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# BEORA – Biotechnology exploitation of orotic acid production

Nuno Duarte Fernandes Lima de Carvalho

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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# BEORA – Biotechnology exploitation of orotic acid production

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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**Preceitos Legais**

De acordo com o disposto no Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto, aprovado por despacho reitoral de 10 de abril de 2015 e publicado em Diário da República, 2ª série, nº 90 de 11 de Maio de 2015, por Despacho n.º 4889/2015, nesta tese foram utilizados os resultados de trabalhos publicados abaixo indicados.

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No cumprimento do Decreto-Lei acima mencionado, o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão de resultados, e na sua redacção.








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Por tudo o que o silêncio da restante página em branco diz e só vocês ouvem.
Pelo carácter e pelas emoções.
É o que se sente.
E eu sinto.
Obrigado.

**Abstract**

The “non-conventional” yeast *Kluyveromyces lactis* has become increasingly attractive for the industry and has been successively used in biotechnology mostly for the production of heterologous proteins. In this study, we demonstrated that the *Klura3*Δ and *Klura5*Δ mutants of the yeast *K. lactis* are able to produce and secrete into the growth medium significant amounts of orotic acid, an intermediate of the *de novo* pyrimidines biosynthetic pathway. Using yeast extract - peptone - glucose (YPD) based media, we optimized production conditions in flask and bioreactor cultures. With cells grown in YPD 5% glucose medium, the best production in flask was obtained using a 1 : 12.5 culture volume / flask volume ratio, 180 rpm, 28 ºC and 200 mM MOPS for pH stabilization at neutral values (initial culture pH at 8.0). The best production in a 2 L bioreactor was achieved at 500 rpm with 1 vvm aeration, 28 ºC and pH 7.0. Under these optimum conditions, similar rates of orotic acid production were obtained and maximum concentration achieved after 96 h was 6.7 g/L in flask and bioreactor cultures. These results revealed an excellent reproducibility between both systems and provided evidence for the biotechnological potential of *Klura3*Δ and *Klura5*Δ strains since the amounts obtained are comparable to the production in flask using a similar mutant of the industrially valuable *Corynebacterium glutamicum* currently used in industry to produce orotic acid.

We have also established the grounds for the development of a low cost production media for industrial scale-up. A media containing 4% (v/v) corn steep liquor (CSL), 3.4% (v/v) molasse, 0.25% (w/v) urea and 200 mM MOPS for pH stabilization at neutral values (initial culture pH at 8.0), which only had a total of 2% (w/v) sugars, allowed the production of 4.5 g/L of orotic acid after 80 h. CSL proved to be very promising as the main low cost constituent of the production media. However, it contains inhibiting factors for the production of orotic acid, thus limiting the concentration of CSL that can be used in the media. Before further development of the industrial media, the inhibition caused by CSL should be first addressed to reduce or eliminate its effects in order to take full advantage of CSL benefits.

A putative link between the pyrimidines biosynthetic pathway and mitochondria led us to assess changes in the oxidative stress markers in the orotic acid producing mutants. Our results show that at latter stages of growth, catalase activity increased in pyrimidines mutants of *K. lactis* with the interruption at any step of the *de novo* pyrimidines pathway. Depending of the growth phase, the *Klura3*Δ mutant displayed a distinct or a more marked phenotype compared to other pyrimidine mutants, suggesting different mechanisms may be involved in this particular mutant. Notably, at latter stages of growth, the high catalase activity exhibited by *Klura3*Δ cells was correlated with an increased resistance to $H_2O_2$. Moreover, deletion of any of the dihydroorotate dehydrogenase genes (DHODases; *Klura1*Δ and *Klura9*Δ) in *Klura3*Δ was shown to alter its phenotype, indicating a possible connection of the *de novo* pyrimidines pathway with oxidative stress response that involves Klura3p, Klura1p and Klura9p.

