JÚLIO CÉSAR CÂMARA ROSA

ENGENHARIA METABÓLICA DA LEVEDURA *Kluyveromyces lactis* PARA SÍNTESE DE ÁCIDO L-ASCÓRBICO (VITAMINA C)

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

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APROVADA: 25 de Abril de 2011

_____ _____

Profª. Denise Mara Soares Bazzolli
(Coorientadora)

Prof. Luciano Gomes Fietto
(Coorientador)

_____ _____

Profª. Elizabeth P. B. Fontes

Prof. Patrick Van Dijck

_____

Profª. Flávia Maria Lopes Passos
(Orientadora)

"A coisa mais indispensável a um homem é reconhecer o uso que deve fazer do seu próprio conhecimento"

Platão

À minha amada mãe, meu refúgio, que sempre me deu forças e inspiração para alcançar meus objetivos.

Ao meu grande e querido amigo Giuseppe pelo total apoio, companheirismo e incentivo.

A todos os amigos do Laboratório de Fisiologia de Microrganismos, pelo companheirismo e amizade durante a incessante busca pelo saber cientifico.

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BIOGRAFIA

JÚLIO CÉSAR CÂMARA ROSA, filho de Marina Câmara e Júlio Maria Peixoto Rosa, nasceu no dia 28 de julho de 1982, na cidade de Piranga, Minas Gerais.

Em agosto de 2000 iniciou o curso superior na Universidade Federal de Ouro Preto (UFOP) e em julho de 2004 graduou-se em Ciências Biológicas Habilitação Licenciatura sendo agraciado como aluno que mais se destacou durante o curso. Em Fevereiro de 2005 obteve o titulo de Bacharel em Ciências Biológicas pela mesma Instituição. Durante a graduação participou de programa de iniciação científica vinculada à linha de pesquisa Biologia Molecular das Hepatites Virais no Laboratório de Bioquímica e Biologia Molecular (LBBM). Durante a graduação também participou de programas de monitorias e de extensão, além de lecionar Biologia na Escola Estadual de Ouro Preto.

Em Março de 2005, ingressou no Programa de pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa (UFV), em nível de mestrado defendendo a dissertação em março de 2007. Em março do mesmo ano ingressou no Programa de Pós-Graduação, em nível de doutorado em Microbiologia Agrícola da UFV. No período de Maio 2009 a Julho 2010 participou do Programa de Doutorado com Estágio no Exterior (PDEE) na *Universiteit Katholieke Leuven* (KUL) na Bélgica financiado pela CAPES. Após a concretização dos trabalhos, submeteu-se à defesa de tese em 25 de abril de 2011.

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RESUMO

ROSA, Júlio César Câmara, D.Sc., Universidade Federal de Viçosa, abril de 2011. **Engenharia metabólica da levedura *Kluyveromyces lactis* para síntese de Ácido L-ascórbico (Vitamina C).** Orientadora: Flávia Maria Lopes Passos. Coorientadores: Denise Mara Soares Bazzolli e Luciano Gomes Fietto.

O Ácido L-ascórbico (L-AA), popularmente conhecido como vitamina C é naturalmente sintetizado pelas plantas a partir de D-glicose por uma via de 10 etapas. L-galactose é o intermediário chave para a biossíntese de L-ascórbico, cuja via de biossíntese foi recentemente elucidada. As leveduras produzem um composto análogo ao L-AA, o ácido D-eritroascórbico, mas em presença de um de seus precursors tais como L-galactose, L galactono-1,4-lactona, ou L-gulono-1 ,4-lactona, as leveduras são capazes de sintetizar o L-AA. Para evitar alimentar a cultura de levedura com o "L" enantiômero, a levedura *Kluyveromyces lactis* CBS2359 foi engenheirada com genes da via de biossíntese de L-galactose: GDP-manose-3 ,5-epimerase (GME), GDP-L-galactose fosforilase (VTC2) e L-galactose-1-fosfato fosfatase (VTC4) isolados de *Arabidopsis thaliana*. Com este objetivo plasmídeos foram construídos visando a integração por recombinação homóloga dos cassetes de expressão no Locus LAC4 (β-galactosidase) Após o processo de transformação, o promotor do gene LAC4 promove a transcrição do gene GME, enquanto que genes VTC2 e VTC4 estão sob o controle dos promotores GPD1 e ADH1 respectivamente provenientes da levedura *S. cerevisiae*. A expressão dos genes da via de biossíntese de L-galactose em *K. lactis* foi determinada por RT-PCR e western blot. As leveduras recombinantes foram capazes de produzir cerca de 23 $mg.L^{-1}$ de ácido L-ascórbico após 48 horas de cultivo, quando cultivados em meio YP suplementado com 2% (p/v) de D-galactose. A biossíntese de L-AA foi

também realizada quando as linhagens recombinantes foram cultivadas em meio de soro de queijo, fonte alternativa rica em lactose proveniente da indústria de laticínios.

Este trabalho é um dos primeiros relatos de engenharia metabólica na levedura *K. lactis* visando a biossíntese de ácido L-ascórbico por um processo fermentativo sem a adição de intermediários precursores no meio de cultura.

ABSTRACT

ROSA, Júlio César Câmara, D.Sc., Universidade Federal de Viçosa, April 2011. **Metabolic engineering of *Kluyveromyces lactis* for L-ascorbic acid (vitamin C) synthesis.** Adviser: Flávia Maria Lopes Passos. Co-Advisers: Denise Mara Soares Bazzolli and Luciano Gomes Fietto.

L-ascorbic acid is naturally synthesized in plants from D-glucose via a ten-step pathway. The branch pathway to synthesize L-galactose, the key intermediate for L-ascorbic biosynthesis, has been recently elucidated. Budding yeast is only able to synthesize L-ascorbic acid if it is cultivated in the presence of one of its precursors: L-galactose, L-galactono-1,4-lactone, or L-gulono-1,4-lactone extracted from plants or animals. To avoid feeding the yeast culture with this "L" enantiomer, we engineered *Kluyveromyces lactis* with L-galactose biosynthesis pathway genes: GDP-mannose-3,5-epimerase (GME), GDP-L-galactose phosphorylase (VTC2) and L-galactose-1-phosphate phosphatase (VTC4) isolated from *Arabidopsis thaliana*. Plasmids were constructed to target the cloned plant genes to the *K. lactis* LAC4 Locus by homologous recombination and the expression was associated to the growth of the cells on D-galactose or lactose. Upon *K. lactis* transformation, GME was under the control of the native LAC4 promoter while VTC2 and VTC4 genes were transcribed by the *S. cerevisiae* promoters GPD1 and ADH1 respectively. The expression in *K. lactis* of the endogenous L-galactose biosynthesis plant genes was determined by RT-PCR and western blotting. The recombinant yeasts were able to produce about 23 $mg.L^{-1}$ of L-ascorbic acid in 48 hours of cultivation when cultured on rich medium with 2% (w/v) D-galactose. We have also successfully evaluated the L-AA production culturing recombinant strains in cheese whey as an alternative source of lactose and which is a waste product during cheese production. This work is the first attempt to engineering *K.*

lactis cells for L-ascorbic acid biosynthesis through a fermentation process without any trace of “L” isomers precursors in the culture medium.

INTRODUCTION

The L-Ascorbic acid or vitamin C is a hydrosoluble vitamin derived from glucose metabolism. It is required for collagen fibers biosynthesis through hydroxylation of proline and lysine residues, and it also confers protection against deleterious effects caused by free radicals. However, humans are not able to synthesize L-ascorbic acid, therefore this essential compound must be obtained from the diet. Currently, most of the commercially available L-ascorbic acid is synthesized from glucose by the Reichstein method. In this process, the synthesis of L-ascorbic acid occurs from D-glucose which involves seven steps, six chemical steps and one fermentation step that presents an overall yield of 50%. Various stages of the Reichstein process use considerable quantities of organic and inorganic solvents and reagents. These include acetone, sulfuric acid and sodium hydroxide. Some companies have employed a modified Reichstein method in an attempt to minimize the use of such solvents.

The use of a biological alternative to chemical synthesis for L-ascorbic acid (L-AA) production is a technological strategy that follows the current order for sustainable development. Thus the interaction between the traditional industry of fermentation and recombinant DNA technology demonstrates that organic compounds can be produced by microorganisms in fermentation processes, although such organic compounds were previously only available through complex methods of extraction from plant or animal tissue with low yields and purity, and sometimes unsafe to be used by humans. So the use of microorganisms, the availability of several sequenced genomes associated to genetic engineering allow us to develop new strategies for L-AA production by fermentation.

Although L-AA naturally occurs in plants, yeasts do not present endogenous genes for its biosynthesis. However, they are capable of synthesizing D-erythroascorbic acid (D-EAA) that has antioxidant properties similar to L-AA. Under appropriate conditions, yeast cells can synthesize L-AA for D-EAA biosynthesis. Research with the yeast *K lactis* has increased due to its ability to assimilate lactose, ethanol and also to synthesize β-galactosidase enzyme (LAC4). The knowledge concerning the induction of LAC4 gene expression in *K. lactis* is the basis for the construction and operation of vectors for heterologous expression of proteins induced by lactose or D-galactose.

This project aimed at engineering *Kluyveromyces lactis* in order to make this yeast able to synthesize L-AA from D-galactosil residue generated by the assimilation of cheese whey or lactose. Genes and strategies for the engineering the L-AA biosynthesis pathway in this yeast have been mapped and cloned. In this study, we targeted genes from *Arabidopsis thaliana* that encode proteins whose enzymatic activities result in the production of the key intermediate L-galactose that are reported from *Arabidopsis thaliana*. In this manner, we evaluated the ability of *K. lactis* to host and express plant genes and also to produce L-AA by fermentation process.

Chapter 1

1. Background

Although required as nutrients in tiny amounts by an organism, vitamins are organic compounds that cannot be synthesized in sufficient quantities, therefore must be obtained from the diet (Lieberman & Breunig, 1990). Moreover, vitamins have diverse biochemical functions such as hormones precursors (vitamin D), antioxidants (vitamin E and C), and mediators of cell signaling and regulators, tissue growth and cell differentiation (vitamin A).

A great number of vitamins (B complex vitamins) function as coenzymes that act as catalysts and substrates in metabolism. In the first case, the vitamins must be bound to enzymes and they are called prosthetic groups (Bolander, 2006). The vitamins are classified by their biological and chemical activities, not their structure. In humans there are two groups of vitamins: 1) water-soluble; which comprises 9 compounds, 8 B vitamins (B complex vitamin) and vitamin C; and 2) fat soluble, represented by vitamins A, D, E and K. Generally the water-soluble vitamins are involved in enzymatic functions and metabolism control by their involvement in substances such as co-enzymes while the fat soluble tends to be used in the development and tissue maintenance (Rashida *et al*, 2009).

1.1 L-Ascorbic Acid (vitamin C)

The L-ascorbic acid (L-AA), widely known as L-xyloascorbic acid, 3-oxo-L-gulofuranolactone (enol form), L-3-ketothreohexuronic acid lactone, antiscorbutic vitamin and vitamin C, has the chemical formula $C_6H_8O_6$ and a molecular weight of 176.12. The vitamin C was discovered in the late 1920's by Albert Von Szent Györgyi who was awarded the Nobel Prize in 1937. Initially, the L-AA was isolated from adrenal glands (Svent-Gyorgi 1928) and afterwards characterized from plant tissues (Herbert et al., 1933; Svirbely & Svent-Gyorgi 1932, 1933; Waugh & King 1932). This acid is the most abundant water-soluble redox compound in plants and eukaryotic algae, and it is an essential compound with important roles as an antioxidant and as a modulator of plant development through hormone signaling (Pastori et al, 2003). It also plays a role in the response against pathogens and confers environmental stress protection as well such as UV radiation, ozone, and others. This compound presents multiple functions and its biosynthesis from D-glucose was first reported in rats (Loewus, 1999).

Structurally, L-AA is one of the rare compounds that present a hydroxyl group so acidic that is completely dissociated at neutral pH (carbon-3 hydroxyl pKa 4.2). This is related to the fact that ascorbic acid comprises two conjugated double bonds and that a resonance form can be written for the deprotonated monoanionic form. Thus, in nature the L-AA can be found in different resonance forms as shown in Figure 1.

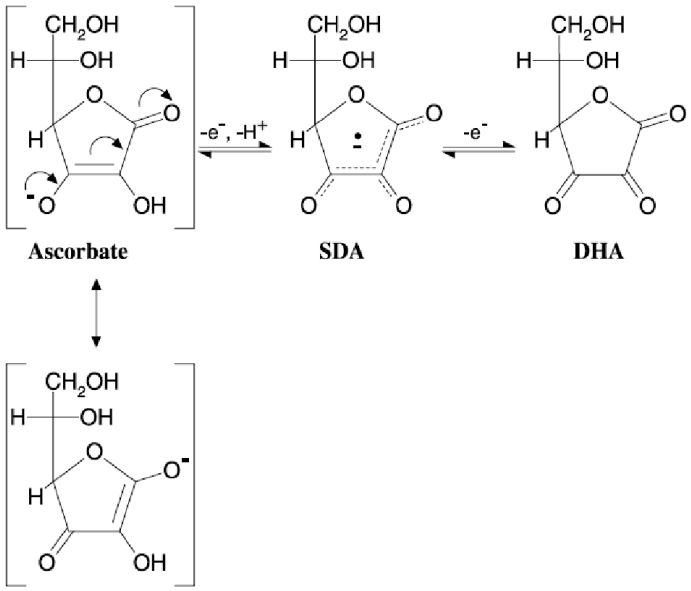

Figure 1. Resonance states of L-Ascorbic acid. Ascorbate, fully reduced form; SDA Semidehydroascorbate, mono-oxidized form; DHA Dehydroascorbate, fully oxidized form. (Source: Linster & Van Schaftingen, 2007).

The ascorbate is the fully reduced form from L-ascorbic acid, and the semidehydroascorbate (SDA) is the result of one electron loss which is much more stable and less reactive than the other free radicals. Further, this compound might be completely oxidized to dehydroascorbate (DHA).

In the early 80's, Poydock *et. al.* reported the anti-cancer role of DHA studying the effect of the mixture of Vitamin C and B_{12} on tumors cells. The studies were performed with malignant murine cell lines *in vitro* and *in vivo* and they found that the L-AA, which they had used, was old and oxidized to DHA. They established that DHA was an active factor against cancer cells instead of L-AA. Therefore, the L-AA can act as powerful antioxidant due to its capacity of easily donate a hydrogen atom and form a relatively stable ascorbyl free radical. As a scavenger of reactive oxygen, ascorbic acid has been shown to be effective against the superoxide radical ion, hydrogen peroxide, the hydroxyl radical and singlet oxygen (Weber et al., 1996). Several chemicals could serve to this purpose; however, the L-AA is considered as an efficient biological antioxidant for aerobic organisms because it is in appropriate amount in the cell, reacts with a variety of free radicals and it is suitable for regeneration (Rose & Bode, 1993; De Tullio & Arrigoni, 2004). Besides being an essential antioxidant in humans, the L-AA has a remarkable function on collagen maturation process acting as lisyl and prolyl hydroxilase enzyme cofactor. These enzymes catalyze the hydroxylation of lysine and proline residues on collagen peptide stabilizing the triple collagen helix which is extremely important in the connective tissue development and maintenance (Pinnel, et al. 1987; May & Qu, 2005).

The recommended dietary allowance (RDA) for vitamin C was set at 75 mg/day for nonsmoking, nonpregnant women and 90 mg/day for nonsmoking men in order to achieve adequate levels of serum ascorbic acid. Serum ascorbic acid concentrations are considered to be adequate if over 28 $\mu mol.L^{-1}$, suboptimal if between 11 and 28 $\mu mol.L^{-1}$, and deficient if lower 11 $\mu mol.L^{-1}$ (Jacob, 1990; Loria et al. 1998), because symptoms of scurvy have been observed just below this level. The connective tissue inside and surrounding the blood vessels becomes impaired, and the vessel wall weakened leading the capillaries rupture and hemorrhages throughout the body. Cartilage and bone require L-ascorbic acid to secrete new matrix among the cells. The failure of the cells to deposit collagen fibrils and intercellular matrix results in periosteal and subperiosteal hemorrhages causing bone pain, inflammatory gingivitis, tooth loosening and eventual loss. In addition, defective bone matrix, cessation of bone growth and failure to ossify

result in musculoskeletal limb pain, limping, swelling over long bones, progressive leg weakness, pseudoparalysis, and fractures.

Since its discovery, the L-AA has considerably reduced the scurvy prevalence in industrialized societies whereas it remains a threat, especially in developing countries (Popovich *et. al.*, 2009). Moreover, the L-AA has an important function on anemia prevention by ensuring the iron uptake in intestinal luminal. It can efficiently convert iron from ferric to the ferrous form in low environmental pH. Furthermore, the vitamin C is able to release iron from ferritin complex and mobilize it from the reticuloendothelial transferrin system which ensure the iron utilization in the erythrocytes.

