Universidade do Minho
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Structural-functional studies of carboxylic acid transporters: novel tools in production optimization using industrial microbes

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Professora Doutora Sandra Paiva

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DECLARAÇÃO

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ABSTRACT

Structural-functional studies of carboxylic acid transporters: novel tools in production optimization using industrial microbes

The rising global energy demands in last century drive to worrying depletion levels of fossil fuels, leading to a growing interest in microbial biofuel synthesis, particularly in model organisms like *Saccharomyces cerevisiae*. The microbial conversion of sugars to biofuels is a promising technology, thus envisaging for more efficient metabolic pathways has been attempted. Indeed byproducts of biomass pretreatment processes and the biofuels themselves are often toxic at industrially-relevant levels., therefore efforts to improve production yield by engineering efflux systems to overcome toxicity problems and secret inhibitory chemicals from the cell has been revealed as a crucial alternative. In this scope, the aim of the present work was to screen a wide range of promising membrane transporters for the transport of carboxylic acids, particularly for xylonic acid, mucic acid, saccharic acid, gluconic acid and xylaric acid in the yeast *S. cerevisiae.* During this study a considerable number of membrane proteins, known as carboxylic acid transporters from different yeasts species were screened and functionally characterized in regard to the transport of mono- and dicarboxylic acids with biotechnological application, such as xylonic and gluconic acids, and saccharic, mucic and xylaric acids, respectively. Among the transporters tested, we have found evidences for the transport of four carboxylic acids, mucic, xylaric, gluconic and saccharic acids with the following specificities: gluconic acid (K_i of 13.2 mM and 40.6 mM), xylaric acid (K_i of 27.6 mM), mucic acid (K_i of 32.9 mM) and saccharic acid (K_i of 24.1 mM and 24.7 mM). These results revealed to be very promising for future work aiming at engineering proper microbial strains with increased ability to export biofuel acids to external medium.

RESUMO

Estudos funcionais/estruturais de transportadores de ácidos carboxílicos: novas ferramentas para otimização da produção em microrganismos industriais.

A crescente procura de combustíveis fósseis a que se assistiu no último século levou a que se atingissem limites de exploração preocupantes, redirecionando a atenção para a síntese microbiana de biocombustíveis, em particular através organismo modelo *Saccharomices cerevisiae*. A conversão microbiana de açúcares em biocombustíveis tem-se revelado uma tecnologia promissora, pelo que novas tentativas de melhorar a eficiência das vias metabólicas envolvidas no processo de síntese têm sido testadas. Tanto os produtos resultantes do processamento de biomassa como os próprios biocombustíveis exercem regularmente um efeito tóxico na produção à escala industrial. Nesse sentido, importantes esforços têm sido levados a cabo para desenvolver novos sistemas de efluxo com o intuito de reduzir problemas de toxicidade e conduzir para o exterior da célula diferentes compostos químicos.

Neste contexto, o objetivo do presente trabalho é desvendar o potencial de diferentes transportadores de membrana para o transporte de ácidos carboxílicos em *S. cerevisiae*.

Ao longo deste estudo, várias proteínas de membrana, conhecidas como transportadores de ácidos carboxílicos provenientes de diferentes espécies de leveduras foram testadas e caracterizadas funcionalmente no que respeita ao transporte de ácidos mono e dicarboxílicos com interesse biotecnológico, tais como ácidos xilónico e glucónico, e ácidos sacárico, mucico e xilárico, respetivamente. De entre os transportadores testados foram encontradas evidências para o transporte de quatro ácidos carboxílicos, a saber os ácidos mucico, xilárico, glucónico e sacárico com as respetivas especificidades: ácido glucónico (K_i de 13,2 mM e 40,6 mM), ácido xilárico (K_i de 27,6 mM), ácido mucico (K_i de 32,9 mM) e ácido sacárico (de 24,1 mM e 24,7 mM). Estes resultados revelam-se bastante promissores para futuros estudos que envolvam a obtenção de estirpes microbianas com capacidade desenvolvida para excretar biocombustíveis.

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ABBREVIATIONS AND ACRONYMS

AMPK - Adenosine Monophosphate-activated Protein Kinase

ald – aldehyde dehydrogenase

COOH – Carboxyl group

dhaB – glycerol dehydratase

DNA - Deoxyribonucleic acid

HCOOH – formic acid

HP – Hidroypropanoic

HPA – Hydroxypropionaldehyde

IUPAC – International Union of Pure and Applied Chemistry

GFP – Green Fluorescence Protein

GRAS – Generally recognized as safe

LDH – Lactate dehydrogenase

K_d - diffusion constant

K_i - inhibition constant

K_m – affinity constant

k_{mobs} – inhibitor's k_m

MFS – Major Facilitator Superfamily

ORF – Open Reading Frame

PfK - Phospho-fructokinase

PLA – Polylactic acid

pmf – Proton motive force

PP1 - Phosphoprotein phosphatase 1

R – Hydrocarbon group

SHS – Sialate:H^+ Symporter

SSS – Solute/ Sodium Symporter Family

TCDB - Transport Classification Database

TDT - Tellurite-resistance/Dicarboxylate Transporter

TMS – Transmembrane segments

V_{max} - maximum velocity

YNB – Yeast nitrogen base

YP – yeast extract peptone

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Table I – Market and current production processes of organic acids**.**

Table II – Organinc acids market demands.

1. INTRODUCTION

1.1 Carboxylic acids: general properties and nomenclature

Carboxylic acids are organic compounds containing a carboxyl group (COOH) and a hydrocarbon group (R) (fig.1.1). These two unlike parts have great impact on the physical, as well as, chemical properties of the molecule as a whole (Abbott *et al.*, 2009).

Carboxylic acids are easily converted into both esters and amides, and the esters and amides are also converted back to the carboxylic acids. These properties make molecules that contain carboxylic acids, amides, and esters important in biochemistry and chemical industry.

The most significant property of carboxylic acids is their behavior as weak acids. They surrender the hydrogen of the carboxyl group, COOH, to bases and establish acid base equilibrium in aqueous solution. The common carboxylic acids share the concentration-dependent corrosive properties of all acids but are not generally hazardous to human health. Like alcohols, carboxylic acids form hydrogen bonds with each other so that even formic acid (HCOOH), the simplest carboxylic acid, is a liquid at room temperature with a boiling point of 101 °C. Acids with saturated straight-chain, R groups, of up to nine carbon atoms are volatile liquids with strong, pungent, and usually unpleasant odors; those with up to four carbons are water-soluble. Acids with larger saturated R groups are waxy, odorless solids. Their water solubility falls off as the size of the hydrophobic, alkane R group increases relative to the size of the water-soluble portion (McMurry *et al.*, 2010).

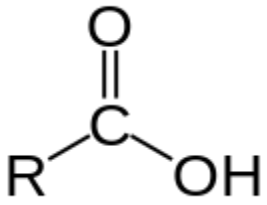


Figure 1.1– Chemical structure of carboxylic acid group (Chemspider, 2013).

Carboxylic acids are named in the International Union of Pure and Applied Chemistry (IUPAC) system by replacing the final *-e* of the corresponding alkane name with *-oic*

acid. Thus, the names corresponding to the one-, two-, and three-carbon acids are methanoic acid, ethanoic acid, and propanoic acid. These names are derived from methane, ethane, and propane. If alkyl substituents or other functional groups are present, the chain is numbered beginning at the end, as in 3-methylbutanoic acid or in 2-hydroxypropanoic acid, which is better known as lactic acid, the acid present in sour milk. Dicarboxylic acids**,** which contain two carboxylic groups, are named systematically by adding the ending *-dioic acid* to the alkane name (the *-e* is retained). Unsaturated acids are named systematically in the IUPAC system with the ending *–enoic* (McMurry *et al.*, 2010).

The IUPAC method is neither the only nor the most-used method for naming acids, primarily because carboxylic acids were among the first organic compounds to be isolated and purified. Organic acids are usually known by common names. Methanoic, ethanoic, and propanoic acids are called formic, acetic, and propionic acids, respectively. These names often refer to a natural source of the acid and are not systematic. Many of the carboxylic acids, especially those with even numbers of carbon atoms ranging from 4 to about 20, exist in combined form in plant and animal fats. These are called *fatty acids*. Another nomenclature method uses letters of the Greek alphabet (α, β, γ, δ,…) to name certain acid derivatives, especially hydroxy, amino, and halogen acids. When Greek letters are used, the carbon atoms, beginning with the one adjacent to the carboxyl group, are labeled α, β, γ, δ. When numbers are used (the IUPAC System), the numbers begin with the carbon in the COOH group (McMurry *et al.*, 2010).

1.2 Carboxylic acids in industry

Carboxylic acids together with enols, phenols, sulfonic acids, mercapto-compounds, and phosphonic acids make up the organic acids group. During centuries they have taken an important role in human development. Their presence goes from ancient to modern foods and beverages, as well as drugs, detergents, cosmetics, polymers, plastics, resins, and many other biochemical or chemical products (Huang *et al*., 2007).

There are two main industrial approaches for the production of organic acids: fermentation and chemical synthesis. Fermentation processes were dominant in the production of organic acids from the end of the nineteenth century until the beginnings of the last century. The main precursor for such monopoly leans on Pasteur's microbiological founds, mainly due to the abolishment of spontaneous generation theory

and the identification of fermentative microorganisms. However, the petrochemical industry revolution, boosted by the first and second world wars in the first half of the late century unveils new chemical synthesis procedures highly competitive, driving the organic acid market until the 90's. Raising concerns about sustainable development and human health safety gave a new opportunity to fermentative industry. With the recombinant DNA (Deoxyribonucleic acid) technology revolution in the last two decades, virtually all organic acids can be produced by microorganisms, unlike chemical synthesis. Meanwhile, the products from fermentation have a higher safety degree, which is a significant advantage to human health. Another advantage of microbial production is the high specificity for production of desirable stereospecific forms of carboxylic acids (Hong and Nielsen, 2012; Huang *et al.*, 2007).

Whether it is fermentation or chemical synthesis, separation, concentration, and purification are necessary for product preparation. More downstream processes are required by fermentation since the carboxylic acid is recovered from culture broth. The related traditional techniques include precipitation and acidification, extraction, crystallization, distillation, ion-exchange, and adsorption. Precipitation and acidification are perhaps the most unfriendly processes to environment, due to solid pollution, particularly by gypsum. Solvent extraction is handicapped by undesirable distribution coefficients and environmental problems due to the use of hazardous solvents. Crystallization is unfavorable due to its low yield, high costs for chemical use, and waste discharge (Sauer *et al.*, 2008).

Currently, carboxylic acids building blocks for biofuels can be obtained via microorganism's fermentative properties, and represent a reliable replacement to petroleum-based fuels (Table I). Producing these biofuels in a cost effective manner often requires the engineering of cell's machinery (Peralta-Yahya *et al.*, 2012).

Table I – Market and current production processes of organic acids (Almeida *et al.*, 2012).

Product	Annual Production (t)	Production process
1,3-Propanediol	130,000	Petrochemical
2,3-Butanediol	1,250,000	Petrochemical
Ethanol	61,000,000	Microbial fermentation
n-Butanol	2,800,000	Petrochemical
Lactic acid	350,000	Microbial fermentation
Succinic acid	16 -30,000	Petrochemical
Citric acid	1,600,000*	Microbial fermentation
Oxalic acid	124,000*	Petrochemical
Mannitol	13,600 - 50,000	Chemical conversion of sugars
Erythritol	20,000 – 23,000	Microbial fermentation

*Estimated values.

Usually biofuel production from microorganisms' metabolism uses as a main nutrient food crops (mainly starch and sucrose), however a danger linkage between fuel and food prices is established. Therefore alternative and cheaper nutrient sources, such as algae biomass, greenhouse gases, lignocellulose and carbon dioxide are preferred. Algae biomass grows in salt water rather than arable land, but its collection and dewatering is challenging. Lignocellulose is a rich source of sugars and highly abundant, however thermal, chemical and biochemical pretreatment are needed for sugar polymers hydrolysis. Through photosynthetic organisms carbon dioxide could be transformed in biofuels (Hong and Nielsen, 2012).

Microbial production is not only related with engineering the selected host and the choice of nutrient source but also factors as energy content, combustion, engine type, quality or ignition delay, cloud point, volatility, lubricity, viscosity, stability, odor, toxicity, water miscibility must be considered (Peralta-Yahya *et al.*, 2012)

In the last decade, several reports from international institutions, such as The United States Department of Energy, the European Group BREW and the European Commission have emphasized the potential of carboxylic acids as key players in enzymatic and chemical catalysis processes (Table II). On the other hand several studies have also shown that the potential of organic acids has been underestimated. Most of the microbial production of these platform chemicals is targeted to food applications and at the moment

it comprises a modest market size, however its huge potential holds a staggering future (Abbott *et al.*, 2009).

Table II – Organinc acids market demands (Sauer *et al.*, 2008).

Number of carbon atoms	Organic acid	Annual production (t)	Annual production by microbial process (t)[b]	Projected market volume (t)	Use (examples)
C_2	Acetic acid	7 000 000	190 000		Vinylacetate for polymers, ethylacetate as 'green' solvent
C_2	Oxalic acid	124 000	–		Synthetic intermediate, complexing agent
C_3	Acrylic acid	4 200 000	–		Polymer production
C_3	3-hydroxypropionic acid	n.a.	–	Up to 3 600 000	Potential substitute for acrylic acid and production of biodegradable polymers
C_3	Lactic acid	150 000	150 000		Food and beverages, biodegradable polymer production
C_3	Propionic acid	130 000	n.a.		Food and feed
C_4	Butyric acid	50 000	n.a.		Therapeutics, aroma, fragrance
C_4	Fumaric acid	12 000	–	>200 000	Food and feed, polyester resins
C_4	Malic acid	10 000	–	>200 000	Potential to replace maleic anhydride
C_4	Succinic acid	16 000	–	>270 000	Potential to replace maleic anhydride, manufacture of tetrahydrofuran, polymers
C_5	Itaconic acid	15 000	15 000		Specialty monomer
C_5	Levulinic acid	450	–	High	Possible precursor for bulk chemicals
C_6	Adipic acid	2 500 000	–		Production of nylon 6,6 esters used as plasticizers and lubricants
C_6	Ascorbic acid	80 000	–		Food additive
C_6	Citric acid	1 600 000	1 600 000		Food additive
C_6	Glucaric acid	n.a.	–	High	Production of new nylons, new building-block
C_6	Gluconic acid	87 000	87 000		Food additive, metal chelator

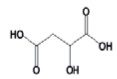
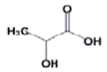
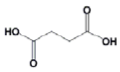
Abbreviation: n.a., no data are available.
[a]These numbers are intended to give the reader an impression of the order of magnitude in which relevant acids are on the market. One should be aware that reliable market data are not often found in the public domain.
[b]The '–' indicates that these acids are not microbially produced on an industrial level to our knowledge.

In this context, the native potential for carboxylic acid production by microorganisms should be engineered and boosted in order to strive with petrochemical production, which is a well-established, productive and robust industry. In order to attain such productive and effective microbes, strain improvement is the most elected approach for such propose. First attempts for microbial strain improvement were based in "classical genetics" by induction of random mutagenesis associated to advanced selection analysis of mutants with higher performance. Indeed, "classical genetics" approach is a well-established tool for the biotechnology industry, but further tools such as recombinant DNA technology and metabolic engineering are required and should be a complementary part of the previous one, allowing manipulation of regulatory, transport and enzymatic functions of the cell (Liu and Jarboe, 2012).

Nowadays, many carboxylic acids are preferentially produced by prokaryotic organisms. However, most of these prokaryotes, such as lactic-acid bacteria have complex nutritional requirements because of reduced ability to synthesize B-type vitamins and amino acids, representing an increased cost, as well as more difficult downstream processing (Wee *et al.*, 2005). Moreover bacterial organisms are generally unable to grow and synthetize

organic acids at the low pH values where these acids are present in their undissociated form. As a solution, acid-tolerant microorganisms, such as the yeast *Saccharomyces cerevisiae* with higher predisposition for growing at lower pH conditions, would reduce the cost for pH titrants and limiting byproduct formation (e.g. gypsum). Additionally, several species of filamentous fungi are known to naturally produce high amounts of carboxylic acids, such as *Aspergillus niger*, which is applied for large-scale citric acid production (Papagianni, 2007), and *Aspergillus flavus*, which natively produces high quantities of malic acid (Battat *et al*., 1991). However, the morphology structure of these fungi holds a barrier for growth and production tuning. The production of aflotoxins, in the case of *A. flavus* also contributes to additional problems in process and product safety (Abbott *et al*., 2009). A comparison between the titers values of the main carboxylic acids obtained by microbial production of E. coli and S. cerevisiae is presented in table III.
In this introduction chapter will be reviewed in more detail the production of carboxylic acids by *S. cerevisiae* and *E. coli*, two important model microorganisms with high potential for the biotechnology industry.

Table III - Production of the carboxylic acids malate, lactate and succinate by *E. coli* and *S. cerevisiae* from glucose (Liu and Jarboe, 2012).

