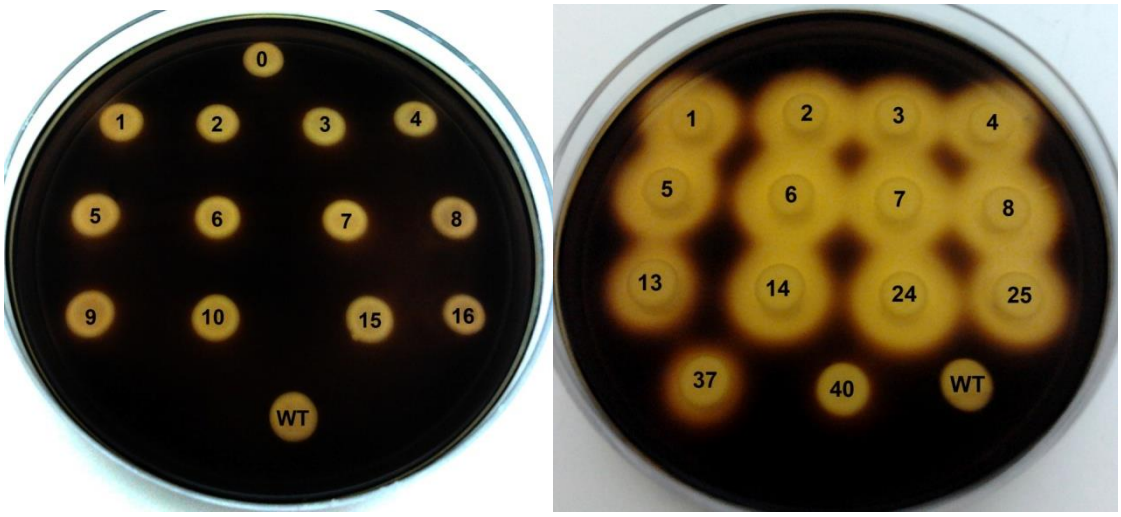


Figure 17 - YPLS plate after iodine staining with *K. lactis* cells transformed with: (A) LAC-EXAMY, (B) LAC-INTAMY, (C) LAC-EXSIG, (D) LAC-INTSIG. *K. lactis* WT was used as a negative control.

The *K. lactis* LAC-INTAMY transformants did not produce clear halos on starch plates (Figure 17 B). As these cells are expected to produce and retain the enzyme intracellularly, their enzymatic activity will only be confirmed on liquid fermentation tests, when it is possible to execute a cell disruption.

On the other hand, the *K. lactis* LAC-EXSIG cells, which were supposed to secrete the enzyme because they contain the α-mating factor leader, could not

export the enzyme and none of the isolated colonies presented clear halos after iodine staining (Figure 17 C). The presence of both the leader sequence and the native signal peptide on this construct might be interfering with protein secretion and proper processing.

Furthermore, the *K. lactis* LAC-INTSIG transformants (Figure 17 D), which were supposed to keep the enzyme intracellular, were able to secrete the enzyme. This indicates that these *Kluyveromyces lactis* cells are able to recognize the bacterial signal peptide present at the *B. subtilis* α-amylase gene.

Signal sequences are usually recognized with low specificity in yeast species. For that reason, foreign signals might also have the possibility of being recognized and processed by the cell. Some foreign proteins have already been successfully secreted by yeast using their own signal peptide or other foreign signals (ROMANOS *et al.*, 1992).

Nevertheless, it is preferred to use a yeast signal sequence because it is difficult to predict if a particular foreign sequence will work. The most widely used yeast signal peptides (also called presequences) are those from acid phosphatase (PHO5) and invertase (SUC2). For some heterologous proteins, the use of a signal peptide alone is sufficient to have good secretion of the product. However, for others the signal peptides are not sufficient to secrete the heterologous protein and they tend to accumulate in the endoplasmatic reticulum or be degraded. In those cases it is necessary the addition of a pro sequence to correctly direct the protein through the secretory pathway. Secretion leaders (preprosequences) are responsible for this guidance through the secretory machinery. The most used secretion leader is the prepro region of the α-mating factor, which contains a signal peptide (19aa) that is initially cleaved at the endoplasmatic reticulum and a prosequence which is processed by Kex2 and STE13 proteases at late Golgi (CAREY, 1996).

The *Kluyveromyces lactis* cells have a very flexible secretion mechanism, and usually the native signal of the heterologous protein is functional in driving the polypeptide through the secretion pathway (MICOLONGHI *et al.*, 2007).

STRASSER *et al.* (1989) used a *Schwanniomyces occidentalis* AMY1 promoter and secretion signal for heterologous production of α-amylase in different yeast species, including *K. lactis*. The AMY1 native signal could be

recognized by *K. lactis* cell and the gene product was readily secreted to the broth.

BARTKEVICIUTE & SASNAUSKAS (2003) used an α-amylase gene from *Bacillus amyloliquefaciens* as a marker gene for the screening of super secreting mutants of *K. lactis*. The initial strain, which had not gone through mutation to enhance secretion, was transformed with a construct containing the *Bacillus* own promoter and signal. The amylase was not expressed sufficiently for halo formation on starch agar plates. However, after random mutation with UV light, some isolated mutants started to produce α-amylase with detectable halos.

The initial strain used in the present dissertation (*K. lactis* CBS2359) with construct LAC-INTSIG could secrete the *B. subtilis* α-amylase with its own signal sequence without going through any mutational procedure to enhance production.

Other authors preferred to express the α-amylase without its original signal peptide. TOKUNAGA *et al.* (1997) expressed a mouse α-amylase in *K. lactis* without its own signal sequence, using instead a 128kDa killer precursor protein. For the expression of a *Rhizopus oryzae* α-amylase in *Kluyveromyces lactis*, LI *et al.* (2011a) constructed a vector containing the yeast α-mating factor leader and only the coding sequence of the mature amylase. However, none of these authors attempted to compare the production with or without the signal peptide.

In a different way, LI *et al.* (2011b) compared the production of a recombinant *Rhizopus oryzae* α-amylase in *Pichia pastoris* with its native signal sequence or with an α-mating factor leader followed by the mature protein sequence. Like other yeast species, *P. pastoris* has a low-specificity requirement for a signal sequence, and the native signal could correctly direct folding, processing and secretion of the protein. Moreover, cells secreting with the native signal had 10% higher productivity than cells with α-factor signal.

PAIFER *et al.* (1994) also compared the production with different signals. They cloned and expressed a bacterial α-amylase gene in *P. pastoris* using two integrative vectors with two different secretion signals: one vector contains the structural gene encoding the mature α-amylase fused to the SUC2 signal and the other contains the α-amylase with its own signal sequence. In both cases, the α-amylases were secreted into the culture medium with high efficiency, around 2.5 and 0.9 g/l respectively.

In relation to the α-amylase from *Bacillus* strains, their signal peptide, like many other proteins secreted by this genus, is unusually long (approximately 31 aa) when compared to those signals typically found in eukaryotes, which are around 20 aa long (RUOHONEN *et al.*, 1987).

The *B. amyloliquefaciens* α-amylase gene with its own secretion signal was transformed in a *Saccharomyces cerevisiae* strain by RUOHONEN *et al.* (1987). The prokaryotic signal sequence could direct the protein to secretion and the signal peptide was correctly cleaved.

Unlike what happened with construct LAC-EXSIG, where the native signal interfered with protein secretion, KARAKAŞ *et al.* (2010) expressed a *Bacillus subtilis* α-amylase in *Pichia pastoris* in a construct containing both the α-mating factor leader and the α-amylase native signal. The protein was correctly secreted to the broth (22mg/L) and presented visible halos on agar starch plates after iodine staining.

5.6 SHAKE FLASK CULTIVATION

The initial shake flask cultivations were performed in Erlenmeyers flasks containing 50 mL of YPL broth. At first the inoculum of each strain was prepared in a 3mL vial containing YPL broth, which was incubated overnight at 30ºC and 150rpm. On the following day, each flask had its optical density measured at 600nm and the right amount of inoculum was calculated to achieve an initial OD_{600} of 0.200 for all strains in the 50mL flasks with YPL. Each yeast culture was incubated at 150 rpm and 30ºC for 48h. Afterwards the cultures had their biomass, protein content and enzymatic activity assayed. Tests were performed with the same strains used in the agar plate screening: *K. lactis* LAC-EXSIG (colonies 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16), LAC-EXAMY (colonies 3A, 23A, 2, 3, 4, 5, 7, 15, 16, 17, 18, 25, 29), LAC-INTSIG (colonies 1, 2, 3, 4, 5, 6, 7, 8, 13, 14, 24, 25, 37, 40) and LAC-INTAMY (colonies 1, 2, 5, 16, 21, 22). Strain *K. lactis* CBS 2359 was used as a negative control for enzymatic activity and was denominated WT (wild type).

5.6.1 Biomass production

After 48h cultivation in YPL broth all strains presented a similar biomass concentration, with approximately 5 g/L of dry weight and OD_{600}≈14. Table 6 presents only the mean values of the selected strains of each transformant and the wild type. Apparently the heterologous enzyme production did not interfere in biomass formation, considering that the wild strain presented equivalent biomass results as the transformants.

Table 6 - Mean results for biomass determination (OD_{600} or dry weight ± standard deviation) of the transformants and WT strain.

	OD600±SD	**Dry weight (g/L)±SD**
LAC-EXAMY	13.06±0.677	4.910±0.254
LAC-EXSIG	14.16±0.710	5.546±0.278
LAC-INTAMY	13.74±0.855	5.341±0.332
LAC-INTSIG	13.12±0.615	5.141±0.241
WT	13.65±0.605	5.286±0.308

5.6.2 Protein content

After performing the biomass measurements, the fermented broth free of cells was analyzed for extracellular protein content. The cell pellet was also collected and those strains which were expected to produce the enzyme intracellularly (LAC-INTAMY and LAC-INTSIG) were lysed and their intracellular protein content measured. Figure 18 and Figure 19 show the protein content of each transformant, the wild strain and of the initial YPL broth. Each bar represents the mean value of a triplicate and the error bars represent ±1 standard deviation. The protein concentration of the intracellular extract is expressed in the graphics as mg of protein per liter of broth and calculation was performed according to the calculation report in Appendix 3.

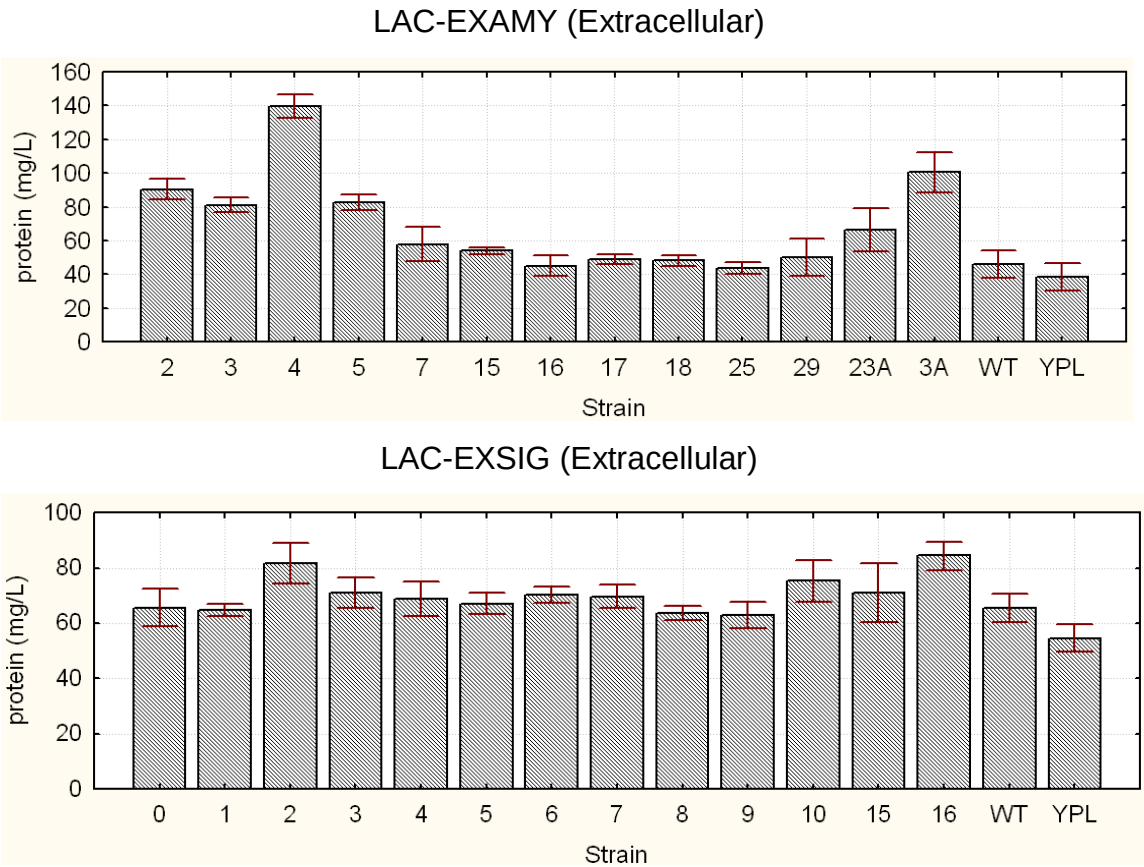


Figure 18 – Protein concentration on Bradford assay of the fermented extracellular broth of transformants LAC-EXAMY and LAC-EXSIG, the WT strain and the non-fermented YPL broth. Bars represent ±1 standard deviation.

The extracellular protein concentration had only a pronounced difference in some LAC-EXAMY colonies. The LAC-EXAMY 2, 3, 4, 5 and 3A were the only ones significantly different from WT and YPL on a Tukey statistical test. The other transformants had a protein content slightly higher or similar to the initial YPL broth and the WT fermentation. It is difficult to follow the enzyme expression based on protein content because the initial fermentation broth contains peptides and proteins. It is problematic to distinguish between proteins from the initial broth, proteins normally secreted by *K. lactis* and the induced α-amylase. Therefore the analysis of enzymatic activity was also performed.

The LAC-INTAMY and LAC-INTSIG transformants had also their intracellular protein measured. Only LAC-INTAMY 1 and 5 were statistically significant when compared to the WT intracellular content.