**Resumo**

A levedura “não-convencional” *Kluyveromyces lactis* tem-se tornado cada vez mais atrativa para a indústria e tem sido usada com sucesso em biotecnologia sobretudo para a produção de proteínas heterólogas. Neste trabalho, demonstramos que os mutantes *Klura3*Δ e *Klura5*Δ da levedura *K. lactis* são capazes de produzir e secretar para o meio de crescimento quantidades consideráveis de ácido orótico, um intermediário da via de síntese *de novo* das pirimidinas. Usando meios de cultura baseados em extrato de levedura - peptona - glicose (YPD), otimizamos as condições de produção em matraz e biorreactor. Com células cultivadas em meio YPD 5% glicose, a melhor produção em matraz foi obtida com uma proporção de 1 : 12.5 para volume de cultura / volume de matraz, 180 rpm, 28 ºC e 200 mM MOPS para a estabilização do pH em valores neutros (pH cultura inicial a 8.0). A melhor produção num biorreator de 2 L foi alcançada a 500 rpm com um 1 vvm de arejamento, 28 ºC e pH 7.0. Sob estas óptimas condições, as taxas de produção de ácido orótico foram semelhantes e a concentração máxima atingida após 96 horas foi de 6.7 g / L em culturas de matraz e biorreactor. Estes resultados revelaram uma excelente reprodutibilidade entre os dois sistemas e fornecem evidências relativamente ao potencial biotecnológico das estirpes *Klura3*Δ e *Klura5*Δ, uma vez que os valores obtidos são comparáveis à produção em matraz com um mutante semelhante do microorganismo *Corynebacterium glutamicum*, que é actualmente usado na indústria para a produção de ácido orótico.

Foram também estabelecidas as bases para o desenvolvimento de um meio de produção de baixo custo para “scale-up” industrial. Um meio contendo 4% (v/v) de água de maceração de milho, 3.4% (v/v) melaço, 0.25% (w/v) ureia e 200mM MOPS para estabilização do pH a valores neutros (pH 8.0 para início da cultura), contendo somente um total de açucares de 2%, permitiu a produção 4.5 g/L de ácido orótico após 80 h. A água de maceração de milho provou ser bastante promissora como o principal constituinte de baixo custo do meio de produção. Contudo, demonstrou-se conter fatores inibitórios para a produção de ácido orótico o que, por esse motivo, limita a sua concentração no meio de cultura. Previamente à continuação com o desenvolvimento do meio industrial, a inibição

causada pela água de maceração de milho deve ser primeiro avaliada para reduzir ou eliminar os seus efeitos de forma a poder usufruir de todas as suas vantagens.

A existência de uma possível ligação entre a via de síntese *de novo* das pirimidinas e a mitocôndria levaram-nos as avaliar as alterações nos marcadores de stress oxidativo nos mutantes produtores de ácido orótico. Os nossos resultados mostram que em fases avançadas do crescimento, há um aumento da actividade da catalase nos mutantes de *K. lactis* com a interrupção em qualquer um dos passos da via de síntese *de novo* das pirimidinas. Dependendo da fase de crescimento, o mutante *Klura3*Δ apresentou um fenótipo diferente ou mais marcado quando comparado com outros mutantes da via das pirimidinas sugerindo que outros mecanismos poderão estar envolvidos neste mutante em particular. Em fases avançadas do crescimento, a elevada actividade de catalase exibida pelas células *Klura3*Δ correlacionou-se com um aumento da resistência ao $H_2O_2$. Mais ainda, a deleção de qualquer um dos genes que codificam para desidroorotato desidrogenases (DHODases; *Klura1*Δ e *Klura9*Δ) no *Klura3*Δ alterou o seu fenótipo, indicando uma possível ligação da via de síntese *de novo* das pirimidinas com a resposta ao stress oxidativo que envolve a Klura3p, a Klura1p e a Klura9p.