Carboxylic acid	Organism	Condition	Titer (g/L)	Yield (g/g)	Productivity g/L/h	Refs
 Malate	*S. cerevisiae*	Aerobic flask	59	0.31	0.19	(82)
	E. coli	Two-stage process	34	1.05	0.47	(85)
 Lactate	*S. cerevisiae*	Anaerobic, batch	70	n/a	0.93	(72)
	E. coli	Anaerobic, batch	118	0.98	2.88	(24)
 Succinate	*S. cerevisiae*	Shake flask	3.62	0.1	n/a	(62)
	E. coli	Anaerobic, batch	83	0.92	0.88	(32)

n/a – not available

1.2.1 Carboxylic acid production in *Escherichia coli*

Among the most widely used biocatalysts, *E. coli*, a gram-negative bacteria, has been explored in different biotechnological processes. This microorganism has several advantages in relation with other cell factories, namely: many feasible genetic tools, which have been developed in the last 30 years; its sensitivity to many antibiotics, which is extremely useful for strain selection and screening; a clear integrome information

facilitating metabolic engineering approaches; a deep knowledge of central carbon metabolism and physiology; it grows fast in minimal media and keeps the ability to metabolize both 5 and 6 carbon sugars (van Maris *et al.*, 2004).

Genetic and metabolic engineering, directed evolution, and classic strain selection have been used in the improvement of *E. coli* strains that produce organic acids building blocks, such as succinic acid, lactic-acid and 3-hidroypropanoic acid (3-HP). Improved titers have been reached by optimization of fermentation conditions and new biological pathways. However, titer limitations occur when fermentation is processed in unbuffered media, allowing acidification phenomena related with raising acid concentration. On the other hand, considerable quantities of base titrant could be used to alkalinize media during the organic acid production and subsequently the anion form of carboxylic acid would prevail. As an approach to solve this bottleneck, metabolic and genetic engineering tools are being employed in order to develop acid tolerance strains, turning fermentation possible at a pH below pKa of the acid produced, reducing the costs with acid and base titrants (Mills *et al*., 2009; Warnecke and Gill, 2005).

One of the most successful examples of large volume chemical production in *E. coli* is lactic acid production. Relying in pentose and hexose consumption, *E. coli* was selected as a favorable host strain to produce optically pure L-lactic acid, which is the ultimate desired precursor for chemical synthesis of polylactic acid (PLA). Through heterologous expression of the L-specific lactic acid dehydrogenase (LDH) gene from *Streptococcus bovis*, a lactic acid producing strain of *E. coli* was created (Wyckoff *et al.*, 1997). Under anaerobic and pH buffered conditions (pH 7.0), high titers (50–75 g/L) were observed, but if pH was allowed to drop with increasing acid production, titers fall to 10–20 g/L (Wyckoff *et al.*, 1997). However, it should be mentioned that in the last approach the undissociated form was predominant (pH < pKa), decreasing the costs of downstream processes. The strain genetic´s background - mainly associated with growth features - also determines lactic acid production efficiency, as for instance *E. coli* K12 selected strains get the equivalent to half production of *E. coli B* strains (van Maris *et al*., 2004; Warnecke and Gill, 2005).

Interesting titers of succinic acid have been collected from *E. coli* fermentation broth. *E. coli* strains were genetically engineered in order to tune their metabolism for succinic acid biosynthetic pathways, avoiding the production of other anaerobic byproducts. Therefore, succinic acid production was increased due the inactivation of the pyruvate-formatelyase and lactate dehydrogenase, impairing the conversion of pyruvate into lactate and formate.

As described for lactic acid production, in pH controlled conditions, titers obtained (50 g/L) were significantly higher than pH unbuffered cultures (Yu *et al*., 2011).

At last, another metabolic engineered *E. coli* for 3HP production was reported. kumar et al. (2013) reported a 2-step pathway, where glycerol was the preferred precursor to be converted in 3HPA (3-hydroxypropionaldehyde) and then in 3HP through a catalysis reaction performed by glycerol dehydratase enzyme (*dhaB* – isolated from *Klebsiella pneumoniae*) and aldehyde dehydrogenase (*ald*). However very diminished titers (0.2 g/L) were reported (van Maris *et al.*, 2004). Thus, five additional pathways were proposed by Selifinova *et al.* relying in 3-HP production directly from glucose, but until the moment no experimental data was reported regarding this new approach. One bottleneck that remains to be solved is the need to produce 3HP at a pH value below the pKa = 4.51 of 3HP, which would decrease the dependency on large amounts of base titrant to retain neutral pH at high titers (Yu *et al.*, 2011).

As discussed previously, different range of tools and biological machinery for carboxylic acid production are available in *E. coli*. However issues regarding organic acid anion toxicity deserve attention for future genetic engineering efforts to circumvent tolerance against the less well characterized metabolic effects associated with increased organic acid anion concentrations (Warnecke and Gill, 2005).

1.2.2 Carboxylic acid production in *Saccharomyces cerevisiae*

The low performance of *S. cerevisiae* to produce organic acids in large quantities through their own machinery is well known, however features like pH tolerance, robustness, simple nutrient requirements and a huge repertoire as an industrial workhorse turns this exceptional living organism to one of the most perfect candidate for such processes. A fundamental approach to increment carboxylic acids production by *S. cerevisiae* is leaned in the flux reduction of ethanol fermentation by artificial evolution, genetic engineering and selection in order to increase the concentration of pyruvate, a critical precursor molecule for organic acid synthesis. Complementary manipulation, such as expression of heterologous enzymes and transporters could boost strain production, specifically for lactate and malate production (Abbott *et al*., 2009).

From centuries ago, *S. cerevisiae* has been used unconscionably in the production of traditional foods and beverages, such as cheese, bread, beer and wine. However after the

industrial revolution its potential was employed in different industrial processes, either in new food manufacture or for metabolite production, covering a high range of applications (Donalies *et al*., 2008). Interestingly, most of these processes still rely on both wild-type or strains from classical genetics improvement. In the last decade, researchers´ efforts were focused in meliorate *S. cerevisiae* for the production of organic acids from lignocellulosic and at the same time maintaining its efficiency and robustness. (Hong and Nielsen, 2012; Steen *et al*., 2008).

Regarding the production of simple carboxylic acids, at the moment there are no reports of industrial application of *S. cerevisiae.* However this organism holds a particular ability to grow extremely well under acidic conditions even at pH values below 3.0, making it a preferred target. The great majority of weak acids (e.g succinate (pKa = 4.21, 5.67), lactate (pKa = 3.86) and malate (pKa = 3.41, 5.05)) at such pH tend to occur in their undissociated form, avoiding the expensive steps of alkali agents addition during fermentation and acidifying process after fermentation. Thus it allows the direct collection of undissociated form demanded by costumers. Moreover, the bread yeast is well adapted to survive in simple chemically defined media, which make the production and downstream processing cheaper. At last, *S. cerevisiae* is recognized as a GRAS (Generally Recognized As Safe) organism, which gives a crucial advantage for production of carboxylic acids applied to medical and food applications (Piper *et al*., 2001).

New approaches have been developed to improve *S. cerevisiae* cells' ability to produce monocarboxylic acids, namely pyruvate and lactate, as well as dicarboxylic acids, namely malate and succinate - natural metabolites resulting from glucose oxidation but poorly excreted to the extracellular medium. These approaches comprise different levels of action: elimination of alcoholic fermentation, which, irrespective of the availability of oxygen, is the major route of sugar dissimilation in batch cultures of wild-type strains (Abbott *et al.*, 2009); engineering fast and efficient metabolic pathways that link the high-capacity glycolytic pathway with the product of choice, taking into account redox and free-energy constraints; engineering of product export; engineering of product, substrate and/or environment tolerance. All these concepts and developments will also be important tools for *S. cerevisiae* as a platform for the production of carboxylic acids.(Abbott *et al*., 2009; Liu and Jarboe, 2012).

1.3 Sugar acids bio-production

Sugar acids are currently generating considerable interest due to their potential as platform chemicals and precursors in the manufacture of biomass derived plastics. Their microbial production is getting important attention as a guaranteed sustainable production process of these sugar acids (Toivari *et al*., 2012b).

Regarding sugar acids, the state of art for microbial production of gluconic, xylonic, xylaric, saccharic and mucic acids will be emphasized.

1.3.1 Gluconic acid microbial production

The Gluconic monocarboxylic acid (Fig. 1.2), also known as pentahydroxycaproic acid is obtained from glucose oxidation. Although this process could be fully conducted by chemical synthesis, fermentation is preferred for industrial scale production. Through metabolism the hexose sugar suffers enzymatic conversion, by glucose oxidase, of the aldheide group in the first carbon to a carboxyl group resulting in glucono-δ-lactone. Then this molecule is hydrolyzed spontaneously or via peroxidase catalysis having as final product gluconic acid (Anastassiadis and Rehm, 2006).

Gluconic acid production dates back to 1922 when this sugar was found in the fermentation broth of *Aspergillus niger.* After that new microorganisms were used for gluconic acid production such as *Pseudomonas, Gluconobacter* and *Acetobacter* bacteria, as well as *Penicillium* fungi. The fermentation process was found highly pH-dependent and addition of calcium carbonate results in increased yield (Ramachandra, *et al.* 2008).

Presently, gluconic acid industrial production is leaned in *A. niger* fed-batch fermentation, at buffered pH around 6.0 – 6.5 and 34º C. After purification, the final product sodium gluconate reaches concentrations around 360 g/L considering a 24 h fermentation time (Ramachandran *et al*., 2008).

Gluconic acid is used in wide range of industrial fields. In food industry, this weak acid is used as flavoring and leavening agent, fat absorption intervenient and mineral supplement. It is used in textile industry to prevent polyamide and polyester desizing and iron deposition. Due to its chelating properties it has several applications in metallurgy industry, concrete resistance and mouthwashes products. The zinc gluconate salt is used in common cold treatment, wound healing and in several diseases caused by zinc

deficiencies, such as mental lethargies, skin changes, delayed sexual maturation and immunodeficiency (Ahuja *et al*., 2007; Biagi *et al*., 2006; Erzinger and Vitolo, 2006).

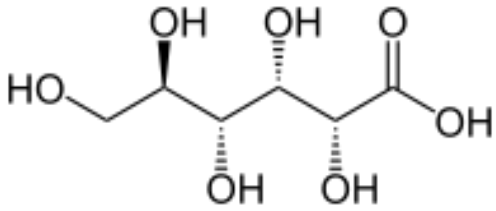


Figure 1.2– Molecular structure of Gluconic Acid (Chemspider, 2013).

1.3.2 Xylonic acid microbial production

D- Xylonic acid (Fig. 1.3) is a monocarboxylic acid produced from D-xylose oxidation. Such process can be addressed by fermentative, chemical and enzymatic processes. Regarding fermentative process, it is carried out by both D-xylose and D-glucose dehydrogenases with a corresponding D-xylonolactone, which is converted into D-xylonate spontaneously or via lactonase (Toivari *et al*., 2012a; Toivari *et al*., 2012b).

In 1898 fermentative production of xylonic acid was reported for the first time. Since then several different bacteria were described as xylonate producers such as *Pseudomonas sp., Acetobacter sp., Aerobacter sp., Gluconobacter sp. and Erwinia sp.,* as well as other fungi species, particularly *Aspergillus niger*. In addition, and quite recently, *E. coli* and some yeast strains, including *S. cerevisiae*, were successfully metabolic engineered for the production of D-xylonate, through the introduction of heterologous D-xylose dehydrogenase genes (Toivari *et al.*, 2010).

Due to structural likeness xylonic acid is recognized as a proper substitute of gluconic acid and thus it has a wide spectrum of applications, among which production of copolyamides, precursor for 1,2,4-butanetriol synthesis and dispersal of concrete. However and unlike gluconic acid, xylonic acid could be biotechnological produced from non-food carbohydrate (Toivari *et al*., 2012a).

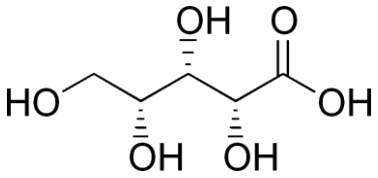


Figure 1.3– Molecular structure of Xylonic acid (Chemspider, 2013).

1.3.3 Saccharic acid microbial production

Saccharic acid (Fig. 1.4), also known as glucaric acid, is a dicarboxylic acid commonly found in vegetables, fruits and mammals. Since 1888, saccharic acid chemical synthesis production is processed in a very expendable way by nitric acid oxidation of D-glucose and recovered as potassium salt. Meanwhile other chemical versions of this process were created in order to increase the range of applications (Denton *et al*., 2011).

Despite the inexistence of microbial production reports at industrial scale, strong efforts have been made to change this scenario. Recently a metabolic engineering approach to glucaric acid production by *E. coli* was reported. For that, the D-glucuronic acid pathway of mammals, which converts D-glucose in D-saccharic acid after a ten enzymatic step conversion, served as basis to achieve it. However this is a quite long pathway and very difficult to mimic in *E. coli*, so it was zipped. The insertion of three genes of distinct organism was required: *INO1* from *S. cerevisiae* gene codifying for myo-Inositol- 1-phosphate synthase; Miox mice gene for expression of *myo*-inositol oxygenase and *udh* from *Pseudomonas syringae* encoding for uronate dehydrogenase. Yields of 1 g/L of saccharic acid were obtained. Further developments will be needed to get a feasible microbial production of this carboxylic acid, but the first results are encouraging (Moon *et al*., 2009).

Among the more demanded saccharic acid applications are medical applications for induction of cholesterol reduction and cancer chemotherapy; chemical platform for polymer production, such as nylon, polyesters and biodegradable fibers (Denton *et al*., 2011; Moon *et al*, 2009; Yu *et al*., 2011).

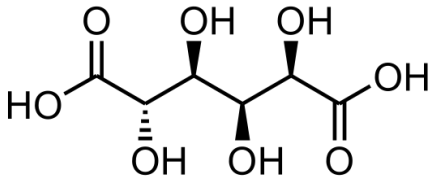


Figure 1.4– Molecular structure Saccharic acid (Chemspider, 2013).

1.3.4 Mucic acid microbial production

Mucic acid or galactaric acid (Fig. 1.5) is a dicarboxylic acid commercially produced by two alternative methods: D-galactose oxidation with nitric acid or electrolytic oxidation of D-galacturonate. At the moment there are no reports of commercial microbial production of mucic acid (Sengoku *et al.*, 2012). However, a new engineered metabolic pathway was reported recently in two filamentous fungi for the production of galactarate from D-galacturonate, a compound of pectin and easily assimilated by these microorganisms. The first step operated was the deletion of genes encoding D-galacturonate reductase, *gar1* and *gaaA* of *Hypocrea jecorina* and *A. niger*, respectively. The deletion of these genes impairs the reduction of D-galacturonate and its conversion in undesirable metabolites. Subsequently, the two fungi were transformed with *udh* gene from *Agrobaterium tumefaciens.* This gene encodes a D-galacturonate dehydrogenase, responsible for oxidation of D-galacturonate in D-galactarolactone, which is spontaneously hydrolyzed in galactarate (mucate). When mucic acid production was assessed, *H. jecorina* revealed better production performance than *A. niger,* approximately 5.9 g/L against 1 g/L of mucate, respectively. Such reduced production levels in *A. niger* should be associated with galactarate catabolism after its formation. However, while in *A. niger* mucate titers are almost the same intracellularly and extracellularly*, H. jecorina* has higher accumulation in cytoplasm than culture media. Here, the media pH associated with proper transport systems are crucial in extracellular accumulation of mucic acid. In fact, higher titers of external mucate were measured when the two filamentous fungi were grown in neutral pH rather than acidic broth. Due to its low solubility, galactaric acid is easily purified (Mojzita *et al*., 2010).

Mucic acid has been used as chelator agent, in skin care products, as a leavening agent, as well as a platform chemical and in polymer synthesis (Mojzita *et al*., 2010; Sengoku *et al*., 2012)

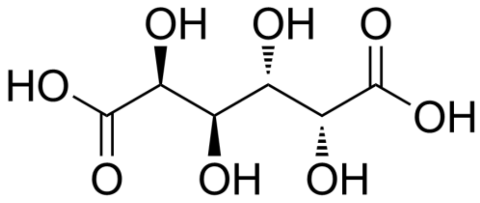


Figure 1.5 – Molecular structure of mucic acid (Chemspider, 2013).

1.3.5 Xylaric acid microbial production

Xylaric acid (fig. 1.6) is a dicarboxylic acid chemically synthetized by nitric acid oxidation of D-xylose or alternatively 5-ketogluconic acid oxidation (Sergienko *et al.*, 2010). At the moment there are no reports about microbial production of xylaric acid.
This hidrocarboxylic acid is known as a precursor of heterometallic, oxide, and porous materials. In addition derivatives of xylaric acid, such as metal complexes, have shown interesting biological properties. *In vitro* assays reported anticancer activity and toxic effect against tumor cells by this class of compounds (Sergienko *et al.*, 2010).

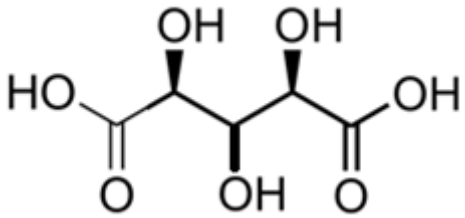


Figure 1.6– Molecular structure of xylaric acid (Chemspider, 2013).

1.4 Plasma membrane carboxylic acid permeases in *Saccharomyces cerevisiae*

The first evidence for a carboxylic acid membrane transporter in yeasts was found in *Schizosaccharomyces pombe* (Osothsilp and Subden, 1986), and later Mae1 was found as

the permease associated with the transport of malic, maleic, oxaloacetic, malonic, succinic and fumaric acids (Grobler et al., 1995).

The first work reporting a monocarboxylate transport system was published in *Candida utilis* (Leão and Van Uden, 1986) and an identical transporter was also reported in *S. cerevisiae* (Cássio *et al*. 1987). In both cases cells grown in lactate revealed activity for an accumulative proton-monocarboxylate symporter, with affinity for L and D-lactate, pyruvate, propionate and acetate and subjected to glucose repression.