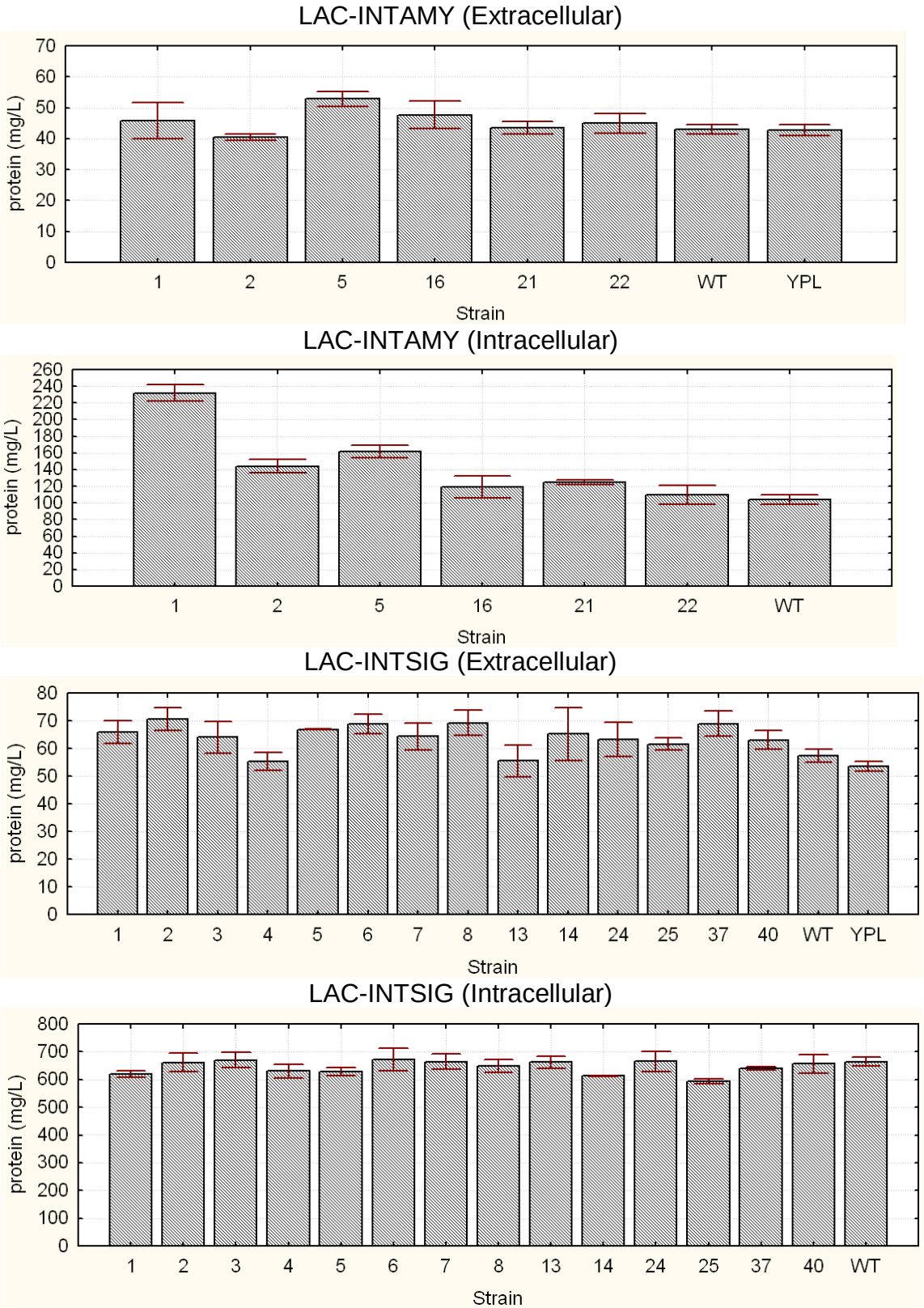


Figure 19 – Protein concentration on Bradford assay of the fermented extracellular broth and intracellular extract of transformants LAC-INTAMY and LAC-INTSIG, the WT strain and the non-fermented YPL broth. Bars represent ±1 standard deviation.

5.6.3 Enzymatic activity

The amylolytic activity can be determined by different methods, such as: quantifying the starch breakage (dextrinizing power), the increase in reducing sugars (saccharifying power), the decrease in viscosity (liquefying power), or the change in optical rotatory power. It is difficult to compare results from different references because each author may adopt different quantification methods, reaction conditions, and enzymatic unit definition. Furthermore, the type of starch used in the assay and its origin (maize, potato, cassava) may influence the results due to different proportion of amylose and amylopectin. As an example, the enzymatic activity from the iodine method is circa 4-6 times higher than those obtained by quantifying the reducing sugar. This happens because endo-acting α-amylases reduce the concentration of starch polymers that are able to bind iodine much more quickly than they produce reducing sugar ends (XIAO *et al.*, 2006). Therefore, care should be taken when comparing the enzymatic activity from each referenced author.

The recombinant α-amylase was correctly secreted by some transformants. The LAC-EXAMY strains presented a high enzymatic activity (approximately 1200 U/L) and most transformants presented a similar activity (Figure 20), except for strains 23A, 16 and 17, which presented no activity. Transformant 4 was selected for shake flask cultivations because it presented a high activity and the highest extracellular protein concentration.

In the liquid cultivation of LAC-EXSIG transformants it could be observed and quantified a low extracellular activity (Figure 20), although they did not present extracellular activity during the agar plate tests. This low enzymatic activity might not have been sufficient to produce a visual observation of a clear halo in the agar plates experiments. As stated before, the *B. subtilis* signal peptide present in this construct might be interfering with protein secretion. This interference in protein secretion can only be confirmed by performing further analyses in other fields of study, such as transcriptomics and proteomics.

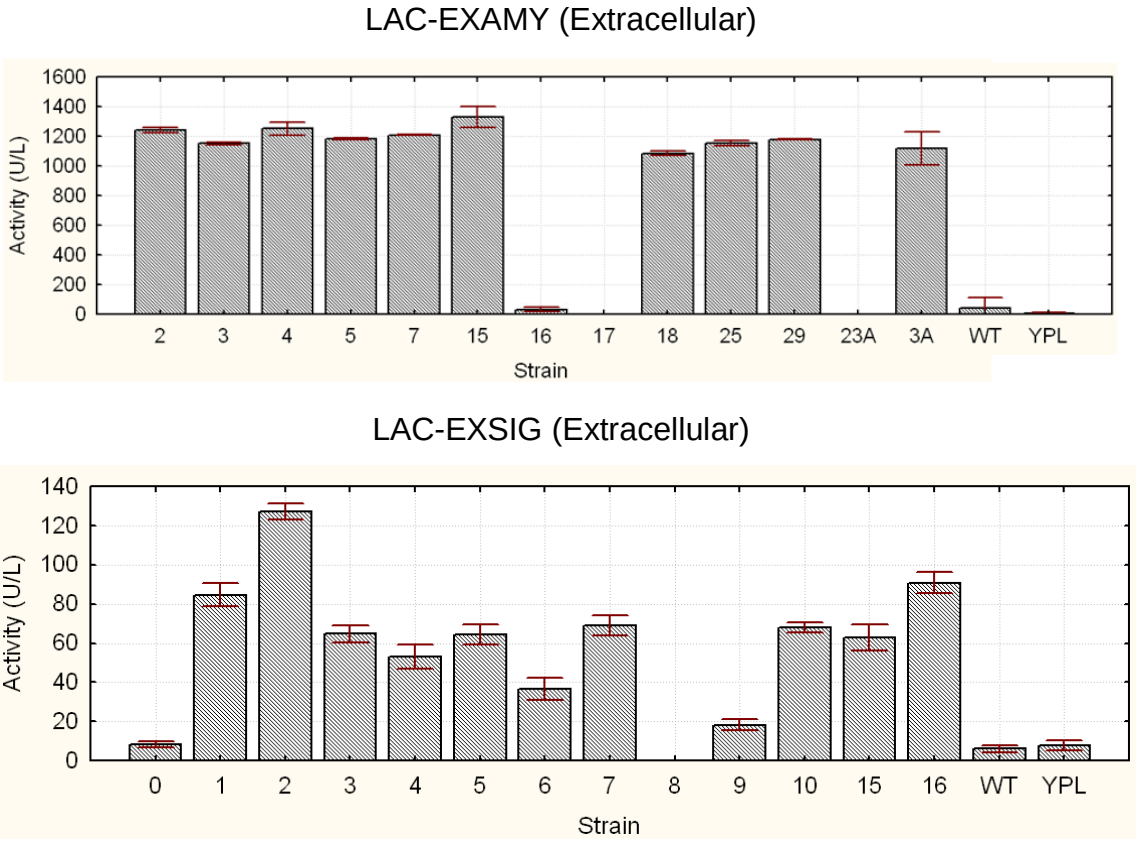


Figure 20 - Enzymatic activity of the fermented extracellular broth of transformants LAC-EXAMY and LAC-EXSIG, the WT strain and the non-fermented YPL broth. Bars represent ±1 standard deviation.

The LAC-INTSIG strains presented a high extracellular activity (Figure 21), comparable to the levels achieved with the LAC-EXAMY transformants, even though they did not have the preprosequence. As stated before, the *K. lactis* cells are able to recognize the bacterial signal peptide and export the α-amylase to the broth with high efficiency.

The isolated transformants containing the LAC-INTAMY construct presented extremely low extracellular and intracellular activities (Figure 21). This strain was intended to produce the intracellular enzyme, permitting the comparison between the strains producing the enzyme intracellularly with those producing extracellularly. The comparison of the results from these different constructs was achieved by calculating the intracellular activity in terms of units of enzyme per liter of fermented broth (calculation report presented in Appendix 3).

The intracellular production of the protein has the advantage of easier concentration, because the initial centrifugation step concentrates cells, and consequently, the desired intracellular protein. However, as it can be seen on the intracellular graphics of Figure 21 andFigure 22, the productivity per litter of broth is not as high as those obtained by the extracellular producers LAC-EXAMY and LAC-INTSIG. Therefore, with the present isolated strains, the best choice for producing the α-amylase is the extracellular production with LAC-EXAMY or LAC-INTSIG transformants.

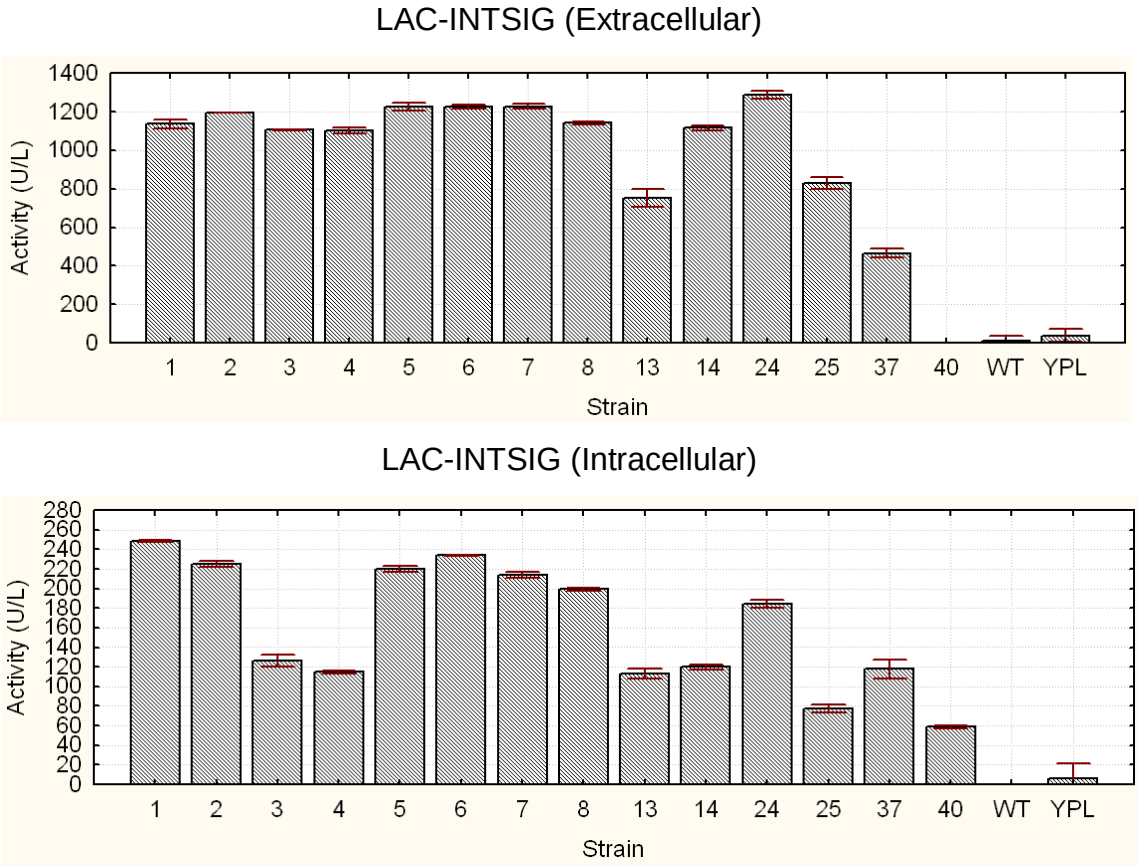


Figure 21 - Enzymatic activity of the fermented extracellular broth and intracellular extract of transformants LAC-INTSIG, the WT strain and the non-fermented YPL broth. Bars represent ±1 standard deviation.

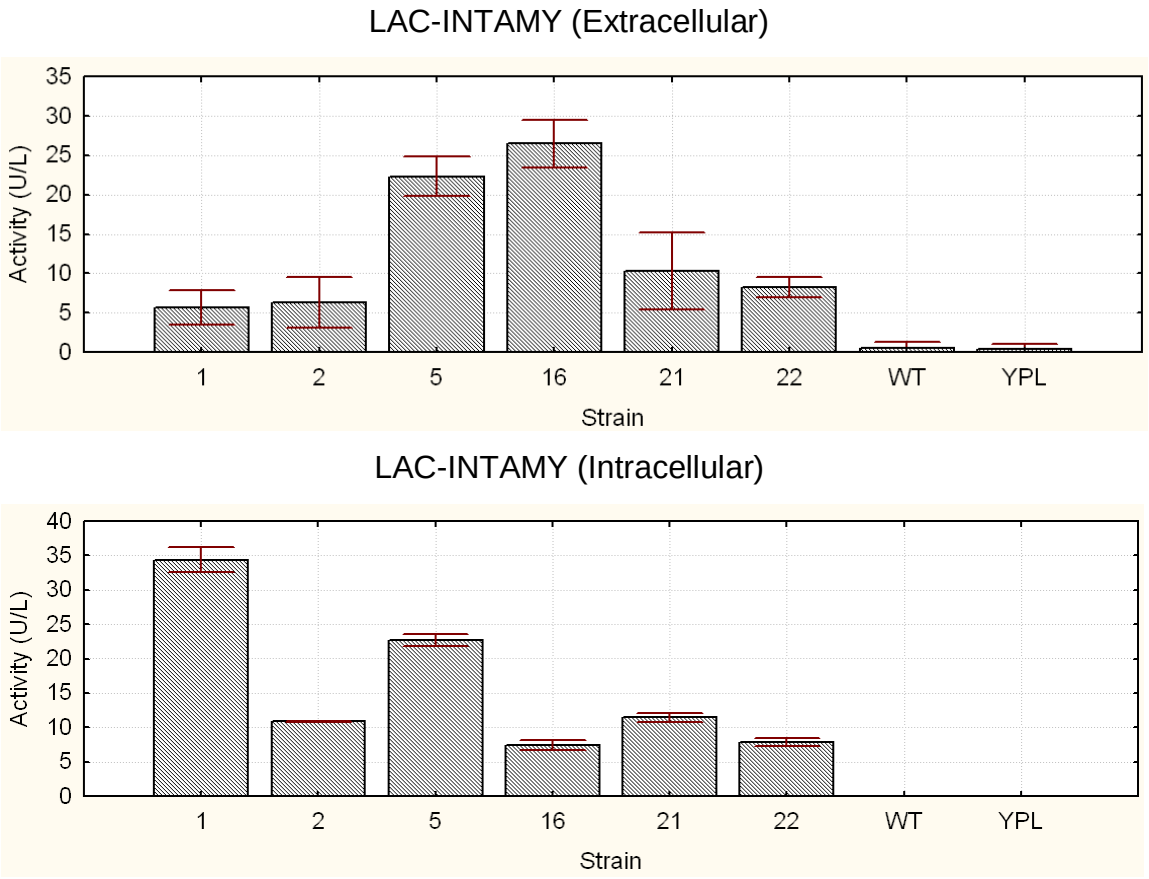


Figure 22 - Enzymatic activity of the fermented extracellular broth and intracellular extract of transformants LAC-INTAMY, the WT strain and the non-fermented YPL broth. Bars represent ±1 standard deviation.