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**List of Abbreviations**

ABS – artificial biosynthesis of sugar nucleotides
ABC – artificial biosynthesis of carbohydrates
ATCase – aspartate transcarbamylase
ATCC – American Type Culture Collection
ATP – adenosine triphosphate
CPSase – carbamylphosphate synthetase
CSL – corn steep liquor
CTP – cytidine triphosphate
CWP – cheese whey powder
DHODase – dihydroorotate dehydrogenase
DNA - deoxyribonucleic acid
DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
EDTA – ethylenediamine tetraacetic acid
5-FU – 5-fluorouracil
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GMP – guanosine monophosphate
GOS – galacto-oligosaccharides
GSH – reduced glutathione
GSSG – oxidized glutathione
GRAS – generally regarded as safe
GTP – guanosine-5'-triphosphate
HPLC – high-performance liquid chromatography
LC – lethal concentration
MOPS – 3-(N-morpholino)propanesulfonic acid
NADH - nicotinamide adenine dinucleotide
NADPH - nicotinamide adenine dinucleotide phosphate
OMP – orotidine-5’-monophosphate
ORF – open reading frame
PCR – polymerase chain reaction
RNA - ribonucleic acid

ROS – reactive oxygen species

SEM / EDS – scanning electron microscopy with X-ray microanalysis exam

SOD – superoxide dismutase

UMP – uridine-5’-monophosphate

UTP – uridine-5’-triphosphate

UV – ultraviolet

WT – wild type

YPD – yeast extract peptone dextrose

# CHAPTER I

## Introduction

## 1.1. The yeast *Kluyveromyces lactis*

The yeast *Kluyveromyces lactis* owes its name to the Dutch microbiologist Albert Jan Kluyver (1888–1956) and to have been originally isolated from milk. Most microorganisms lack the enzymes to metabolize lactose and, besides lactic acid bacteria, enterobacteria and some filamentous fungi, *K. lactis* is one of the few exceptions that can grow in lactose as sole carbon source. For that reason, it can be found in milk and constitutes the predominant eukaryote during cheese production, which by decreasing the whey pH allows better conditions for the growth of lactic acid bacteria.

*K. lactis* is closely related to *Saccharomyces cerevisiae* [1] (Figure 1.1) and shares similar morphologic and life cycle characteristics. It is a unicellular ascomyceteous yeast that divides by polar budding, having similar generation time as wild type strains of *S. cerevisiae*. *K. lactis* has a life cycle supported by two mating types, a stable haploid and a semi-stable diploid phase, and can be induced to produce tetrads by nutrient starvation [2]. However, interest in *K. lactis* arose from its distinctive physiological properties compared to the classical yeast model *S. cerevisiae*. Along with the fission yeast *Schizosaccharomyces pombe* and the non-fermenting *Yarrowia lipolytica*, *K. lactis* is one of the most studied "non-conventional" yeasts and has driven increased interest into fundamental research studies [3, 4].

### 1.1.1. Varieties and episomal DNA

Two different varieties of *K. lactis* can be found: the domestic *K. lactis* var. *lactis* [5] and its wild relative *K. lactis* var. *drosophilarum* [6]. The taxonomic difference between the two varieties is based arbitrarily on phenotypic and ecological characteristics [7]. However, the most obvious metabolic difference is that the wild variety has lost the ability to ferment lactose, reflecting the silencing of *KlLAC4* and *KlLAC12* encoding β-galactosidase and lactose permease, respectively [8]. Therefore, for basic research purposes, the *K. lactis* research community has chosen to work with strain *K. lactis* var. *lactis* CBS 2359 (DSM 70799 from DSMZ).

*K. lactis* var. *lactis* is also the variety predominantly used in biotechnology. Unless named otherwise, further mention to *K. lactis* will be referring to *K. lactis* var. *lactis*. Some *K. lactis* strains contain a cytoplasmic linear DNA plasmid pair, pGKL1 and pGKL2, conferring the "killer" phenotype [9]. These plasmids are responsible for the production of an anti-yeast toxin complex known as zymocin. The toxin itself is encoded by pGKL1 whereas pGKL2 is responsible for the maintenance of pGKL1.