Another monocarboxylate transporter was identified in *S. cerevisiae* growing in ethanol or acetate, however, and unlike the previous one, it has only specificity for propionate, acetate and formate (Casal *et al.*, 1996). Others permeases were identified in yeast species such as *Torulaspora delbrueckii, Zigosaccharomyces bailii* and *Dekkera anomala* (for review see Casal *et al*., 2008). It is also noteworthy, the report of a distinct monocarboxylate uniporter identified on *K. marxianus* (Casal et al., 2008; Queiros et al., 2007).

Yeast cells establish an electrochemical gradient through their biological membranes. As the name mentions, this gradient comprises a pH gradient and an electrical potential difference, with the inside of the cell (cytoplasm) alkaline and negatively charged relatively to the outside. The transport of protons or cationic particles and anions make up the proton motive force (*pmf*), which will provide energy for cells´ activity. These net translocations are mediated by cells´ machinery, such as proton-pumping ATPases, solute export systems and respiration-driven proton translocation (Fig. 1.7) (Casal et al., 2008). A strictly coordinated regulation is then applied by microorganisms, reacting spontaneously to every extracellular pH change in order to maintain their internal homeostasis, usually at neutral values. The *pmf* is also maintained constant across the cell plasma membrane, even in acidophilic bacteria like *Thermoplasma acidophilum* and *Picrophilus oshimae* exposed at different pH deviations. At very low pH conditions the intracellular compartment can become positive (+120 mV) (van Maris *et al.*, 2004).

At intracellular pH values ranging from 6.0 to 8.0 carboxylic acids are predominantly in the anion or dissociated form according to the equation $pH = pKa + \log (A^-/HA)$, passive diffusion of these solutes is dependent on their solubility and their permeability across the membrane. Therefore, only specific transport proteins in the membrane allow export of this weak acid form.

When microorganisms redirect their metabolism to weak acid production, the pH homeostasis is maintained by the export of the undissociated and dissociated (more

predominant) acid forms. This export can be achieved in two, energetically equivalent, ways: (1) uniport of the undissociated acid, or (2) symport of the anion with a proton. To maintain electroneutrality and intracellular pH homeostasis, anions and protons do not need necessarily to be transported by the same transporter. Thus, when the anions are exported via a uniport system, the resulting surplus protons can be excreted via proton-pumping ATPases. On the other hand, when secondary transport is coupled to the export of additional protons, the resulting *pmf* has to be used for *pmf*-driven processes, such as the generation of ATP by ATPase (Casal *et al*., 2008; Stratford *et al*., 2013; van Maris *et al*., 2004).

Yet all these principles are specifically applied to carboxylates transport. However further attention is needed once export energetics could diverge from the previous one. Regarding the uniport of the monovalent anion it follows the same energetic balance of the anion form of monocarboxylic acids. However, the divalent anion transport by primary or secondary transporters implies the additional transport of the remaining proton by proton-pumping ATPases in order to maintain the electrochemical gradient (Abbott *et al.*, 2009).

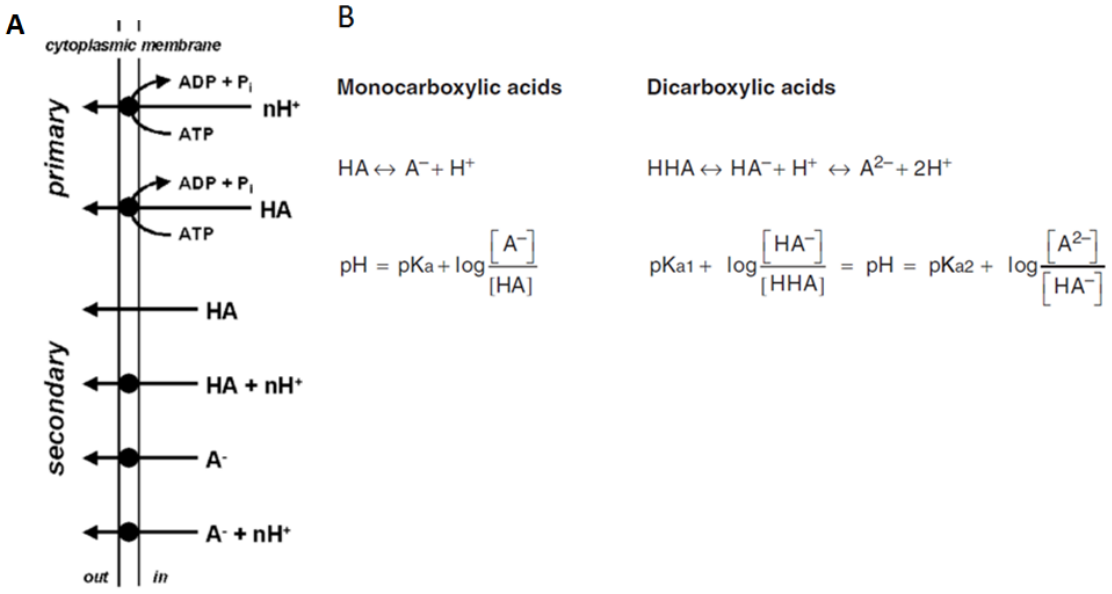


Figure 1.7– A) Schematic overview of the mechanisms involved in weak organic acid export via primary and secondary transport. B) The distribution between undissociated acid (HA or HHA) and dissociated anions (A^-, HA^- and A^{-2}) and protons (top equations) is determined by the pH of the environment and the pKa of the carboxylate groups as described in the equations. Adapted from Abbot *et al.*, 2009; Maris *et al.*, 2004.

1.4.1 ScJen1 transporter and homologues

The *S. cerevisiae JEN1* was the first gene found to be related with transport of monocarboxylic acids in fungi and described as a lactate/pyruvate/acetate/propionate-H^+ symporter (ScJen1) (Fig. 1.8). This permease is a member of the Major Facilitator Superfamily (MFS) (TCDB (Transport Classification Database) 2.A.1), with 12 putative transmembrane segments (TMS), included in the Sialate:H^+ Symporter (SHS) Family (TCDB 2.A.1.12) . A *S. cerevisiae jen1*Δ strain is unable to uptake lactate by a mediated mechanism, however the transformation of such strain with a plasmid carrying the *JEN1* ORF (Open Reading Frame) reestablishes lactate uptake. Jen1p is located at cell plasma membrane (Paiva et al., 2002) and biochemical studies reveal the ability of Jen1p to transport lactate in both intact cells and isolated vesicles (Soares-Silva *et al*., 2003)

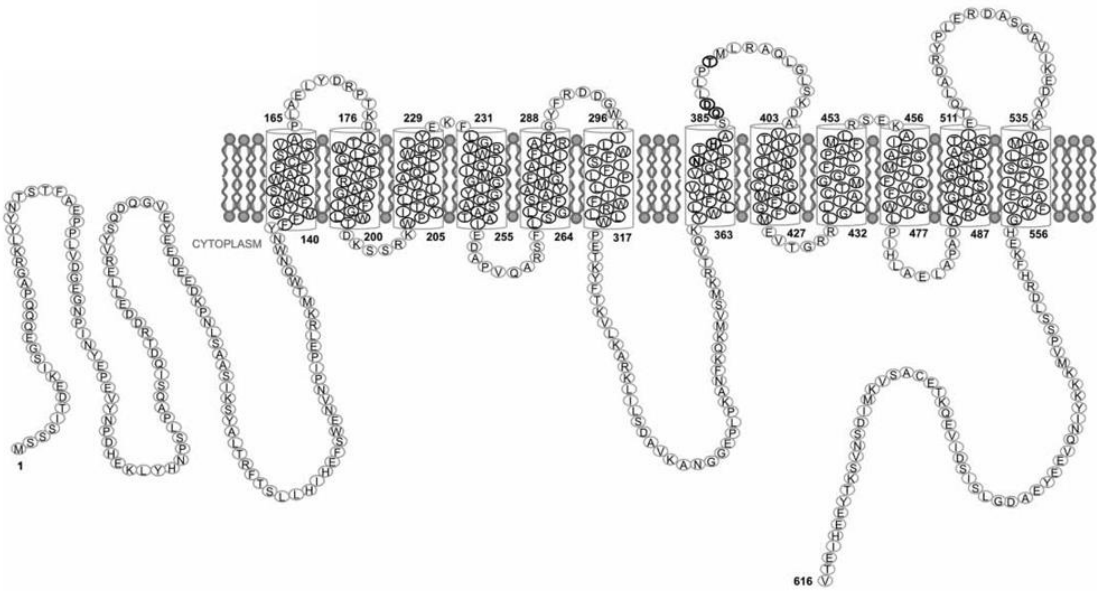


Figure 1.8 - Predicted topology of Jen1p, built by the TMHMM software (http://www.cbs.dtu.dk/services/ TMHMM-2.0/), with the location of the identified motif highlighted. The protein topology shown is composed of 12 transmembrane helices and cytoplasmic N- and C-tails. Adapted from Soares-Silva *et al.*, 2007.

A conserved domain ^{379}NXX[S/ T]HX[S/T]QDXXXT^{391} present in the seventh TMS was identified between hemiascomycetes *JEN1* homologues. Relying in a site directed

mutagenesis approach, it was possible to describe the role of this sequence in transport ability and substrate affinity. In detail: mutants D387, H383 and N379 accounts for reduction of lactate and pyruvate uptake; the residues T391 and Q386 are crucial to affinity properties; the bond between D387 and H383 are important for the protein structure. In other Jen1p homologues from different species, such as filamentous fungi, bacteria and archea it was confirmed the presence of this conserved domain, however and until now no functional study was performed in those organisms (Soares-Silva *et al*., 2007)., Other amino acids residues involved in substrate binding were found in TMS-V and TMS-XI of Jen1p: N501 residues are critical for physiological function; F270 and Q498 are involved in substrate specificity and transport kinetics; S266, S271, A272, Y273 play an important role in transport characteristics Soares-Silva *et al.* (2011). In these studies LacY crystallographic structure was used to obtain a hypothetical 3D structure of Jen1p and posteriorly a refined model leaning in Jen1p similarity with GlpT permease was obtained (Fig. 1.9). This model confirmed the previous functional studies, showing the internal pore of amino acids responsible for substrate or co-substrate interaction, as well as anticipating a functional role for R188, which was confirmed by further functional studies (Soares-Silva *et al*., 2007; Soares-Silva *et al*., 2011).

The regulation of *JEN1* expression is mediated by external signals, such as glucose and lactate pulses, a repressor and an activator of *JEN1* expression, respectively. Moreover, it is also identified that Mig1p and Mig2p acted as repressors of this membrane protein (Bojunga and Entian, 1999) and the main transcription factors able to counteract glucose repression mechanisms are the Cat8p, Snf1p, HAP2/3/4/5 complex (Lodi *et al*., 2002). Similar with others proteins related with the metabolism of non-fermentable carbon sources, aerobic conditions are needed for *JEN1* expression (Lodi *et al*., 2002). It was also reported that *JEN1* expression can result in three different mRNA transcripts, the -31, +392 and +972 (Andrade *et al*., 2005).

Glucose addition to lactic acid grown cells also triggers Jen1 endocytosis and degradation involving the ubiquitination machinery (Paiva *et al.*, 2002). However, Becuwe *et al*. (2012b) have shown how this signaling pathway works for Jen1 transporter in the budding yeast (Fig.1.1). The environmental signal will trigger the transporter endocytosis by the glucose signaling pathway. The main players are the Snf1- homologue of AMPK (Adenosine Monophosphate-activated Protein Kinase) – and the Glc7/Reg1, a phosphoprotein phosphatase 1 (PP1). In the absence of glucose, Snf1 kinase phosphorylates the protein adaptor Rod1/Art4, impairing its ubiquitylation. However,

when glucose is added, Reg1 phosphatase is free to dephosphorylate Art4 adaptor, allowing that Rsp5 ubiquitylates the Rod1 under coordination of 14-3-3 proteins. Although these advances, the right location for Art4 to contact with Jen1 transporter was not found, indeed it was not detected significant amounts of Rod1 in plasma membrane in the presence of glucose. However a transitory membrane permanency should not be discarded. Other possible location of Art4 could be at the endosome level, in fact others authors report the ubiquitination of iron transporter (Fet3/FTr1 complex) in this compartment (Becuwe *et al*., 2012a; Becuwe *et al*., 2012b).

Meanwhile, another monocarboxylate transporters were identified in *Candida albicans* (CaJen1), *K. lactis* (KLJen1), all sharing similar mechanisms of repression and induction, actually CaJen1 is under the control of Cat8p transcription factor, and KlJen1 is under control of Fog2p, a homologue of *S. cerevisiae* Snf1p, and KlCat8 (Lodi *et al.*, 2004; Soares-Silva *et al.*, 2004).

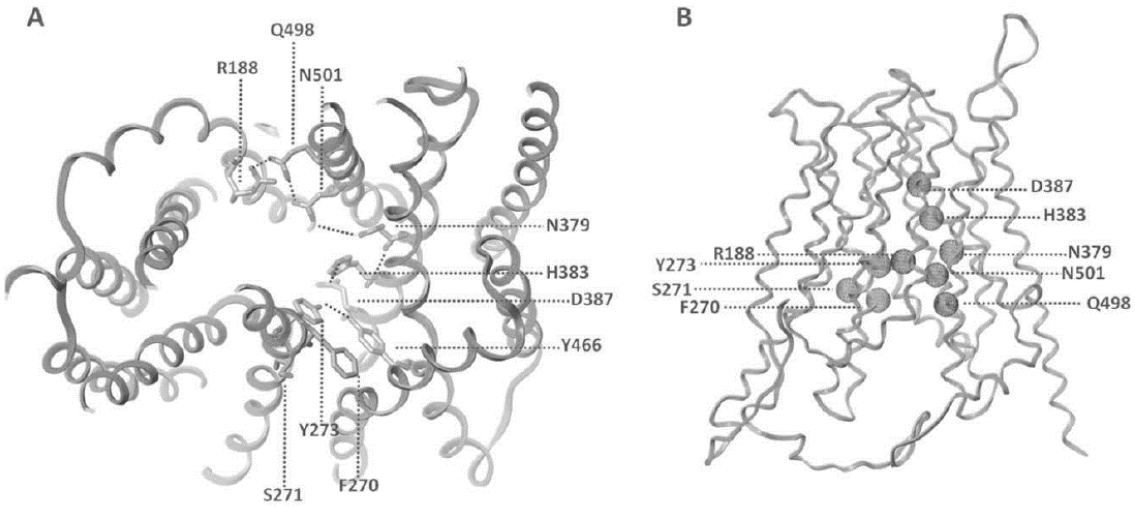


Figure 1.9 - Overall structure of the Jen1p model. A) A section of the transporter as viewed from the periplasmic side. Visible is the spiral rim-like network of hydrogen bonds that is formed. B) Side view of the protein. Residues that define the translocation pathway are illustrated as spheres. Adapted from Soares-Silva *et al.*, 2011.

The increased income of yeast sequenced genomes made possible the construction of a Jen1p phylogenetic tree, comprising the homologues of Hemiascomycetes and Euascomycetes fungi (Fig. 1.10). Permeases functionally characterized as monocarboxylate transporters were allocated in the Jen1 cluster. On the other hand, putative transporters genetically related with KlJen2, a characterized dicarboxylate

transporter, were incorporated in Jen2 cluster. An additional cluster was described including six homologue members of *Yarrowia lipolytica*.(Casal *et al*., 2008; Lodi *et al*., 2007).

The yeast *K. lactis* carries in its genome two genes, *KlJEN1* and *KLJEN2* encoding a monocarboxylate (lactate/pyruvate) and a dicarboxylate transporter, respectively. The KlJen1 permease shares the same structural and functional identity with ScJen1, as well as the same physiological role and regulation mechanisms. It was reported that *KLJEN1* knockout results in limited growth upon lactate or pyruvate as sole source of carbon. As expected, the lactate uptake is severely affected, though not completely abolished. This could mean that internal presence of lactic acid is needed and guaranteed in order to induce the expression of critical players on lactate metabolism. Like ScJen1, the monocarboxylate permease of *K. lactis* is repressed by glucose and induced by lactate (Queiros *et al.*, 2007). The dicarboxylate transporter KlJen2 is a D,L-malate transporter. Despite its homology with ScJen1, functional and physiological roles are not shared. Indeed, deletion of *KLJEN2* does not affect the yeast growth upon lactate or pyruvate growth conditions and KlJen2 was associated to succinic acid transport. Heterologous expression in *S. cerevisiae,* also reveals succinate and fumarate transport beyond malate uptake, which was never been reported in this species. Moreover, it allowed KlJen2 location on cell membrane through GFP (Green Fluorescence Protein) tagged mutants. Regarding KlJen2 regulation, glucose repression is intrinsically related with strain type. While *K. lactis* PM4-4B have their carboxylic acid uptake repressed in glucose growth conditions, *K. lactis GG1888* maintains expression of both, KlJen1 and KlJen2 even in presence of glucose. Furthermore, KlJen2 is expressed in succinate, ethanol and acetate (Lodi *et al.*, 2004; Queiros *et al.*, 2007). All evidences suggest that KlJen1 and KlJen2 resulted from gene duplication and that evolutionary pressure made them paralogues proteins (Lodi *et al.*, 2004).