Kim et al. (2008) described an inhibitory effect of vitamin C on replicative senescence. Vitamin C was found to inhibit p53-induced senescence in human bladder cancer EJ cells. Senescence-like phenotypes induced by p53 which comprises morphological changes and irreversible cell cycle arrest were not observed in EJ cells treated with vitamin C. Besides, recent findings have reported the effect of L-AA on cell signaling and transcriptional factor activation as a perspective research (De Tullio and Arrigoni 2004, Bremus *et. al.*, 2006).

In summary, L-AA is an important compound involved in several physiological functions associated with tissue protection against harmful oxidative products that have been implicated with many chronic disorders, including cardiovascular disease and cancer as well.

1.2 Biosynthesis of L-Ascorbic Acid

In 1934, Reichstein and Grüssner introduced a biochemical method known as Reichstein process, which allows the production of L-AA from glucose (Figure 2).

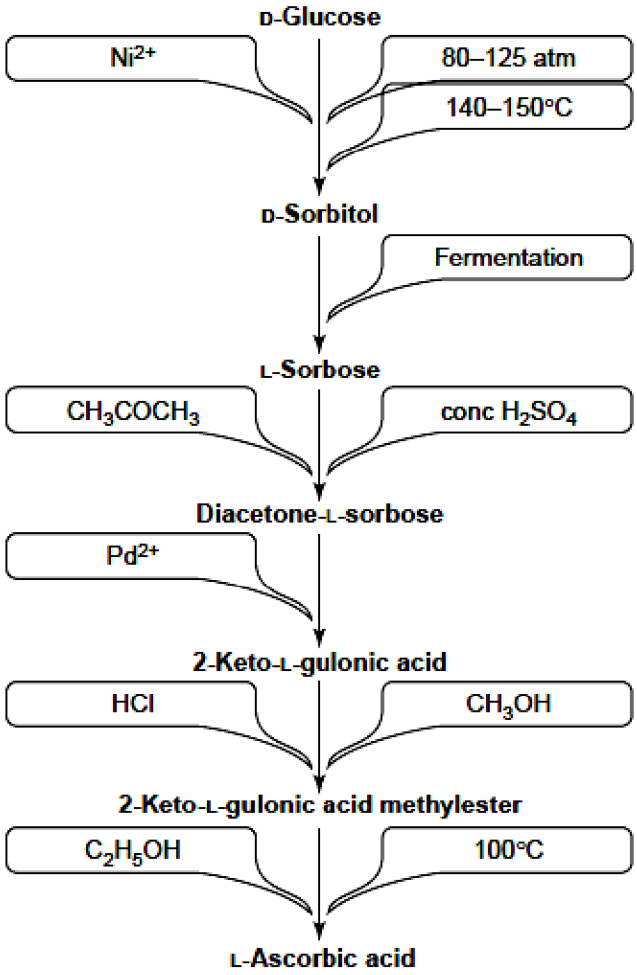

Figure 2: Reichstein Process for L-AA production from D-glucose.

This process was patented and sold for Hoffmann-La Roche in 1935. The first product containing L-AA sold was called Cebion, Merck. Currently, a considerable part of production is still carried out using this process. The production of L-AA is estimated around 110,000 tonnes per year, generating a worldwide market of approximately $ 600 million (Boudrant, 1990, Chotani et al. 2000; Macauley et al. 2001;). L-AA synthetically synthesized is used for different applications: i) pharmaceutical industry (50%) - production of supplements, additives for cosmetic and pharmaceutical preparations (Chen et al., 2005); ii) food industry (25%) - to prevent food discoloration by oxidation of pigments (Mihalev et al., 2004); iii) the beverage industry (15%) - as antioxidant agent; iv) animal feed industry (10%) - supplement in animal feed (Madej & Grzeda, 2000).

Various stages of the Reichstein process use considerable amounts of solvents, organic and inorganic reagents. These include acetone, sulfuric acid and sodium

hydroxide. Although some of these components can be recycled, there is still a need of more environmental control. Some companies such as BASF, Merck and ADM have adopted a modified Reichstein method in an attempt to minimize the use of such solvents. The modified method includes two stages of fermentation using microorganisms. Both processes consume a large quantity of energy and employ high temperatures and pressure. These economic factors arouse interest in the use of an economically viable alternative process for L-AA production. Thus the use of microorganisms along with recent innovations in biochemistry and molecular biology allows us to develop new strategies for fermentation (Bremus et al., 2006).

L-AA is naturally produced in plants and its biosynthetic pathway has been completely elucidated recently. All genes encoding enzymes with their respective enzymatic activities have been identified and partially characterized (Smirnoff & Wheeler, 2000; Linster et al., 2007). Wheeler et al. (1998) have previously reported the presence of L-galactose biosynthesis pathway in plants from GDP-D-mannose. Three other pathways for L-AA production in plants have been described (Hancock and Viola 2005): the L-Gulose pathway (Wolucka & Van Montagu 2003), the D-Galacturonic acid pathway (Agius et al. 2003), and the Myoinositol pathway (Lorence et al. 2004), but these seem to be of minor importance. However, the L-galactose pathway is recognized as the main route for L-AA biosynthesis (Valpuesta et al., 2004; Ishikawa et al. 2006). The Figure 3 illustrates the L-AA biosynthesis pathway in plants.

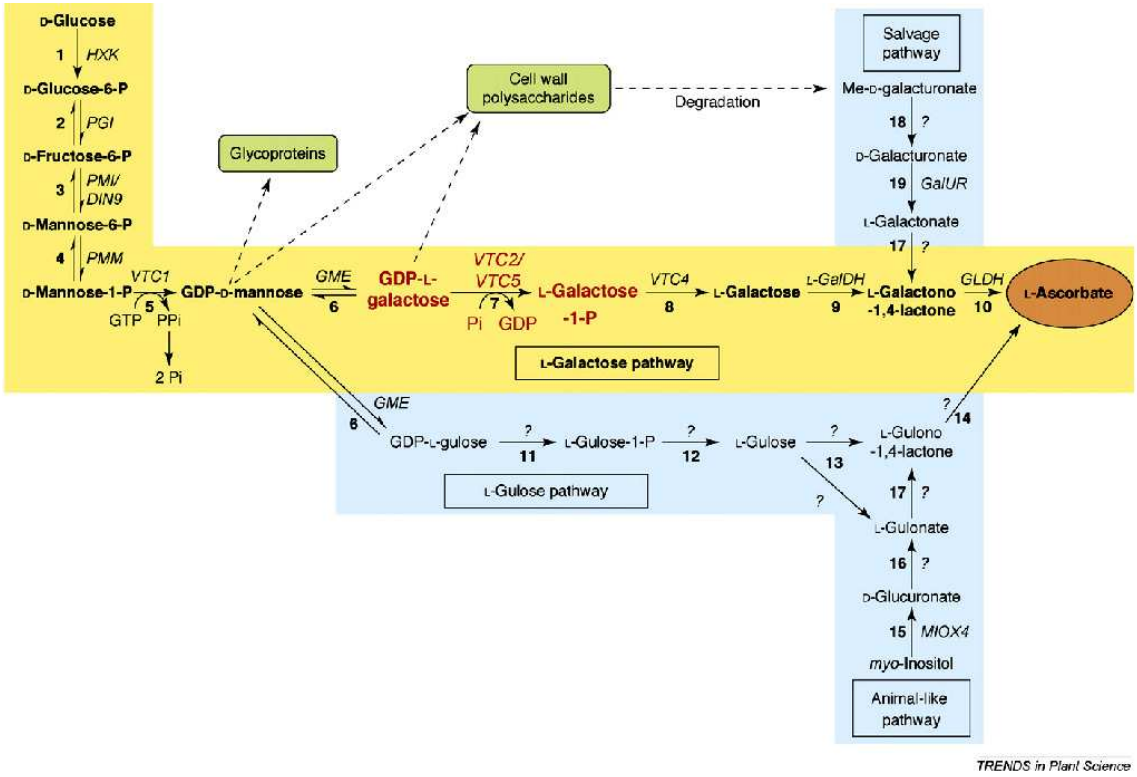

Figure 3. Biosynthetic routes to L-ascorbate in higher plants. The major L-galactose pathway is highlighted in yellow with intermediates in bold type; alternative proposed biosynthetic routes are highlighted in blue. Designations are given in italics for genes encoding known enzymes of the pathways; question marks indicate possible reactions where the gene and the specific enzyme have not yet been identified. The central reaction (and the first committed step for L-ascorbate synthesis) in the L-galactose pathway, which is catalyzed by VTC2 and VTC5, is shown in red. Enzymes catalyzing the numbered reactions are: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphomannose isomerase (PMI); 4, phosphomannomutase (PMM); 5, GDP-D-mannose pyrophosphorylase; 6, GDP-D-mannose 30,50-epimerase (GME); 7, GDP-L-galactose phosphorylase; 8, L-galactose-1-P phosphatase; 9, L-galactose dehydrogenase; 10, L-galactono-1,4-lactone dehydrogenase (GLDH); 11, nucleotide pyrophosphatase or sugar-1-P guanylyltransferase; 12, sugar phosphatase; 13, sugar dehydrogenase; 14, L-gulono-1,4-lactone dehydrogenase/oxidase; 15, myo-inositol oxygenase; 16, urinate reductase; 17, aldonolactonase; 18, methylesterase; 19, D-galacturonate reductase. (Source: Linster & Clarke, 2008)

GDP-D-mannose is epimerized into GDP-L-galactose by GDP-mannose-3,5-epimerase (Wolucka & Van Montagu, 2003). The epimerization of D to L-substrates, which is much less common in nature, is crucial to generate the first intermediate galactose enantiomer in L-AA pathway. The GDP-L-galactose phosphorilase (VTC2) is a member of GalT/Apa1 branch of the histidine triad protein superfamily that catalyzes the conversion of GDP-L-galactose to L-galactose-1-phosphate in a reaction that consumes inorganic phosphate and produces GDP (Linster et al. 2007). The VTC2

might present both enzymatic and regulatory function in L-AA biosynthesis pathway in plants (Müller-Moulé, 2008). The third enzyme is L-galactose-1-phosphate phosphatase (VTC4) which is a bifunctional enzyme that affects both ascorbate as well as myoinositol biosynthesis pathway, although it shows selective preference for L-galactose 1-phosphate (Torabinejad et al., 2009).

Humans, non-humans primates and a few other mammals are not capable to produce L-ascorbic acid due the last enzyme from that pathway, L-gulono-1,4-lactone oxidase, which is highly mutated and non functional. Thus this important antioxidant must be incorporated into the human diet (Chatterjee, 1973; Valpuesta et al 2004). The L-AA is widely found in nature, mostly in rich fresh fruits and leafy vegetables such as guava, mango, papaya, cabbage, mustard leaves and spinach (Tee et al., 1997). The table 1 shows the nutritional value of L-AA in some foods.

TABLE 1. Nutritional value of L-AA in vegetable foods. Source: Davey *et. al.*, (2000)

Source	*mg* $(100g)^{-1}$	$\mu molg^{-1}$ *fresh weight*
Acerola (west indian cherry)	1300	73.00
Apple	2–10	0.11–0.56
Apricot	7–10	0.39–0.56
Avocado	15–20	0.84–1.12
Banana	10–30	0.56–1.68
Blackberry	15	0.84
Broccoli	113	6.35
Broccoli (cooked)	90	5.05
Brussels sprouts	87–109	4.94–6.12
Cabbage (raw)	46–47	2.64
Cauliflower	64–78	3.63–4.38
Cauliflower (cooked)	55	3.09
Carrot	6	0.34
Cranberry	12	0.67
Cherry	5–8	0.28–0.45
Blackcurrant	200–210	11.2–11.8
Redcurrant	40	2.25
Damson	3	0.17
Gooseberry	40	2.25
Gourd	8	0.45
Passion fruit	25	1.40
Grapefruit	40	1.18
Guava	230–300	13.1–16.8
Horseradish	120	6.74
Kale	186	1.01
Kale (cooked)	62	3.48
Kiwi	60	3.41
Lemon	50	2.84
Lettuce	15	0.85
Lime	25	1.40
Loganberry	30	1.68
Lychee	45	2.55
Melon	10–35	0.57–1.97
Orange	50	2.84
Orange (juice)	50	2.84
Tangerine	30	1.68
Peach	7–31	0.39–1.76
Peach (canned)	6	0.34
Pepper (green)	128	0.72
Plum	3	0.17
Pea	25	1.40
Pear	3–4	0.17–0.23
Pineapple	12–25	0.68–1.40
Pineapple (canned)	12	0.68
Pomegranate	6	0.34
Potato (new)	30	1.68
Potato (Oct, Nov)	20	1.14
Potato (Dec)	15	0.85
Potato (Jan, Feb)	10	0.57
Potato (Mar, May)	8	0.45
Potato (boiled)	16	0.90
Quince	15	0.84
Raspberry	25	1.40
Rosehip	1000	5.62
Spinach	51	2.86
Spinach (cooked)	28	1.57
Strawberry	59–60	3.37
Tomato	20–25	1.14–1.40
Tomato (juice)	16	0.90
Watercress	68–79	3.82–4.44

Animal sources of this vitamin such as meat, poultry, eggs and dairy products contain tiny amounts that are not significant sources. The L-AA content in foods is highly influenced by season, transportation to the market, shelf life, time storage, cooking practices and chlorination of the water.

1.3 - Yeasts and L-ascorbic acid production

The yeast does not naturally present genes in the biosynthesis of L-ascorbic acid (L-AA), but they are able to synthesize D-erythroascorbic acid. The D-EAA production is observed when yeasts are grown in the medium with D-aldoses such as D-glucose, D-galactose, D-mannose or D-arabinose (Murakawa et al., 1977). Because of its ability to donate one or two electrons, L-AA is important in different biochemical functions such as antioxidant, enzyme cofactor and it is involved in controlling the growth of plants (Moser & Bendich, 1991, Huh et al. 2001). The D-EAA, a similar five-carbon, has a reducing power similar to L-AA plus it has the potential to stimulate collagen production, but has no anti scurvy activity (HANCOCK et al., 2000). The D-EAA has been detected in different filamentous fungi species such as *Neurospora crassa*, *Sclerotinia sclerotium*, and *Lipomyces starkeyi,* and in yeast as *Saccharomyces cerevisiae* and *Candida* species. *In Candida albicans* and *S. cerevisiae* the biosynthesis of D-EA from D-arabinose has been established (Figure 4).

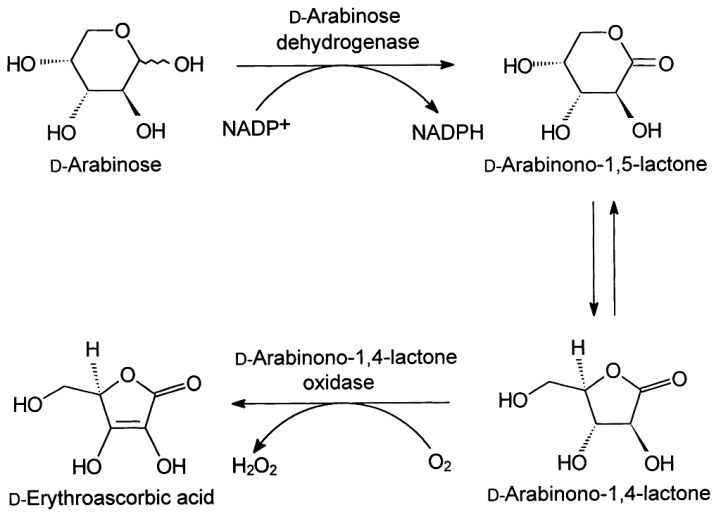

Figure 4: Proposed D-EAA biosynthesis pathway in yeast. (Source: Kim et al. 1998)

The last two steps of the biosynthesis of L-AA have some similarities with the biosynthetic pathway in yeast that leads to formation of erythroascorbic acid. D-Arabinose is converted to D-arabino-1,4-lactone by the enzyme D-arabinose dehydrogenase, which is subsequently converted into D-AAS by the enzyme D-arabinose-1 ,4-lactone oxidase (Hancock et al., 2002; SAUER et al., 2004). The enzymes D-arabinose dehydrogenase and D-arabinose-1 ,4-lactone oxidase showed broad substrate specificity in vitro, ie they are not only capable of oxidizing D-arabinose as well as L-galactose, L-fucose and L-xylose (Hancock et al., 2000). Structural motifs of the molecules changed during the conversion are similar to structural motifs modified during the conversion of L-galactose pathway L-Galactono-1,4-lactone to L-ascorbic acid (Sauer et al., 2004; Figure 5).