The yeast *Kluyveromyces lactis* has already been used for the production of some recombinant α-amylases. One of the first reports is from STRASSER *et al.* (1989), who produced a recombinant *S. occidentalis* α-amylase in *K lactis*. They used a mineral medium (YNB) and achieved an enzymatic activity of 30.5 U/L. It has been observed in previous tests in our laboratory that the *K. lactis* strain CBS2359 does not grow well and does not express the enzyme in a mineral medium such as YNB (data not shown), therefore cultivations in mineral media were not performed in this work.

TOKUNAGA *et al.* (1997) attempted to produce a mouse α-amylase in *K. lactis* using a complex medium (similar to YPX) containing galactose as the inducer, and obtained a maximum activity of 0.527 U/mL. LI *et al.* (2011a) produced a *R. oryzae* α-amylase in *K. lactis* in the same medium and obtained 22.4 U/mL (approximately 20 mg/L of extracellular protein). LI *et al.* (2011b) also

attempted to produce the same *R. oryzae* enzyme in another microorganism (*Pichia pastoris*) and obtained 46.2 U/mL (41.1 mg/L of extracellular protein).

The *B. subtilis* α-amylase gene was used by KARAKAŞ *et al.* (2010) to produce the enzyme in *P. pastoris*. They achieved 22 mg/L of extracellular protein (44.34 U/mL of enzymatic activity) in a complex medium. MORAES *et al.* (1995) also introduced a *B. subtilis* α-amylase in *S. cerevisiae* and produced 38 U/mL in YNB medium.

In the present dissertation, the maximum enzymatic activity obtained by the transformed cells in shake flasks (circa 1200 U/L) is far below the ones reported in the literature. One of the reasons for this difference might be due to the diversity of activity determination assays and unit definitions. Moreover, the present work did not use a selected strain with good secretory capabilities, such as the commercial strain GG799, or an engineered strain with lower protease activity. Therefore, further improvements can be achieved with the isolated transformants by improving its genetic characteristics and secretion capability (decreasing protease levels and increasing folding-helper chaperones), and adjusting its fermentation conditions (physicochemical parameters and fermentation operation mode).

5.6.4 Enzyme production in different carbon sources

In order to observe the behavior of enzymatic expression in the presence of different carbon sources, strain LAC-EXAMY 4 was cultivated in YPX medium (where X stands for glucose, sucrose, starch, lactose or galactose). Samples were taken periodically to analyze biomass, sugar, and protein concentration, as well as pH variation and enzymatic activity.

Glucose, sucrose and lactose were quickly consumed by the cells, and biomass rapidly increased in these cultures (Figure 23 and Figure 24). When cultured in galactose as the only carbon source, the strain presented a slower growth rate. Growth on glucose tends to be faster than growth on galactose because the conversion of galactose into a glycolytic intermediate needs additional energy and catabolic steps (known as the Leloir pathway). The doubling time observed in the literature for *K. lactis* CBS2359 in YPX medium is

78 min for lactose, 84 min for glucose and 108 min for galactose (RUBIO-TEXEIRA, 2005).

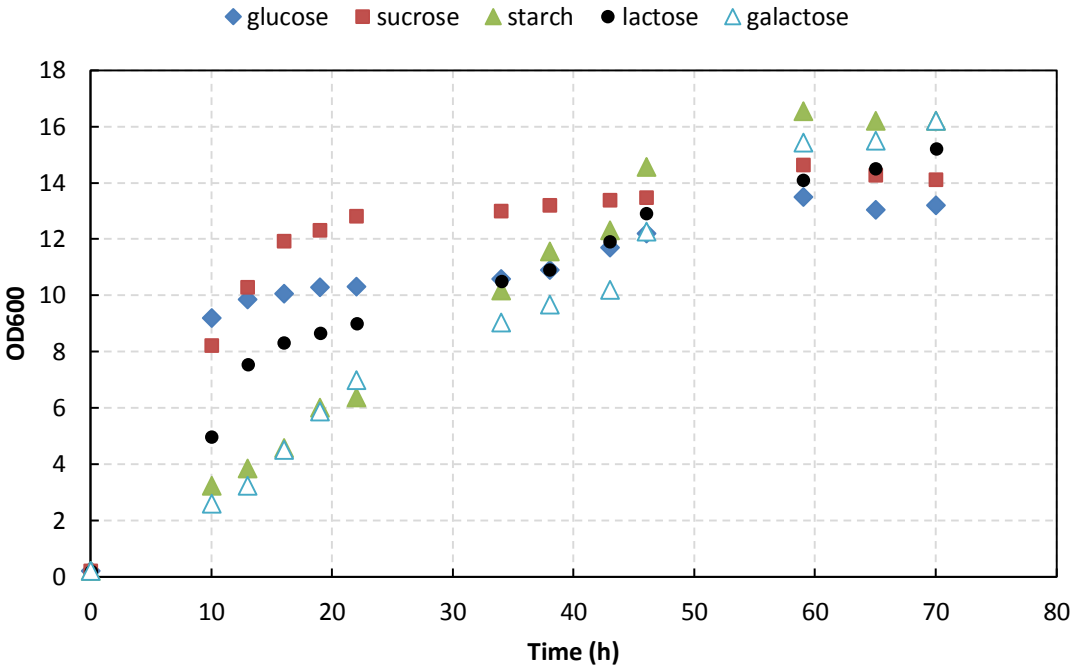


Figure 23 - Biomass production (OD_{600}) of *K. lactis* LAC-EXAMY 4 when grown on glucose, sucrose, lactose or galactose as the only carbon source.

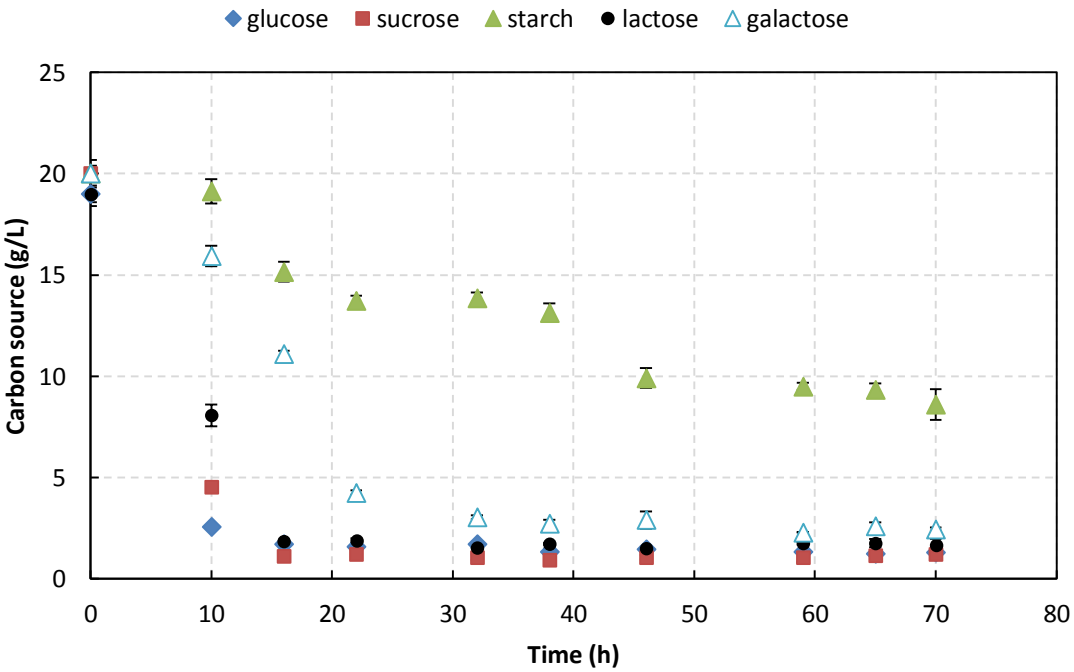


Figure 24 - Carbon source consumption by *K. lactis* LAC-EXAMY 4 when grown on glucose, sucrose, lactose or galactose as the only carbon source. Bars represent ± 1 standard deviation.

This transformant was also able to grow on starch as the only carbon source. *K. lactis* cells do not naturally produce α-amylase, however, as will be discussed subsequently, the expression of the recombinant protein is not totally repressed and a basal expression (leakage) of the enzyme allows the cells to break up the starch molecule and use it as the carbon source.

During carbon consumption it was observed in all cultures, except for starch, a decrease in pH to values around 5.8 (Figure 25). Subsequently, the pH increased until it reached approximately 7.5.

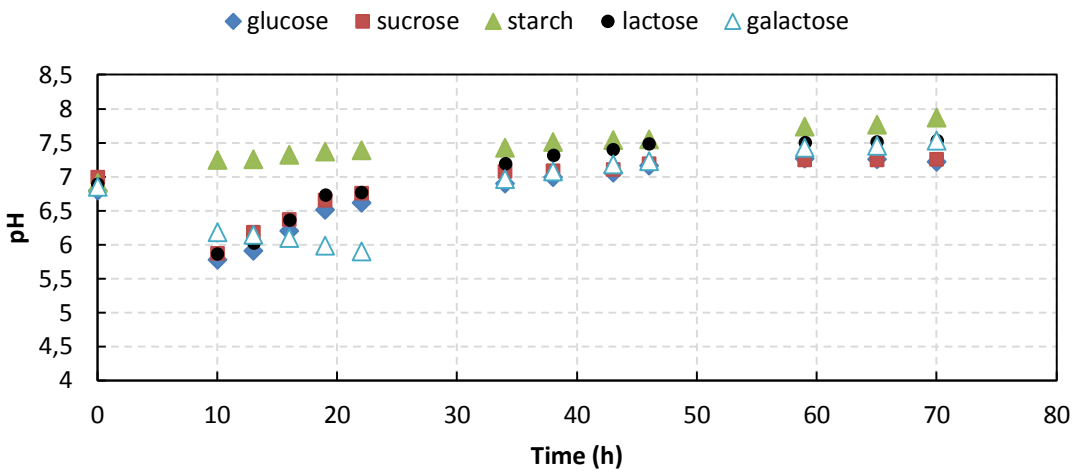


Figure 25 - pH profile during *K. lactis* LAC-EXAMY 4 growth on glucose, sucrose, lactose or galactose as the only carbon source.

Extracellular protein production was accompanied in all flasks by the Bradford assay and enzymatic activity by the iodine-starch method. Both results presented a similar profile (Figure 26 and Figure 27), with cultures containing galactose and lactose producing more extracellular protein and presenting higher enzymatic activity. Nevertheless, the cultures without induction also presented a basal expression of the enzyme. The LAC4 promoter is induced in the presence of galactose or lactose in the growth medium, but it is not totally repressed in the absence of the inducers (VAN OOYEN *et al.*, 2006). Glucose might repress expression of the LAC regulon in some, but not all, *K. lactis* strains, but this repression is less pronounced than it is observed in other species, such as *S. cerevisiae* (RUBIO-TEXEIRA, 2005).

LI *et al.*, (2011a) also attempted to cultivate a recombinant *K. lactis* in different carbon sources. The galactose medium presented a high enzymatic production due to its induction of the LAC4 promoter. However, their lactose medium presented no induction at all, presenting only a basal expression similar to other carbon sources (glucose, starch, maltose and glycerol). It was not explained why this happened.

In the present work the lactose substrate could successfully induce enzyme production, allowing its use as a carbon source in further studies because it is cheaper than galactose and can be obtained from industrial waste such as cheese way. Cheese way is the byproduct obtained during cheese making, where 1 kg of cheese generates nearly 9 kg of whey. Annually 145 million tons of liquid whey are produced, but only about 50% of it is treated and transformed in other food products (SISO, 1996).

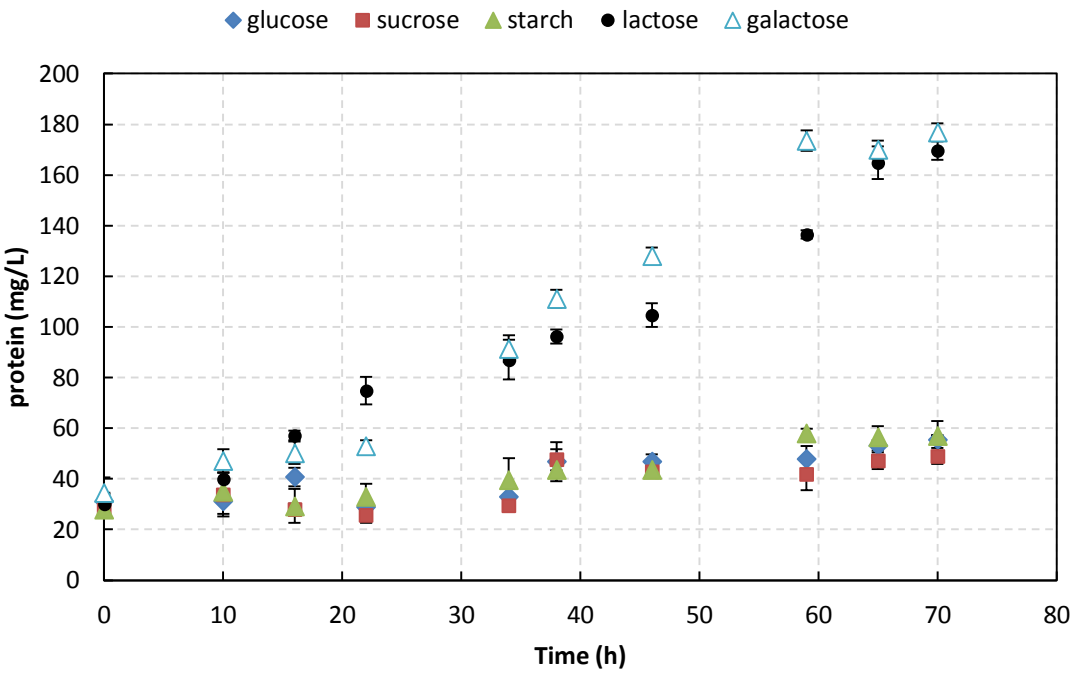


Figure 26 - Protein production of *K. lactis* LAC-EXAMY 4 when grown on glucose, sucrose, starch, lactose or galactose as the only carbon source. Bars represent ± 1 standard deviation.