Similarly to *K. lactis, C. albicans* also displays two important players on carboxylate transport, the *CaJEN1* and *CaJEN2* genes (Lodi *et al.*, 2007). The *CaJEN1* encodes a monocarboxylate transporter. Unlike pyruvate and propionate, acetate triggers the inhibition of DL-lactic acid transport through CaJen1 in a noncompetitive manner, which could unveil distinct uptake mechanisms, as well as competition for co-substrates, most likely protons. Expression analysis of CaJen1 has shown that not all possible substrates are acting as inducers. In fact only DL-lactic acid, pyruvic acid and glycerol induce CaJen1 expression. Heterologous expression of CaJen1 in *S. cerevisiae* restored the

lactate uptake in a Δ*jen1* strain, after replacement of leucine by serine, at position 217, according to the codon usage of each specie (Soares-Silva *et al.*, 2004).

CaJen2 is also localized at the plasma membrane, and the knock-out of the encoding gene, *CaJEN2*, resulted in the absence of malic and succinic acids uptake. In addition, the heterologous expression of *CaJEN2* in *S. cerevisiae* conferred to cells the ability to uptake succinic acid. Upon glucose growth conditions CaJen2 permease is internalized, however differences regarding signaling response were reported between yeast species, since in *C. albicans* permease endocytosis occurs far faster than in *S. cerevisiae* (Vieira *et al*., 2010).

The *Schizosaccharomyces pombe* permease, Mae1, was the first membrane protein associated to dicarboxylate transport in yeasts (Osothsilp and Subden, 1986). According the TCDB classification, Mae1 corresponds to a 10 transmembrane domains grouped in Tellurite-resistance/Dicarboxylate Transporter (TDT) Family (TC 2.A.16). Functional studies of Mae1 unveil its role in malate and C4 dicarboxylic acids (maleic, oxaloacetic, malonic, and succinic acids) transport found in glucose-grown cells. The symport process was described as reversible and dependent on substrate and proton gradient across cell membrane. In addition, further studies show non-competitive inhibition of Mae1 by fumarate and α-ketoglutarate either in *S. pombe* or in *S. cerevisiae* expressing heterologously this permease. Besides, no genetic homology was found with KlJen2 (Casal *et al*., 2008; Vieira *et al*., 2010).

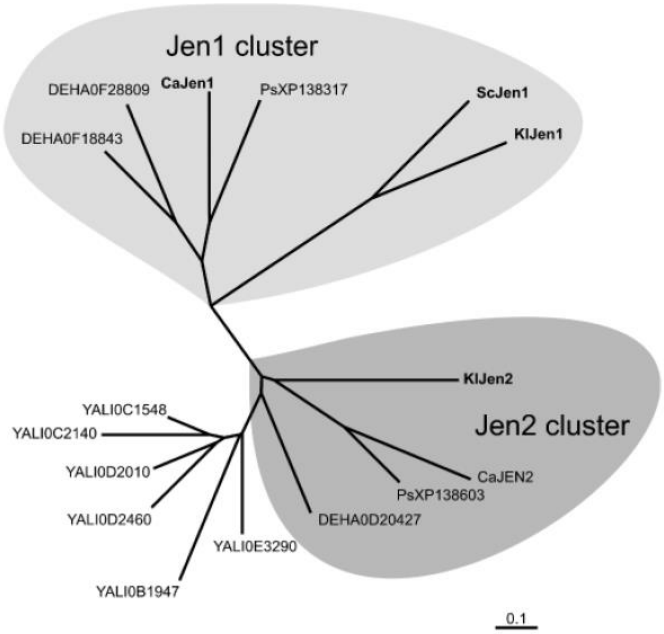


Figure 1.10- Phylogenetic tree of ScJen1p homologues. The sequences used were obtained from Genolevures, except for the *Pichia stipitis* and *Candida albicans* homologues which were obtained by homology search with BLASTP (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The permeases with characterized function are highlighted in bold. Adapted from Casal *et al.*, 2008.

1.4.2 Ady2 transporter and homologues

The ability to use acetic acid, an end-product of fermentation, as a carbon and energy source by aerobic metabolism is well known in yeasts. In order to identify carriers of acetic acid in *S. cerevisiae*, Paiva *et al*. (2004) performed a whole genome expression analysis in yeast cells grown on acetate under glucose starvation conditions. Indeed, this nutrition triggers the expression of genes involved in metabolic and physiological changes, such as aerobic oxidation, active mitochondria division, and transcription of novel permeases. As a result of this approach, a new gene – *ADY2/YCR010c* (TCDB 2.A.96) – was identified and described as a member of YaaH family by the transport classification database (TCDB). The disruption of ADY2 in S. cerevisiae resulted in acetate active transport abolishment. Further microarray analyses of ady2Δ strains showed that this gene is not a critical regulator of acetate response, however it is undoubtedly connected to acetate transport and a likely candidate to be a membrane

protein (Paiva *et al.*, 2004). Two other homologues of Ady2 exist in S. cerevisiae, YDR384c and YNR002c, which have five to seven predicted transmembrane domains (Casal, *et al*., 2008).

Later, an attempt to exploit the potential of *S. cerevisiae* to bioprocess lactate, by heterologous expression of *LDH* genes, highlighted the role of Ady2 in the efflux, as well as in the uptake of this weak acid. During the first 16 hours of fermentation Ady2 was associated to lactic acid extrusion and after that, owing to glucose privation, a switch in the transport functions of this permease occurs, as it starts to pump in the weak acid inside the cell (Pacheco *et al*., 2012).

The homologue AcpA of the filamentous fungus *Aspergillus nidulans* has been shown to be an acetate transporter which is expressed in germinating conidia. Ady2p and AcpA share 50% of amino acid sequence identity. A second *A. nidulans* homologue, AlcS, is more distantly related to Ady2 but is of unknown function (Robellet *et al*., 2008).

In the bacteria domain, the homologue of Ady2 is YaaH and gives the name to this family of transporters. The YaaH family member of *E. coli* is a carboxylic acid transporter (the *E. coli* YaaH protein 2.A.96.1.1) with six predicted transmembrane domains and encoded by y*aaH* gene. The YaaH family members share a conserved motif at the first predicted transmembrane: N-P-[AV]-P-[LF]-G-L-x-[GSA]-F (Sá-Pessoa *et al.,* 2013). The deletion of *yaaH* compromises the uptake of both acetic acid and succinic acid. This ability to transport either a monocarboxylic acid or a dicarboxylic acid was reported for the first time in above mentioned work, since other YaaH family members were associated exclusively to acetate and other monocarboxylates transport. The amino acid residues Leu131 and Ala164 were identified as critical players in lactate specificity and transport. The assignment of this protein as an acetate succinate transporter led to its new nomenclature as SatP: the Succinate-Acetate Transporter Protein (Sá-Pessoa *et al.*, 2013). Besides YaaH/SatP, another acetate transporter (evolutionary distinct from the YaaH family) called ActP and formerly known as YjcG has been reported in *E. coli*. ActP has been classified as a member of the solute/sodium Symporter Family (SSS Family), consisting of 549 amino acids arranged as 14 putative TMS (Sa-Pessoa et al., 2013).

1.4.3 Carboxylic acids toxicity in *S. cerevisiae*

Carboxylic acids are synthetized inside the cell with the anion prevailing over the neutral form due to the alkali environment of the cytoplasm. As expected, the dissociated forms raises their concentration and unless the organism reacts with effective efflux processes to reduce such anion levels, which is not the case of *S. cerevisiae,* an extensive range of toxic effects will be triggered (Mira *et al.*, 2010; Piper *et al.*, 2001).

The toxic effects reported in *S. cerevisiae* include: increased high turgor pressure; stimulation of free radical production and subsequently severe oxidative stress, which is the main stress cause in *S. cerevisiae* under aerobic conditions; cytosol acidification due to proton accumulation, affecting different metabolic pathways and functions.

In spite of these antimicrobial effects, carboxylic acids are often associated with the anion accumulation and intracellular acidification (Russell, 1991), being likely to admit that these weak acids have their own particular mechanisms of action to induce stress. As a matter of fact, studies about acetate and sorbate toxicity in *S. ceresisiae* (Bracey *et al*., 1998) have reported the effect of acetate toxicity by anion and proton accumulation, while sorbate affected respiratory function and cell membrane disturbance due to the hydrophobicity of the molecule. Interestingly, *S. cerevisiae* growth inhibition occurs at acetic acid concentrations between 80–150 mM and at sorbate concentrations ranging 1–3 mM, however the pKa is identical for both acetate (pKa = 4.75) and sorbate (pKa = 4.76), but unlike sorbate, acetate causes a noticeable reduction in the internal pH (Arneborg *et al*., 2000). Meanwhile, membrane stress induction was correlated with monocarboxylic acids with a more lipophilic structure (Mira *et al*., 2010).

Under sorbate and benzoate stress conditions, *S. cerevisiae* experiences a significant reduction in ATP levels, which is mainly related with phospho-fructokinase (PfK) reaction impairment and subsequently glycolysis yield is harshly affected. As a consequence reactive oxidation species formation takes place in electron transport chain dysfunctional mitochondria (Pearce *et al*., 2001).

1.4.4 Mechanisms to avoid a futile cycle of diffusional entry and active extrusion of organic acids

When the anion form of a weak acid, after its mediated export, faces the extracellular media, at a pH value below the acid pKa, it will assume the undissociated form. Then the protonated acid form would cross the plasma membrane by simple diffusion, which would result in an endless and energetically expensive cycle of export and uptake processes. However, *S. cerevisiae* has developed limitation mechanisms to constrict the flux of such cycle (Piper *et al*., 2001).

Although the precise action mechanisms regarding this regulation processes in *S. cerevisiae* are not understood, the ability of the yeast to control the distribution of carboxylic acids between intracellular and extracellular media is well known, particularly reported to benzoate (Piper *et al*., 1998). Evidences point to an additional mechanism that complements the anion extrusion, which acts upon organic acid diffusion. It has been proposed that mannoproteins could restrict the porosity of *S. cerevisiae* cell wall. Actually, *Zigosaccharomyces bailii* under benzoate stress conditions shows an uptake reduction of the protonated form around 40 %, however the propionate uptake was not affected (Piper *et al*., 2001).

1.5 Outline of the thesis

Carboxylic acids owing to their chemical and biological properties are a preferential target for industrial microbial production. Besides all advances in metabolic engineering, some important troubleshoots remain to be solved. In this scenario, product toxicity appears as one of the major limitations in microbial cell factories.

The main goal of this work is to exploit membrane transporters of mono- and dicarboxylates as new tools for exporting internal produced carboxylic acids in order to reduce their toxic effects in the yeast model organism, *S. cerevisiae*.

More specifically, this work will explore a well characterized collection of ScJen1p mutants to indirectly assess the transport of xylonic, mucic and saccharic acids. Additionally strains of *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Candida albicans* will be also screened for transport of the previous carboxylic acids plus gluconic and xylaric acids. Once such evidences for the transport of these compounds are found, heterologous expression of described or putative

carboxylic acids transporters in these species will be performed in the budding yeast. Detailed analysis of transport kinetics, particularly inhibition kinetics, will allow to discriminate the type of interaction between carboxylic acids and transporter proteins.

The present work was developed under the scope of the 2nd year of the Masters Course in Molecular Genetics at the Department of Biology of the University of Minho under the supervision of Professor Margarida Casal and Professor Sandra Paiva. The experimental work was performed in the Laboratory of Molecular Biotechnology at the Molecular and Environmental Biology Centre/Department of Biology and in a close collaboration with VTT Finland Research Institute.

2. MATERIAL AND METHODS

2.1 Biological Material

The yeast strains and the plasmids used in this work are listed in tables IV and V respectively.

Table IV – Yeast strains used in this work.

Strain	Genotype	Source/reference
Saccharomyces cerevisiae		
W303-1A	MATα *ade2 leu2 his3 trp1 ura3*	Thomas and Rothstein (1989)
*jen1*Δ	W303-1A; *JEN1::KanMX4*	M. Casal collection
*ady2*Δ	W303-1A; *Ady2::KanMX4*	Paiva *et al*. (2004)
jen1Δ ady2Δ	W303-1A; *JEN1::KanMX4 ADY2::HphMX4*	Soares-Silva *et al*. (2007)
W303-1A-p416GPD	*jen1Δ ady2Δ* transformed with p416GPD	Soares-Silva et al. (2007)
W303-1A-p416JEN1	*jen1Δ ady2Δ* transformed with p416GPD*::JEN1*	Soares-Silva *et al.* (2003)
jen1Δady2Δ–pUG35*CaJEN1*	*jen1Δady2Δ* transformed with pUG35*::CaJen1*	Soares-Silva *et al.* (2004)
jen1Δady2Δ-p416GPD*CaJEN2*	*jen1Δady2Δ* transformed with p416GPD*::CaJen2*	Vieira *et al.* (2010)
jen1Δady2Δ-p416GPD-*KlJEN1*	*jen1Δady2Δ* transformed with p416GPD*::KlJen1*	Lodi *et al.* (2004)
jen1Δady2Δ-p416GPD-*KlJEN2*	*jen1Δady2Δ* transformed with p416GPD*::KlJen2*	Lodi *et al.* (2004)
Candida albicans		
RM1000	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG*	Negredo *et al*. (1997)
Kluyveromyces lactis		
CBS 2359	Type strain	
Yarrowia lipolytica		
ISA 1718	Type strain	
Debaryomyces hansenii		
CBS767	Type strain	

Table V – Plasmids used in this work.

Plasmid	Features	Source/reference
p416GPD	*CEN, AMP$_R$, URA3*	Mumberg *et al*. (1995)
pDS1	p416GPD*::JEN1, CEN, AMP$_R$, URA3, GPD prom, JEN1*	Soares-Silva et al, 2007

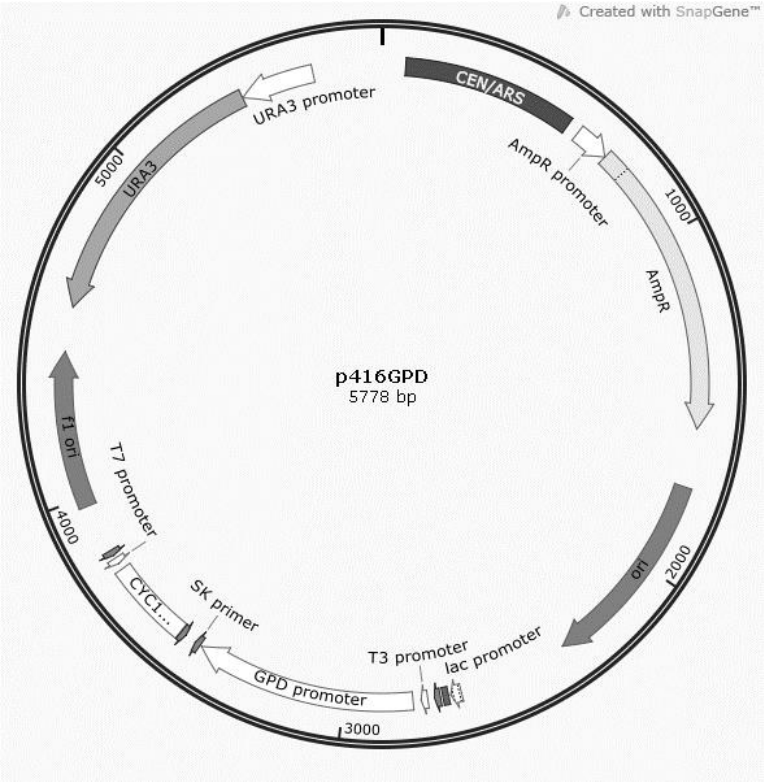


Figure 2.1– p416GPD vector elements.

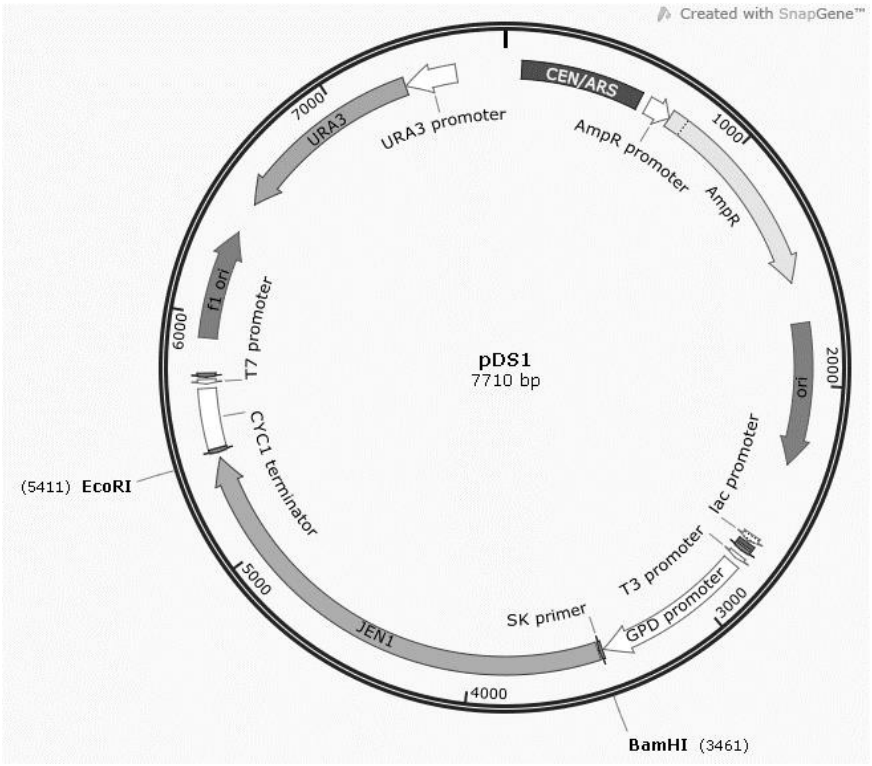


Figure 2.2– pDS1 (p416GPD*::JEN1*) vector elements.