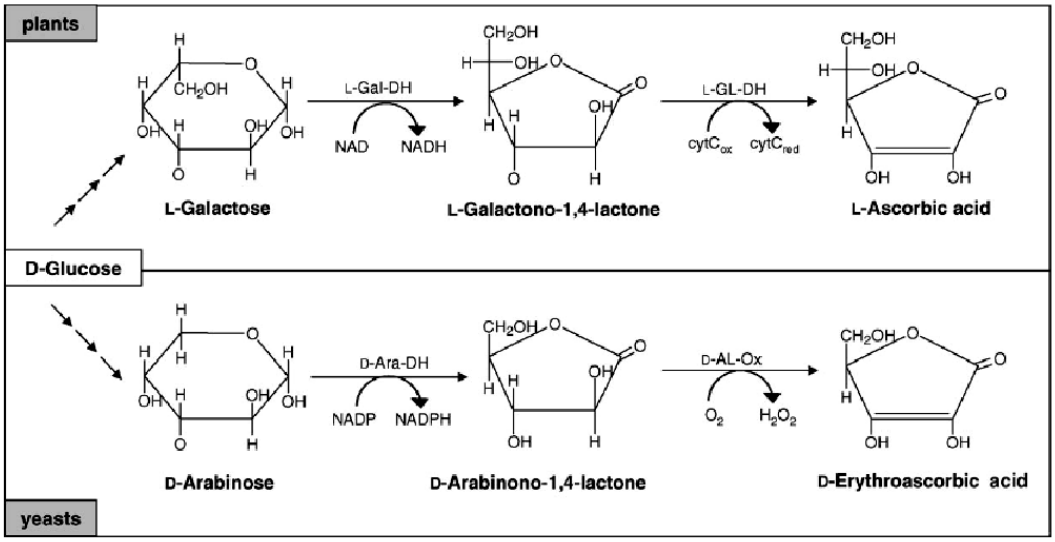

Figure 5: Comparison of the final steps in the biosynthesis of L-AA in plants and analogue D-EA in yeast. (Source: Bremus et al. 2006)

The kinetic parameters of enzymes D-arabinose dehydrogenase (ARA) and D-arabinona-1,4 lactone oxidase (ALO) have been determined in vitro and the results demonstrated low substrate specificity (Table 2). In the presence of L-Galactono-1,4-lactone, intermediate biosynthetic pathway of L-AA in plants, ALO is able to catalyze the production of ascorbic acid (Lee et al. 1999).

Table 2. Arabinose dehydrogenase Kinetic parameters from *S. cerevisiae* and *C. albicans* D-EAA biosynthetic pathway for different substrates.

Species	*Candida albicans*				*Saccharomyces cerevisiae*			
Substrate	D-arabinose	L-galactose	L-fucose	L-xylose	D-arabinose	L-galactose	L-fucose	L-xylose
K_m (mM)	29	91	29	37	161	180	98	24
k_{cat} (s^{-1})	68	53	70	63	194	241	222	163
Inferred product	D-arabinono-1,4-lactone	L-galactono-1,4-lactone	L-fucono-1,4-lactone	L-xylono-1,4-lactone	D-arabinono-1,4-lactone	L-galactono-1,4-lactone	L-fucono-1,4-lactone	L-xylono-1,4-lactone
Reference	Kim et al. 1996				Kim et al. 1998			

In *Saccharomyces cerevesiae*, Huh et al. 1998 reported the identification, sequence analysis, inactivation and overexpression of the gene ALO1 which coding for D-arabinona-1, 4 lactone oxidase (ALO). The sequence analysis revealed that ALO1 has a putative domain for the covalent FAD molecule and that this same domain was found in oxygen-dependent oxidoreductases. Spickett et al. (2000) found that the production of analogues of L-AA is strongly influenced by the aeration of the culture. These data suggest that probably the key regulatory enzyme pathway may be dependent on the dissolved oxygen levels. When mutants of *S. cerevisiae* Δalo1 mutant cells were grown in the presence of oxidizing agent (H_2O_2) cells were more sensitive to the agent while the overexpression made cells more resistant (Figure 6).

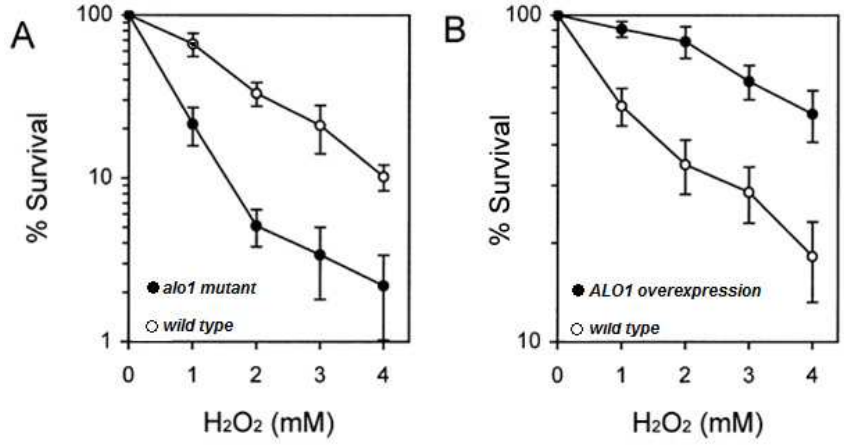

Figure 6: Effects of ALO1 gene inactivation (A) and overexpression (B) on the survival rate of cells in different concentrations of H_2O_2. (Source: adapted from Huh et al. 1998)

However, when they analyzed the ALO1 transcription in the same oxidizing conditions, it was not observed any changes in transcription levels even in relation to the wild type. Thus the hypothesis that the gene is regulated in response to oxidative stress is ruled out. Physiologically, alo1 mutant strains showed no auxotrophy unlike

gsh1 mutants that are deficient in glutathione biosynthesis, a well known antioxidant. The cells grew normally in minimal medium as well as in complex medium whereas the cells overexpressing the gene ALO1 showed a slight difference in growth rate compared to the wild type. When oxidative stress was induced by addition of t-BHP (ter-butyl hydroperoxide - organic peroxide) the intracellular D-EAA levels were drastically reduced, but increased levels of D-EAA did not prevent the loss of glutathione during oxidative stress. In addition, the sub-lethal oxidative stress did not result in a high rate of biosynthesis of D-EAA even when the precursors were added (Spickett et. al. 2000). These results suggest that D-EAA does not play a crucial role in adaptive oxidative stress response considering the high levels of intracellular glutathione in yeast. However, the D-EAA must play a role in defense systems in order to assist non-enzymatic glutathione intracellular pool under oxidative stress.

Lee et al. (2001) established that NADH-cytochrome b5 reductase mitochondrial plays a crucial role in reducing free radical-D eritroascorbil in *S. cerevisiae*. Similar to L-AA, D-EA has the potential to protect both cytosolic and membrane components against damage caused by oxidative stress. The D-EAA is almost as readily oxidized in aqueous system (Shao et al. 1993) and has a similar system to L-AA in order to maintain its redox system (Figure 7).

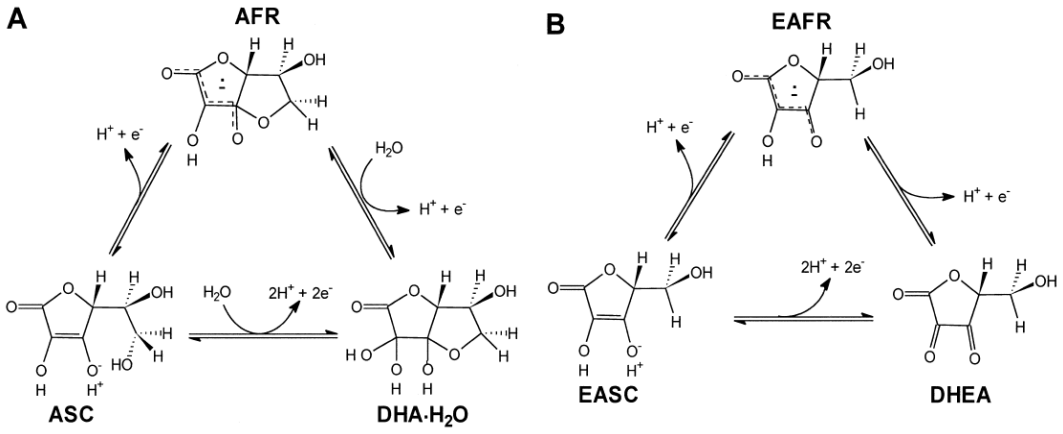

Figure 7. L-AA (A) and D-EAA (B) redox equilibrium. (Source: Lee et al.2001)

It is assumed that the balance EASC / EAFR is maintained by a putative NADH-D-erythroascorbyl oxidase free radical although no record of purification and characterization of NADH-reductase in *S. cerevesiae* have been reported.

It has been reported in mammalian cells the presence of NADH-cytochrome b5 reductase, encoded by the gene MCR1, and their participation in the regeneration of L-

AA from the ascorbyl free radical. Lee et al. (2001) showed that this enzyme is present in the outer membrane of mitochondria and reduces EAFR radical (D-erythroascorbyl free radical) in EASC (D-EAA). However, the overexpression and interruption of CYB5, single cytochrome b5 gene reported in *S. cerevisiae*, did not cause any change in NADH-cytochrome b5 reductase. Nevertheless, the overexpression of MCR1 resulted in an increased oxidative stress resistance whereas the opposite was observed when the gene was interrupted. Furthermore, the content of D-EAA in mcr1 mutant cells was higher compared with the *alo1* mutants, but they were more sensitive to stress. These data indicate that the NADH-cytochrome b5 reductase has another function besides the reduction of -D erythroascorbyl free radical. Although it is possible that D-EAA plays an important role as an antioxidant with protective effect, one possible explanation is that D-EAA acts as an essential component for other biochemical processes, and it is normally produced in limiting concentrations and its depletion during oxidative stress reduces cell viability. Possibly, at low concentrations, D-EAA is required as a cofactor for iron dependent hydroxylases as it is known for L-AA in plants and animals (Davies et al. 1991, Sipckett et al. 2000) or required for the synthesis of oxalate as reported in *S. sclerotiorum* (Loewus et al. 1995).

Huh et al. (2001) reported that Δ*alo1 Candida albicans* are more sensitive to oxidative stress and its overexpression has increased the survival rate during oxidative stress. In addition, *alo1* mutants showed an attenuated virulence and hyphal growth deficiency. It is suggested that the D-EAA in *C. albicans* plays an important role as an antioxidant, whereas systems of defense against oxidizing agent is essential to resist the immune system during host infection and as a virulence factor. In addition, the D-EAA can be required as a component of the signal transduction pathway involved in the transition to hyphal growth as well as an integral normal cell wall during host infection.

Huh et al. (2008) found that D-EAA activates cyanide-resistant respiration in *C. albicans*. This respiratory pathway is mediated by an alternative oxidase (AOX) that accepts electrons from the ubiquinone pool and reduces O_2 to H_2O. Increased levels of D-EAA induce the expression of AOX, playing an important role in the activation of this alternative respiratory pathway.

In *Neurospora crassa* the levels of D-EA were higher when the cells were under nitrogen deprivation. D-EAA levels were also elevated in mutants for adenylate cyclase, and much lower when cAMP was added. It is suggested that in filamentous fungi the cAMP intracellular pool affects the biosynthesis of D-EA in response to nitrogen stress (Dumbrava & Pall, 1987).

These evidences reveal that D-EA can participate in different biochemical processes, and have a protective function against oxidative stress. It is synthesized at very low levels when compared to other cellular antioxidants such as glutathione peroxidase, catalase, superoxide dismutase and also to lipophilic antioxidants (ubiquinol, α-tocoferol, etc.). The functions of D-EA can vary from organism to organism whose mechanisms of regulation of the biosynthesis pathway might have diverged during the evolutionary process to improve environmental adaptations. Thus, considering the lack of information regarding the regulation of the biosynthesis of D-EA, the elucidation of these mechanisms in a particular organism requires further study.

1.4 *Kluyveromyces lactis*

The genus *Kluyveromyces* was originally established by van der Walt (1956) to accommodate the new isolated yeast specie *Kluyveromyces polysporus*, an unusual yeast that formed large asci containing great numbers of ascospores. Later, van der Walt (1965) transferred yeasts species that had been previously assigned to *Saccharomyces* into this new group. Currently, *Kluyveromyces* is classified within the major group *Saccharomyces* complex from Saccharomycotina subphyla. More than 1000 species have been described in this subphyla. The *Saccharomyces* complex is primarily comprised by *Saccharomyces* and *Kluyveromyces* species. The Figure 8 presents the phylogenetic relationship among the yeasts.

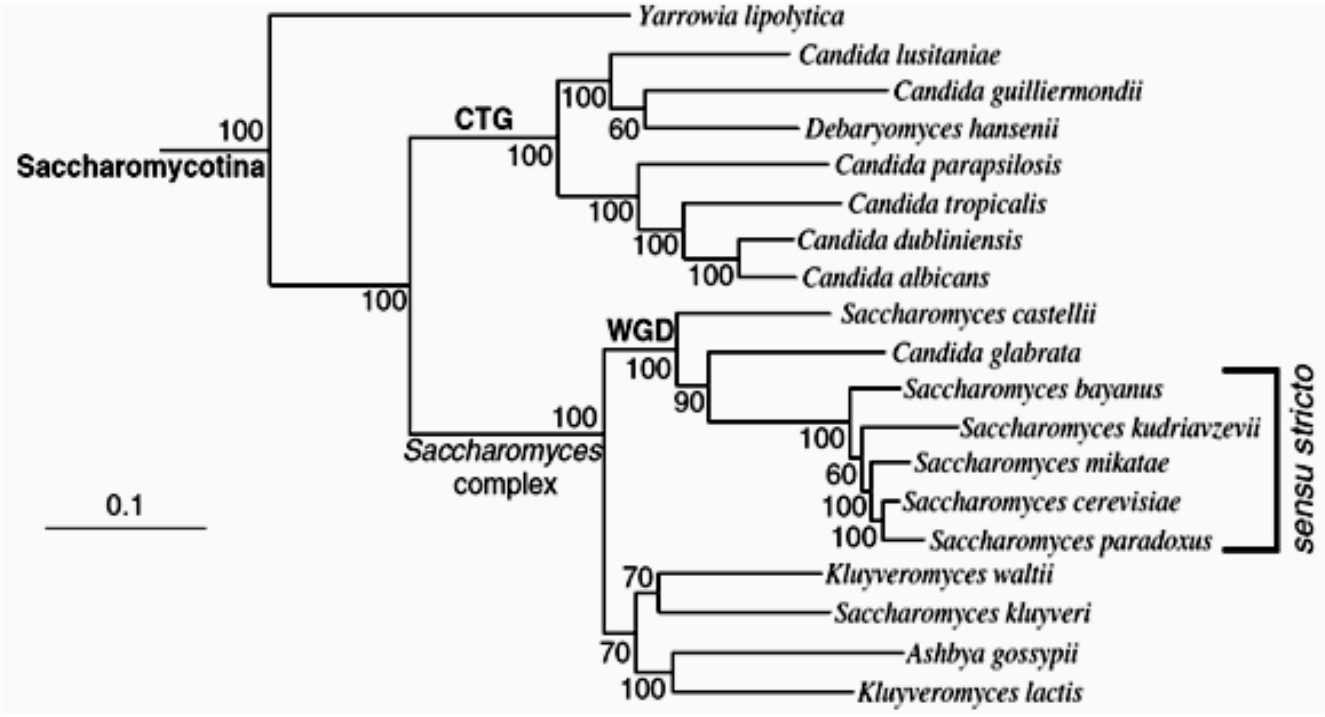

Figure 8: Phylogenetic relationship among the sequenced yeasts genomes. The tree is a maximum-likelihood phylogeny reconstruction using the concatenated sequences of 153 genes that are universally presented in the genomes shown. Bootstrap percentages are shown for all nodes. (Source: adapted from Scannel et al., 2007).

Besides the major *Saccharomyces* complex, Saccharomycotina subphyla comprises two other clusters: one consists of yeasts species that translate CTG codons as serine than leucine, a reassignment that occurred more than 170 million years ago (Massey et al., 2003; Miranda et al., 2006), which includes *Candida* species, *Debaryomyces hansenii* and *Lodderomyces elongisporus* (Fitzpatrick, et al., 2006); and the cluster comprising only the yeast *Yarrowia lipolytica* (Dujon et al., 2004). The major phylogenetic division within the *Saccharomyces* complex is between those yeasts whose common ancestor underwent whole genome duplication (WGD; Dujon et al., 2004; Kellis et al., 2004) and those that diverged prior to this event.