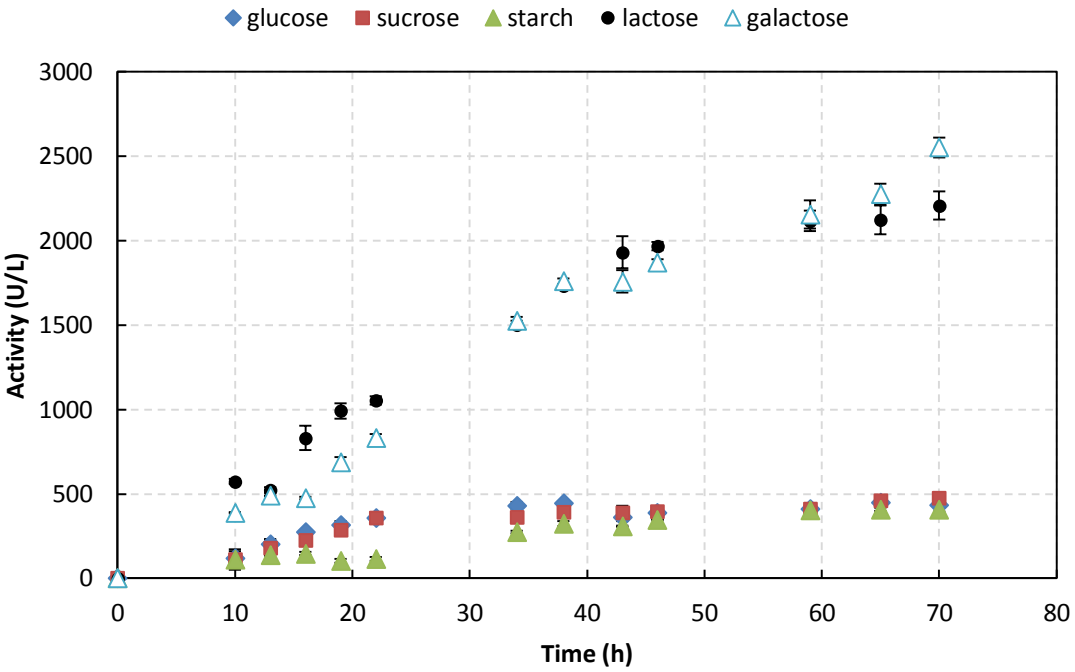


Figure 27 - Enzyme production of *K. lactis* LAC-EXAMY 4 when grown on glucose, sucrose, starch, lactose or galactose as the only carbon source. Bars represent ± 1 standard deviation.

Some kinetic parameters of each culture were summarized in Table 7. As already seen on the graphics presented before, the glucose has the highest maximum specific growth rate (0.3827 h^{-1}). Growth rates on sucrose and lactose are also good, indicating that the strain could be cultured in substrates such as sugar cane molasses (rich in sucrose) and cheese whey (rich in lactose). The potential use of these low cost substrates stimulates its scale up and industrial application. BARBA *et al.* (2001) cultivated *K. lactis* in cheese whey and presented a specific growth rate (μ_{max}) of 0.29-0.43 h^{-1}, which is in the same range of those obtained here.

The biomass yield ($Y_{X/S}$) was high, ranging between 0.38 and 0.73. Crabtree positive yeasts usually have low biomass yield (0.1-0.2 g/g) because they also produce some byproducts (mainly ethanol) (PORRO *et al.*, 2005). As *K. lactis* is a Crabtree negative yeast, with a prevalence of an oxidative metabolism, the biomass yield obtained is higher (RUBIO-TEXEIRA, 2005).

Lactose and galactose had the highest product yield (127.35 and 145.39 U/g, respectively), which is five times greater than the yield without

induction. When LI *et al.* (2011a) cultured the recombinant *K. lactis* in different carbons sources, the induced enzymatic activity in galactose broth was only two times higher than in those without induction.

Table 7 – Kinetic parameters and yield on different carbon sources after 70h culture.

	Glucose	**Sucrose**	**Starch**	**Lactose**	**Galactose**
Maximum Specific Growth Rate (μ_{max})[a]	0.3827	0.3714	0.2784	0.3212	0.2564
Product Yield ($Y_{P/S}$)[b]	24.52	25.33	35.63	127.35	145.39
Biomass Yield ($Y_{X/S}$)[c]	0.3802	0.3829	0.7267	0.4479	0.4714
Maximum Productivity (R_m)[d]	17.20	16.24	11.11	57.40	47.70

[a] h^{-1}
[b] Units of enzyme per grams of carbon source
[c] grams of dry biomass per grams of carbon source
[d] U/L/h

In conclusion, the monitoring of fermentation in different carbon sources permitted visualizing that even without the presence of an inducer the cells are able to produce the α-amylase. However, the presence of lactose and galactose greatly enhances product yield.

The versatility of substrates and the GRAS status of *K. lactis* cells and some of their enzymes permits their use in various pharmaceutical and food applications and stimulates the scale up of the process. Not only are natural *K. lactis* enzymes considered GRAS (such as lactase), but also some recombinant enzymes, such as the heterologous bovine prochymosin produced in industrial-scale at DSM Food Specialties (VAN OOYEN *et al.*, 2006).

It was observed that during fermentation the pH varies from 5.8 to 7.5. Maybe the use of a buffered medium or the control of pH in a bioreactor might affect protein production, increase enzyme stability, and inhibit the attack of proteases.

It was also observed that the fermentation is limited by carbon after circa 10 h. The use of a fed-batch fermentation could improve both the biomass and the product yield. Fed-batch is the most common fermentation strategy for recombinant microorganisms because it allows obtaining at first a high biomass concentration and then a high product formation during the induction phase (PORRO *et al.*, 2005).

5.7 PARTIAL CHARACTERIZATION OF THE ENZYME

5.7.1 Determination of optimal pH and temperature

Cells of LAC-EXAMY 4 and LAC-INTSIG 24 were cultivated in YPL and the fermented supernatant free of cells was used for enzymatic determination. LAC-EXSIG 2 and LAC-INTAMY 1 were also cultivated in YPL, but in this case the cells pellet was collected and lysed to obtain the intracellular enzyme. *B subtilis* was cultivated in BAP broth and the fermented supernatant was used to determine the optimal pH and temperature of the natural enzyme.

The extracts containing the enzymes had their activity assayed in different conditions and response surfaces were fitted to data to obtain optimum values of temperatures and pH. This analysis was employed because it permits to observe the interaction between pH and temperature variation at the same time. This kind of information is important for process instrumentation and control, making it possible to establish a range where pH and temperature can vary without significant loss of activity (RODRIGUES & IEMMA, 2005). The response surfaces and contour plots obtained are presented in Figure 28 to Figure 32. The enzymatic activity was plotted as a relative activity: the highest activity value obtained was set as 100% and the other values were calculated based on this assumption.

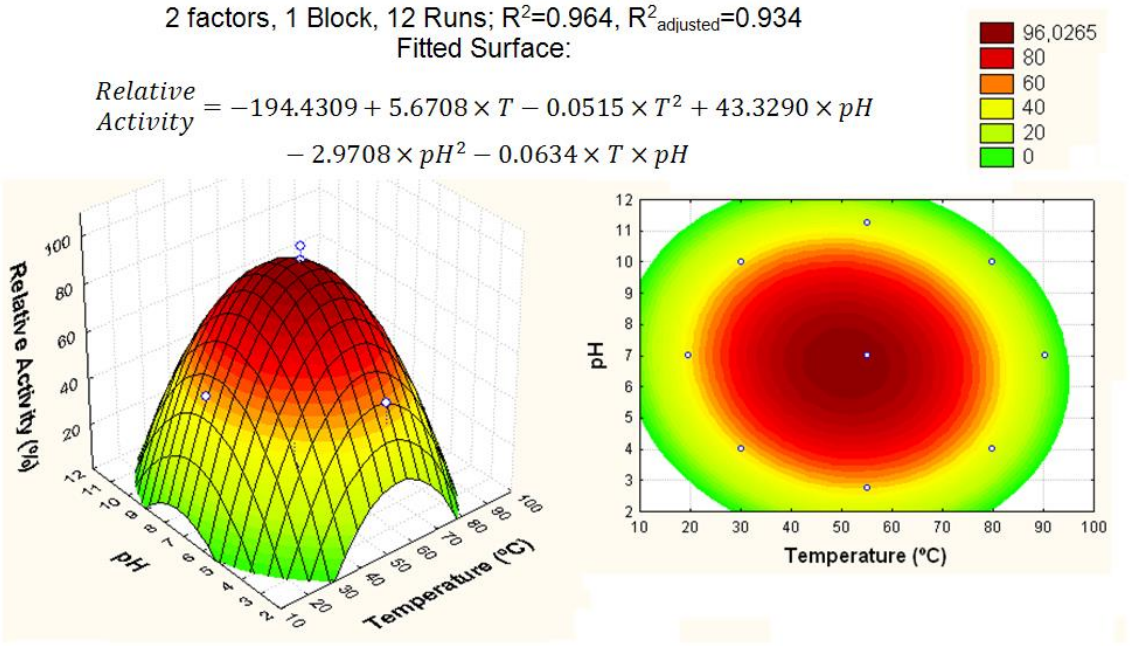


Figure 28 - Response surface and contour plot of LAC-EXAMY 4 relative enzymatic activity as function of pH and temperature.

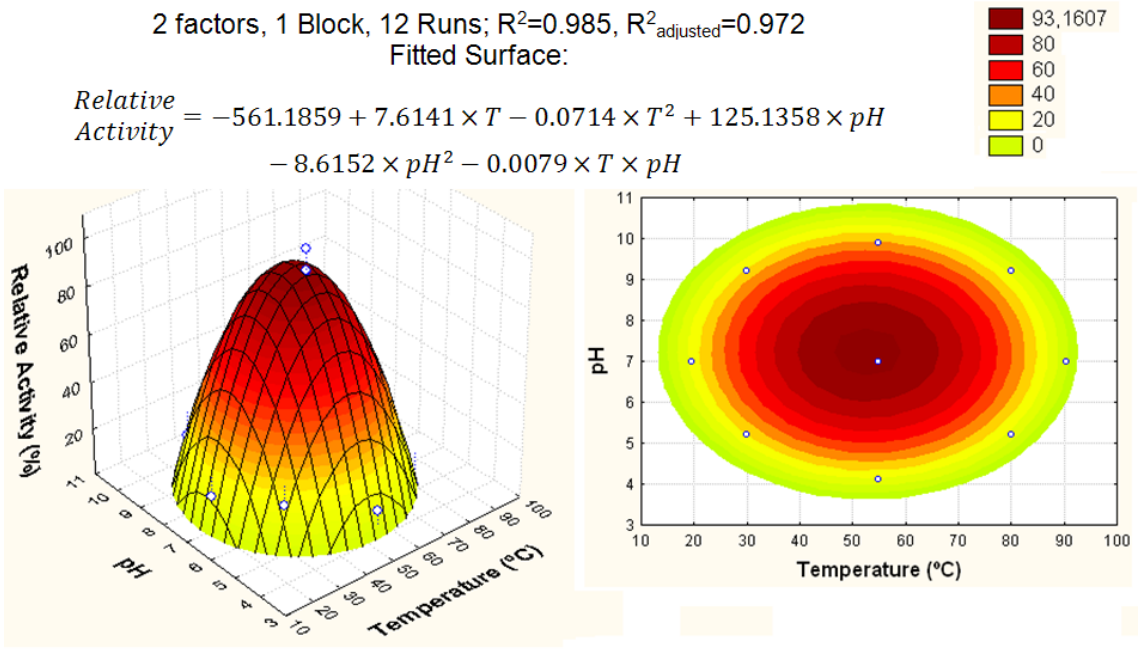


Figure 29 - Response surface and contour plot of LAC-EXSIG 2 relative enzymatic activity as function of pH and temperature.

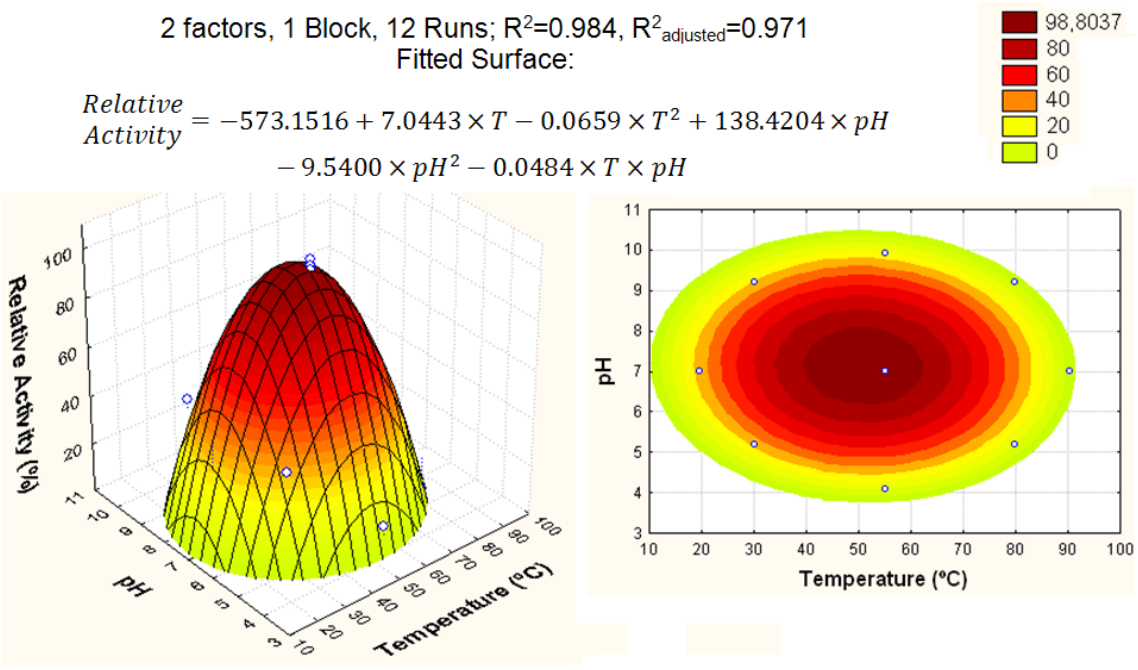


Figure 30 - Response surface and contour plot of LAC-INTAMY 1 relative enzymatic activity as function of pH and temperature.

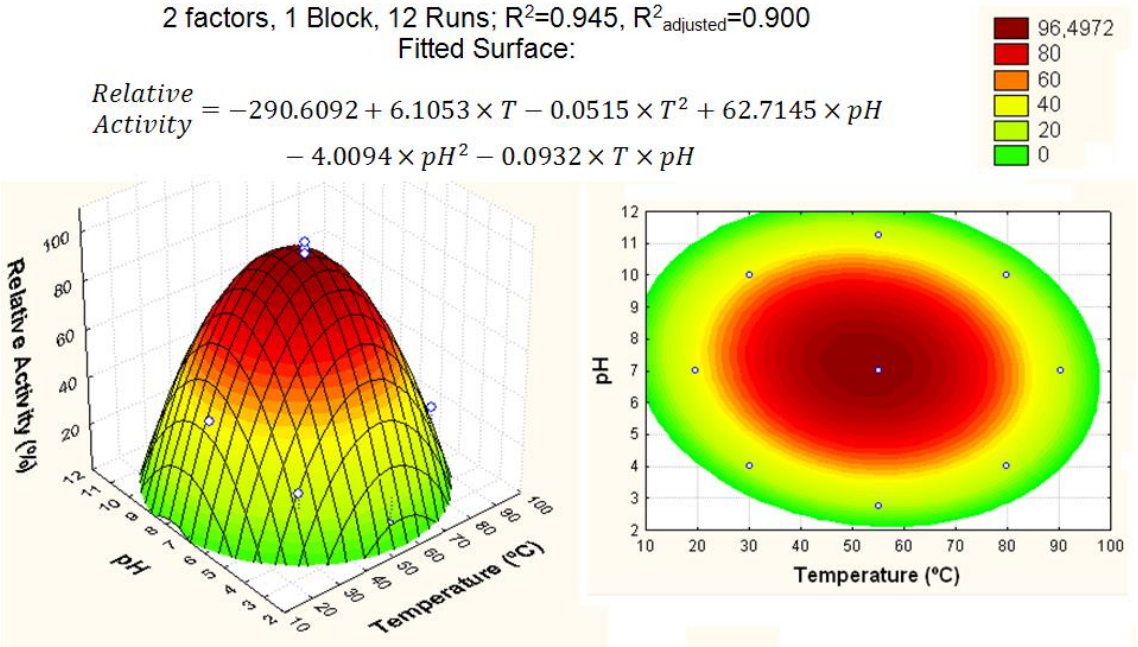


Figure 31 - Response surface and contour plot of LAC-INTSIG 24 relative enzymatic activity as function of pH and temperature.