2.2 Yeast cells culture conditions

The yeast cultures were maintained on slants of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v) or minimal media with required supplements for growth. Yeast cells were grown in yeast nitrogen base (Difco), 0.67%, w/v (YNB medium), supplemented with adequate requirements for prototrophic growth, or in yeast extract (1%, w/v), peptone (1%, w/v) (YP medium). Carbon sources were glucose (2%, w/v), lactic acid (0.5%, v/v, pH 5.0), pyruvic acid (0.5%, w/v, pH 5.0), succinic acid (1%, w/v, pH 5.0) and malic acid (1%, w/v, pH 5.0). All culture media were autoclaved (20 minutes, 120 ^{0}C, 1 atm), although some media components such as amino acids, nucleotides and yeast nitrogen base were filter sterilized and added to autoclaved media. The growth in liquid media was performed in Erlenmeyer flasks, with quintuple capacity of the volume of culture medium utilized. Cultures were always harvested during the exponential phase of growth. The cultures were incubated at proper temperature (as given in tables and legends) in an orbital incubator at 200 rpm. The growth evaluation in liquid medium was followed by OD measurement at the wavelength of 640 nm. For derepression conditions glucose-grown cells were centrifuged, washed twice in sterile

deionized water and cultivated into fresh YNB medium supplemented with lactic acid or succinic acid for 4 h. For drop tests, cells were grown on YNB Glu-Ura media, until a OD640 of 0.1 was reached. A set of three 1:10 serial dilutions was performed and 3 ml of each suspension was inoculated in the desired medium, using YNB Glu-Ura as a control. Cells were incubated at 18°C for 10 days or alternatively at 30°C for 3 days (Soares-Silva *et al*., 2011).

2.3 Transport assays

Transport assays are meant to access the intracellular accumulation of radiolabelled molecules to infer about functional features of cell membrane transporters. Leaning in uptake rates of radiolabelled substrates *per se* or in conjugation with non-labeled molecules, this technical approach allows the determination of different transport kinetics parameters, such as, K_m (affinity constant), V_{max} (maximum velocity), K_d (diffusion constant) and K_i (inhibition constant) (Casal *et al*., 1995). The affinity of the transport is crucial information to run correctly the uptake assays, once inappropriate concentrations of measurable substrate could result in miscalculation of substrate transport, either by transport saturation or absence of transport due excessive or reduced concentrations of radiolabelled substrate (Soares-Silva *et al.,* 2007).

For uptake measurements cells were harvested at specific time points (as given in figure legends) by centrifugation (5000 rpm, 2 minutes), washed twice in ice-cold deionised water and resuspended in ice-cold deionised water to a final concentration of about 25–35 mg dry weight/mL, using 1.5 mL eppendorf tubes containing 60 µL of 0.1 M KH2PO4 buffer, at pH 5.0 and 30 µL of the yeast cell suspension. After 2 minutes of incubation at 26 ^{0}C, the reaction started by the addition of 10 µL of a solution of radiolabelled substrate, at the desired pH and concentration, rapidly mixed in the vortex, and incubated at 26 ^{0}C. After specific time points (as given by legends and figures), 100 µL of non-labelled substrate at 100 mM was added, quickly mixed by vortexing and chilled on ice, to stop the reaction. The reaction solutions were centrifuged for 5 minutes at 13200 rpm. The supernatant was carefully rejected, the pellet was ressuspended in 1 mL of deionized cold water and centrifuged for 5 minutes at 13200 rpm. The resulting pellet was resuspended in 1 mL of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). To evaluate the non-specific adsorption and the diffusion component, a mixture of labelled and non-labelled substrates 1000-fold concentrated was

used. The values estimated represent less than 5 % of the total incorporated radioactivity. The inhibition assays were carried out by adding simultaneously the labelled and nonlabelled substrates.

The following radioactive labelled substrates were utilized, D,L - [^{14}C] lactic acid (Perkin Elmer) and [^{14}C]-succinic acid (Moravek Biochemicals). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer, with dpm correction. Non-specific ^{14}C adsorption to the cells, as well as the diffusion component, was determined by adding a mixture of labelled and unlabelled acid 1000-fold concentrated. The inhibition constant (K_i) was deduced by the effect of different concentrations of the non-labelled inhibitor in uptake velocities. The transport kinetics best fitting the experimental initial uptake rates were determined by a computer-assisted non-linear regression analysis (using GraphPad Prism version 6.0 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The data shown are mean values of at least three independent experiments, with three replicas of each.

3. RESULTS

3.1 Seeking for novel production pathways of sugar acids in *S. cerevisiae*

The rising global energy demands in last century has driven to worrying depletion levels of fossil fuels, leading to a growing interest in microbial biofuel synthesis, particularly in model organisms like *S. cerevisiae*. The microbial conversion of sugars to biofuels is a promising technology and besides engineering attempts for more efficient metabolic pathways, the byproducts of biomass pretreatment processes and the fuels themselves are often toxic at industrially-relevant levels. Therefore, efforts to improve production yield by engineering efflux systems to overcome toxicity problems and secret inhibitory chemicals from the cell has been revealed as a crucial alternative. Towards that end a wide range of promising membrane transporters for the transport of carboxylic acids will be screened, particularly xylonic, mucic, saccharic, gluconic and xylaric acids.

3.1.1 Screening a ScJen1p mutant collection for potential export properties of xylonic, mucic and saccharic acids

In order to select and test different transporters, a collection of mutants (Fig. 3.1) constructed from ScJen1p monocarboxylic acid transporter was screened for promising candidates. Noteworthy, this collection resulted from structural and functional studies of Jen1p (Soares-Silva et al., 2007; Soares-Silva et al., 2011). The main reason behind the choice of this collection relies in the existence of mutants with boosted uptake performance for monocarboxylic acids or with ability to transport dicarboxylic acids, specifically succinic acid, as well as, the chemical structure likeness between the previous mentioned organic acid and the two dicarboxylic acids under examination, mucic and saccharic acids. During the process of selection two different variants were beared in mind: changes in uptake rate and change of substrate specificity. Subsequently and according to the drop tests which allow to infer about changes in: i) substrate specificity – from monocarboxylates to dicarboxylates; and ii) kinetic parameters for lactic acid uptake rates (by the detection of significant alterations in kinetic parameters displayed by the mutants in the mentioned studies). The following Jen1p mutants were selected: i) with

alteration in substrate specificity (F270G, F270A, and F270QS271Q) and ii) with alteration for lactate uptake (A272G, Y284Q, Y284A, Q386A, Q498A, S271Q);

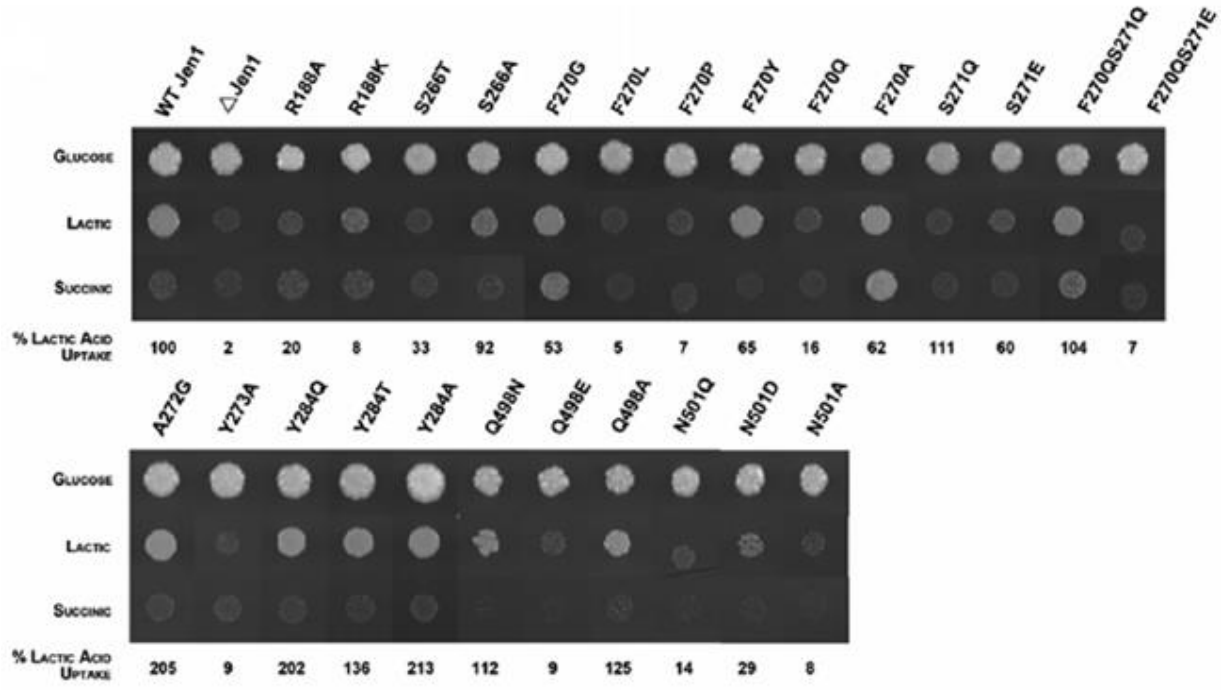
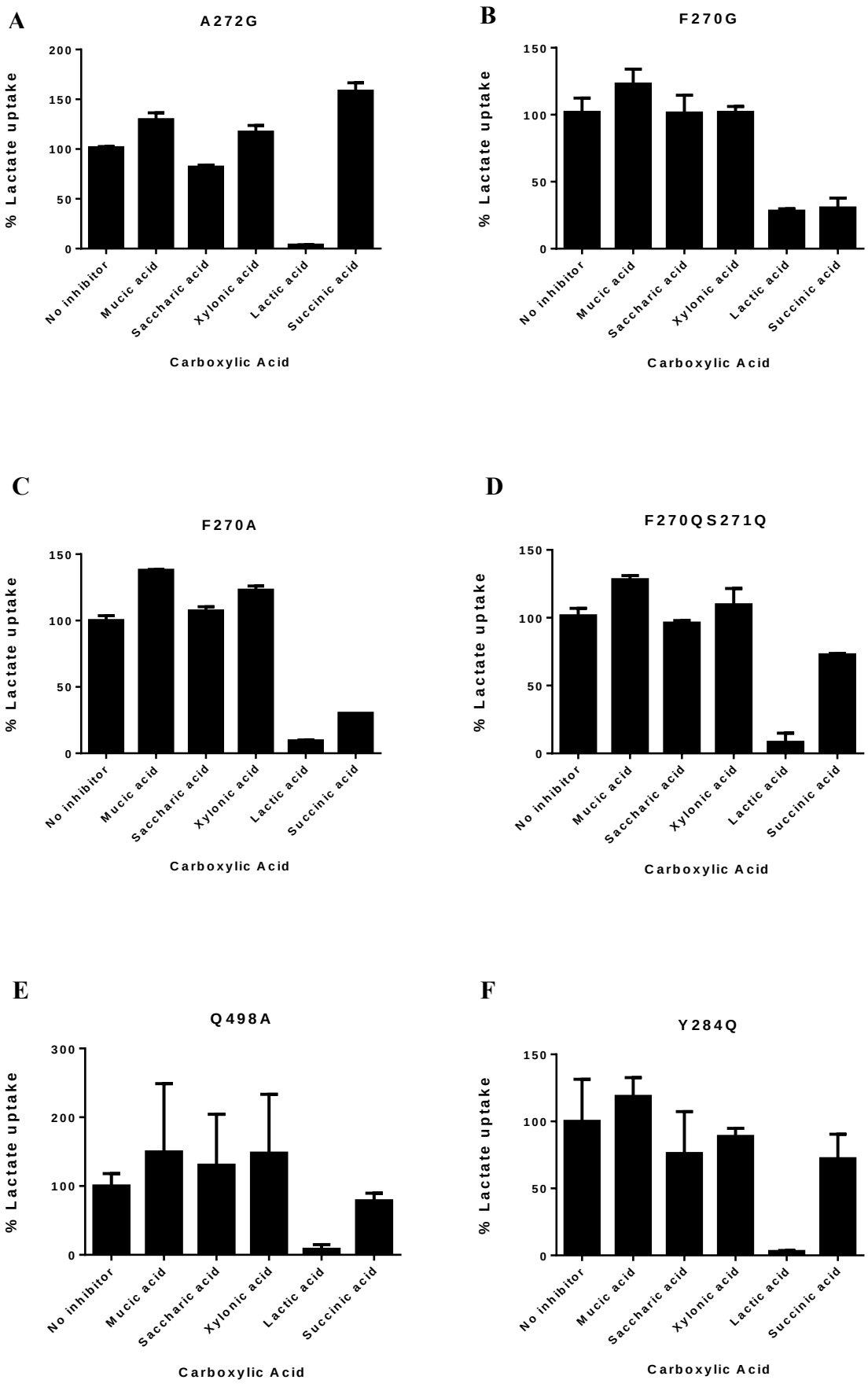


Figure 3.1- Growth test of control strains and mutants on glucose (2%), lactic acid (0.5%) or succinic acid (1%). All strains are isogenic (*S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ) expressing from a low copy plasmid either wild-type Jen1 (WT-Jen1) or the relevant *JEN1* alleles. The negative control is a strain carrying an empty vector (ϕ*JEN1*). (Soares-Silva *et al*., 2011)

The transport of radiolabeled lactic acid, the known substrate with high affinity for ScJen1p, was accessed in the presence of the carboxylic acids with biotechnological interest (xylonic, mucic and saccharic acids) in cells of the mutants strains collection mentioned. This preliminary test allowed the identification of potential inhibitors of lactic acid uptake, as indicative for the interaction of the substrates as competitors for the same membrane transporter (Fig. 3.2).


A
A272G
% Lactate uptake
200
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid
B
F270G
% Lactate uptake
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid
C
F270A
% Lactate uptake
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid
D
F270QS271Q
% Lactate uptake
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid
E
Q498A
% Lactate uptake
300
200
100
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid
F
Y284Q
% Lactate uptake
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
Xylonic acid
Lactic acid
Succinic acid
Carboxylic Acid

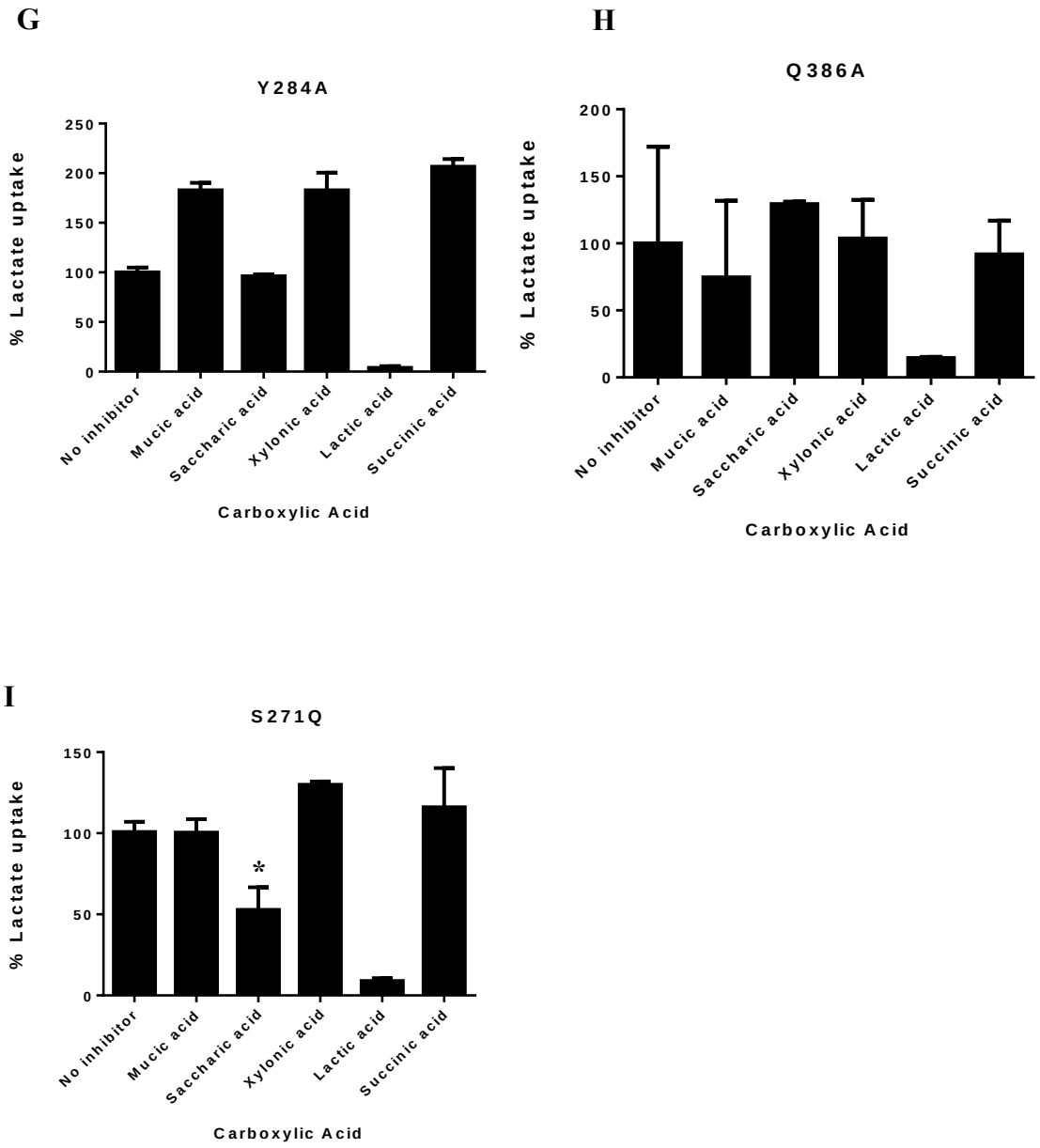


Figure 3.2. Relative capacity (%) of ^{14}C-lactic acid uptake (60 mM), after 1 minute incubation with non-labelled carboxylic acids (10 mM), at pH 5.0 and 30 ºC, acting as inhibitors for the transport of lactate in Jen1p mutants: A, A272G; B, F270G; C, F270A; D, F270QS271Q; E, Q498A; F, Y284Q; G, Y284A; H, Q386A; I, S271Q. The data shown are mean values of at least two independent experiments and the error bars represent standard deviation.* Significant differences ($p < 0,05$).