The WGD in yeast is estimated to have occurred in the ancestor of the Saccharomyces *sensu stricto* species complex around 100 million years ago (Dietrich et al., 2004) and has been proposed to have led to evolution of an efficient fermentation system in this lineage (Piskur & Langkjaer 2004; Piskur et al., 2006). Most of the post WGD yeast species primarily carry out fermentation to generate energy under aerobic conditions (Merico et al., 2007) due to glucose repression of respiration pathways (Wang et al., 2004; Santangelo 2006), phenomena known as crabtree-positive.

However, the pre-WGD yeast species such as *Kluyveromyces lactis* predominantly generate energy for fast cellular growth through respiration pathway. The regulation of primary carbon metabolism in *K. lactis* differs noticeably from *Saccharomyces cerevisiae* and reflects the dominance of respiration over fermentation typical for the majority of yeasts. The low glucose repression of respiration, a high capacity of respiratory enzymes and a tight regulation of glucose uptake in *K. lactis* are key factors determining physiological differences to *S. cerevesiae* (Breunig et al., 2000).

The yeasts within the genus *Saccharomyces*, *Kluyveromyces* belong to the family Saccharomycetaceae, originated from a common ancestor, and the ability of these yeasts to assimilate galactose as carbon source is determined by the presence of genes comprising the GAL regulon (Schaffrath & Breunig , 2000; Bhat & Murthy, 2001; Rubio-Texeira, 2005). The system for internalization and hydrolysis of sources of galactose has differently evolved between these yeasts. The assimilation of lactose (O-β-D-galactopiranosil -1.4 - β - D-glucose) by *K. lactis* is due the presence of two genes LAC12 and LAC4 encoding the Lactose permease and β-galactosidase (lactase) respectively (Sreekrishna & Dickson, 1985). *K. lactis* has been used as a source of lactase (β-galactosidase) since the earlier fifties mainly to provide the enzyme for lactose hydrolysis in dairy products for lactose intolerant consumers. The LAC4 and LAC12 genes are divergently transcribed from an intergenic region of about 2.6 Kb, which contains an upstream activator sequence (UAS). The activating sequence contributes synergistically to the activation of both genes to allow the binding of transcriptional activator Lac9/KlGal4p. The presence of lactose / galactose increases the transcription of the LAC / GAL regulon. The galactose together with an ATP molecule activates the sensing function of the KlGal1p (galactokinase) which is the first active enzyme of the metabolic pathway for conversion of galactose into glucose-6-phosphate intermediate of the glycolytic pathway (Leloir Pathway). Figure 9 shows the simplified scheme for LAC/GAL regulation in *K. lactis.*

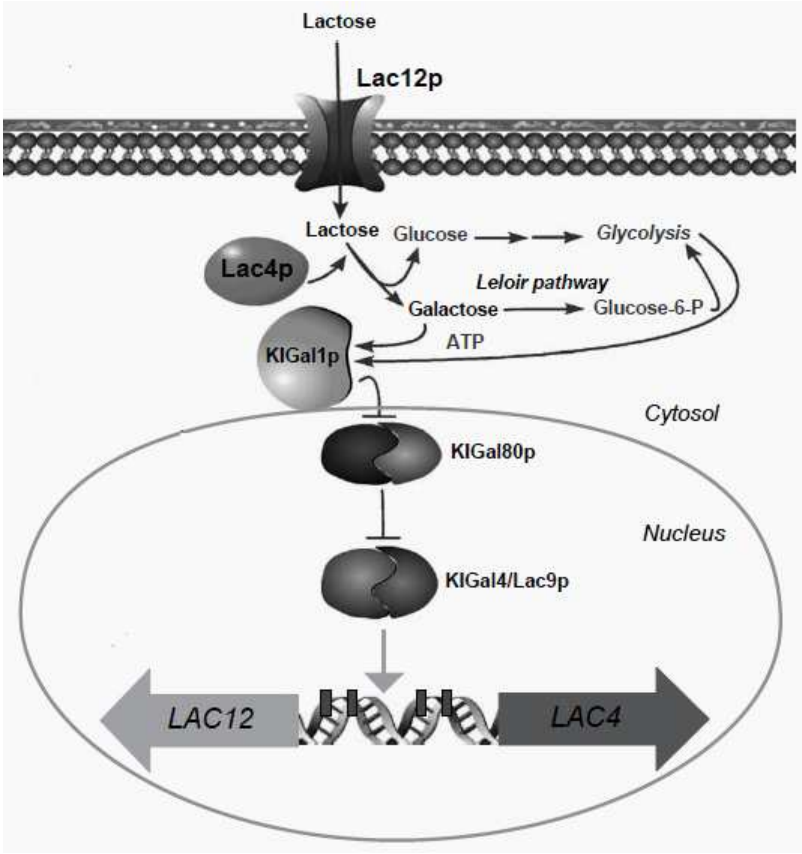

Figure 9. Model for the regulation of lactose permeabilization and hydrolysis in *Kluyveromyces*. Lactose and/or galactose enter the cells through basal levels of the lactose permease, Lac12p. Cytosolic Lac4 β-galactosidase hydrolyzes lactose into glucose and galactose. (Source: Adapted from Rubio-Teixeira, 2006).

After interacting with galactose and ATP, Gal3p undergoes a conformational change that allows the sequestration of Gal80p to the cytosol, reducing its intranculear levels. Thus the transcriptional activator Gal4p which acts as a dimer promotes the transcription of the LAC / GAL regulon (Pilauri et al. 2005).

In a scientifical and biotechnological perspective, *Kluyveromyces lactis* is one of the most important non-Saccharomyces yeasts. The knowledge about the induction and expression of the gene LAC4 encoding β-galactosidase associated to its metabolism properties is the basis for construction and operation of vectors for heterologous proteins expression induced by lactose or galactose in this yeast. The biotechnological significance of *K. lactis* LAC genes is built on its history of safe use in the food industry. Additionally, the complete genome sequence of *K. lactis* available online (http://cbi.labri.fr/Genolevures/elt) and its closely phylogenetic relationship with *S. cerevesiae* allow us to employ the same genetic engineering techniques to construct

efficient systems for proteins expression in *K. lactis* (Dujon, et. al., 2004). High-level of gene expression can be approached from either integrative or episomal strategies allowing mitotic stability or multicopy dosage effects of the foreign gene. Both approaches have been successfully exploited for bovine prochymosin and human serumalbumin (van den Berg et al., 1990; Fleer et al., 1991).

Colussi & Taron (2005) developed an integrative vector for expression and secretion of recombinant proteins, called pKLAC1, whose integration occurs in the promoter region of the gene LAC4. The vector pKLAC1 is a component of an expression system that was developed from basic research at New England Biolabs, Inc. and DSM Biologics Company B.V. This vector harbors a fungal acetamidase selectable marker gene (amdS) which allows the use of non-auxothrophic *K. lactis* strain and non-antibiotic selection. Read et al. (2007) demonstrated that the high frequency of multicopy integration associated with the use of acetamide selection can be exploited to rapidly construct expression strains that simultaneously produce multiple heterologous proteins

The ability of *K lactis* to assimilate lactose as carbon source allows the employment of the LAC genes in bioremediation processes for the conversion of whey lactose. Whey is a major by-product from the dairy industry and its composition varies depending on the cheese. It is estimated that for every kilogram of cheese around 9 kg of whey are produced (Yang & Silva, 1995). This by-product contains about 6.0 to 6.5% total solids, and about 4.5 to 5.0% lactose, 0.8 to 1.1% protein, 0.03 to 0.1% fat, 0.5 to 0.8% of ash and 0.2 to 0.8% lactic acid.

Whey is used to produce ricotta, brown cheeses and many other products for human consumption. Indeed, it is also an additive in many processed foods, including breads and commercial pastry, and in animal feed. Whey proteins consist primarily of α-lactalbumin and β-lactoglobulin. Due to the high concentration of organic compounds, mainly represented by lactose (approximately 70% of total solids) and proteins (approximately 20% of total solids), the whey places a high value of biological oxygen demand (BOD) during the wastewater treatment. The ultrafiltration process is considered as an alternative way to minimize the polluting effects of whey. Furthermore, it is suitable for obtaining a protein concentrate due to its high nutritional and functional value. (Siso, 1996;)

Thus exploiting the *Kluyveromyces lactis* fermentation capacity, the conversion of lactose from cheese whey into valuable industrial compounds is a project of great social and economic interest.

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Chapter 2

Metabolic Engineering of *Kluyveromyces lactis* for L-ascorbic acid (vitamin C) synthesis

Key words: ***Kluyveromyces lactis,*** **L-ascorbic acid, L-galactose, metabolic engineering**

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Metabolic Engineering of *Kluyveromyces lactis* for L-ascorbic acid (vitamin C) synthesis

ABSTRACT

L-ascorbic acid is naturally synthesized in plants from D-glucose via a ten-step pathway. The branch pathway to synthesize L-galactose, the key intermediate for L-ascorbic biosynthesis, has been recently elucidated. Budding yeast is only able to synthesize L-ascorbic acid if it is cultivated in the presence of one of its precursors: L-galactose, L-galactono-1,4-lactone, or L-gulono-1,4-lactone extracted from plants or animals. To avoid feeding the yeast culture with this "L" enantiomer, we engineered *Kluyveromyces lactis* with L-galactose biosynthesis pathway genes: GDP-mannose-3,5-epimerase (GME), GDP-L-galactose phosphorylase (VTC2) and L-galactose-1-phosphate phosphatase (VTC4) isolated from *Arabidopsis thaliana*. Plasmids were constructed to target the cloned plant genes to the *K. lactis* LAC4 Locus by homologous recombination and the expression was associated to the growth of the cells on D-galactose or lactose. Upon *K. lactis* transformation, GME was under the control of the native LAC4 promoter while VTC2 and VTC4 genes were transcribed by the *S. cerevisiae* promoters GPD1 and ADH1 respectively. The expression in *K. lactis* of the endogenous L-galactose biosynthesis plant genes was determined by RT-PCR and western blotting. The recombinant yeasts were able to produce about 23 $mg.L^{-1}$ of L-ascorbic acid in 48 hours of cultivation when cultured on rich medium with 2% (w/v) D-galactose. We have also successfully evaluated the L-AA production culturing

recombinant strains in cheese whey as an alternative source of lactose and which is a waste product during cheese production. This work is the first attempt to engineering *K. lactis* cells for L-ascorbic acid biosynthesis through a fermentation process without any trace of “L” isomers precursors in the culture medium.

1. INTRODUCTION

The enediol ascorbate or L-ascorbic acid (L-AA), known as Vitamin C, is an important metabolite in many organisms. In eukaryotes, L-AA is essential for a variety of cellular functions (21) such as i) scavenger of free radicals (44); ii) reducing agent (3), iii) cofactor for enzyme activity (31, 33), iv) intermediate on catecholamines biosynthesis and v) limiting growth factor in plant development (2). Most of the commercially available vitamin C is synthetically synthesized by the Reichstein process, using D-glucose as start material (13).

L-AA is naturally produced in plants where its biosynthetic pathway has been completely elucidated (25, 39). In most cases, GDP-D-mannose is converted into L-galactose, which is further converted into L-AA (45). Although there may be alternative routes (1, 26), this pathway is recognized as the main route for L-AA biosynthesis (16, 43). There are three enzymes required for the conversion of GDP-D-mannose into L-galactose. The GDP-mannose 3,5 epimerase (GME) catalyzes the conversion of GDP-D-mannose to GDP-L-gulose or to GDP-L-galactose, depending whether the epimerization occurs on 5'- carbon or on both 3'- and 5'- carbon of GDP-D-mannose respectively (46). GDP-L-gulose seems to represent the minor part of the products (around 25% under equilibrium) and can also be converted to L-AA (27). The epimerization of D to L-substrates, which is rare in nature, is a crucial step to generate the galactose enantiomer in the L-AA pathway. GDP-L-galactose is then converted to L-galactose-1-phosphate by GDP-L-galactose phosphorylase, encoded by the VTC2 gene (9). This gene encodes a member of GalT/Apa1 branch of the histidine triad protein superfamily that catalyzes the conversion of GDP-L-galactose to L-galactose 1-phosphate in a reaction that consumes inorganic phosphate and produces GDP (25). The VTC2 may present both enzymatic and regulatory functions in the L-AA biosynthesis pathway in plants (30). The third enzyme is L-galactose-1-phosphate phosphatase encoded by VTC4 (23) which is a bifunctional enzyme that plays a role in both ascorbate as well as myoinositol biosynthetic pathways, although it shows selective

preference for L-galactose 1-phosphate (42). The resulting L-galactose is then the main precursor for L-AA biosynthesis.

Yeasts are known to produce the 5-carbon ascorbic acid analogue, D-erythroascorbic acid (D-EAA), which is synthesized from D-arabinose. Although D-EAA does not show any anti scurvy activity, its physicochemical properties and biological activities are quite similar to those of L-AA. For this reason D-EAA can replace L-AA in some industrial applications (15, 37). Surprisingly, the structural motifs of the enzymes involved in the D-EAA biosynthetic pathway resemble those of the pathway in plants that converts L-galactose into L-AA. D-EAA pathway enzymes from *Candida albicans* and *Saccharomyces cerevisiae* have shown to be able to convert a broad range of substrates besides D-arabinose including L-galactose into their respective galactonic acids in vitro (19, 20). Furthermore, L-AA production in yeasts was achieved when appropriate precursors such as L-galactose, L-galactono-1,4-Lactone, L-gulono-1,4-lactone were exogenously supplied in the growth medium (35). Thus, isolation of genes involved in L-galactose production in plants provides biochemical support to guide the metabolic capacity of industrial microorganisms to produce L-AA by fermentation (13).

Attempts have been made to synthesize L-AA in genetically modified microorganisms. Sauer et al. (35) observed a high production of vitamin C in the culture supernatant of *S. cerevisiae* cells expressing the L-galactose dehydrogenase (LDGH) and D-arabinose-1, 4-lactone oxidase (ALO1) from yeast or the L-galactona-1, 4-lactone dehydrogenase (AGD) from *Arabidopsis thaliana* when cultivated in a medium containing 250 $mg.L^{-1}$ L-galactose. Further, Branduardi et al. (5) have engineered this strain with GME and VTC4 from *A. thaliana* and also with L-fucose guanylyltransferase from *Rattus norvegicus* FGT in order to convert D-glucose to L-AA completing the L-AA pathway in *S. cerevisiae*. The L-AA production conferred an increased stress tolerance under oxidative conditions.

Kluyveromyces lactis is one of the most important non-*Sacharomyces* yeast species used as an eukaryotic model and tool for biotechnological applications including an alternative host for heterologous gene expression. *K. lactis* has the ability of growing, by respiration, on a wide range of substrates, including lactose with low glucose repression (4). The genome has been completely sequenced and the Lac-Gal regulon with the induced genes for lactose transport and hydrolysis has been extensively studied (36). Many heterologous expression systems have been developed, based on the lac4 promoter with the production of lysozyme (17), serum albumin (7), thermostable

bacterial xylanase (47) and heparin sulfate sulphotransferase (48) as examples. The potential use of *K. lactis* as a host for protein expression associated to its physiological properties suggest that this yeast could also be used for large-scale protein production in the food and pharmaceutical industry. Furthermore, its ability to express and process heterologous proteins makes this yeast well suited for multiple proteins expression such as the enzymes involved in L-galactose metabolism from plants. Considering the high costs of using non-physiological substrates in the L enantiomer form for industrial applications, herein, we report the construction of *K. lactis* strains capable to convert D-galactose or lactose into L-galactose, the main intermediate metabolite of the L-AA pathway in plants, and its simultaneous conversion into L-ascorbic acid.

2. MATERIAL AND METHODS

2.1 Cell strains and growth medium.

Escherichia coli TOP10 cells [F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-] were used to amplify the plasmids. *E. coli* cells were grown on Luria Bertani (LB) medium (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl, pH 7.5) with or without 100$\mu g.mL^{-1}$ ampicilin at 37°C. *E. coli* TOP10 cells harboring the recombinant vector pGEM T easy were grown on solid LB medium supplemented with 1mM isopropyl β-D-thiogalactopyranoside (IPTG) and 40 $\mu g.mL^{-1}$ 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-Gal). *Kluyveromyces lactis* CBS2359 strain was used as host for protein expression on this work. YPD medium (20 $g.L^{-1}$peptone, 10 $g.L^{-1}$ Yeast extract, 20 $g.L^{-1}$Dextrose) or YPGal (20 $g.L^{-1}$peptone, 10 $g.L^{-1}$ Yeast extract, 20 $g.L^{-1}$ Galactose) were routinely used for obtaining biomass of the recombinant and parental yeast strains at 30°C. For solid medium 20 $g.L^{-1}$ agar was added. YCB (Yeast Carbon Base - Sigma) medium supplemented with 5 mM acetamide and YPD containing 200 $\mu g.mL^{-1}$ geneticin were used to select *K. lactis* cells transformed with the vectors constructed on this work. Cheese whey, YNB (Yeast Nitrogen Base - Sigma) or YP medium supplemented with 20 $g.L^{-1}$ galactose or lactose was used to grow the cells for L-ascorbic acid measurements.