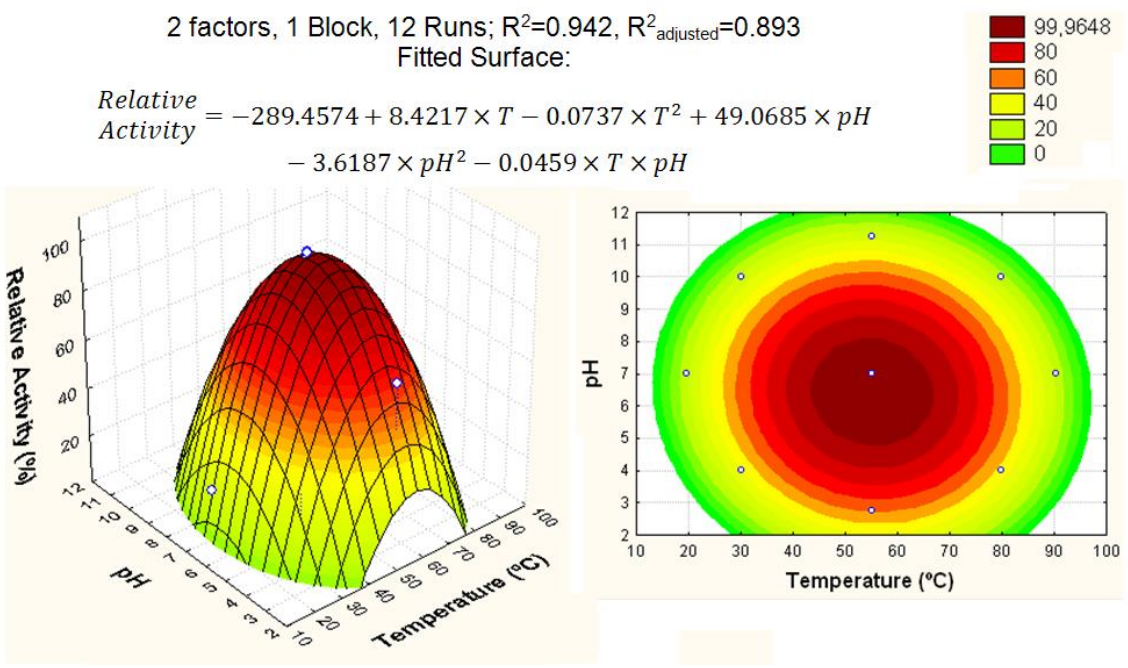


Figure 32 - Response surface and contour plot of *B. subtilis* α-amylase relative enzymatic activity as function of pH and temperature.

As showed in Figure 29 and Figure 30, it was later observed in the experiments with the intracellular extract of LAC-EXSIG and LAC-INTAMY that the buffer used to adjust the pH was not strong enough to correct the pH value to those presented in Table 5 of Materials and Methods. The (-1) point had a pH of 5.2 instead of 4.0; the (+1) point had a pH of 9.2 instead of 10.0; the (-1.41421) point had a pH of 4.1 instead of 2.75; and the (+1.41421) point had a pH of 9.9 instead of 11.24.

Based on the obtained surface equations of each enzyme, the critical points with highest activity were calculated and the values of optimal temperatures and pH are summarized in Table 8. The optimal temperature for the recombinant α-amylases was slightly lower than the one for the natural *B. subtilis* α-amylase. In another way, the optimal pH of the recombinant enzymes was somewhat higher.

Table 8 – Estimated optimal temperature and pH for the α-amylases produced.

	Optimal Temperature (ºC)	Optimal pH
LAC-EXAMY 4	50.92	6.75
LAC- EXSIG 2	52.93	7.24
LAC-INTAMY 1	50.84	7.13
LAC-INTSIG 24	52.77	7.21
B. subtilis	55.14	6.43

This initial characterization of the enzyme is not ideal, because it uses the crude extract of the fermented broth or the intracellular extract to measure the enzymatic activity. To improve these results it is suggested that the enzymes are purified in a future study and then retested for the optimal pH and temperature. Also, the pH variation that occurred with the intracellular buffer in LAC-INTAMY and LAC-EXSIG could have interfered in the estimated values of these two experiments. It would also be interesting to perform a validation of the estimated optimal points by performing a test in these optimal conditions and comparing to the projected values.

The values reported in the literature for the optimal temperature and pH of other α-amylases from *Bacillus subtilis* (Table 9) vary depending on the source. However, they tend to fluctuate in a certain common range (the mean observed temperature was 60 ºC and the pH was 6.5).

Many of these authors used bacterial strains isolated from the environment. These isolated strains might contain mutated forms of the amylolytic enzyme, which could cause these differences in enzymatic properties. Furthermore, in the case of the recombinant producers, differences in signal peptide processing, gene construction (protein truncation of C-terminal) and post-translational glycosylation might alter protein molecular size and activity.

Table 9 - *B. subtilis* α-amylase characteristics from different studies.

Producing strain	Strain isolated from	Gene from	Optimal Temperature (ºC)	Opti-mal pH	Molecular Weight (kDa)	Reference
B. subtilis US116	soil	-	65	6.0	60	MESSAOUD *et al.*, 2004
B. subtilis	-	-	65-70	6.0	46	MITSUIKI *et al.*, 2005
B. subtilis	canned sausages	-	55	6.5	-	MITRICA & GRANUM, 1979 *
B. subtilis	-	-	60-65	6.8	55	YAMANE *et al.*, 1973 *
B. subtilis 65	soil	-	60-65	6.0	68	HAYASHIDA *et al.*, 1988 *
B. subtilis JS-2004	-	-	70	8.0	-	ASGHER *et al.*, 2007
B. subtilis AX20	soil	-	55	6.0	139-149 (78kDa ho-modimer)	NAJAFI *et al.*, 2005
Bacillus subtilis 1	soil	-	45.0	6.0	-	ABDEL-NABY *et al.*, 1998
B. subtilis PY22	-	-	50	-	-	KARAKAŞ *et al.*, 2010
P. pastoris	-	*B. subtilis* PY22	60	7.0	50-64 (degly-cosylated)	KARAKAŞ *et al.*, 2010
E. coli	-	*B. subtilis* (truncated gene)	50	6.5	48	MARCO *et al.*, 1996 **
B. subtilis Ba-04	-	-	50	5.4-6.4	-	SALVA & MORAES, 1995 ***
B. subtilis	flour mill waste	-	80	5.6	54.78	UGURU *et al.*, 1998 ***

* Cited by SATYANARAYANA *et al.*, 2006

** Cited by GUPTA *et al.*, 2003

*** Cited by PANDEY *et al.*, 2000

Another great difference observed among studies is the molecular size. Usually multiple forms of bacterial α-amylases and fungal glucoamylases are present in the culture supernatant free of cells. Specifically in *B. subtilis*, the α-amylase may present different molecular sizes due to the action of proteases which act at the C terminal of the protein and cause a truncation of 186aa (MITSUIKI *et al.*, 2005). Estimating the molecular size of the recombinant proteins produced in this dissertation based on their nucleotide sequence, the predicted non-glycosylated α-amylase should have around 72.39 kDa (with signal peptide) or 68.89 kDa (mature protein).

Among these reviewed articles, the α-amylase most similar to the ones presented in this work is the enzyme produced with *B. subtilis* PY22 by KARAKAŞ

et al. (2010). It presented an optimal temperature of 50 ºC and had a gene sequence 99% identical to the sequence from strain 168 (which is also called strain OI1085, whose sequence is identical to the ones from EXAMY and EXSIG). These researchers also cloned the *B. subtilis* PY22 *amyE* gene to produce the enzyme in *Pichia pastoris*. This recombinant enzyme had optimal activity at 60 ºC and pH 7.0 and presented a molecular weight of 50-64 kDa after deglycosylation.

6 CONCLUSIONS AND PERSPECTIVES

The present work achieved its main objective which was to produce a *Bacillus subtilis* α-amylase in *Kluyveromyces lactis*. The vectors were correctly constructed, except for INTAMY, which presented a non-synonymous point mutation verified by sequencing. Yeast cells were successfully transformed and positive colonies were detected by PCR and enzymatic activity on agar plates. At this point it could be observed that the bacterial signal sequence was recognized by the yeast cell and that the presence of both the leader sequence and the signal sequence interfered with proper protein secretion. Shake flask cultivations were also performed to see their performance in submerged fermentation. One of the strains was also selected to be cultivated in different carbon sources. It presented a faster growth in glucose (μ_{max}=0.3827 h^{-1}), but also presented good growth in sucrose and lactose, enabling its cultivation in substrates such as sugarcane molasses and cheese whey. Higher enzyme production was achieved with lactose (2207 U/L) and galactose (2552 U/L) as the carbon source. A basal expression of approximately 438 U/L was observed even without induction. The recombinant enzymes were partially characterized to determine their optimum pH and temperature. They ranged between 50.84-52.93 ºC and pH 6.75-7.24, which are comparable to other *B. subtilis* α-amylases reported in the literature. In this range of temperature the α-amylase would still not be suitable for ethanol production, but it could be used in other industrial procedures such as food processing and textile treatment.

The insights obtained in this project incite further investigations to continue this work, which can be divided in three main branches:

Molecular Biology

- Perform a codon optimization of the α-amylase gene, altering rare codons to better reflect the codon usage of *K. lactis*, thus, facilitating the translational step and possibly enhancing expression.
- Do a protein design, changing amino acids to enhance protein activity and stability.
- Investigate why the LAC-EXSIG strains could not secrete the enzyme by performing proteomic and transcriptomic analysis. Also, proteomic and

transcriptomic experiments might be useful to identify stressful conditions suffered by the cells during fermentation.

- The α-amylase signal sequence, as observed in the LAC-INTSIG transformants, can be recognized by the yeast and used as a novel tool for signaling heterologous protein secretion in *K. lactis*. Its usefulness can be tested at first with a reporter gene, such as GFP or luciferase, and then with another exogenous gene for the production of the desired recombinant protein.
- Engineer a *K. lactis* strain with reduced level of proteases and enhanced number of chaperones, to confer a super-secreting phenotype.

Fermentation

- Study a better broth composition for optimal growth and enzyme production. Also, study the use of byproducts such as cheese way and sugar cane molasses as substrate.
- Scale-up fermentation to a bioreactor.
- Optimize culture operation in a fed-batch mode with a two-step fermentation: at first a growth phase to accumulate biomass and then and induction phase for enzyme production.
- Use other strains of *K. lactis* as host for enzyme production and even other yeasts, such as *Pichia pastoris* and *Saccharomyces cerevisiae*.
- Perform sequential fermentations reusing the same biomass. After an induction phase, the fermented broth is separated from cells and they are used again for another cycle of induction.

Biochemistry

- Establish a series of purification steps to obtain the protein with the desired purity for industrial application and for biochemical characterization.
- Perform a better characterization of the enzyme, determining its molecular weight (analyzing if occurs correct processing of signal peptide and if the protein is glycosylated), temperature and pH stability (thus, determining the best storage conditions), kinetic parameters (K_m and v_{max}), calcium ions dependency, inhibition by other molecules, among others properties.
- Immobilization of the enzyme for re-utilization in subsequent cycles.

7 REFERENCES

ABDEL-NABY, M. A.; HASHEM, A. M.; ESAWY, M. A.; ABDEL-FATTAH, A. F. Immobilization of Bacillus subtilis α-amylase and characterization of its enzymatic properties. **Microbiological research**, v. 153, n. 4, p. 319–325, 1998.

AIBA, S.; KITAI, K.; IMANAKA, T. Cloning and expression of thermostable α-amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*. **Applied and Environmental Microbiology**, v. 46, n. 5, p. 1059–1065, 1983.

ALI, M. BEN; KHEMAKHEM, B.; ROBERT, X.; HASER, R.; BEJAR, S. Thermostability enhancement and starch breakdown-profile change of the maltohexaose-forming-amylase of *B. stearothermophilus* US100 strain. **Biochemical Journal**, v. 394, p. 51–56, 2006.

ALIKHAJEH, J.; KHAJEH, K.; NADERI-MANESH, M.; RANJBAR, B.; SAJEDI, R. H.; NADERI-MANESH, H. Kinetic analysis, structural studies and prediction of pKa values of *Bacillus* KR-8104 α-amylase: The determinants of pH-activity profile. **Enzyme and Microbial Technology**, v. 41, n. 3, p. 337–345, 2007.

ALTINTAŞ, M. M.; ÜLGEN, K. O.; KIRDAR, B.; ÖNSAN, Z. I.; OLIVER, S. G. Improvement of ethanol production from starch by recombinant yeast through manipulation of environmental factors. **Enzyme and Microbial Technology**, v. 31, n. 5, p. 640–647, 2002.

ARRUDA, A. **Utilização do promotor do gene PGK1 de *Pichia pastoris* para expressão heteróloga**. Master dissertation presented to the graduation program in Cellular Biology of Universidade de Brasília, 2008.

ASGHER, M.; ASAD, M. J.; RAHMAN, S. U.; LEGGE, R. L. A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. **Journal of Food Engineering**, v. 79, n. 3, p. 950–955, 2007.

AUSUBEL, F. M.; BRENT, R.; KINGSTON, R. E.; *et al.* (EDS.). **Current Protocols in Molecular Biology**. 1st ed. London: John Wiley & Sons Inc, 2003.

BARBA, D.; BEOLCHINI, F.; RE, G. DEL; GIACOMO, G. DI; VEGLIÓ, F. Kinetic analysis of Kluyveromyces lactis fermentation on whey: batch and fed-batch operations. **Process Biochemistry**, v. 36, n. 6, p. 531–536, 2001.

BARTKEVICIUTE, D.; SASNAUSKAS, K. Studies of yeast *Kluyveromyces lactis* mutations conferring super-secretion of recombinant proteins. **Yeast (Chichester, England)**, v. 20, n. 1, p. 1–11, 2003.

BAYSAL, Z.; UYAR, F.; AYTEKIN, C. Solid state fermentation for production of α-amylase by a thermotolerant Bacillus subtilis from hot-spring water. **Process Biochemistry**, v. 38, p. 1665–1668, 2003.

BEMILLER, J.; WHISTLER, R. (EDS.). **Starch: Chemistry and Technology**. 3rd ed. New York: Elsevier, 2009.

BERTOLDO, C.; ANTRANIKIAN, G. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. **Current Opinion in Chemical Biology**, v. 6, n. 2, p. 151–160, 2002.