Unlike what it was previously expected, xylonic acid, a monocarboxylic acid, did not reveal any affinity or influence in uptake rates of lactic acid in the yeast strains that carry the mutant alleles of Jen1p with increased uptake rates such as A272G, Y284Q, Y284A, Q386A, Q498A, S271Q. Furthermore, and as foreseen, there was no inhibitory effect in the lactate uptake rate for the yeast strain´s group carrying Jen1p mutant alleles with change of substrate specificity for dicarboxylic acids.

Regarding mucic acid, both mutants groups, with increased uptake rate and altered substrate specificity, did not show any inhibition in the lactate uptake rates and therefore any interaction between this carboxylic acid and the mutant transporters should take place.

At last, and concerning the lactate transport rates in the presence of saccharic acid, only the strain expressing Jen1p-S271Q allele transporter revealed a significant decrease in lactate uptake rate. The remaining mutant strains did not show inhibition effects for the radiolabeled substrate in conjugation with saccharic acid.

Once S271Q mutant strain showed evidences for a likely interaction with saccharic acid, we assessed the kinetics for lactic acid uptake in the absence and in the presence of saccharic acid, and subsequently infer about the inhibitory profile revealed, as well as the cells ability of this mutant to transport saccharic acid.

The uptake of several concentrations of lactic acid was assessed in the presence of 17 or 25 mM saccharic acid. The linearization of an ordinary saturation kinetics (Eadie-Hoffstee chart) (Fig. 3.3) shows different K_m values (x axis interception) and same V_{max} (y axis interception) for the transport of lactic acid *per se* or in the presence of distinct concentrations of saccharic acid. Thus a clear competitive inhibition pattern is supported, which points to the ability of S271Q to transport efficiently saccharic acid.

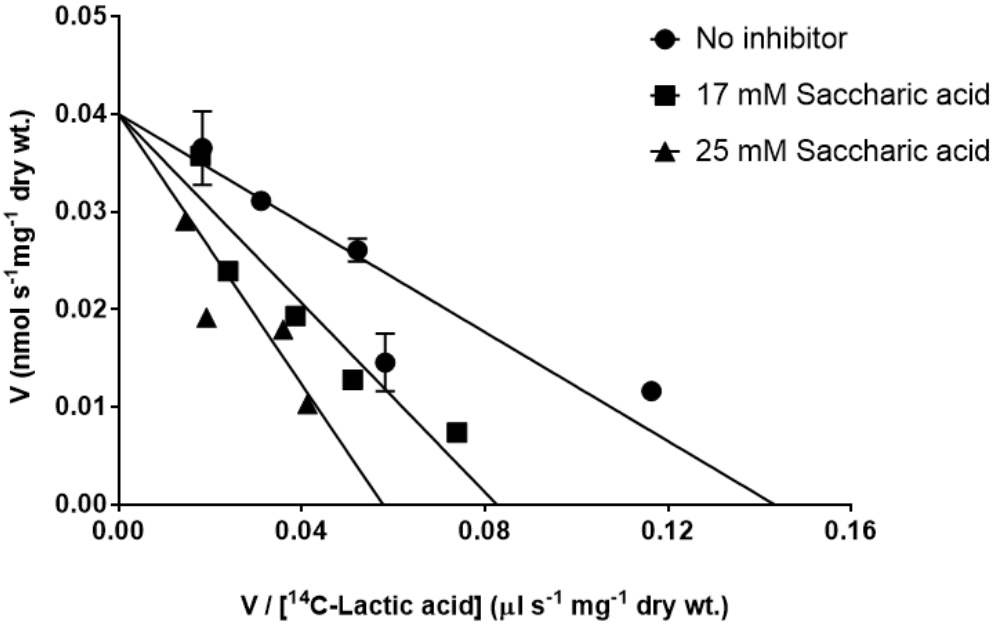


Figure 3.3- Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-lactic acid as a function of the acid concentration at pH 5.0, 30 ºC for *S. cerevisiae jen1*Δ *ady2*Δ p416GPDJen1p-S271Q: ●, no inhibitor; in the presence of saccharic acid 20 mM (■) and 30 mM (▲). The data shown are mean values of at least three independent experiments and the error bars represent standard deviation

Further information from a computational docking interaction simulation, kindly provided by Machado and Casal (unpublished data), also supported the previous results. Here an additional interaction between saccharic acid and an amino acid residue (tryptophan 149) close to the transporter predicted pore was found for the S271Q mutant in relation with Jen1p native transporter (see appendix A.1, A.2).

3.1.2 Screening Jen1p homologous proteins in different yeast strains for potential export properties of carboxylic acids

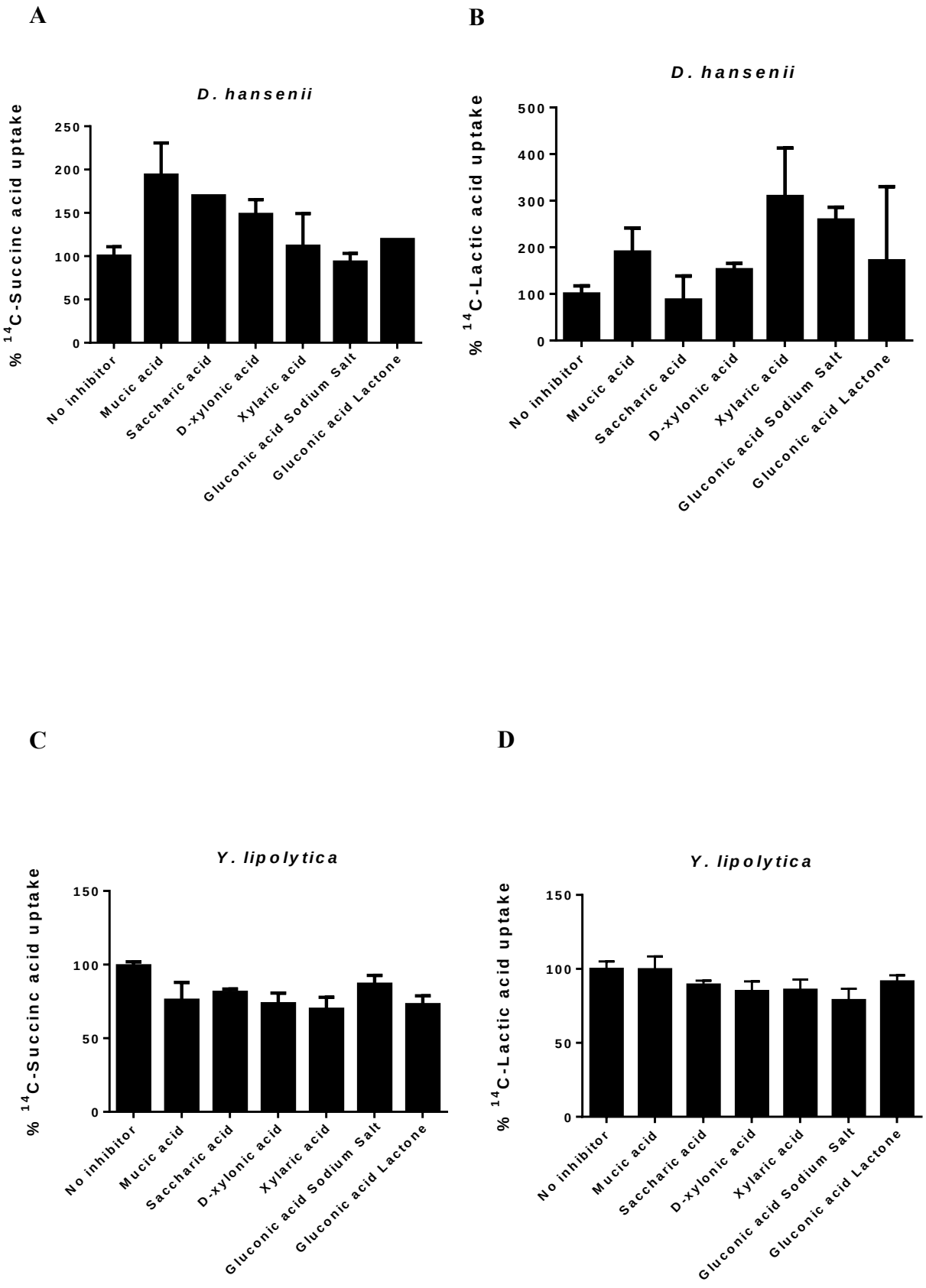
In order to identify additional membrane transporters with potential features to be exploited as carboxylic acids exporters in cell factories, a restrict group of yeast type strains was selected. The selection of such yeast species relies in Jen1p phylogenetic tree constructed from genomes sequences and comprising the homologues of Hemiascomycetes and Euascomycetes fungi (Lodi *et al*., 2007; Casal *et al*., 2008). In such study permeases functionally characterized as monocarboxylate transporters were allocated in the Jen1 cluster. On the other hand, putative transporters genetically related

with KlJen2, a characterized dicarboxylate transporter, were incorporated in Jen2 cluster. An additional cluster was described including six homologue members of *Yarrowia lipolytica* (Casal et al. 2007). The following yeast species were selected for further studies: *Yarrowia lipolytica, Kluyveromyces lactis, Candida albicans and Debaryomices hansenii*. Moreover other carboxylic acids were added to our screening - gluconic acid and xylaric acid - beyond the ones previous mentioned, xylonic, saccharic and mucic acids.

As first screening approach the uptake rates of radiolabeled substrate, both monocarboxylic and dicarboxylic acids – lactic acid and succinic acid, respectively – were assessed in the presence of the above mentioned carboxylic acids for the several yeast species (Fig. 3.4). According to our hypothesis such approach allows the identification of the most likely transporter able to have an inhibitory in inhibition effect, making possible the distinction for monocarboxylate and dicarboxylate transporters.

Regarding the uptake rates for radiolabeled succinic and lactic acid presented by *D. hansenii* (Fig 3.4 A,B) and *Y. lipolytica* (Fig 3.4 C,D) no inhibition effect in uptake rates was found for the tested compounds. One plausible explanation for such results is the absence of interaction of all membrane transporters with the carboxylic acids used as inhibitors. However, due to the existence of a considerable number of putative carboxylic acids transporters in such species, specifically in *Y. lipolytica*, one can also postulate that, even if one of the known putative permease is interacting with the carboxylic acids in test, a dilution effect could mask this phenomenon.

In respect to *K. lactis* (Fig 3.4 E,F) and *C. albicans* (Fig 3.4 G,H) there was no inhibition effects in the uptake rate of lactic acid. On the other hand, ^{14}C-succinic acid uptake, when in added in combination with the non-labelled carboxylic acids, considerable affected in both yeast species was found. It is noteworthy that both yeast species display an identical inhibition profile. Mucic acid, saccharic acid, xylonic acid, and gluconic acid lactone have a greater inhibition effect in radiolabeled succinic acid uptake, specially the gluconic acid lactone. The remaining carboxylic acids, xylaric acid and gluconic acid sodium salt appear to not interfere so markedly in ^{14}C-succinic uptake, however a slight inhibition was detected.


A
D. hansenii
% 14C-Succinc acid uptake
250
200
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
D-xylonic acid
Xylaric acid
Gluconic acid Sodium Salt
Gluconic acid Lactone
B
D. hansenii
% 14C-Lactic acid uptake
500
400
300
200
100
0
No inhibitor
Mucic acid
Saccharic acid
D-xylonic acid
Xylaric acid
Gluconic acid Sodium Salt
Gluconic acid Lactone
C
Y. lipolytica
% 14C-Succinc acid uptake
150
100
50
0
No inhibitor
Mucic acid
Saccharic acid
D-xylonic acid
Xylaric acid
Gluconic acid Sodium Salt
Gluconic acid Lactone
D
Y. lipolytica
% 14C-Lactic acid uptake
150
100
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0
No inhibitor
Mucic acid
Saccharic acid
D-xylonic acid
Xylaric acid
Gluconic acid Sodium Salt
Gluconic acid Lactone

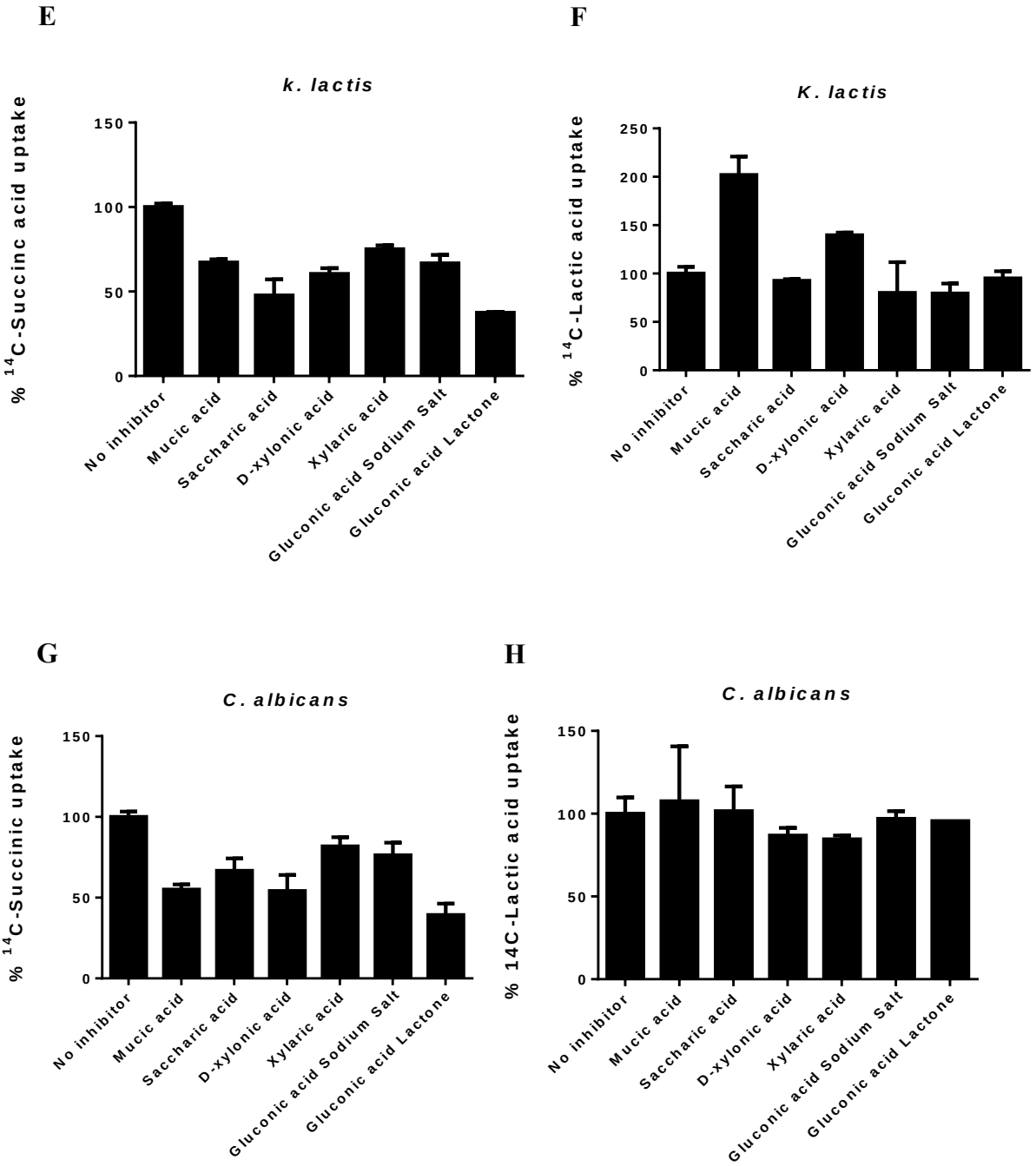


Figure 3.4- Relative capacity (%) of radiolabelled carboxylic acid uptake, after 20 seconds incubation with non-labelled carboxylic acids (10 mM), at pH 5.0 and 30 ºC, acting as inhibitors for the transport in: **A**) *D. hansenii* (^{14}C-succinic acid 20mM); **B**) *D. hansenii* (^{14}C-lactic acid uptake 60 mM); **C**) *Y. lipolytica* (^{14}C-succinic acid 20mM); **D**) *Y. lipolytica* (^{14}C-lactic acid uptake 60 mM); **E**) *K. lactis* (^{14}C-succinic acid 20mM); **F**) *K.lactis* (^{14}C-lactic acid uptake 60 mM); **G**) *C. albicans* (^{14}C-succinic acid 20mM); **H**) *C. albicans* (^{14}C-lactic acid uptake 60 mM).