2.2 L-ascorbic acid pathway genes amplification.

L-Ascorbic acid pathway genes from *Arabidopsis thaliana*, GDP-D-Mannose 3',5'-Epimerase [AtGME (E.C. 5.1.3.18)], GDP-L-Galactose Phosphorylase [AtVTC2(E.C.2.7.7.220], L-Galactose-1-Phosphate Phosphatase [AtVTC4 (E.C. 3.1.3.23) were amplified using *A. thaliana* cDNA, kindly provided by Dr. Filip Rolland (K.U. Leuven, Belgium), as a template. L-AA pathway proteins were tagged with the Flag Tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). Phusion High Fidelity DNA polymerase was used for PCR amplification and primers are listed on Table 1. Amplification cycles comprised 5 minutes 95°C, 1 minute 95°C, 30 seconds Tm^x, 90 seconds 72°C, 5 minutes 72°C. Tm^x was 58°C for AtGME, 66°C for AtVTC2 and 60°C for AtVTC4 amplification.

2.3 Construction of plasmid vectors.

Maps of the plasmids used on this study are shown in Figure 1**.** pKLAC1 plasmid (7) was used as starting point. pMB7-A (10) was used as template for hisG fragments amplification, 1 minute 94°C, 1 minute 63°C, 1 minute 68°C (34 cycles), with the primers hisGI-F and hisGI-R, hisGII-F and hisGII-R. HisG fragments were subcloned into pGEM T easy Vector and further transferred to pKLAC1 generating the plasmid pKLhisG2. The repeat hisG sequences flank the *amdS* (acetamidase) marker for its removal by homologous recombination in the counterselection procedure. Bidirectional promoter in the pBEVY-L vector (28), ScGPD and ScADH1 fused promoters, and the ADH2 sequence terminator were amplified using the primers GPDADH1-F and GPDADH1-R in the following amplification cycles: 20 sec 98°C, 20 sec 63°C, 45 sec 72°C (34 cycles). The resulting 1405 bp fragment was subcloned into the pGEM vector linearized by AatII and NdeI, generating the plasmid pGDPADH1. The AtVTC4 gene was inserted into pGPDADH1 linearized by EcoRI and KpnI. Finally, the AtVTC4 expression cassette, under the control of the ADH1 promoter was cut out from the pGPDADH1 vector and cloned into pKLhisG2, linearized with HindIII and NotI. Afterwards, the AtVTC2 gene was released from the pGEM Vector with NotI and StuI digestion and transferred to pKLhisG2, linearized with the same restriction sites resulting in the vector pKlVTc. pKLAC1 was digested with HindIII and XhoI, followed by treatment with Klenow enzyme and also with T4 DNA ligase to destroy the signal secretion sequence of the alpha mating factor. AtGME gene was released from

pGEM vector with the XhoI and StuI and inserted into the SalI and StuI sites from pKLAC1 α-mating factor free vector generating the vector pKLJC/GME. LoxP-KanMX-LoxP cassette was amplified by polymerase chain reaction using pYX012 vector (Novagen) as template and the primers KanMX-F and KanMX-R in the following amplification cycles: 3 minutes 98°C, 20 sec 98°C, 20 sec 63°C, 45 sec 72°C (34 cycles). The cassette was further inserted into BsrGI and XmaI site from pKLJC/GME vector. All ligation reactions were performed with Rapid DNA Ligation Kit from Roche®.

2.4 Yeast transformation.

Kluyveromyces lactis transformation was carried out according to Kooistra et. al. (22) with some modifications. Fresh CBS2359 cells were plated on YPD agar medium and incubated overnight at 30°C. An isolated colony was grown in 2 mL YPD culture at 30°C, 200 rpm overnight. 50 mL YPD were inoculated with this 2mL pre-cultured cells to start $O.D_{600}$ 0.0025 per mL (0,1 O.D.). When $O.D_{600}$ reached approximately 1, the cells were harvested at 1,075 x *g* for 5 minutes at 4°C and washed with 25 mL sterile ice-cold electroporation buffer EB (10 mM tris-HCl, pH 7.5, 270 mM sucrose and 1mM $MgCl_2$). 25 mL YPD medium containing 25 mM DTT and 20 mM HEPES pH 8.0 were added and further incubated at 30°C for 30 minutes without shaking. Cells were collected at 1,075 x *g* for 5 minutes at 4°C and washed with 10 mL sterile ice-cold EB buffer. Cells were resuspended in 0.2 mL ice-cold EB and added to 60 µL aliquots of competent cells. To each aliquot 50 µg SS-DNA (Salmon Sperm DNA) plus 2µg transforming DNA was added and kept on ice for 15 minutes. The mixture was transferred to a chilled electroporation cuvette (2 mm) and eletroporated at 1KV, 25 µF and 400 Ohm. Immediately, 1 mL YPD was added and the mixture was incubated at 30°C for 3 hours, 200 rpm. The cells were harvested at 1,075 x *g* for 5 minutes at 4°C and washed with sterile water. Cells were plated on selective agar plates and kept at 30°C for 2 days.

2.5 Total DNA extraction and yeast transformants screening.

Cells were grown in 2 mL YPD at 30°C to saturation. The biomass were collected by centrifugation, resuspended in 0.2 mL lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH8, 1 mM EDTA) and transferred to a 2 mL screwcap tube. Afterwards, 0.2 mL PCI [phenol pH 6.7- chloroform-isoamylalcohol (25:24:1)] and 0.3 g glass beads were added. The cells were broken using the fastprep machine, speed 6 for 20 sec followed by centrifugation at 17,968 x *g* for 10 minutes. The supernatant was transferred to a new tube, 0.5 mL ethanol was added and kept at -20°C for at least 20 minutes. The total DNA was pelleted by centrifugation at 17,968 x *g* for 10 minutes, washed with 70% ethanol and dried at room temperature. The DNA samples were dissolved in 30 µL nuclease-free H_20 and kept on -20°C. The correct cassette integration into the LAC4 locus was confirmed by colony PCR or by using their total DNA as template. For colony PCR, isolated colonies obtained on selective media were transferred to fresh selective agar media for the isolation of single colonies. Single colonies were picked up with a sterile toothpick and dissolved in 100 µL 0.01M NaOH and kept at room temperature for 45 minutes. A 1.5 µL aliquot of this sample or 1 µL from total purified DNA was used as a template for a 50 µL PCR reaction. The specific primers used to detect the single or multiple cassette insertions into the LAC4 promoter locus are indicated in Table 2. The amplification cycles comprised 5 minutes 98°C, 45 seconds 98°C, 30 seconds 58°C, 1 minute 72°C (35 cycles) and 5 minutes 72°C.

2.6 Total RNA extraction from yeast and RT-PCR

The cells were grown overnight in 5 mL YPGal medium at 30°C, 200 rpm. The cells were pelleted by centrifugation and the supernatant was discarded. The total RNA from recombinant *K. lactis* yeast cells was extracted using the Trizol® method (Invitrogen). The cDNA synthesis from the total RNA extracted was achieved using the Reverse Transcription System from Promega®. A 2 µL cDNA aliquot from each sample was used in a 50 µL PCR reaction in order to qualitatively detect mRNA expression of the L-AA pathway plant genes inserted into *K. lactis* genome. The RT-PCR was performed using the same primers and amplification cycles used for plant genes amplification.

2.7 Protein extraction, immunoprecipitation and western blotting

The recombinant cells were precultured overnight in 3 mL YPGal, 200 rpm at 30°C and used to inoculate 50 mL YPGal. When the culture reached the OD_{600} of 5, the cells were pelleted by centrifugation at 1,075 x *g*, 4°C for 5 minutes and washed with ice-cold Phosphate buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 at pH 7.3). Protein extraction was carried out with glass beads in lysis buffer containing 1x PBS, 0.001% Triton X-100, 8.7% glycerol, 25 mM $MgCl_2$, 10 mM EDTA (pH 7), 10 mM dithiotreitol, 100 mM NaF, 4 mM Na_3VO_4, 1 mM β-glycerophosphate and one tablet of Complete Protease Inhibitor Cocktail (Roche). Total protein content was measured according to Bradford, (1975) using bovine serum albumin (BSA) as standard. An aliquot, comprising 400 to 500 µg total protein extract, was used for flag tagged protein immunoprecipitation with monoclonal anti-FLAG antibodies (M2, Sigma-Aldrich) by incubation with Protein G agarose (Roche) for 3 hours at 4°C. SDS sample buffer (5X: 250 mM Tris–HCl, 10% SDS, 0.5% bromophenol blue, 1.4 M β-mercapto-ethanol) was added after three wash steps and stored at -20°C.

Proteins were separated by SDS-polyacrylamide gel electrophoresis on the NUPAGE Novex Bis-Tris mini Gel system (Invitrogen®). Separated proteins were transferred to nitrocellulose membrane (HybondC extra, Amersham) and detected by incubation with monoclonal anti Flag antibodies and horseradish peroxidase-conjugated anti mouse IgG secondary antibodies (Amersham) and detected using the Supersignal West Pico Luminol solution (Thermo Scientific). Immunoblots chemiluminescence was imaged using Fujifilm LAS-4000 mini, and the accompanying software Image Reader LAS-4000, (Life Science Fuji Photofilm Co., Ltd).

2.8 Measurement of intracellular L-galactose formation.

Recombinant cells precultured in 3 ml YPGal were used to inoculate 50 mL YPGal, 30°C, 200 rpm for 24 hours. The cells were harvested by filtration on nitrocellulose filters 0.45 µm, transferred to 8 mL methanol/chloroform (5 mL MeOH/3mL Chloroform) and kept at -20°C overnight. Aliquots from the supernatant were taken, transferred to 2mL tubes and cleared by centrifugation at 13,201 x *g,* 4°C for 10 minutes. Fractions of the supernatant were dried by speedvac and resuspended in 1 mL milliQ H_2O. Charged compounds were removed from the sample using Dowex

ion-exchange resins (1:1 v/v) 50WX8-200 (Sigma-Aldrich) and 1X8 200 (Acros Organics) The samples were used immediately for HPLC analysis (CarboPac PA1 anion-exchange column, 10 µm, 4 x 250 mm, DIONEX, eluent: 100 mM and 16 mM NaOH, flow rate: 1mL.min^{-1}, detection: pulse amperometry ED40 gold electrode) using pure D-galactose (Sigma-Aldrich, G0750) and L-galactose (Sigma, G7134) as standards.

2.9 Determination of L-Ascorbic acid.

For intracellular L-ascorbic acid determination, yeast cells were pregrown in 3 mL YP or YNB medium supplemented with 2% (w/v) galactose or lactose. These cells were used to inoculate 50 mL of either medium at an initial optical density of 0.1. The cells were grown for 24 hours, harvested by centrifugation at 2,988 x *g* for 5 minutes at 4°C and washed once with ice cold distilled H_2O. The cell pellet was resuspended in about twice the volume with ice cold 10% (w/v) trichloroacetic acid, vortexed vigorously for 2 minutes and kept on ice for 20 minutes. The supernatant was cleared from cell debris by centrifugation. L-ascorbic acid was determined spectrofotometrically according the method adapted from Sullivan et Clarke (40): 135 µL of sample was mixture with 40 µL 85 % (v/v) H_3PO_4, 675 µL 0.5% (w/v) α'α' dipyridyl and 135 µL 1% (w/v) $FeCl_3$. After incubation at room temperature for 10 minutes the absorbance at 525 nm was measured and the L-ascorbic acid concentration was calculated using the L-AA standard curve.

2.10 Statistical analysis.

The L-AA measurement experiments were carried out at least three times. Herein, we reported mean values as well as for L-AA standard curve. Student's t-test was performed with $p < 0.05$.

TABLE 1: List of primers used on this study

Name	*Sequence* (5'→3')	*Restriction site*
GME-F	**CTCGAG**ATGGGAACTACCAATGGAACAG	XhoI
GME-RFlag	**CCCGGCGGCCG**TCACTTGTCATCGTCATCCTTGTAATCCTCTTTTCCATCAGCCGCG	NotI
VTC2-F	**GCGGCCGC**ATGTTGAAAATCAAAAGAGTTCCGACC	NotI
VTC2-RFlag	**AGGCCT**TCACTTGTCATCGTCATCCTTGTAATCCTGAAGGACAAGGCACTCGGCGGC'	StuI
VTC4-F	**CTCGAG**ATGGCGGACAATGATTCTCTAG	XhoI
VTC4-RFlag	**AGGCCT**TCACTTGTCATCGTCATCCTTGTAATCTGCCCCTGTAAGCCGC	StuI
VT4-F	CGACTC**GGTACC**ATGGCGGACAATGATTCTCTAG	KpnI
VT4-R	CGACTC**GAATTC**TCACTTGTCATCGTCATCCTTG	EcoRI
hisG I – F	**TGTACA**CCAGTGGTGCATGAACGC	BsrGI
hisG I – R	**ACATGT**CTAGGGATAACAGGGTAATATAGACATGG	BsrGI
hisG II – F	CGACTC**CCCGGG**CCAGTGGTGCATGAACGC	XmaI/SmaI
hisG II – R	CGACTC**CTGCAG**CTAGGGATAACAGGGTAATATAGACATGG	PstI
KanMX-F	CGACTC**TGTACA**CTGAAGCTTCGTACGCTGCA	BsrGI
KanMX-R	CGACTC**CCCGGG**ATCACCTAATAACTTCGTATAGCATACATTATAC	SmaI
GPDADH1-F	CGACTC**CATATG GCGGCCGC**GTCGAAACTAAGTTCTTGGTGTTTTAAAACT	NdeI /NotI
GPDADH1-R	CGACTC**GACGTC AAGCTT**GGCATGCGAAGGAAAATGAGA	AatII / HindIII
KlACT1-F	ATGGATTCTGAGGTCGCTGC	
KlACT1-R	TTAGAAACACTTCAAGTGAACGATGG	
P1	ACACACGTAAACGCGCTCGGT	
P2	ATCATCCTTGTCAGCGAAAGC	
P3	ACCTGAAGATAGAGCTTCTAA	
P4	GGTACCCCTAGGAGATCTAGCTC	

Underlined are shown the Flag Tag sequence.
In bold are represented the restriction site.
In blue, the stop codon.

3. RESULTS

3.1 Isolation and cloning of the L-ascorbic acid pathway genes from *Arabidopsis thaliana*.

A cDNA library from *Arabidopsis thaliana* leaves was used as template to amplify the three genes required for L-galactose synthesis for the L-ascorbic acid (L-AA) pathway (see Matherials and Methods). The amino acid sequences encoded by the corresponding amplified genes AtGME, AtVTC2, AtVTC4 were determined and verified to be the same as those in the *A thaliana* genome database. The three genes were cloned in *K. lactis* expression vectors (Fig. 1).