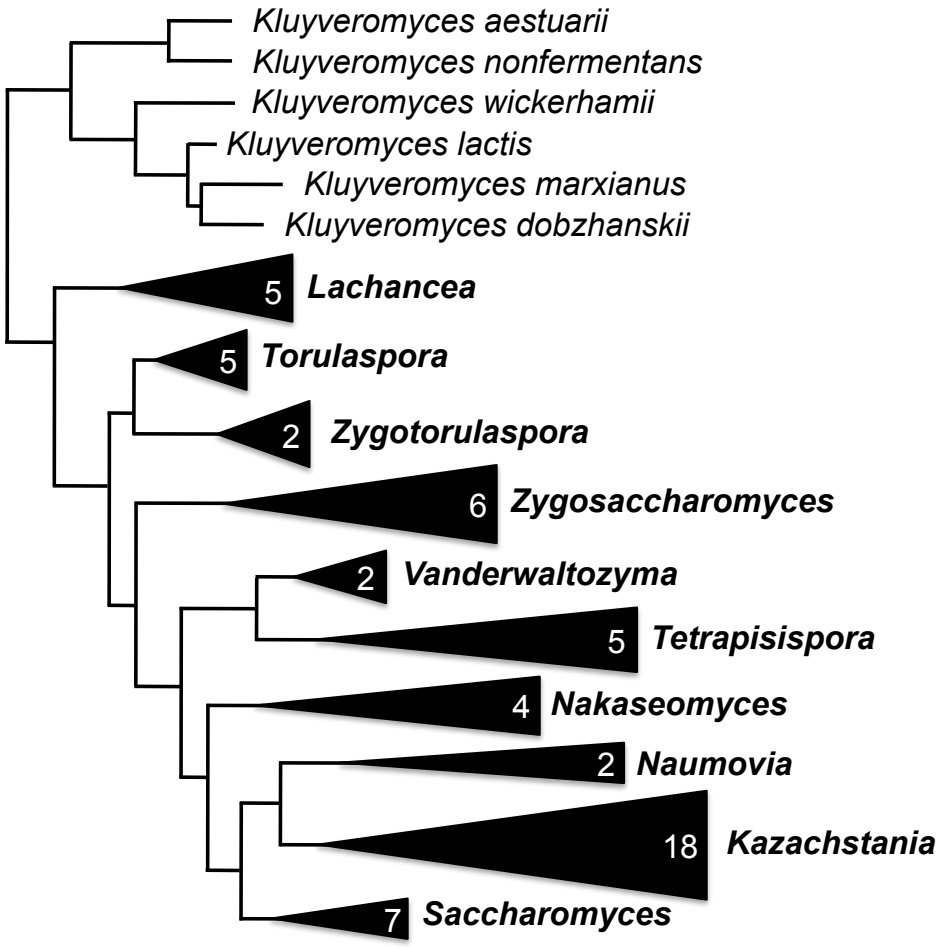


**Figure 1.1** Phylogenetic placement of species currently assigned to *Kluyveromyces* within the 11 clades of the Saccharomycetaceae. The tree is modified from that published by Kurtzman [10], which was based on a maximum parsimony analysis of sequences from the small-subunit rRNA gene, part of the internal transcribed spacer/5.8S region, three regions of the large-subunit rRNA gene, elongation factor 1K, the mitochondrial small-subunit rRNA gene and cytochrome oxidase II. The total number of species recognized in each genus proposed by Kurtzman [10] is shown. A complete branching network is given only for species in the genus *Kluyveromyces*.