BIROL, G.; ÖNSAN, Z. I.; KIRDAR, B.; OLIVER, S. G. Ethanol production and fermentation characteristics of recombinant Saccharomyces cerevisiae strains grown on starch. **Enzyme and Microbial Technology**, v. 22, n. 8, p. 672–677, 1998.

BÖER, E.; STEINBORN, G.; KUNZE, G. Yeast expression platforms. **Applied Microbiology and Biotechnology**, v. 77, p. 513–523, 2007.

BOLHUIS, A.; TJALSMA, H.; SMITH, H. E.; DE JONG, A.; MEIMA, R.; VENEMA, G.; BRON, S.; VAN DIJL, J. M. Evaluation of bottlenecks in the late stages of protein secretion in Bacillus subtilis. **Applied and Environmental Microbiology**, v. 65, n. 7, p. 2934–2941, 1999.

BURGESS, R. R.; DEUTSCHER, M. P. (EDS.). **Methods in Enzymology - Volume 463 - Guide to Protein Purification**. 2nd ed. San Diego: Academic Press, 2009.

CALADO, V.; MONTGOMERY, D. **Planejamento de Experimentos usando o Statistica**. Rio de Janeiro: E-Papers Serviços Editoriais, 2003.

CAREY, P. R. (ED.). **Protein Engineering and Design**. London: Academic Press, 1996.

CEREGHINO, G. P. L.; CEREGHINO, J. L.; ILGEN, C.; CREGG, J. M. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. **Current Opinion in Biotechnology**, v. 13, n. 4, p. 329–332, 2002.

CHOI, D. B.; PARK, E. Y. Enhanced production of mouse α-amylase by feeding combined nitrogen and carbon sources in fed-batch culture of recombinant *Pichia pastoris*. **Process Biochemistry**, v. 41, n. 2, p. 390–397, 2006.

DEMAIN, A. L.; VAISHNAV, P. Production of recombinant proteins by microbes and higher organisms. **Biotechnology Advances**, v. 27, n. 3, p. 297–306, 2009.

DONG, G.; VIEILLE, C.; SAVCHENKO, A.; ZEIKUS, J. G. Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. **Applied and Environmental Microbiology**, v. 63, n. 9, p. 3569–3576, 1997.

FUJIMOTO, Z.; TAKASE, K.; DOUI, N.; MOMA, M.; MATSUMOTO, T.; MIZUNO, H. Crystal structure of a catalytic-site mutant alpha-amylase from Bacillus subtilis complexed with maltopentaose. **Journal of molecular biology**, v. 277, n. 2, p. 393–407, 1998.

FUWA, H. A new method for microdetermination of amylase activity by the use of amylose as the substrate. **The Journal of Biochemistry**, v. 41, n. 5, p. 583–603, 1954.

GALDINO, A. S.; ULHOA, C. J.; MORAES, L. M. P.; *et al.* Cloning, molecular characterization and heterologous expression of AMY1, an α-amylase gene from Cryptococcus flavus. **FEMS Microbiology Letters**, v. 280, n. 2, p. 189–194, 2008.

GRAF, A.; DRAGOSITS, M.; GASSER, B.; MATTANOVICH, D. Yeast systems biotechnology for the production of heterologous proteins. **FEMS Yeast Research**, v. 9, n. 3, p. 335–348, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19341379>. .

GUPTA, R.; GIGRAS, P.; MOHAPATRA, H.; GOSWAMI, V. K.; CHAUHAN, B. Microbial α-amylases: a biotechnological perspective. **Process Biochemistry**, v. 38, n. 11, p. 1599–1616, 2003.

HORVÁTHOVÁ, V.; JANECEK, S.; STURDÍK, E. Amylolytic enzymes: molecular aspects of their properties. **General physiology and biophysics**, v. 20, n. 1, p. 7–32, 2001.

ITO, H.; FUKUDA, Y.; MURATA, K.; KIMURA, A. Transformation of intact yeast cells treated with alkali cations. **Journal of Bacteriology**, v. 153, n. 1, p. 163–168, 1983.

JORGENSEN, S.; VORGIAS, C. E.; ANTRANIKIAN, G. Cloning, sequencing, characterization, and expression of an extracellular alpha-amylase from the hyperthermophilic archaeon Pyrococcus furiosus in Escherichia coli and Bacillus subtilis. **The Journal of Biological Chemistry**, v. 272, n. 26, p. 16335–16342, 1997.

JUGE, N.; ANDERSEN, J. S.; TULL, D.; ROEPSTORFF, P.; SVENSSON, B. Overexpression, purification, and characterization of recombinant barley α-amylases 1 and 2 secreted by the methylotrophic yeast Pichia pastoris. **Protein Expression and Purification**, v. 8, n. 2, p. 204–214, 1996.

KARAKAŞ, B.; İNAN, M.; CERTEL, M. Expression and characterization of *Bacillus subtilis* PY22 α-amylase in *Pichia pastoris*. **Journal of Molecular Catalysis B: Enzymatic**, v. 64, n. 3-4, p. 129–134, 2010.

KATO, S.; ISHIBASHI, M.; TATSUDA, D.; TOKUNAGA, H.; TOKUNAGA, M. Efficient expression, purification and characterization of mouse salivary alpha-amylase secreted from methylotrophic yeast, Pichia pastoris. **Yeast**, v. 18, n. 7, p. 645–655, 2001.

KIM, D.-O.; PARK, K.; SONG, J.-W.; SEO, J.-H. Enhanced production of maltotetraose-producing amylase by recombinant Bacillus subtilis LKS88 in fed-batch cultivation. **Journal of Microbiology and Biotechnology**, v. 7, n. 6, p. 417–422, 1997.

KONSOULA, Z.; LIAKOPOULOU-KYRIAKIDES, M. Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from Bacillus subtilis. **Process Biochemistry**, v. 41, p. 343–349, 2006a.

KONSOULA, Z.; LIAKOPOULOU-KYRIAKIDES, M. Thermostable α-amylase production by Bacillus subtilis entrapped in calcium alginate gel capsules. **Enzyme and Microbial Technology**, v. 39, p. 690–696, 2006b.

LEHNINGER, A. L.; NELSON, D. L.; COX, M. M. **Princípios de Bioquímica**. 4th ed. São Paulo: Sarvier (Almed), 2006.

LI, S.; SHEN, W.; CHEN, X.; SHI, G.; WANG, Z. Secretory expression of *Rhizopus oryzae* α-amylase in *Kluyveromyces lactis*. **African Journal of Biotechnology**, v. 10, n. 20, p. 4190–4196, 2011a.

LI, S.; SING, S.; WANG, Z. Improved expression of Rhizopus oryzae α-amylase in the methylotrophic yeast Pichia pastoris. **Protein Expression and Purification**, v. 79, n. 1, p. 142–148, 2011b.

LIAO, B.; HILL, G. A.; ROESLER, W. J. Amylolytic activity and fermentative ability of *Saccharomyces cerevisiae* strains that express barley α-amylase. **Biochemical Engineering Journal**, v. 53, n. 1, p. 63–70, 2010.

LINDEN, A.; NIEHAUS, F.; ANTRANIKIAN, G. Single-step purification of a recombinant thermostable α-amylase after solubilization of the enzyme from insoluble aggregates. **Journal of Chromatography B: Biomedical Sciences and Applications**, v. 737, n. 1-2, p. 253–259, 2000.

MANONMANI, H. K.; KUNHI, A. A. M. Interference of thiol-compounds with dextrinizing activity assay of a-amylase by starch-iodine colour reaction : Modification of the method to eliminate this interference. **World Journal of Microbiology and Biotechnology**, v. 15, p. 485–487, 1999.

MASUKO, T.; MINAMI, A.; IWASAKI, N.; MAJIMA, T.; NISHIMURA, S.-I; LEE, Y. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. **Analytical biochemistry**, v. 339, p. 69–72, 2005.

MESSAOUD, E. BEN; ALI, M. BEN; ELLEUCH, N.; MASMOUDI, N. F.; BEJAR, S. Purification and properties of a maltoheptaose- and maltohexaose-forming amylase produced by Bacillus subtilis US116. **Enzyme and Microbial Technology**, v. 34, p. 662–666, 2004.

MICOLONGHI, C.; CORSI, E.; CONTE, R.; BIANCHI, M. M. Heterologous products from the yeast Kluyveromyces lactis: exploitation of KlPDC1, a single-gene based system. In: MENDEZ-VILLAS, A. (Ed). **Communicating Current Research and Educational Topics and Trends in Applied Microbiology**. FORMATEX, p. 271-282, 2007.

MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, v. 31, n. 3, p. 426–428, 1959.

MINISTÉRIO DO DESENVOLVIMENTO INDÚSTRIA E COMÉRCIO EXTERIOR. Aliceweb. Available in: <http://aliceweb2.mdic.gov.br/>. Accessed in: 08/06/12.

MITSUIKI, S.; MUKAE, K.; SAKAI, M.; GOTO, M.; HAYASHIDA, S.; FURUKAWA, K. Comparative characterization of raw starch hydrolyzing α-amylases from various strains. **Enzyme and Microbial Technology**, v. 37, n. 4, p. 410–416, 2005.

MONTAÑO, I. D. C. **Otimização dinâmica do cultivo semi-contínuo de *Pichia pastoris* recombinante para produção de enzimas heterólogas**. Master dissertation presented to the graduation program in Chemical Engineering of Universidade Federal de São Carlos, 2010.

MONTESINO, R.; GARCÍA, R.; QUINTERO, O.; CREMATA, J. A. Variation in N-linked oligosaccharide structures on heterologous proteins secreted by the methylotrophic yeast Pichia pastoris. **Protein expression and purification**, v. 14, n. 2, p. 197–207, 1998.

MORAES, L. M. P. DE; ASTOLFI-FILHO, S.; OLIVER, S. G. Development of yeast strains for efficient utilisation of starch: evaluation of constructs that express a-amylase and glucoamylase separetely or as a bifunctional fusion proteins. **Applied Microbiology and Biotechnology**, v. 43, p. 1067–1076, 1995.

MUKHERJEE, A. K.; BORAH, M.; RAI, S. K. To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by Bacillus subtilis DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α -amylase in laundry deterge. **Biochemical Engineering Journal**, v. 43, p. 149–156, 2009.

NAJAFI, M. F.; DEOBAGKAR, DILEEP; DEOBAGKAR, DEEPTI. Purification and characterization of an extracellular α-amylase from Bacillus subtilis AX20. **Protein expression and purification**, v. 41, p. 349–354, 2005.

NAKANO, A.; LEE, C. Y.; YOSHIDA, A.; MATSUMOTO, T.; SHIOMI, N.; KATOH, S. Effects of methanol feeding methods on chimeric α-amylase expression in continuous culture of Pichia pastoris. **Journal of Bioscience and Bioengineering**, v. 101, n. 3, p. 227–231, 2006.

NIGAM, P.; SINGH, D. Enzyme and microbial systems involved in starch processing. **Enzyme and Microbial Technology**, v. 17, p. 770–778, 1995.

NIU, D.; ZUO, Z.; SHI, G.-Y.; WANG, Z.-X. High yield recombinant thermostable α-amylase production using an improved Bacillus licheniformis system. **Microbial Cell Factories**, v. 8, n. 58, 2009.

PAIFER, E.; MARGOLLES, E.; CREMATA, J.; MONTESINO, R.; HERRERA, L.; DELGADO, J. M. Efficient expression and secretion of recombinant alpha amylase in Pichia pastoris using two different signal sequences. **Yeast**, v. 10, n. 11, p. 1415–1419, 1994.

PANDEY, A.; NIGAM, P.; SOCCOL, C. R.; SOCCOL, V. T.; SINGH, D.; MOHAN, R. Advances in microbial amylases. **Biotechnology Applied Biochemistry**, v. 31, p. 135–152, 2000.

POLITZER, K.; BON, E. P. S. Enzimas Industriais e Especiais. Available in: <http://www.anbio.org.br/pdf/2/tr03_enzimas.pdf>. Accessed in: 08/08/12.

PORRO, D.; SAUER, M.; BRANDUARDI, P.; MATTANOVICH, D. Recombinant Protein Production in Yeasts. **Molecular Biotechnology**, v. 31, p. 245–259, 2005.

PRAKASH, O.; JAISWAL, N. α-Amylase: an ideal representative of thermostable enzymes. **Applied Biochemistry and Biotechnology**, v. 160, n. 8, p. 2401–2414, 2010.

RAJAGOPALAN, G.; KRISHNAN, C. α-Amylase production from catabolite derepressed Bacillus subtilis KCC103 utilizing sugarcane bagasse hydrolysate. **Bioresource Technology**, v. 99, p. 3044–3050, 2008.

RASHID, N.; AHMED, N.; HAIDER, M. S.; HAQUE, I. Effective solubilization and single-step purification of *Bacillus licheniformis* α-amylase from insoluble aggregates. **Folia Microbiologica**, v. 55, n. 2, p. 133–136, 2010.

RODRIGUES, M. I.; IEMMA, A. F. **Planejamento de Experimentos e Otimização de Processos - Uma estratégia sequencial de planejamentos**. 1st ed. Campinas, SP: Editora Casa do Pão, 2005.

ROMANOS, M. A; SCORER, C. A; CLARE, J. J. Foreign gene expression in yeast: a review. **Yeast (Chichester, England)**, v. 8, n. 6, p. 423–88, 1992.

ROY, J. K.; RAI, S. K.; MUKHERJEE, A. K. Characterization and application of a detergent-stable alkaline α-amylase from Bacillus subtilis strain AS-S01a. **International Journal of Biological Macromolecules**, v. 50, n. 1, p. 219–229, 2012.

RUBIO-TEXEIRA, M. A comparative analysis of the GAL genetic switch between not-so-distant cousins: *Saccharomyces cerevisiae* versus *Kluyveromyces lactis*. **FEMS Yeast Research**, v. 5, p. 1115–1128, 2005.

RUOHONEN, L.; HACKMAN, P.; LEHTOVAARA, P.; KNOWLES, J. K. C.; KERANEN, S. Efficient secretion of Bacillus amyloliquefaciens a-amylase cells by its own signal peptide from Saccharomyces cerevisiae host. **Gene**, v. 59, p. 161–170, 1987.

RYDBERG, E. H.; SIDHU, G.; VO, H. C.; HEWITT, J.; CÔTÉ, H. C. F.; WANG, Y.; NUMAO, S.; MACGILLIVRAY, R. T. A.; OVERALL, C. M.; BRAYER, G. D.; WITHERS, S. G. Cloning, mutagenesis, and structural analysis of human pancreatic α-amylase expressed in Pichia pastoris. **Protein Science**, v. 8, p. 635–643, 1999.

SAMBROOK, J.; RUSSELL, D. W. **Molecular Cloning - A Laboratory Manual - Vol. 1, 2 and 3**. Third Edit ed. New York: Cold Spring Harbor Laboratory Press, 2001.

SARROUH, B.; SANTOS, T. M.; MIYOSHI, A.; DIAS, R.; AZEVEDO, V. Up-To-Date Insight on Industrial Enzymes Applications and Global Market. **Journal of Bioprocessing & Biotechniques**, S4:002, 2012.

SATYANARAYANA, T.; RAO, J. L. U. M.; EZHILVANNAN, M. α-Amylases. In: A. Pandey; C. Webb; C. R. Soccol; C. Larroche (Eds.); **Enzyme Technology**. 1st ed., Delhi: Springer - Asiatech, p.189–220, 2006.