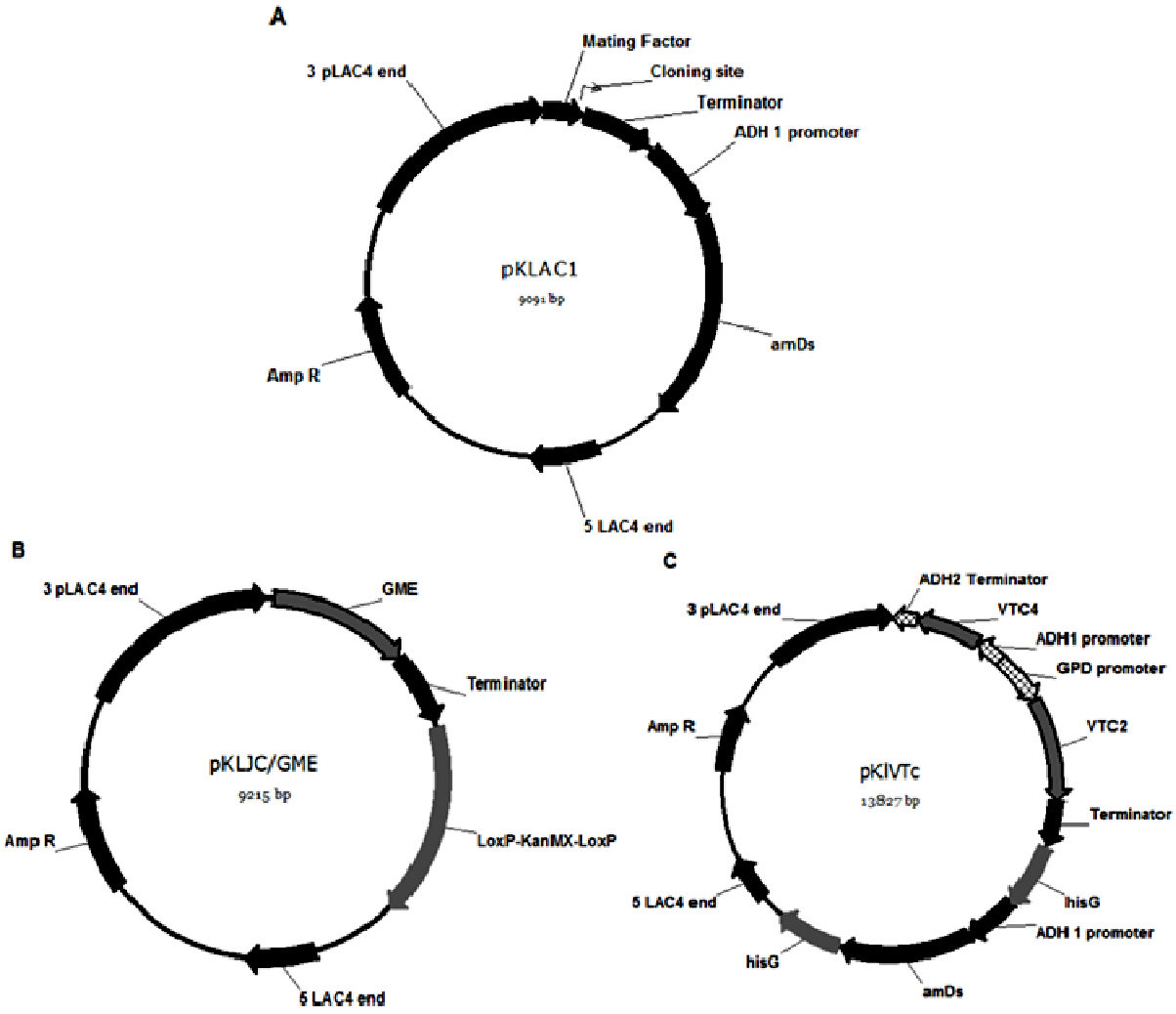

FIG. 1. Maps of the plasmid vectors used for L-AA genes expression. pKlJC/GME (B) and pKlVTc (C) vectors are derived from pKLAC1(A). The vectors contain the 5´ and 3´ ends of the LAC4 promoter separated by DNA encoding β-lactamase (Amp^R) and the pMB1 origin (ori). The yeast ADH1 promoter drives expression of an acetamidase selectable marker gene (*amdS*) which is flanked by hisG into pKlVTc plasmid. ADH1 and GPD promoters from *Saccharomyces cerevesiae* drive the transcription of AtVTC4 and AtVTC2 respectively. The cassette LoxP-KanMX-LoxP in the pKLJC/GME vector confers resistance to geneticin.

The codons of the plant genes were not optimized for expressing in *K. lactis* as Carbone et al. (6) reported that *Saccharomyces* sp. and plants shared the same preferred codons, supporting *K. lactis* as a host for unmodified plant genes expression.

3.2 *Kluyveromyces lactis* strains expressing L-AA genes.

To obtain strains producing L-AA, *K. lactis* CBS2359 cells were transformed with Sac II linearized pKLJC/GME and pKlVTc vectors constructed in this work (Fig 1). Strain JVC1-5, was obtained by transformation of *K. lactis* CBS2359 cells with the pKLJC/GME vector. The strains JVC1-51, JVC1-52, JVC1-53 were derived from JVC1-5 by transformation with pKlVtc vector containing the AtVTC4 and AtVTC2 expression cassette. The JVC3-2 strain was generated by one step transformation with both plasmids. The JVC2-1 and JVC2-2 strains were constructed by transformation of the *K. lactis* CBS2359 cells with the pKlVTc vector. All yeast strains used in this work are listed in table 2.

TABLE 2: Yeasts strains used in this study

Strain	Markers	Cassette expression	Plasmids	Reference
CBS 2359	Wild type	-	-	Genolevures consortium*
JVC1-5	Kan^R	AtGME	pKLJC/GME	This study
JVC1-51	Kan^R, amDs	AtGME, AtVTC2, AtVTC4	pKLJC/GME, pKlVTc	This study
JVC1-52	Kan^R, amDs	AtGME, AtVTC2, AtVTC4	pKLJC/GME, pKlVTc	This study
JVC1-53	Kan^R, amDs	AtGME, AtVTC2, AtVTC4	pKLJC/GME, pKlVTc	This study
JVC2-1	amDs	AtVTC2, AtVTC4	pKlVTc	This study
JVC2-2	amDs	AtVTC2, AtVTC4	pKlVTc	This study
JVC3-2	Kan^R, amDs	AtGME, AtVTC2, AtVTC4	pKLJC/GME, pKlVTc	This study

Kan^R cassette conferring resistance to Geneticin; amDs acetamidase marker.
**Kluyveromyces lactis* strain used for Genome sequencing by the Génolures consortium (www.genolevures.org)

The selection of *K. lactis* cells transformed with pKlVtc was achieved by growth on YCB agar medium containing 5 mM acetamide. The vector harbors the amDs marker which has been reported to favor transformants with more than one integration event into the genome (34). Correct integration into the *K. lactis* LAC4 locus was confirmed by PCR analysis using the primers P1, P2, P3 and P4 (Table 1). Figure 2 provides a schematic overview of the resulting genomic organization of the integrated plasmids at the *K. lactis* LAC4 chromosomal locus. Primer P1 was designed to anneal at the chromosomal LAC4 promoter upstream of the vector integration site and the reverse primers P2 and P4 anneal to pKlVTc and pKLJC/GME expression cassettes sequence respectively. When multiple copies of the cassette are integrated in tandem at the same locus a 2.3 kb fragment will be amplified by using the forward primer P3 in combination with either reverse primers P2 or P4 for each vector. Single and multiple insertions from each cassette were detected by the presence of 2.4 kb and 2.3 kb amplicons respectively. The insertion of the cassette into the LAC4 locus by homologous recombination duplicates the LAC4 promoter region so that it can be targeted by another cassette resulting in multiple copies integration. Although the analysis does not indicate the number of integrated copies, the transformants analyzed harbor multiple copies of each cassette in tandem at the LAC4 locus.

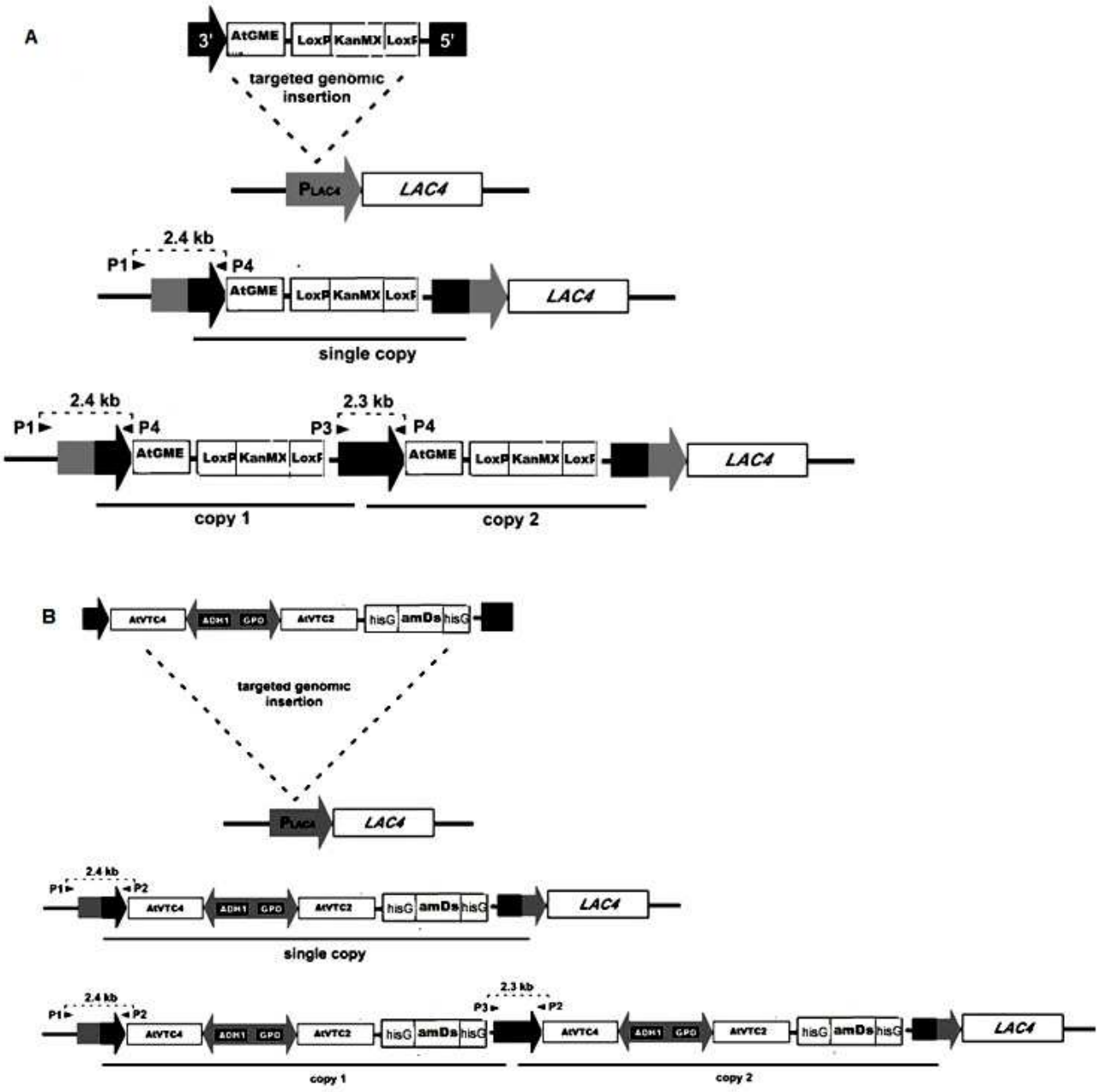

FIG. 2. Integration of SAC II linearized vectors into *K. lactis* LAC4 locus upon transformation. (A) Single and multiple copies of GME cassette detected by PCR using designed primers P1 and P3 (2.4 kb), P3 and P4 (2.3 kb) respectively; B) single and multiple copies of AtVTC2 and AtVTC4 cassette detected by PCR using the primers P1 and P2 (2.4 kb) and P3 and P2 (2.3 kb) respectively.

The AtGME gene is under the control of the inducible LAC4 promoter upon integration by homologous recombination. The strong constitutive *S. cerevisiae* promoters GPD1 and ADH1 drive the transcription of the AtVTC2 and AtVTC4 respectively. The expression analysis of L-AA pathway plant genes in *K. lactis* recombinant cells was peformed by RT-PCR and the flag-tagged proteins from total protein extract were immunoprecipitated and blotted against monoclonal anti-Flag antibody (Fig. 3). All JVC1-5 derived strains, JVC1-51, JVC1-52 and JVC1-53, are

expressing the L-galactose pathway genes, AtGME, AtVTC2, AtVTC4. The JVC1-5 only expresses AtGME and the JVC2-1 and JVC2-2 strains are the control strains for AtVTC2 and AtVTC4 expression.

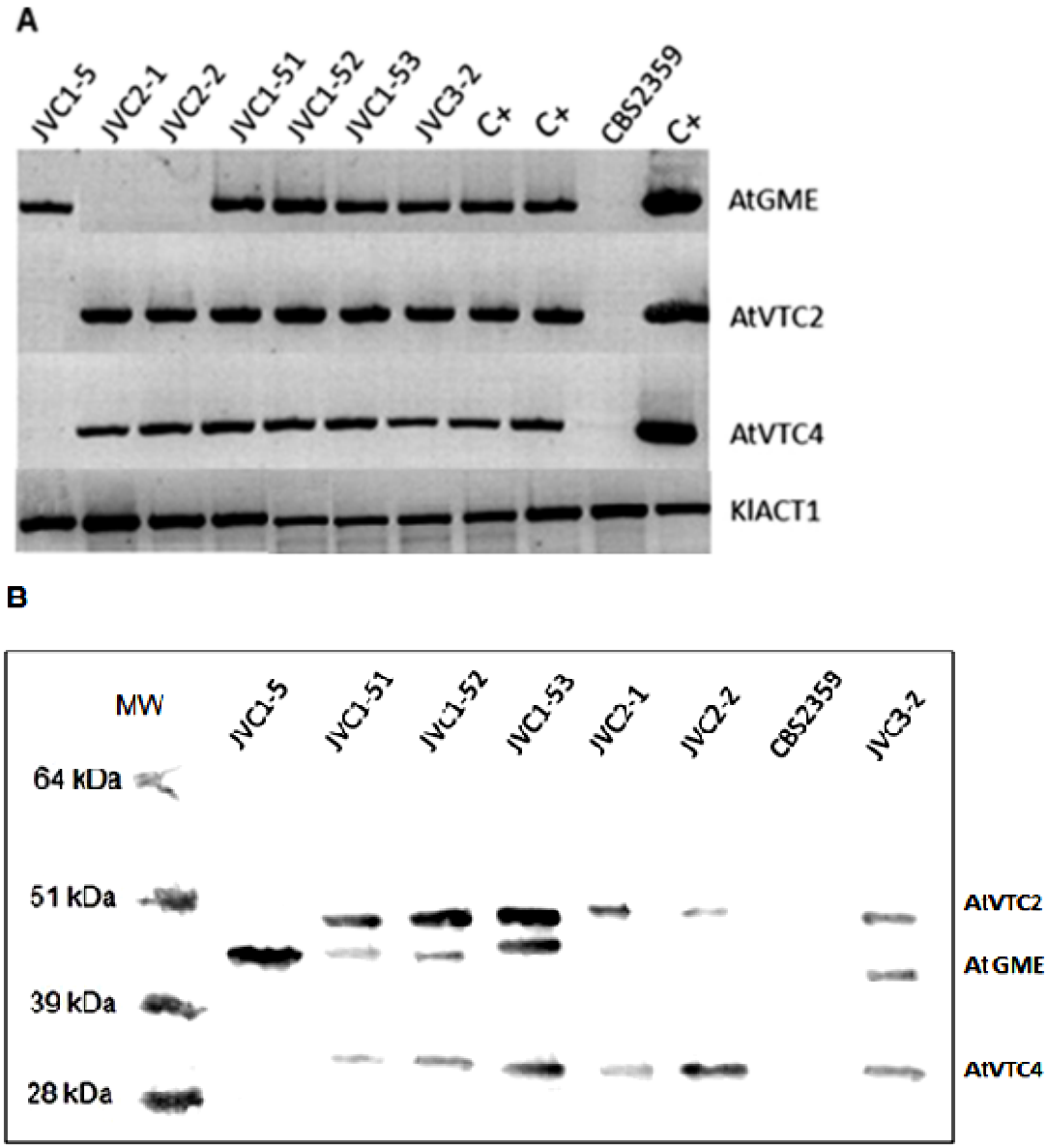

FIG. 3. Expression analysis of L-AA pathway plant genes by recombinant *K. lactis* cells. A- RT-PCR using cDNA from *K. lactis* cells transformed with three early L-AA pathway plant genes from Arabidopsis *thaliana*. C+ - the cDNA from *A. thaliana* leaves and plasmids harboring the corresponding genes was used as template for control in RT-PCR. KlACT1 actin gene from *K. lactis* CBS2359 was used as a control for RNA quality. B – Western blotting of flag-tagged immunoprecipitated proteins from *K. lactis* recombinant cells using monoclonal anti-flag antibody. *K. lactis* CBS2359 strain was used as negative control. The RNA extraction and total protein extraction were carried out from cells grown in YP medium with 2% (w/vol) D-Galactose after 24 hours incubation at 30°C, 200 rpm.

Simultaneous expression of the proteins AtGME (43.8 kDa), AtVTC2 (49 kDa) and AtVTC4 (30 kDa) in the engineered JVC3-2 and JVC1-5 derived strains should result in the production of L-galactose, when lactose or D-galactose are used as the carbon source in the growth medium. To address whether the plant genes integrated into the *K. lactis* genome would allow the cells to produce L-galactose from GDP- mannose we analyzed the L-galactose content in the recombinant strains grown in YP medium supplemented with 2% (w/v) D-galactose and in YP medium with 2% (w/v) lactose for 24 hours at 30°C, 200 rpm. Since we could not detect its intracellular production through HPLC analysis, the expression, as shown by western blot analysis, of the AtGME, AtVTC2 and AtVTC4 in *K. lactis* cells did not result in any measurable L-galactose biosynthesis (data not shown). This suggests that L-galactose was immediately converted into L-AA by the D-EAA enzymes thereby preventing its intracellular accumulation. Hence, the recombinant strains were screened for L-AA production. They were grown in YP or in YNB medium supplemented with 2% (w/v) D-galactose or lactose and grown for 48 hours, before the level of L-AA was determined. As one of the aims of our work is to use cheese whey, which is the waste product during cheese production, as an alternative source of lactose, we also evaluated the L-AA production culturing recombinant strains in cheese whey. Figure 4 shows the L-AA intracellular accumulation by the *K. lactis* strains that we engineered in this study. In the untransformed strain, low levels of L-AA could be measured in either minimal or rich medium supplemented with D-galactose. The accumulation of intracellular L-AA in JVC1-5 derived strains or in the JVC3-2 strain was 4 to 7-fold higher, but only when cultivated in YP medium and not in minimal medium with D-galactose as carbon source (Fig. 4A). When cells were cultivated in both YP and YNB medium with lactose as the sole carbon source, the L-AA accumulation was lower, but still a two-fold increase of L-AA was present in the JVC1-52 and JVC3-2 strains (Fig. 4B). However, when cheese whey was used as substrate all recombinant strains showed a significant intracellular L-AA accumulation compared to YNB medium (Fig. 4B).