Studies using these strains provided insights into new mechanisms of microbial competition [11] and played an important role in the development of *K. lactis* biology, because at that time the only plasmids known in yeast were the 2 μm circular DNA and the double-stranded killer RNA of *S. cerevisiae*. pGKL1 and pGKL2 have been thoroughly studied and represent a model system for analyzing the fundamentals of linear plasmid replication and gene expression [12-14]. Also, the fact that *K. lactis* secreted a high-molecular weight killer toxin retained the attention of the bioindustry that was looking for an efficient system to produce recombinant proteins in a secreted form. Later, and after a deliberate search, a 2 μm type circular plasmid pKD1 was discovered in *K. lactis* var. *drosophilarum* [15] that was capable of replicating in *K. lactis* var. *lactis*. This discovery was of considerable importance for *K. lactis* research by offering a replicating vector system equivalent to the *S. cerevisiae* 2 μm vectors and opened the way for the development of other derivatives vectors with improved characteristics.

### 1.1.2. Carbon and energy metabolism

Regulation of primary carbon and energy metabolism has been one of the main focus of *K. lactis* research since it reflects the adaption of *K. lactis* to aerobic conditions and contrasts with the well-studied but exceptional physiology of *S. cerevisiae* [16]. *K. lactis* assimilates a wider variety of carbon sources but the utilization of lactose is its characteristic trait and has been studied extensively. The studies on the lactose regulon in *K. lactis* illustrated how the regulatory system of this unicellular eukaryote differs from the bacterial lactose operon. This regulon, a close variation of the galactose regulon of *S. cerevisiae*, was shown to involve many genetically unlinked positive and negative regulatory genes [16, 17]. *K. lactis* displays the Kluyver effect, i.e., its growth on certain mono- and oligosaccharides (such as galactose, raffinose, sucrose and maltose) occurs only under respiratory conditions and not under oxygen-limiting conditions. Yeasts displaying this effect are yet capable of fermenting glucose as well as one or more components of these oligosaccharides [18]. This has been associated with low permease activities for these sugars, which are not sufficient to ensure the high substrate flow that is necessary for fermentative growth [19].

*K. lactis* is a so-called “petite-negative” yeast in which maintenance of mitochondrial DNA and mitochondrial protein synthesis is essential [20]. It has a respiratory-fermentative metabolism and most strains can grow on fermentable carbon sources when respiration is blocked [21]. Nevertheless, *K. lactis* appears to be an obligate aerobe that is not able to grow under strict anaerobic conditions [22]. *S. cerevisiae* is a prototypical Crabtree-positive yeast since when provided with glucose it produces ethanol even under conditions of oxygen saturation [23]. On the other hand, *K. lactis* favors respiration over alcoholic fermentation and is Crabtree-negative due to the absence of aerobic ethanol formation [24, 25], thus increasing ATP and biomass yield when compared to Crabtree-positive yeasts [26]. The absence of glucose repression of respiration illustrates a major difference in the regulation of glucose metabolism as compared to *S. cerevisiae*. Once the central metabolite glucose-6-phosphate is produced, its further consumption differs significantly between *K. lactis* and *S. cerevisiae*. Whereas in the latter, most of the glucose consumed is metabolized through glycolysis producing NADH, in *K. lactis* it is mainly channeled into the pentose phosphate pathway [27] and as a consequence there is a higher production of NADPH in the cytosol. Another important difference in energy metabolism between *K. lactis* and *S. cerevisiae* concerns the fate of cytosolic NADH and NADPH, which needs to be oxidized to maintain the redox balance. A significant part of NADPH reoxidation is carried out in *K. lactis* by mitochondrial external alternative dehydrogenases, which can use both NADH and NADPH, in contrast to the enzymes of *S. cerevisiae* that are NADH-specific [28, 29]. In fact, one of the molecular keys supporting the difference in the Crabtree phenotype of the two yeast species lies in the mechanisms involved in the reoxidation of the NADPH [28].