It would be expected that ^{14}C-lactic acid uptake should be inhibited by monocarboxylic acids, like xylonic and gluconic acids, and radiolabeled succinic acid transport inhibited by the dicarboxylic acids, such as saccharic, mucic and xylaric acids. However these results found were based in a first screening and further studies were need to complement these first observations. It is relevant to mention that the dicarboxylate permeases found in *K. lactis* and in *C. albicans* belong to the Jen2 cluster and they are most probably the main players in a potential interaction with the carboxylic acids here found in these preliminary assays. In this scenario, the transporters that fit these phylogenetic properties are KlJen2 and CaJen2.

3.1.3 Accessing carboxylic acid export properties in *S. cerevisiae* expressing heterologously CaJen1, CaJen2, KlJen1 and KlJen2

Taking into account the previous results, the next step of this study was the use of the mutant strain of *S. cerevisiae jen1*Δ *ady2*Δ background, expressing in a episomal plasmid the monocarboxylic or dicarboxylic acids transporters from *C. albicans* – the CaJen1 or the CaJen2 (Fig. 3.5) - or from *K. lactis* - the KlJen1 or the KlJen2 (Fig. 3.7). Hence, each of the transporters were tested individually and moreover they were expressed in yeast, an organism used in microbial production of biofuels.

Regarding the uptake assays with *S. cerevisiae* pUG35::CaJen1 (Fig. 3.5A), no inhibition in the ^{14}C-lactic acid uptake was found. Actually, this result supports the previous results with radiolabeled lactic acid in *C. albicans* where no inhibition was found too.

As expected only the *S. cerevisiae* strain expressing CaJen2 (Fig. 3.5B) shows inhibition of radiolabeled substrate uptake, since this transporter is recognized as a major player in dicarboxylates uptake in *C. albicans*, and subsequently succinic acid (Vieira *et al*., 2010). While in *C. albicans* ^{14}C-succinic acid uptake assays uptake inhibition for all carboxylic acids was found, in *S. cerevisiae* p416GPD::CaJen2 the transport of radiolabeled substrate was significantly affected only in the presence of mucic acid, xylaric acid and gluconic acid lactone. It is likely that *S. cerevisiae* cell's machinery could affect some of the structural and functional properties of CaJen2 transporter, reducing its affinity for xylaric and gluconic acid sodium salt, or eventually these acids enter by another carboxylate transporter other than CaJen2p or CaJen1p.

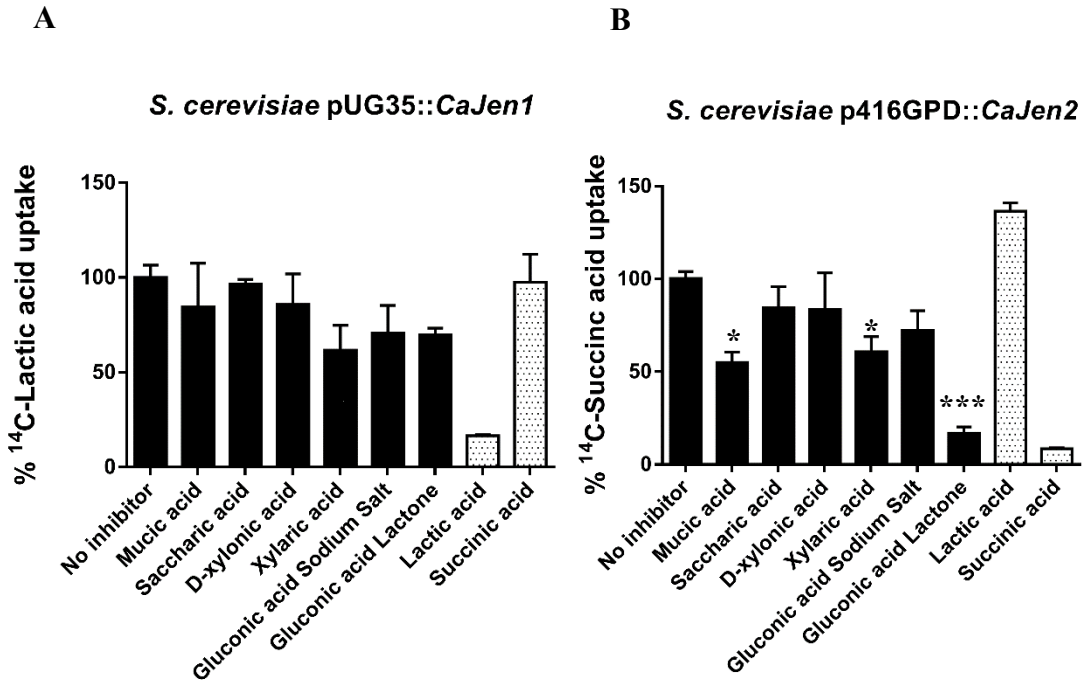
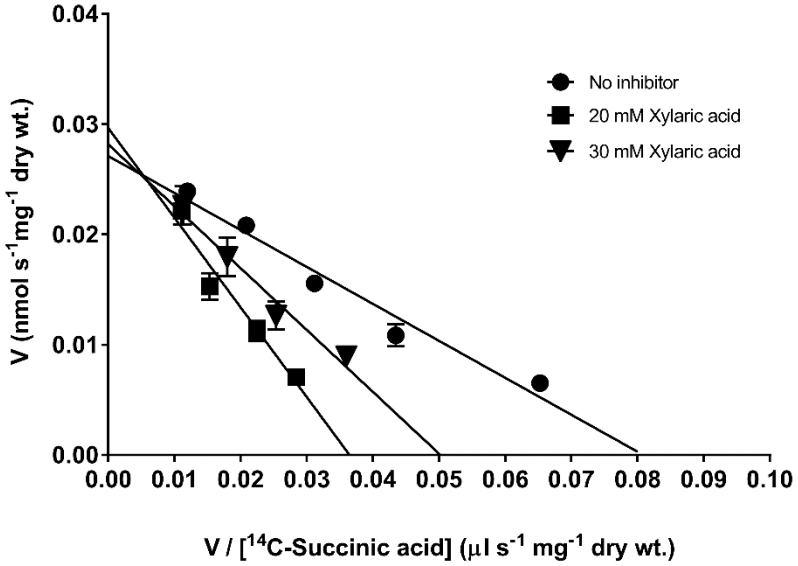


Figure 3.5- Relative capacity (%) of radiolabelled carboxylic acid *ady2*Δ expressing in *trans* distinct Jen1 *C. albicans* homologues. The cells were incubated during 1 minute with non-labelled carboxylic acids (10 mM), at pH 5.0 and 30 ºC, acting as inhibitors of the labeled substrate. **A**) ^{14}C-lactic acid uptake (60 μM) in cells transformed with pUG35::*CaJen1* plasmid. **B)** ^{14}C-succinic acid uptake (20 μM) in cells transformed with p416GPD::*CaJen2*. The data shown are mean values of at least two independent experiments and the error bars represent standard deviation. .* Significant differences ($p \leq 0,05$)

After identification of CaJen2 as a transporter associated with the inhibition of radiolabeled succinic acid, it was of fundamental importance to assess the type of inhibition present for the carboxylic acids tested (mucic acid, xylaric acid and gluconic acid lactone). A detailed kinetic characterization of *S. cerevisiae* p416GPD::CaJen2 was performed (Fig. 3.6). These studies revealed that mucic acid, xylaric acid and gluconic acid lactone present a profile that fits with a competitive inhibition kinetics. The linearization of saturation kinetics through Eadie-Hoffstee plots show ^{14}C-succinic acid uptake alone or in the presence of mucic acid, xylaric acid and gluconic acid lactone displays different K_m values (x axis interception) and similar V_{max} (y axis interception). Which indicates that all these carboxylic acids share the same binding site in the Cajen2 transporter with succinic acid and therefore their share for the same transporter.

A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20 mM Xylaric acid
30 mM Xylaric acid
V (nmol s-1mg-1 dry wt.)
V / [14C-Succinic acid] (µl s-1 mg-1 dry wt.)
0.00
0.01
0.02
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0.04
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0.06
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0.08
0.09
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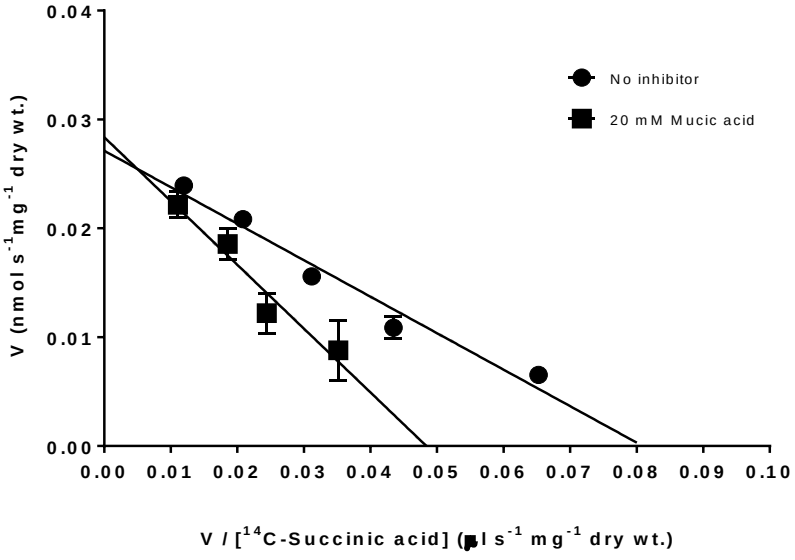
B


No inhibitor
20 mM Mucic acid
V (nmol s-1mg-1 dry wt.)
V / [14C-Succinic acid] (µl s-1 mg-1 dry wt.)
0.00
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C

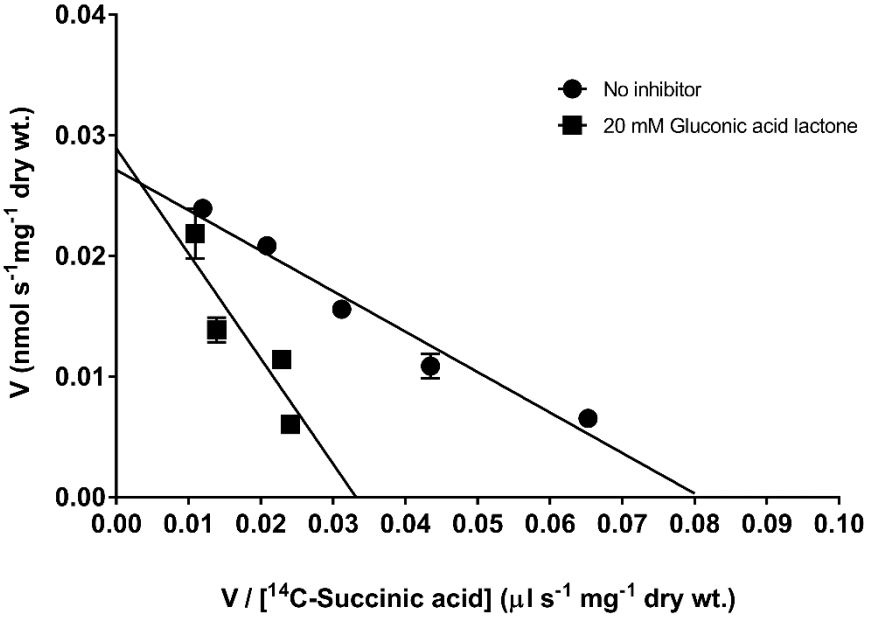

Figure 3.6- Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 30 ºC. **A**) *S. Jcerevisiae jen1Δ ady2*Δ p416GPD*CaJen2*: ●, no inhibitor; in the presence of xylaric acid 20 mM (■) and 30 mM (▲). **B)** *S. cerevisiaejen1*Δ *ady2*Δ p416GPD*CaJen2*: ●, no inhibitor; in the presence of saccharic acid 20 mM (■); and 30 mM (▲). **C**) *S. cerevisiae jen1*Δ *ady2*Δ p416GPD*CaJen2*: ●, no inhibitor; in the presence of gluconic acid 20 mM (■) and 30 mM (▲). The data shown are mean values of at least three independent experiments and the error bars represent standard deviation

Concerning the uptake assays with *S. cerevisiae* p416GPD::KlJen1 (Fig. 3.7A), and similarly with *S. cerevisiae* p416GPD::CaJen1 and *K. lactis* ^{14}C-lactic acid uptake rates any inhibition was found for the acids tested.

However, and as expected only the *S. cerevisiae* strain expressing KlJen2 (Fig. 3.7B) showed inhibition of radiolabeled succinic acid uptake, since this transporter is recognized as a major player in dicarboxylates uptake in *K. lactis*, such as succinic acid (Lodi *et al.*, 2004, Queirós et al 2007). In *K. lactis* ^{14}C-succinic acid uptake assays were inhibited by all carboxylic acids. In accordance with these results in *S. cerevisiae* p416GPD::KlJen2 the transport of radiolabeled ^{14}C-succinic acid was significantly affected but only in the presence of two carboxylic acids, saccharic acid and gluconic acid lactone. It is likely that *S. cerevisiae* cell's machinery could affect some of the structural and functional properties of KlJen2 transporter, reducing its affinity for the remaining carboxylic acids.

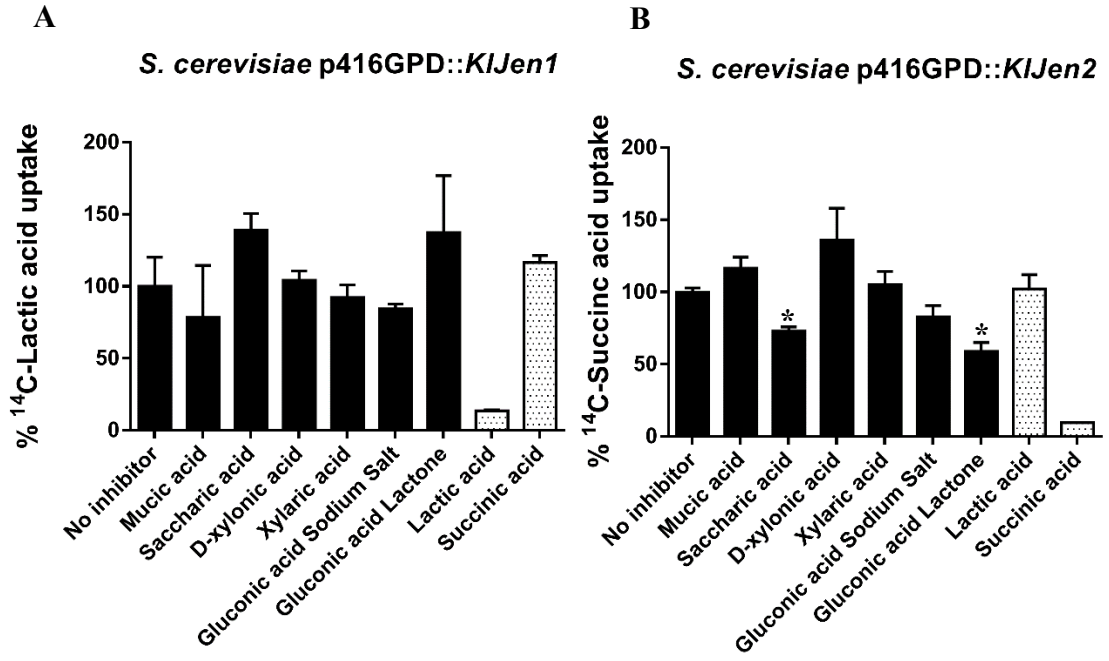

Figure 3.7- Relative capacity (%) of radiolabelled carboxylic acid uptake after 1 minute incubation with non-labelled carboxylic acids (10 mM), at pH 5.0 and 30 ºC, acting as competitors for the transport in strains expressing heterologously the carboxylate transporter. A) ^{14}C-lactic acid uptake (60 μM) in *jen1*Δ *ady2*Δ strain transformed with p416GPD::*KlJen1*; B) ^{14}C-succinic acid (20 μM) *jen1*Δ *ady2*Δ strain carrying p416GPD::*KlJen2*. The data shown are mean values of at least two independent experiments and the error bars represent standard deviation. .* Significant differences ($p \leq 0,05$).

After confirmation of the transporter - KlJen2 - associated with the transport of succinic acid, it was critical to determine the type of interaction between the carboxylic acids (saccharic acid and gluconic acid lactone) and the transporter. In order to determine if the mentioned carboxylic acids are acting as substrates, a kinetic characterization of *S. cerevisiae* p416GPD::KlJen2 was performed (Fig. 3.8).

The linearization of saturation kinetics trough Eadie-Hoffstee plots shows ^{14}C-succinic acid uptake alone or in the presence of saccharic acid and gluconic acid lactone displays different K_m values (x axis interception) and similar V_{max} (y axis interception), which is compatible with a competitive inhibition kinetics profile. Such carboxylic acids share the same binding site in Kljen2 transporter with succinic acid.