SCHAFFRATH, R.; BREUNIG, K. D. Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. **Fungal Genetics and Biology**, v. 30, n. 3, p. 173–190, 2000.

SHARMA, A. D.; SINGH, J. A nonenzymatic method to isolate genomic DNA from bacteria and actinomycete. **Analytical Biochemistry**, v. 337, n. 2, p. 354–356, 2005.

SHIBUYA, I.; TAMURA, G.; SHIMA, H.; ISHIKA, T.; HARA, S. Construction of an α-amylase/glucoamylase fusion gene and its expression in Saccharomyces cerevisiae. **Bioscience, Biotechnology, and Biochemistry**, v. 56, n. 6, p. 884–889, 1992.

SIDHU, G. S.; SHARMA, P.; CHAKRABARTI, T.; GUPTA, J. Strain improvement for the production of a thermostable α-amylase. **Enzyme and Microbial Technology**, v. 21, n. 7, p. 525–530, 1997.

SISO, M. The biotechnological utilization of cheese whey: A review. **Bioresource Technology**, v. 57, n. 1, p. 1–11, 1996.

SOUZA, P. M. DE; MAGALHÃES, P. DE O. E. Application of microbial α-amylase in industry - a review. **Brazilian Journal of Microbiology**, v. 41, p. 850–861, 2010.

STRASSER, A.; SELK, R.; DOHMEN, R. J.; *et al.* Analysis of the a-amylase gene of *Schwanniomyces occidentalis* and the secretion of its gene product in transformants of different yeast genera. **European journal of Biochemistry**, v. 184, p. 699–706, 1989.

SUMITANI, J.-I.; NAGAE, H.; KAWAGUCHI, T.; ARAI, M. Bacillus animal type α-amylase: cloning and sequencing of the gene, and comparison of the deduced amino acid sequence with that of other amylases. **Journal of Fermentation and Bioengineering**, v. 85, n. 4, p. 428–432, 1998.

SUN, H.; ZHAO, P.; GE, X.; XIA, Y.; HAO, Z.; LIU, J.; PENG, M. Recent advances in microbial raw starch degrading enzymes. **Applied Biochemistry and Biotechnology**, v. 160, n. 4, p. 988–1003, 2010.

TJALSMA, H.; ANTELMANN, H.; JONGBLOED, J. D. H.; BRAUN, P. G.; DARMON, E.; DORENBOS, R.; DUBOIS, J.-Y. F.; WESTERS, H.; ZANEN, G.; QUAX, W. J.; KUIPERS, O. P.; BRON, S.; HECKER, M.; VAN DIJIL, J. A. Proteomics of protein secretion by Bacillus subtilis: separating the “secrets” of the secretome. **Microbiology and Molecular Biology Reviews**, v. 68, n. 2, p. 207–233, 2004.

TOKUNAGA, M.; ISHIBASHI, M.; TATSUDA, D.; TOKUNAGA, H. Secretion of Mouse a-amylase from *Kluyveromyces lactis*. **Yeast**, v. 13, p. 699–706, 1997.

TULL, D.; GOTTSCHALK, T. E.; SVENDSEN, I.; KRAMHØFT, B.; PHILLIPSON, B. A.; BISGÅRD-FRANTZEN, H.; OLSEN, O.; SVENSSON, B. Extensive N-glycosylation reduces the thermal stability of a recombinant alkalophilic *Bacillus* α-amylase produced in *Pichia pastoris*. **Protein Expression and Purification**, v. 21, n. 1, p. 13–23, 2001.

USDA. Corn: Food, seed, and industrial use. Available in: <http://www.ers.usda.gov>. Accessed in: 8/8/2012.

VAN OOYEN, A. J. J.; DEKKER, P.; HUANG, M.; OLSTHOOM, M. M. A.; JACOBS, D. I.; COLUSSI, P. A.; TARON, C. H. Heterologous protein production in the yeast Kluyveromyces lactis. **FEMS Yeast Research**, v. 6, n. 3, p. 381–392, 2006.

VERMA, G.; NIGAM, P.; SINGH, D.; CHAUDHAR, K. Bioconversion of starch to ethanol in a single-step process by coculture of amylolytic yeasts and Saccharomyces cerevisiae 21. **Bioresource Technology**, v. 72, n. 3, p. 261–266, 2000.

VIEILLE, C.; ZEIKUS, G. J. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. **Microbiology and Molecular Biology**, v. 65, n. 1, p. 1–43, 2001.

WONG, D. W. S.; BATT, S. B.; LEE, C. C.; ROBERTSON, G. H. Increased expression and secretion of recombinant α-amylase in Saccharomyces cerevisiae by using glycerol as the carbon source. **Journal of Protein Chemistry**, v. 21, n. 6, p. 419–425, 2002.

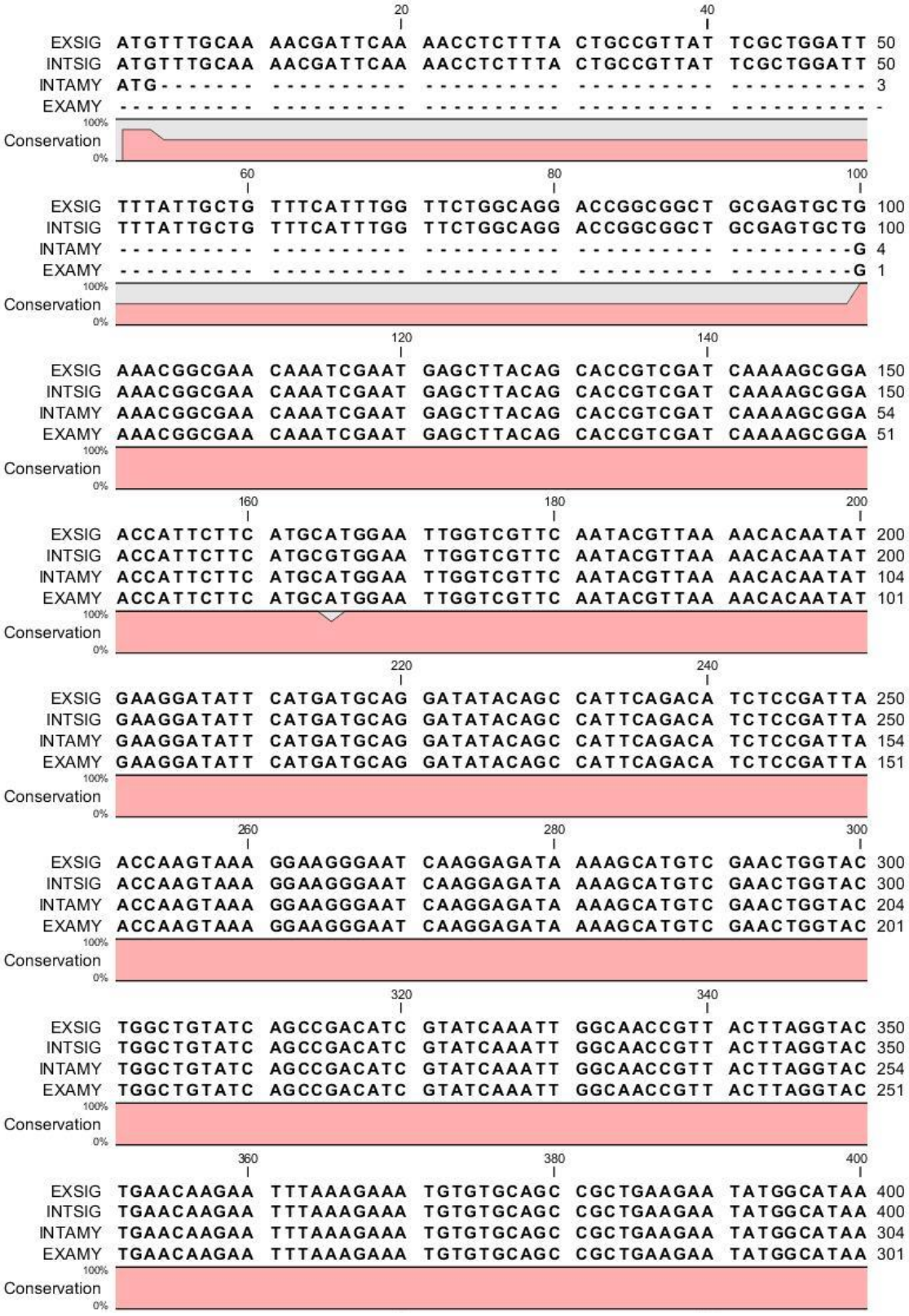
XIAO, Z.; STORMS, R.; TSANG, A. A quantitative starch – iodine method for measuring alpha-amylase and glucoamylase activities. **Analytical Biochemistry**, v. 351, p. 146–148, 2006.

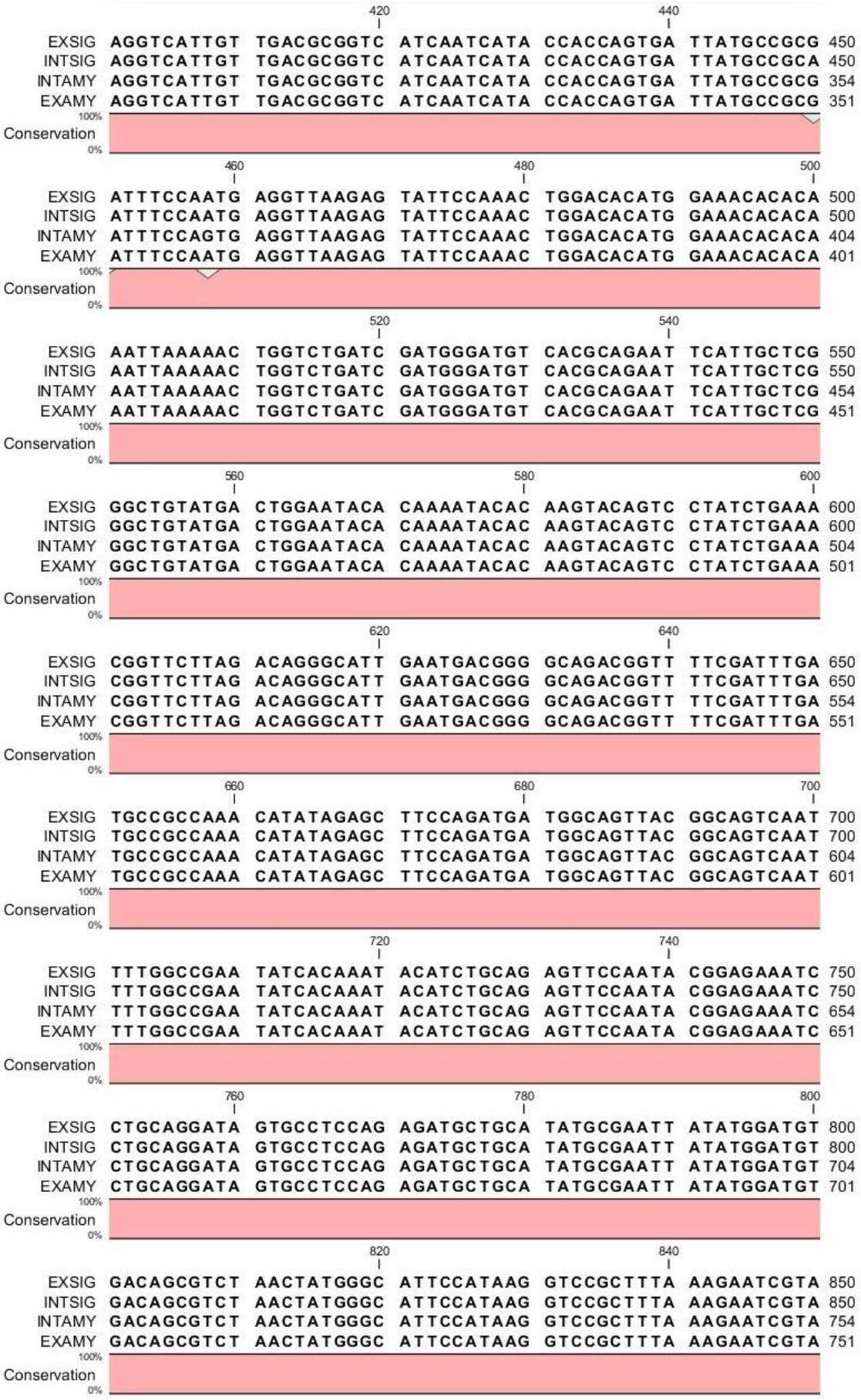
ZHANG, X.; XIA, Z.; ZHAO, B.; CEN, P. Enhancement of production of cloned α-amylase by lactic acid feeding from recombinant Saccharomyces cerevisiae using a SUC2 promoter. **Biotechnology Letters**, v. 23, p. 259–262, 2001.

ZIMBRO, M. J.; POWER, D. A.; MILLER, S. M.; WILSON, G. E.; JOHNSON, J. A. (EDS.). **Difco & BBL Manual - Manual of Microbiological Culture Media**. 2nd ed. Sparks: Becton, Dickinson and Company, 2009.