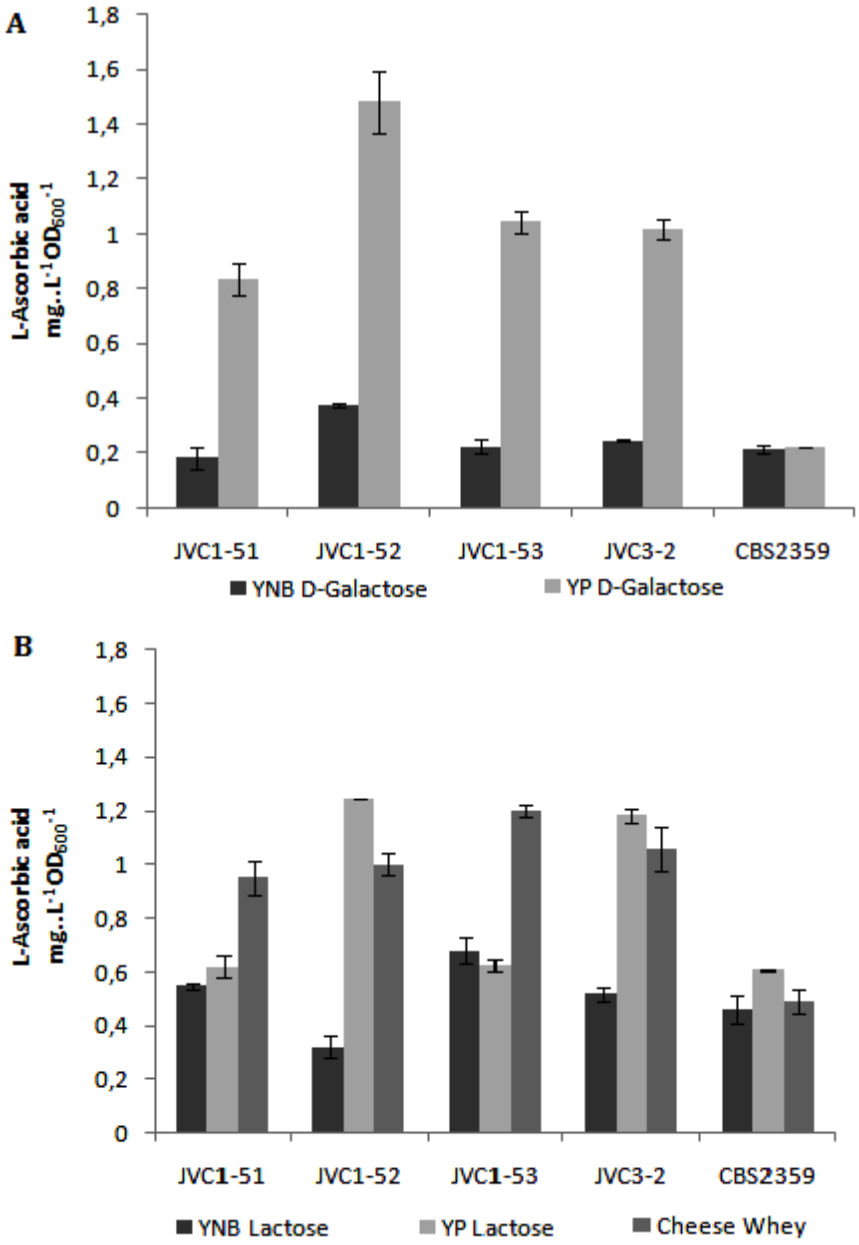

FIG. 4. Intracellular L-ascorbic acid per cell mass unit of *K. lactis* strains harboring plant genes to convert D-Galactose or Lactose to L-ascorbic acid. Transformed yeast cells were grown on cheese whey (B), mineral medium (0.67% [wt/vol] YNB) and on rich (YP) medium (20 $g.L^{-1}$peptone, 10 $g.L^{-1}$ Yeast extract) for 48 h (initial OD_{600} 0.05) supplemented with 2% (wt/vol) D-galactose (A) or lactose (B). CBS2359 parental strain was taken as control. No L-ascorbic acid could be detected in the supernatant from culture broth of the recombinant strains.

4. Discussion

In Figure 5 we present an overview of the L-AA pathway as we have engineered it in *K. lactis*.

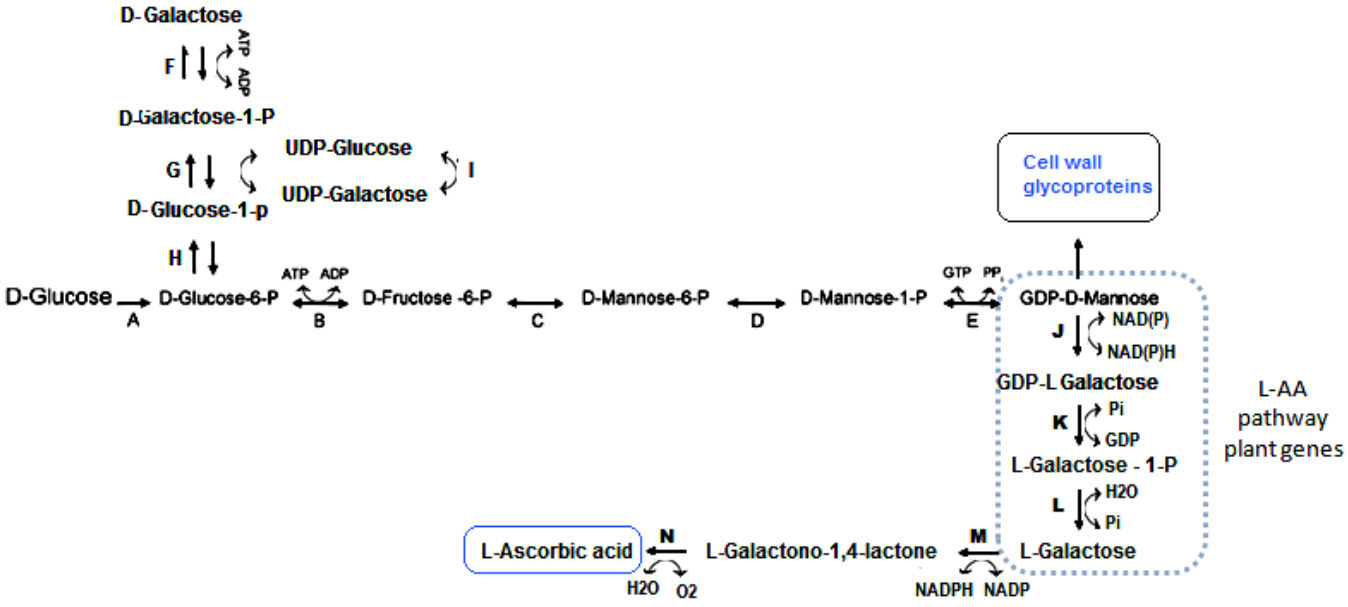

FIG. 5. L-AA pathway engineered in *K. lactis* cells using D-galactose or lactose as substrate. Lactose is first hydrolyzed by the β-galactosidase enzyme into D-Glucose and D-galactose which are promptly metabolized. A- hexokinase, B - glucose-6-phosphate isomerase, C – mannose-6-phosphate isomerase, D – phosphomannomutase, E – mannose-1-phosphate guanyltransferase, F – Galactokinase, G – galactose-1-phosphate urydyltransferase, H – phosphoglucomutase, I – UDP-galactose-1-epimerase, **J- GDP-mannose 3,5 epimerase, K – GDP-L-galactose phosphorylase, L- L-Galactose 1 phosphate phosphatase,** M – D-arabinose dehydrogenase, N – D-arabinono 1,4 lactone oxydase.

The insertion of the L-AA pathway plant genes into the *K. lactis* genome creates an alternative route to metabolize GDP-mannose, which is naturally produced in yeasts for cell wall construction (18). GDP-mannose undergoes epimerization to GDP-L-galactose by GME activity. VTC2 and VTC4 convert GDP-L-galactose in L-galactose that can be used as substrate for L-AA biosynthesis by D-EAA pathway enzymes. The D-EAA pathway is the only known route which contains enzymes able to metabolize non-physiological substrates such as L-galactose (13). Considering cofactors enzymes requirements, the new GDP-mannose branched pathway apparently would not affect the cell redox balance. The NADP(H) molecule produced in the reaction catalyzed by AtGME could be easily recycled by the *K. lactis* redox control system or being used in biosynthetic pathways. Besides the Glutathione/Thiorredoxin reductase system, two alternative dehydrogenases in the external mitochondrial membrane (NDE1 and NDE2) are the main source of cytosolic NADPH reoxidation in *K. lactis* cells (11). NADPH reoxidation is extremely important to maintain the pentose phosphate pathway which has been reported more active in *K. lactis* compared to *S. cerevesiae* (12).

Lactose and galactose metabolism seem to have different effects on this branched pathway. Probably, glucose released from lactose hydrolysis by β-galactosidase activity, may somehow affect the activity of the L-galactose pathway enzymes. Moreover, Lactose seems to stimulate the D-EAA accumulation in *K. lactis* cells more efficient than galactose since we could detect a high L-AA background in CBS2359 (Figure 4A and B). This background in *K. lactis* CBS2359 may be due to D-erythroascorbic acid synthesis naturally occurring in this yeast, which is much higher than in *S. cerevesiae*. This idea is also supported by Porro & Sauer (32).

Cheese Whey represents 85-95% of the milk volume retaining about 85% of milk nutrients such as lactose, soluble proteins, lipids and minerals. It also contains appreciable quantities of lactic and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (38). We suggest that this rich medium can provide intermediate metabolites that could be promptly assimilated reducing metabolic flux towards biosynthetic pathways such as cell wall biosynthesis. Likely, cheese whey (and also the YP medium) might enhance the flux of GDP-mannose towards to L-galactose formation and its subsequent conversion into L-AA.

The downstream L-galactose metabolism is the bottleneck for L-AA biosynthesis throughout this pathway since D-EAA enzymes regulation in yeast has not extensively been elucidated. The D-EA production is observed when yeasts are grown in some sources of D-aldoses such as D-glucose, D-galactose, D-mannose or D-arabinose (29).

The kinetic parameters of enzymes D-arabinose dehydrogenase (ARA2) and D-arabinono-1, 4 lactone oxydase (ALO1) has been determined in vitro and the results have demonstrated low substrate specificity (24). The ALO1 gene has a putative domain for the covalent FAD molecule similar to the domain found in oxygen-dependent oxidoreductases. Spickett et al. (41) found that the production of L-AA analogues is strongly influenced by the aeration of the culture. Probably the key regulatory enzyme pathway may be dependent on the dissolved oxygen levels. Also, when *S. cerevisiae alo1*Δ was grown in the presence of H_2O_2 cells were more sensitive while the overexpression leads to resistance. However no changes in the transcription levels of ALO1 gene were observed under the same conditions. Thus, transcriptional and post translation regulation of the genes from D-EAA pathway in yeast must be considered in this process. Thus, the engineering of D-EAA genes in *K. lactis* strains might be the main target in order to improve L-AA biosynthesis.

This work is the first attempt of engineering *K. lactis* cells for L-ascorbic acid biosynthesis by fermentation taking advantage of its natural ability to grow on lactose and without any exogenously addition of its precursors in the growth medium. By the insertion of the genes for L-galactose pathway from *A. thaliana*, we engineered *K. lactis* strains capable of converting lactose and D-galactose into L-galactose, a rare sugar which is one of the main precursor for L-AA production. Three other pathways for L-AA production in plants have been described (14): the L-Gulose pathway (46), the D-Galacturonic acid pathway (1), and the Myoinositol pathway (26), but these seem to be of minor importance. Thus, we developed yeast strains with great industrial potential to biologically produce L-AA exploiting their fermentation abilities.

Cheese whey represents an environmental problem due to its high volumes produced. Besides, the high organic matter content, mainly lactose, exhibits a biochemical oxygen demand (BOD) of 30-50 g. L^{-1} and a chemical oxygen demand (COB) of 60 – 80 g L^{-1} (38, 8). Considering its fermentation capacity, the recombinant *K. lactis* strains can convert lactose from cheese whey to valuable compounds such as L-AA. Moreover, since L-AA acid producing yeast strains have an improved stress resistance and robustness (5) these strains can also be used as host for producing heterologous proteins with industrial interest in biotechnological processes.

5. ACKNOWLEDGEMENTS

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7. CONCLUSÕES

No presente trabalho foi realizada a engenharia metabólica da levedura *Kluyveromyces lactis* visando a produção do ácido L-ascórbico (L-AA).

Embora as leveduras naturalmente não apresentem genes de biossíntese do L-AA, elas são capazes de sintetizar o ácido D-eritroascórbico (D-EA) que apresenta propriedades antioxidantes similares ao L-AA. Em plantas, o ácido L-ascórbico é sintetizado a partir de D-glicose, sendo L-galactose, o intermediário chave. Os genes GME, VTC2 e VTC4 que estão envolvidos na via de biossíntese do isômero L-galactose a partir de GDP-manose foram isolados e corretamente amplificados a partir de uma biblioteca de cDNA proveniente de folhas de *Arabidopsis thaliana*.

Os plasmideos pKlJC/GME e pKlVTc construídos neste trabalho direcionaram a integração dos cassetes de expressão dos genes na região promotora do gene LAC4 da célula hospedeira.

A transformação de células de *K. lactis* com os plasmideos contendo os genes de biossíntese de L-galactose resultou em múltiplas integrações *in tandem* na região promotora do gene LAC4.

A expressão dos genes GME, VTC2 e VTC4 não resultou em acúmulo intracelular de L-galactose. Porém, as linhagens de *K. lactis* recombinantes foram capazes de sintetizar o ácido L-ascórbico quando cultivadas em meio YP suplementado com 2% (p/v) galactose ou lactose ou em meio de soro de queijo. O soro de queijo apresenta um alto teor matéria orgânica principalmente lactose. Apesar de inúmeros estudos sobre o potencial nutricional, funcional e tecnológico do soro de queijo, nenhuma opção para seu aproveitamento tem sido suficiente para evitar seu despejo sem tratamento nos cursos de água especialmente pelas pequenas e médias empresas. A conversão da lactose do soro de queijo em compostos que apresentam um valor industrial e comercial agregado constitui-se uma alternativa para minimizar os efeitos poluentes do soro.

Portanto ao final deste trabalho, aplicando as técnicas de engenharia genética e de biologia molecular associada à indústria de fermentação, foi desenvolvida uma nova estratégia para produção biológica do ácido L-ascórbico (vitamina C).