### 1.1.3. *K. lactis* as model organism

While *S. cerevisiae* preferably ferments in the presence of glucose, *K. lactis* favors respiratory growth. As opposed to *S. cerevisiae* cells, it has been described the strong resemblance to human of the regulatory circuits of *K. lactis* responses to hypoxic conditions [30]. Many yeast genes related to the hypoxic responses are related to human diseases [31]. Also unlike *S. cerevisiae*, *K. lactis* has not

undergone whole genome duplication and thus, does not produce enzymatic isoforms from paralogous genes. The existence of different protein isoforms makes the analysis of *in vivo* protein functions or complementation assays with human proteins challenging. Therefore, *K. lactis* has been considered a model more suitable than other yeasts to study basic cellular processes related to human diseases such as Alzheimer's and Parkinson's [30, 32].

## 1.2. *K. lactis* in biotechnology

Despite their higher relative complexity, yeasts tend to have many beneficial, industrially attractive traits relative to commonly used bacteria such as *E. coli* [33-36]. A few of these attributes include: growth to high cell densities on a wide variety of carbon sources, ability to perform a variety of post-translational modifications, potential to compartmentalize reactions in organelles, high secretion capacity, and a lack of susceptibility to infectious agents like bacteriophage [37]. *S. cerevisiae* is currently the dominant yeast in protein biochemistry, metabolic engineering and the production of fermented beverages. Over the years, *K. lactis* has received increasing attention from bioindustries, having now emerged as one of the most important non-*Saccharomyces* yeast species for industrial biotechnology, especially for the industrial production of proteins [4, 38, 39].

### 1.2.1. Food industry

*K. lactis* has a GRAS (Generally Regarded as Safe) status making it an attractive production organism in the food industry [40, 41]. It possesses a broader and diversified catabolic repertoire compared to *S. cerevisiae*, thus enabling lower cost bioprocesses. In particular, the ability of *K. lactis* to use lactose allows it to grow on whey, which is an inexpensive waste product from the dairy industry. This is a major advantage over other types of yeasts due to the resulting economic and ecological benefits. The whey produced in industry constitutes a major environmental problem because of its high organic load [42, 43]. On the other

hand, it represents a cheap carbon source for *K. lactis* growth, since the price for the traditionally used molasses has increased considerably over the past decades.
The Crabtree-negative phenotype of *K. lactis* is of great relevance for industrial production of metabolites and heterologous proteins since it can be advantageous for increasing biomass accumulation in aerated cultures without losing overflow carbon to undesired ethanol production. Swinkels *et al*. first reviewed in 1993 the use of *K. lactis* as a host for heterologous protein expression [41]. Eight proteins had been successfully secreted from *K. lactis* at that time. Since *K. lactis* rapidly achieves high culture densities and produces high yields of recombinant proteins, currently, nearly 100 heterologous proteins have been produced successfully in *K. lactis* and large-scale cultures of up to 140 $m^3$ have been reported [39]. Proteins produced by *K. lactis* originate from bacteria, fungi, viruses, plants, and mammals, emphasizing the ability of *K. lactis* to efficiently produce a diverse range of heterologous proteins.
Some of the most important current applications of *K. lactis* include the industrial production of proteins on an industrial scale in the food industry. Due to its ability to grow on lactose, *K. lactis* has been the natural choice for the production of its ß–galactosidase since the 1950s. This enzyme is used to produce lactose-free dairy products allowing the growing number of lactose-intolerant individuals to consume milk-based products. It is also used to produce pre-biotic galacto-oligosaccharides (GOS) by exploiting its transglycosylation activity [44, 45]. GOS are thought to reduce the level of cholesterol and to prevent colon cancer [46, 47] and are also used as low-calorie sweeteners [48]. ß-galactosidase is now produced on an industrial-scale and marketed under several trade names from distinct companies. To improve its production, vector systems, genetically modified yeast strains and adjustment of growth parameters are intensively investigated [49-52].
As a host for heterologous protein production, *K. lactis* is best known for its use in commercial production of recombinant bovine chymosin [53]. Showing a higher specific activity than traditional rennet, this milk-clotting enzyme is widely employed in the production of cheese. Bovine chymosin was the first heterologous enzyme originating from a higher eukaryote that was produced at low cost in a microorganism. The process developed for its industrial-scale production was widely recognized as a major biotechnological achievement and is now one of the

most widely used processes in the food industry. Other commercially relevant proteins produced using *K. lactis* include inulinase [54], phospholipase B [55], chitinase [56], xylanase [57], brazzein [58], glucoamylase [59] and α-L-rhamnosidase [60].