8 APPENDIX

Appendix 1 – DNA sequence alignment




420
440
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INTSIG AGGTCATTGT TGACGCGGTC ATCAATCATA CCACCAGTGA TTATGCCGCA 450
INTAMY AGGTCATTGT TGACGCGGTC ATCAATCATA CCACCAGTGA TTATGCCGCG 354
EXAMY AGGTCATTGT TGACGCGGTC ATCAATCATA CCACCAGTGA TTATGCCGCG 351
100%
Conservation
0%
460
480
500
EXSIG ATTTCCAATG AGGTTAAGAG TATTCCAAAC TGGACACATG GAAACACACA 500
INTSIG ATTTCCAATG AGGTTAAGAG TATTCCAAAC TGGACACATG GAAACACACA 500
INTAMY ATTTCCAGTG AGGTTAAGAG TATTCCAAAC TGGACACATG GAAACACACA 404
EXAMY ATTTCCAATG AGGTTAAGAG TATTCCAAAC TGGACACATG GAAACACACA 401
100%
Conservation
0%
520
540
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INTSIG AATTAAAAAC TGGTCTGATC GATGGGATGT CACGCAGAAT TCATTGCTCG 550
INTAMY AATTAAAAAC TGGTCTGATC GATGGGATGT CACGCAGAAT TCATTGCTCG 454
EXAMY AATTAAAAAC TGGTCTGATC GATGGGATGT CACGCAGAAT TCATTGCTCG 451
100%
Conservation
0%
560
580
600
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INTSIG GGCTGTATGA CTGGAATACA CAAAATACAC AAGTACAGTC CTATCTGAAA 600
INTAMY GGCTGTATGA CTGGAATACA CAAAATACAC AAGTACAGTC CTATCTGAAA 504
EXAMY GGCTGTATGA CTGGAATACA CAAAATACAC AAGTACAGTC CTATCTGAAA 501
100%
Conservation
0%
620
640
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INTSIG CGGTTCTTAG ACAGGGCATT GAATGACGGG GCAGACGGTT TTCGATTTGA 650
INTAMY CGGTTCTTAG ACAGGGCATT GAATGACGGG GCAGACGGTT TTCGATTTGA 554
EXAMY CGGTTCTTAG ACAGGGCATT GAATGACGGG GCAGACGGTT TTCGATTTGA 551
100%
Conservation
0%
660
680
700
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INTSIG TGCCGCCAAA CATATAGAGC TTCCAGATGA TGGCAGTTAC GGCAGTCAAT 700
INTAMY TGCCGCCAAA CATATAGAGC TTCCAGATGA TGGCAGTTAC GGCAGTCAAT 604
EXAMY TGCCGCCAAA CATATAGAGC TTCCAGATGA TGGCAGTTAC GGCAGTCAAT 601
100%
Conservation
0%
720
740
EXSIG TTTGGCCGAA TATCACAAAT ACATCTGCAG AGTTCCAATA CGGAGAAATC 750
INTSIG TTTGGCCGAA TATCACAAAT ACATCTGCAG AGTTCCAATA CGGAGAAATC 750
INTAMY TTTGGCCGAA TATCACAAAT ACATCTGCAG AGTTCCAATA CGGAGAAATC 654
EXAMY TTTGGCCGAA TATCACAAAT ACATCTGCAG AGTTCCAATA CGGAGAAATC 651
100%
Conservation
0%
760
780
800
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INTSIG CTGCAGGATA GTGCCTCCAG AGATGCTGCA TATGCGAATT ATATGGATGT 800
INTAMY CTGCAGGATA GTGCCTCCAG AGATGCTGCA TATGCGAATT ATATGGATGT 704
EXAMY CTGCAGGATA GTGCCTCCAG AGATGCTGCA TATGCGAATT ATATGGATGT 701
100%
Conservation
0%
820
840
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INTSIG GACAGCGTCT AACTATGGGC ATTCCATAAG GTCCGCTTTA AAGAATCGTA 850
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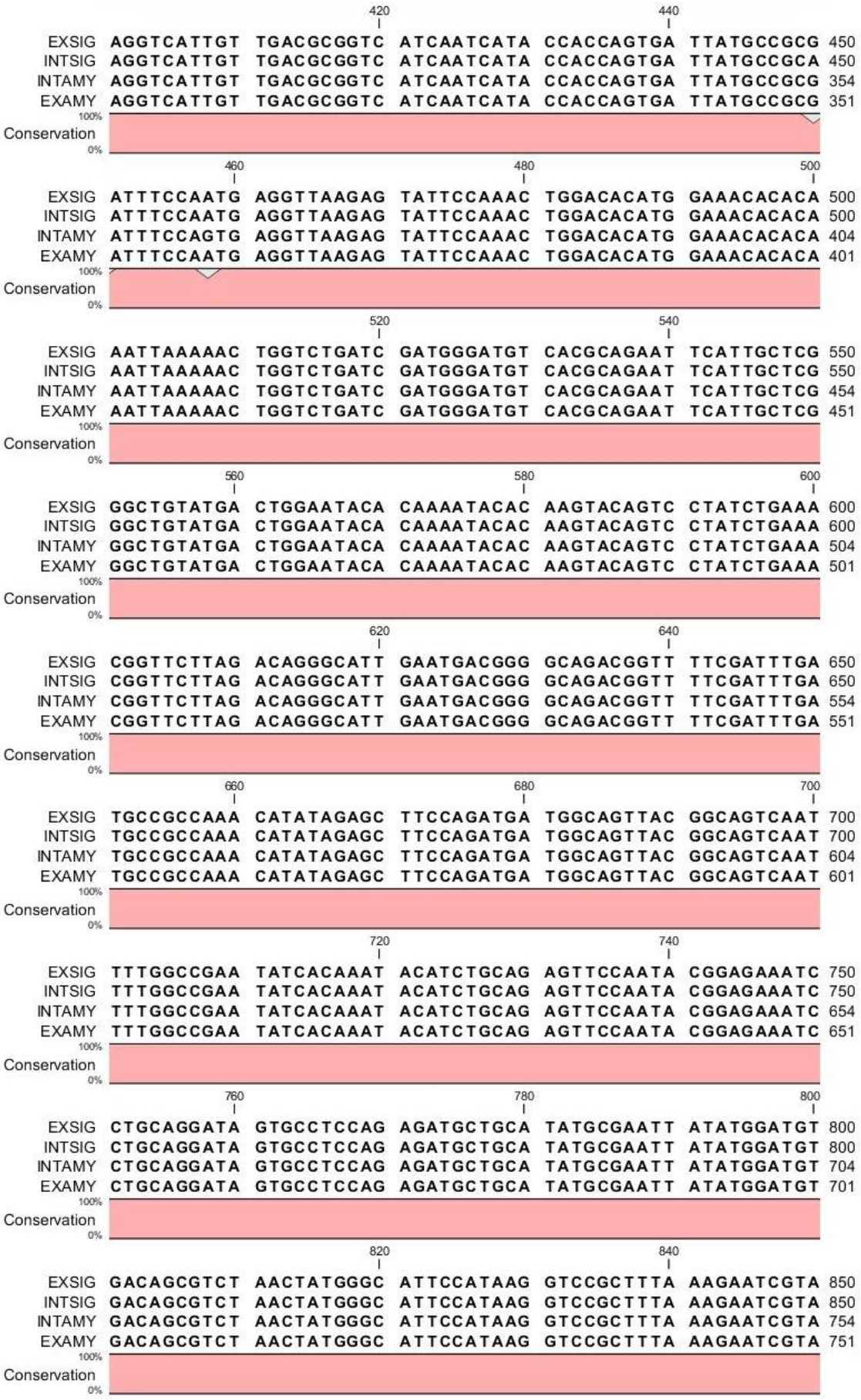
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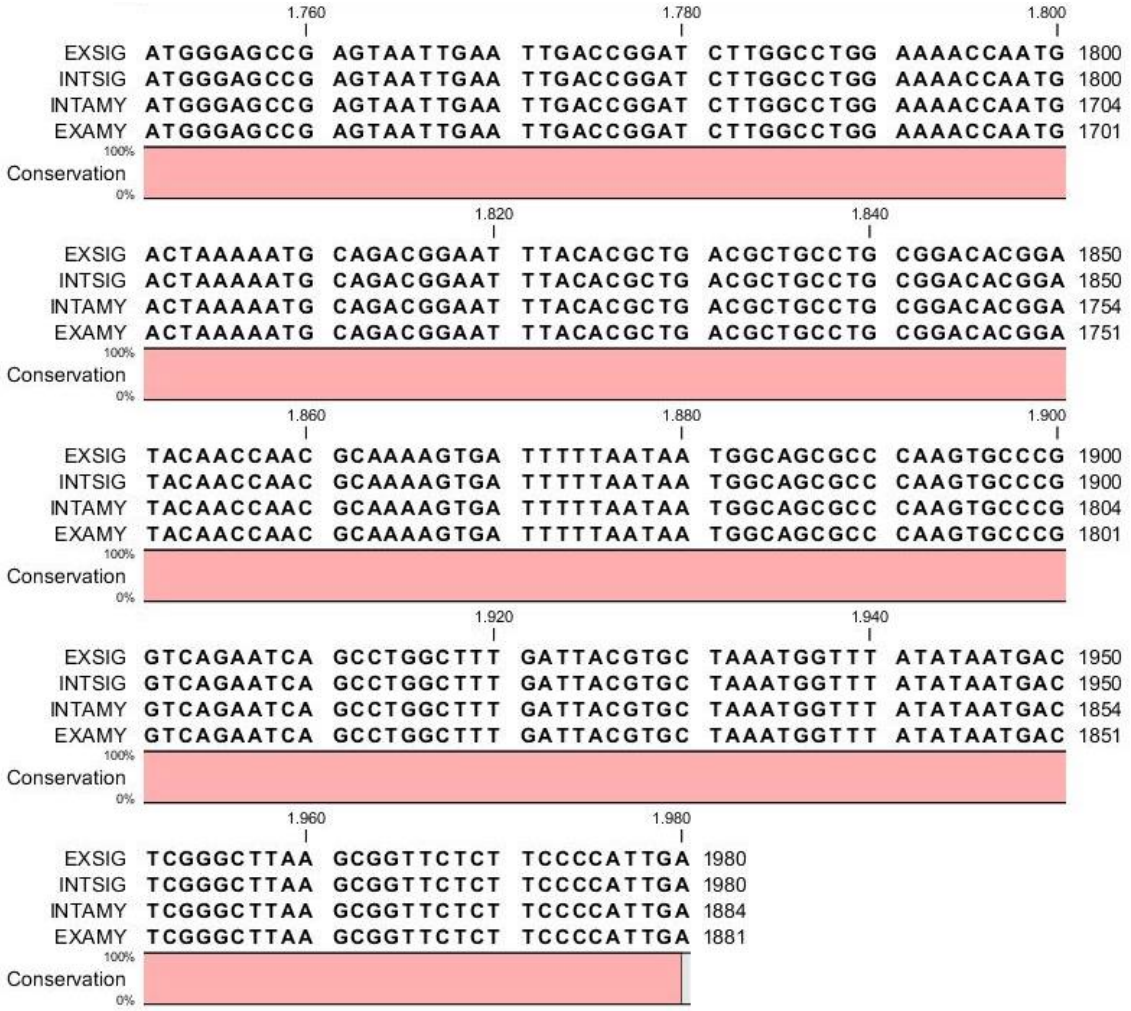
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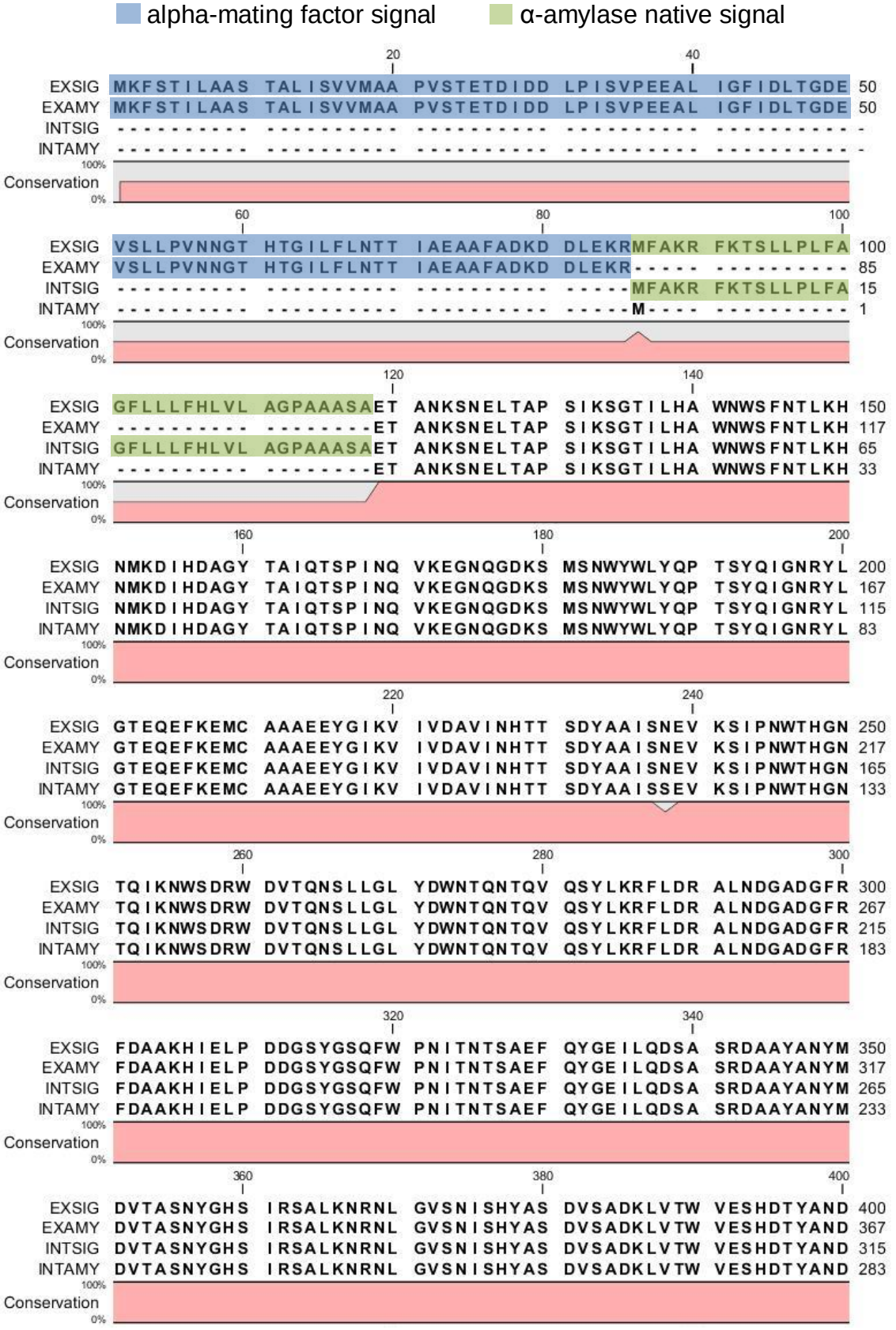
Appendix 2 – Protein sequence alignment, with alpha-mating factor signal for EXSIG and EXAMY.

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Conservation
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Appendix 3 – Calculation report.

Intracellular protein concentration or intracellular enzymatic activity:

The intracellular protein concentration was obtained as described below. The absorbance reading of the Bradford assay was used in the standard curve to calculate a concentration in mg of protein per L of intracellular extract. This result was multiplied by the dilution factor of the sample, the volume of intracellular extract, the cell concentration in the broth (in g of cell per L of broth) and divided by the amount of cells used for extraction (g of cell):

$$\underset{Result}{Curve}\left(\frac{mg\ protein}{L\ intracell.}\right) \times \underset{Factor}{Dilution} \times \underset{Extracted}{Volume}\ (L\ intracell.) \times \frac{1}{g\ cells} \times \frac{g\ cells}{L\ broth}$$

The enzymatic activity was obtained in a similar way. The absorbance reading of the iodine assay was used to calculate the starch concentration based on a standard curve and this value was used to calculate the enzymatic activity in U per L of intracellular extract. One enzymatic unit (U) was defined as the amount of enzyme necessary to hydrolyze 0.1 mg of soluble starch per minute at the reaction conditions (50ºC, 0.1M phosphate buffer pH 7.0, 2% soluble starch). Therefore:

$$1U = \frac{0.1\ mg\ of\ hydrolysed\ starch}{1min}$$

$$\frac{U}{L} = \frac{\{[Starch_{20g/L}] - [Sample]\}\left(\frac{mg}{L}\right) \times Vol_{starch}(\mu L)}{time\ (\min) \times Vol_{sample}(\mu L)}$$

Where: [*Starch*$_{20g/L}$] is the absorbance of the tube without enzymatic reaction; [*Sample*] is the starch concentration of the tube where the enzymatic reaction occurs; *Vol*$_{starch}$ is the volume of starch solution used for reaction; *time* is the amount of time the reaction occurred; and *Vol*$_{sample}$ is the sample volume used in reaction.

This result was multiplied by the dilution factor of the sample, the volume of intracellular extract, the cell concentration in the broth (in g of cell per L of broth) and divided by the amount of cells used for extraction (g of cell):

$$\underset{Result}{Activity}\left(\frac{U}{L\ intracell.}\right) \times \underset{Factor}{Dilution} \times \underset{Extracted}{Volume}\ (L\ intracell.) \times \frac{1}{g\ cells} \times \frac{g\ cells}{L\ broth}$$