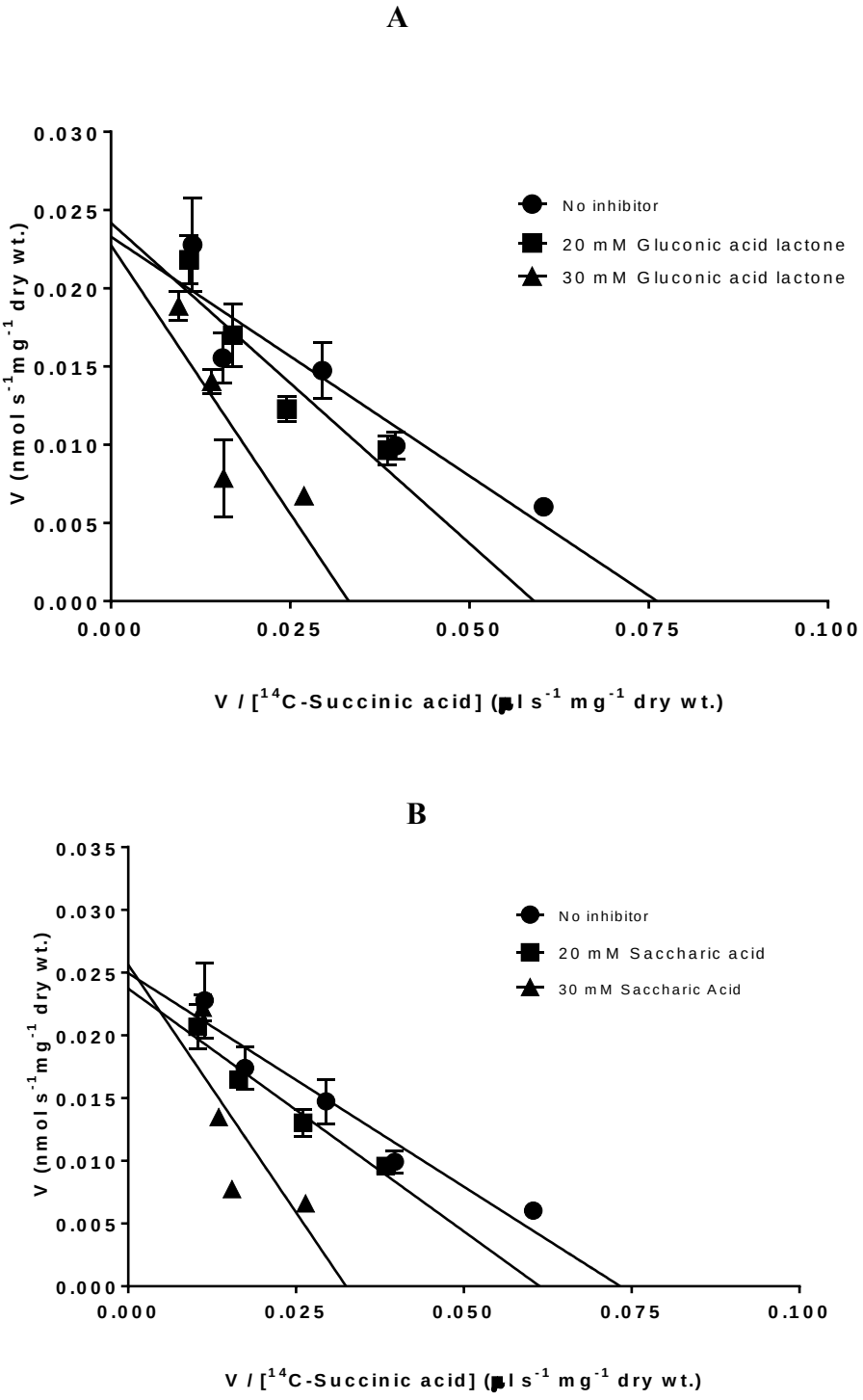

Figure 3.8- Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 30 ºC.**A**) *S. cerevisiae jen1*Δ *ady2*Δ p416GPD*KlJen2*: ●, no inhibitor; in the presence of gluconic acid 20 mM (■) and 30 mM (▲). **B)** *S. cerevisiae jen1*Δ *ady2*Δ p416GPD*KlJen2*: ●, no inhibitor; in the presence of saccharic acid 20 mM (■); and 30 mM (▲). The data shown are mean values of at least three independent experiments and the error bars represent standard deviation.

3.2 Analysis of inhibition constant (K_i) for the distinct sugar carboxylic acid transporters

To further characterize the selected transporters expressed in *S. cerevisiae* with potential properties to act as export pumps as well as compare the affinity to the substrate between transporters, the inhibition constant (K_i) values were estimated (Table VI). They were obtained according to the following formula: $K_i = (k_m * [I]) / (k_{mobs} - k_m)$, where [I] corresponds to inhibitor concentration, and k_m and k_{mobs} corresponds to k_m value of the uptake kinetics in the absence or presence of inhibitor, respectively.

The CaJen2 transporter seems to be the most versatile protein. Evidences were found for the transport of three carboxylic acids, mucic, xylaric and gluconic acids. Among them, CaJen2 has higher affinity for gluconic acid (K_i value of 13.2 mM), followed by xylaric (K_i value of 27.6 mM) and mucic acid (K_i value of 32.9 mM).

Similarly to CaJen2, KlJen2 transporter also shows affinity for gluconic acid (K_i value of 40.6 mM) however in a noticeable lower affinity. Additionally KlJen2 reveals affinity for saccharic acid (K_i value 24.1 mM).

At last Jen1p-S271Q only reveals affinity for saccharic acid presenting a similar K_i (value of 24.7 mM) to KlJen2 for the same carboxylic acid.

Table VI - K_i values (mM) for the sugar carboxylic acids transporters expressed in *S. cerevisiae.*

Transporter	Carboxylic acid	K_i (mM)
CaJen2	Mucic acid	32.9
	Gluconic acid lactone	13.2
	Xylaric acid	27.6
KlJen2	Gluconic acid lactone	40.6
	Saccharic acid	24.7
Jen1pS271Q	Saccharic acid	24.1

4. DISCUSSION

4.1 Final remarks

During this study a considerable number of carboxylic acid transporters, belonging to different yeasts species were screened and functionally characterized regarding the ability to transport sugar acids, such as xylonic and gluconic acids (as monocarboxylic acids) and saccharic, mucic and xylaric acid (as dicarboxylic acids). Our objective was to explore the use of these transporters as exporters in model and industrial microorganisms. In spite of the major advances in metabolic engineering, the main strategy to improve microbial production, until the last decade lays in redirecting the intracellular metabolic pathways of the microorganisms towards the production of a target compound. This approach proves to be effective, but also presents limitations, especially when the production yields reaches significant toxic levels, the production can be hardly compromised (Foo and Leong, 2013). As a solution for this microbial cell factories bottleneck, plasma membrane transporters have been explored as extrusion systems to decrease such toxic levels on intracellular medium, allowing a considerable boost in production, as well as a lesser expensive product's purification (van Maris *et al*., 2004; Warnecke and Gill, 2005).

The yeast *S. cerevisiae* was used in this study as a model organism carrying natively or recombinant engineered transporters aiming to assess their impact in carboxylic acid production. Beyond all the knowledge about *S. cerevisiae* as model organism (domestication from ancient times, first eukaryote sequenced genome, straight forward homologous recombination, huge number of knocked-out library strains, etc…) other important physiological features specifically for the production of carboxylic acids were also crucial factors that contributed to its selection (Abbott *et al.*, 2009). Among them are the ability of *S. cerevisiae* cells to grow in very acidic environmental conditions (pH values ranging from 3 and 4), which represents a high advantage in relation to other model organisms like *E. coli* (Liu and Jarboe, 2012). In a scenario where a noticeable amount of the carboxylic acid produced in the cytoplasm's cell is exported in its anion form to the growth media, it is important to assure that it does not return in its undissociated form. Therefore an extracellular pH value below the carboxylic acid's pK_a is needed, usually between 4 and 5, in order to maintain the organic acid in its deprotonated form (Piper *et al.*, 2001; Stratford *et al.*, 2013).

In this work the search for potential carboxylic acid extrusion transporters started with a mutant alleles collection obtained from structural and functional studies of ScJen1p, a lactate/proton symporter belonging to the lactate/pyruvate:H(+) symporter subfamily (TC#2.A.1.12.2) of the Major Facilitator Superfamily (Soares-Silva et al., 2007). Such ScJen1p mutants were reported as having altered functional properties, such as increased uptake velocity, increased substrate affinity and altered substrate specificity, namely for dicarboxylic acids (Soares-Silva et al., 2007), which made this collection a preferential target to search for potential efflux transporters.

Among all the mutants tested (A272G, Y284Q, Y284A, Q386A, Q498A, S271Q, F270G, F270A, F270QS271Q), only Jen1p-S271Q shows to be an interesting candidate for yeasts strains metabolic engineered to produce saccharic acid. The ^{14}C-lactic acid uptake in *S. cerevisiae* expressing Jen1p-S271Q showed a reduction in the presence of saccharic acid. Further kinetic analysis revealed a competitive inhibition profile, with a K_i value of 24.1 mM, demonstrating that Jen1p-S271Q affinity for saccharic, subsequently its transport across the plasma membrane. Further information from a computational docking interaction simulation (kindly provided by Frederico Machado and Casal, 2013, see appendix A) also supported the results obtained. Beyond an interaction of saccharic acid with the arginine 188 amino acid residue, an additional interaction between the carboxylic acid and another amino acid residue, tryptophan 149, close to the transporter predicted pore was found for the S271Q mutant in relation with Jen1p native transporter (figure A.1, A.2).

Aiming at identifying other transporters able to export sugar acids, the study's scope turned to carboxylic acids transporters of other yeasts species, with homology to ScJen1p. A phylogenetic study provided the needed information to identify potential protein candidates. The resultant Jen1p phylogenetic tree comprises homologues of Hemiascomycetes and Euascomycetes fungi. Permeases functionally characterized as monocarboxylate transporters were allocated in the Jen1 cluster. On the other hand, putative transporters genetically related with KlJen2, a characterized dicarboxylate transporter, were incorporated in a Jen2 cluster. An additional cluster was described including six homologue members of *Yarrowia lipolytica* (Lodi *et al.*, 2007). The following yeast species were selected *D. hansenii*, *Y. lipolytica*, *K. lactis* and *C. albicans* for further studies. When radiolabeled lactic acid and succinic acid uptake rates were assessed in the presence of the other carboxylic acids, no inhibition effects were detected for ^{14}C-lactic acid uptake for the above mentioned yeast species. On the other hand ^{14}C-

succinic acid uptake was inhibited in *C. albicans in* the presence of mucic acid, xylaric acid and gluconic acid lactones, as well as in *K. lactis* in the presence saccharic acid and gluconinc acid lactone.

S. cerevisiae strains expressing mono and dicarboxylic acids transporters of *C. albicans* (CaJen1 and CaJen2) (Soares-Silva *et al,* 2004; Vieira *et al.*, 2010) and of *K. lactis* (KlJen1 and KlJen2) (Lodi *et al.*, 2004) were used to assess the radiolabeled acids uptake. The results have shown an inhibitory effect in the radiolabeled succinic acid uptake in the *S. cerevisiae* strains transformed with the dicarboxylate transporters. A detailed kinetic characterization revealed a competitive inhibition profile for all carboxylic acids previously mentioned. In respect to *S. cerevisiae* p416GPD::CaJen2 the K_i values were: 13.2 mM, 27.6 mM and 32.9 mM for gluconic acid lactone, xylaric acid and mucic acid, respectively. Meanwhile the K_i values for *S. cerevisiae* p416GPD::KlJen2 were: 27,6 mM for saccharic acid and 40,6 mM for gluconic acid lactone.

An overview upon this study reveals three carboxylic acid transporters – Cajen2, KlJen2 and Jen1p-S271Q with interesting properties to be used as efflux transporters regarding the microbial production of mucic, saccharic, xylaric and gluconic acids (Fig. 4.1).

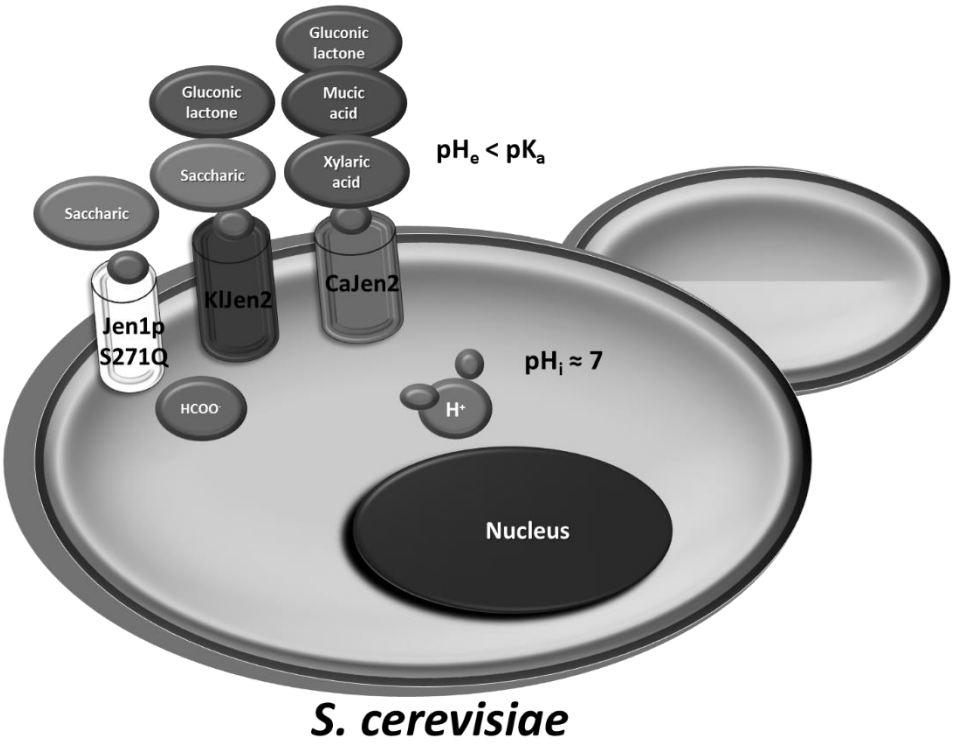

Figure 4.1- Overview of carboxylic acids transport properties found for CaJen2, KlJen2 and Jen1p-S271Q membrane transporters.

4.2 Future perspectives

In order to continue the present study it is relevant to proceed with other complementary approaches and strategies.

Similarly to what was done with ScJen1p, additional functional and structural studies are needed for the remaining transporters, in order to fully clarify the role of specific amino acids residues in the transporter's function and structure as well as to contribute for the development of new mutant versions of this transporters with boosted properties to export carboxylic acids.

One approach to follow is to extend the modulation and computational docking simulation to CaJen2 and KlJen2 which would provide additional information to unveil the main players in the ligand-transporter interaction. Rational design of mutations based on these models would allow a fine tuning of transporter properties for the desired industrial application.

Preliminary production tests for engineered *S. cerevisiae* strains should be accomplished, in order to assess the impact of such carboxylic acid transporters in the extracellular carboxylic acids titers.

Moreover other carboxylic acids transporters, such as Ady2p, should be screened for export properties.

Important achievements were made during this study, however it comprises the very first steps of a long path towards an effective and profitable application of carboxylic acid transporters as extrusion systems in industrial settings.

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APPENDIX

Protein/substrate docking simulation

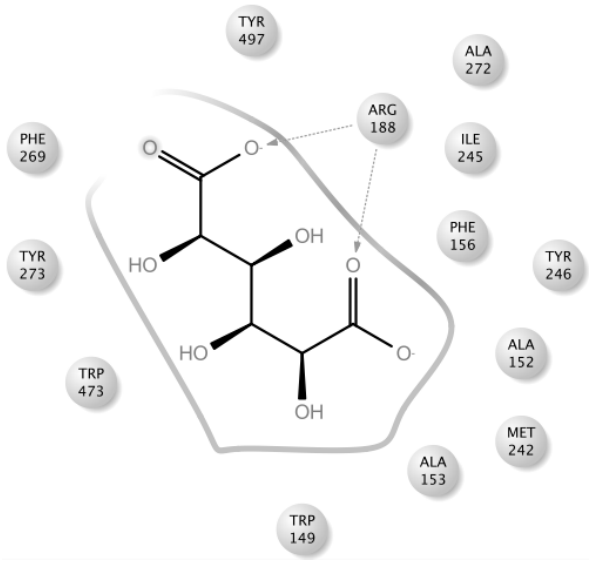

Figure A.1- Docking Jen1p/saccharic acid simulation and illustration on molecular interactions between transporter and carboxylic acid.

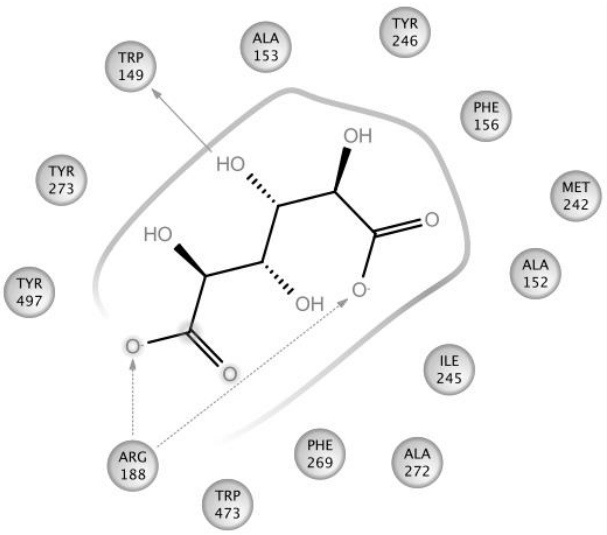

Figure A.2- Docking Jen1p-S271Q/saccharic acid simulation and illustration on molecular interactions between transporter and carboxylic acid.