Appendix

Arabidopsis thaliana **GME (GDP-D-Mannose 3',5'-Epimerase)**

LOCUS NM_122767.3 1134 pb mRNA

```
   1 ATGGGAACTA CCAATGGAAC AGACTATGGA GCATACACAT ACAAGGAGCT AGAAAGAGAG
  61 CAATATTGGC CATCTGAGAA TCTCAAGATA TCAATAACAG GAGCTGGAGG TTTCATTGCA
 121 TCTCACATTG CTCGTCGTTT GAAGCACGAA GGTCATTACG TGATTGCTTC TGACTGGAAA
 181 AAGAATGAAC ACATGACTGA AGACATGTTC TGTGATGAGT TCCATCTTGT TGATCTTAGG
 241 GTTATGGAGA ATTGTCTCAA AGTTACTGAA GGAGTTGATC ATGTTTTTAA CTTAGCTGCT
 301 GATATGGGTG GTATGGGTTT TATCCAGAGT AATCACTCTG TGATTATGTA TAATAATACT
 361 ATGATTAGTT TCAATATGAT TGAGGCTGCT AGGATCAATG GGATTAAGAG GTTCTTTTAT
 421 GCTTCGAGTG CTTGTATCTA TCCAGAGTTT AAGCAGTTGG AGACTACTAA TGTGAGCTTG
 481 AAGGAGTCAG ATGCTTGGCC TGCAGAGCCT CAAGATGCTT ATGGTTTGGA GAAGCTTGCT
 541 ACGGAGGAGT TGTGTAAGCA TTACAACAAA GATTTTGGTA TTGAGTGTCG AATTGGAAGG
 601 TTCCATAACA TTTATGGTCC TTTTGGAACA TGGAAAGGTG GAAGGGAGAA GGCTCCAGCT
 661 GCTTTCTGTA GGAAGGCTCA GACTTCCACT GATAGGTTTG AGATGTGGGG AGATGGGCTT
 721 CAGACCCGTT CTTTTACCTT TATCGATGAG TGTGTTGAAG GTGTACTCAG GTTGACAAAA
 781 TCAGATTTCC GTGAGCCGGT GAACATCGGA AGCGATGAGA TGGTGAGCAT GAATGAGATG
 841 GCTGAGATGG TTCTCAGCTT TGAGGAAAAG AAGCTTCCAA TTCACCACAT TCCTGGCCCG
 901 GAAGGTGTTC GTGGTCGTAA CTCAGACAAC AATCTGATCA AAGAAAAGCT TGGTTGGGCT
 961 CCTAATATGA GATTGAAGGA GGGGCTTAGA ATAACCTACT TCTGGATAAA GGAACAGATC
1021 GAGAAAGAGA AAGCAAAGGG AAGCGATGTG TCGCTTTACG GGTCATCAAA GGTGGTTGGA
1081 ACTCAAGCAC CGGTTCAGCT AGGCTCACTC CGCGCGGCTG ATGGAAAAGA GTGA
```

Protein Sequence

Number of residues: 377;
Molecular weight: 42.8 kDa

```
"MGTTNGTDYGAYTYKELEREQYWPSENLKISITGAGGFIASHIARRLKHEGHYVIASDWKKNEHMTEDM
FCDEFHLVDLRVMENCLKVTEGVDHVFNLAADMGGMGFIQSNHSVIMYNNTMISFNMIEAARINGIKRFF
YASSACIYPEFKQLETTNVSLKESDAWPAEPQDAYGLEKLATEELCKHYNKDFGIECRIGRFHNIYGPFG
TWKGGREKAPAAFCRKAQTSTDRFEMWGDGLQTRSFTFIDECVEGVLRLTKSDFREPVNIGSDEMVSMNE
MAEMVLSFEEKKLPIHHIPGPEGVRGRNSDNNLIKEKLGWAPNMRLKEGLRITYFWIKEQIEKEKAKGSD
VSLYGSSKVVGTQAPVQLGSLRAADGKE"
```

Appendix A: *Arabidopsis thaliana* GME mRNA sequence and corresponding protein sequence. GME was amplified by PCR in this study using specific primers and cDNA from *A. thaliana* leaves as template.

Arabidopsis thaliana VTC2 (GDP-L-Galactose Phosphorylase)

LOCUS NM118819 1329 bp mRNA

```
   1 ATGTTGAAAA TCAAAAGAGT TCCGACCGTT GTTTCGAACT ACCAGAAGGA CGATGGAGCG
  61 GAGGATCCCG TCGGCTGTGG ACGGAATTGC CTCGGCGCTT GTTGCCTTAA CGGGGCTAGG
 121 CTTCCATTGT ATGCATGTAA GAATCTGGTA AAATCCGGAG AGAAGCTTGT AATCAGTCAT
 181 GAGGCTATAG AGCCTCCTGT AGCTTTTCTC GAGTCCCTTG TTCTCGGAGA GTGGGAGGAT
 241 AGGTTCCAAA GAGGACTTTT TCGCTATGAT GTCACTGCCT GCGAAACCAA AGTTATCCCG
 301 GGGAAGTATG GTTTCGTTGC TCAGCTTAAC GAGGGTCGTC ACTTGAAGAA GAGGCCAACT
 361 GAGTTCCGTG TAGATAAGGT GTTGCAGTCT TTTGATGGCA GCAAATTCAA CTTCACTAAA
 421 GTTGGCCAAG AAGAGTTGCT CTTCCAGTTT GAAGCTGGTG AAGATGCCCA AGTTCAGTTC
 481 TTCCCTTGCA TGCCTATTGA CCCTGAGAAT TCTCCCAGTG TTGTTGCCAT CAATGTTAGT
 541 CCGATAGAGT ATGGCCATGT GCTGCTGATT CCTCGTGTTC TTGACTGCTT GCCTCAAAGG
 601 ATCGATCACA AAAGCCTTTT GCTTGCAGTT CACATGGCTG CTGAGGCTGC TAATCCATAC
 661 TTCAGACTCG GTTACAACAG CTTGGGTGCT TTTGCCACTA TCAATCATCT CCACTTTCAG
 721 GCTTATTACT TGGCCATGCC TTTCCCACTG GAGAAAGCTC CTACCAAGAA GATAACTACC
 781 ACTGTTAGTG GTGTCAAAAT CTCAGAGCTT CTAAGTTACC CTGTGAGAAG TCTTCTCTTT
 841 GAAGGTGGAA GCTCTATGCA AGAACTATCT GATACTGTTT CAGACTGCTG TGTTTGCCTT
 901 CAAAACAACA ACATTCCTTT CAACATTCTC ATCTCTGATT GTGGAAGGCA GATCTTCTTA
 961 ATGCCACAGT GTTACGCAGA GAAACAGGCT CTAGGTGAAG TGAGCCCGGA GGTATTGGAA
1021 ACACAAGTGA ACCCAGCCGT GTGGGAGATA AGTGGTCACA TGGTACTGAA GAGGAAAGAG
1081 GATTACGAAG GTGCTTCAGA GGATAACGCG TGGAGGCTCC TTGCGGAAGC TTCTCTGTCG
1141 GAGGAAAGGT TTAAGGAGGT TACTGCTCTC GCCTTTGAAG CCATAGGTTG TAGTAACCAA
1201 GAGGAGGATC TTGAAGGAAC CATAGTTCAT CAGCAAAACT CTAGTGGCAA TGTTAACCAG
1261 AAAAGCAACA GAACCCATGG AGGTCCGATC ACAAATGGGA CGGCCGCCGA GTGCCTTGTC
1321 CTTCAGTGA
```

Protein Sequence

Number of residues: 442;

Molecular weight: 49.0 kDa

“MLKIKRVPTVVSNYQKDDGAEDPVGCGRNCLGACCLNGARLPLYACKNLVKSGEKLVISHEAIEPPVAF
LESLVLGEWEDRFQRGLFRYDVTACETKVIPGKYGFVAQLNEGRHLKKRPTEFRVDKVLQSFDGSKFNFT
KVGQEELLFQFEAGEDAQVQFFPCMPIDPENSPSVVAINVSPIEYGHVLLIPRVLDCLPQRIDHKSLLLA
VHMAAEAANPYFRLGYNSLGAFATINHLHFQAYYLAMPFPLEKAPTKKITTTVSGVKISELLSYPVRSLL
FEGGSSMQELSDTVSDCCVCLQNNNIPFNILISDCGRQIFLMPQCYAEKQALGEVSPEVLETQVNPAVWE
ISGHMVLKRKEDYEGASEDNAWRLLAEASLSEERFKEVTALAFEAIGCSNQEEDLEGTIVHQQNSSGNVN
QKSNRTHGGPITNGTAAECLVLQ”

Appendix B: *Arabidopsis thaliana* VTC2 mRNA sequence and corresponding protein sequence. VTC2 was amplified by PCR in this study using specific primers and cDNA from *A. thaliana* leaves as template.

Arabidopsis thaliana **VTC4 (L-galactose-1-phosphate phosphatase/ inositol or phosphatidylinositol phosphatase)**

LOCUS NM111155 816 bp mRNA

```
  1 ATGGCGGACA ATGATTCTCT AGATCAGTTT TTGGCTGCCG CCATTGATGC CGCTAAAAAA
 61 GCTGGACAGA TCATTCGTAA AGGGTTTTAC GAGACTAAAC ATGTTGAACA CAAAGGCCAG
121 GTGGATTTGG TGACAGAGAC TGATAAAGGA TGTGAAGAAC TTGTGTTTAA TCATCTCAAG
181 CAGCTCTTTC CCAATCACAA GTTCATAGGA GAAGAAACTA CAGCTGCATT TGGTGTGACA
241 GAACTAACTG ACGAACCAAC TTGGATTGTT GATCCTCTTG ATGGAACAAC CAATTTCGTT
301 CACGGGTTCC CTTTCGTGTG TGTTTCCATT GGACTTACGA TTGGAAAAGT CCCTGTTGTT
361 GGAGTTGTTT ATAATCCTAT TATGGAAGAG CTATTCACCG GTGTCCAAGG GAAAGGAGCA
421 TTCTTGAATG GAAAGCGAAT CAAAGTGTCA GCTCAAAGCG AACTTTTAAC CGCTTTGCTC
481 GTGACAGAGG CGGGTACTAA ACGAGATAAA GCTACATTAG ACGATACAAC CAACAGAATC
541 AACAGTTTGC TAACCAAGGT CAGGTCCCTT AGGATGAGTG GTTCGTGTGC ACTGGACCTC
601 TGTGGCGTTG CGTGTGGAAG GGTTGATATC TTCTACGAGC TCGGTTTCGG TGGTCCATGG
661 GACATTGCAG CAGGAATTGT TATCGTGAAA GAAGCTGGTG GACTCATCTT TGATCCATCC
721 GGTAAAGATT TGGACATAAC ATCGCAGAGG ATCGCGGCTT CAAACGCTTC TCTCAAGGAG
781 TTATTCGCTG AGGCGTTGCG GCTTACAGGG GCATGA
```

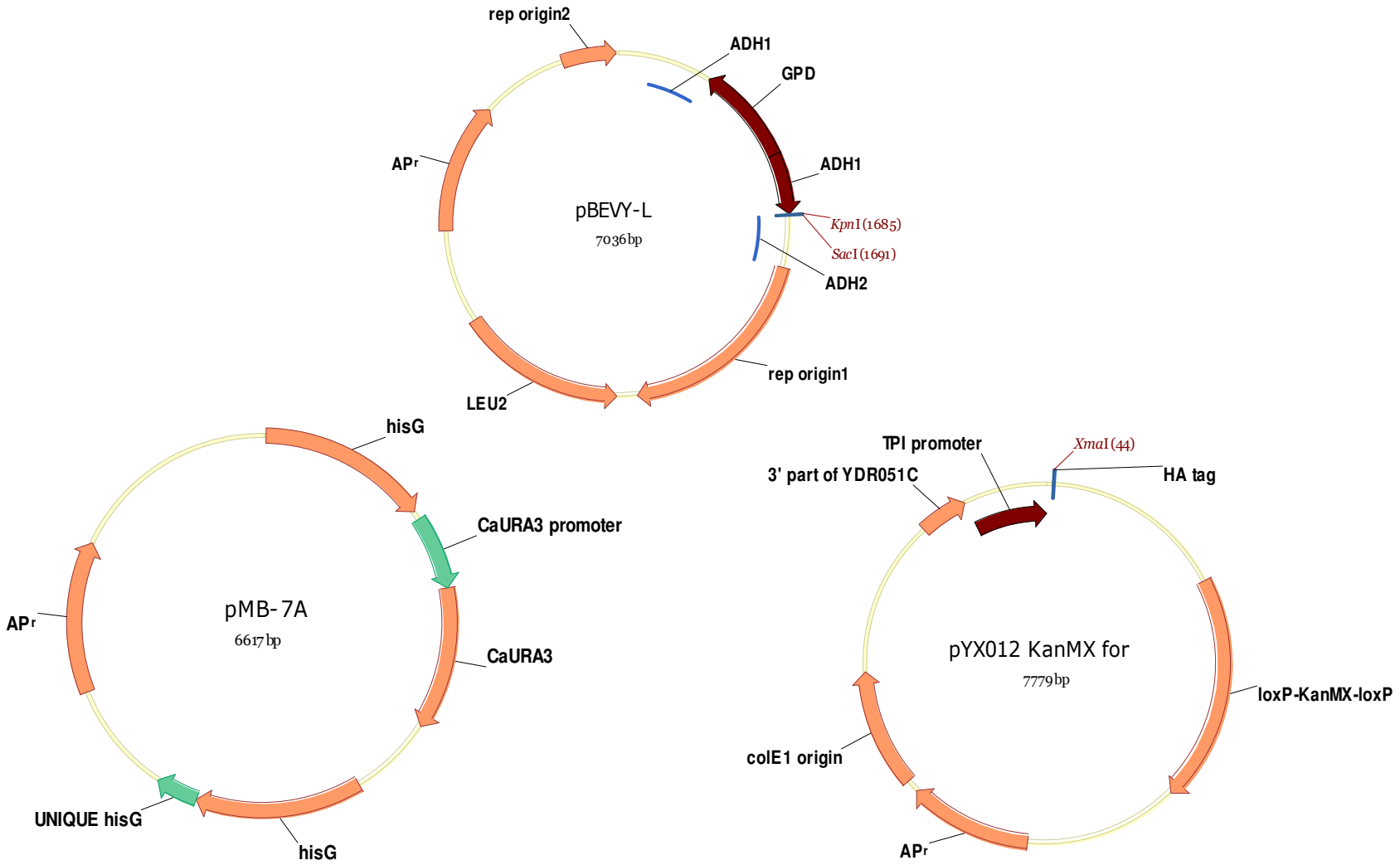
Protein Sequence

Number of residues: 271;

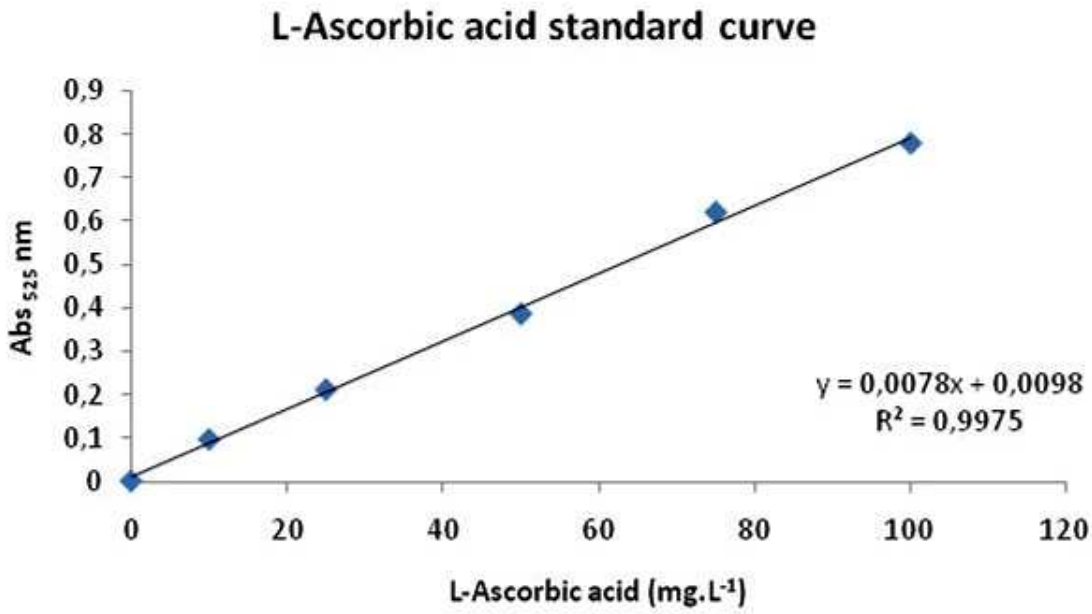
Molecular weight: 29.1 kdal

```
“MADNDSLDQFLAAAIDAAKKAGQIIRKGFYETKHVEHKGQVDLVTETDKGCEELVFNHLKQLFPNHKFI
GEETTAAFGVTELTDEPTWIVDPLDGTTNFVHGFPFVCVSIGLTIGKVPVVGVVYNPIMEELFTGVQGKG
AFLNGKRIKVSAQSELLTALLVTEAGTKRDKATLDDTTNRINSLLTKVRSLRMSGSCALDLCGVACGRVD
IFYELGFGGPWDIAAGIVIVKEAGGLIFDPSGKDLDITSQRIAASNASLKELFAEALRLTGA”
```

Appendix C: *Arabidopsis thaliana* VTC4 mRNA sequence and corresponding protein sequence. VTC4 was amplified by PCR in this study using specific primers and cDNA from *A. thaliana* leaves as template.



Appendix D: Plasmids pBEVY-L, pMB-7A and pYX012 KanMX used as template for GPD-ADH1 fused promoters, His and LoxP-KanMX-LoxP cassette in vitro amplification, respectively.



Appendix E: L-Ascorbic acid standard curve used as reference to estimate intracellular L-AA content in recombinant *K. lactis* strains