### 1.2.2. Pharmaceutical industry

Given the success as a host for protein expression in the food industry, *K. lactis* was later introduced for large-scale production of therapeutic heterologous proteins in the pharmaceutical industry. Numerous proteins of pharmaceutical relevance have been produced in *K. lactis* to high titer. That was the case of human interleukin 1-β, used to treat autoimmune disorders [61, 62], interferon-α, used in cancer therapy [63, 64] and for the treatment of hepatitis [65], macrophage colony-stimulating factor (M-CSF), used to combat disorders of the hematopoietic system [66], and insulin precursors, used for diabetes therapy [67]. Furthermore, *K. lactis* is also proficient in the production of β-lactoglobulin [68], human serum albumin [69-71] and single-chain Fv antibodies [72, 73]. One drawback to the use of *K. lactis* (and other yeast and fungal expression systems) for the production of therapeutics is that secreted proteins that become glycosylated will bear a non-human glycosylation pattern due to its tendency to hyperglycosylate [74]. However, advances in the area of humanizing yeast protein glycosylation pathways have been applied in *K. lactis* demonstrating it can be modified to produce enzymes that mimic human glycosylation, as previously achieved in *P. pastoris* and *S. cerevisiae* [75, 76].

### 1.2.3. Metabolites production using *K. lactis*

*K. lactis*, and other few non-conventional yeasts, have served as long-standing industrial protein production platforms. However, their utility in the small molecule chemical space is sometimes limited to native metabolite overproduction phenotypes. Nevertheless, several metabolites are produced commercially in *K. lactis*. These include lactic acid and glycolic acid [77], the sugar acid D-xylonic

acid (D-gluconic acid), which is derived from D-xylose [78], and D-arabitol, which is produced directly from whey [79].

## 1.3. Strain improvement and *K. lactis* molecular tools

The development of suitable production strains requires the selection and engineering of a strain with the most appropriate properties. Over the past few decades, several factors have contributed to the success of *S. cerevisiae*, namely the existence of a well-established classical genetics, the availability of fast and efficient transformation procedures, a large number of integrative or autonomously replicating vectors, and the exploitation of its efficient homologous recombination machinery to obtain isogenic strains with specific gene deletions and other modifications. Due to their close phylogenetic relationship and taking advantage of the advances in the *S. cerevisiae* genetic tools, similar procedures were rapidly adapted and made available to *K. lactis*. As for all other model organisms, the completion of the genome sequencing project greatly enhanced the application of these techniques [80].

### 1.3.1. Host strain

Several *K. lactis* strains are readily available from various sources such as DSMZ (www.dsmz.de), CBS (www.westerdijkinstitute.nl) and ATCC (www.atcc.org) and natural isolates can be obtained from a variety of dairy products [41]. For protein and metabolite production, the choice of the host strain, and thus the genetic background, is of paramount importance to achieve maximum yield. For example, having examined over 50 isolates, it was demonstrated that the ability of *K. lactis* to secrete recombinant human serum albumin is greatly variable in a strain-dependent manner [41, 69, 81]. Strain GG799, an haploid food industry isolate, has a track record of excellent protein synthesizing and secretory capabilities and is included in a commercially available expression kit (New England Biolabs, Massachusetts, USA). In addition, this strain presents little glucose repression of the β-galactosidase (*KlLAC4*) promoter and therefore,

*KlLAC4*-based expression is high in standard yeast growth media with glucose as the carbon source.

### 1.3.2. Genetic tools and engineering

Strain genomes can be altered by random mutagenesis followed by screening for a desired trait. However, oriented genetic modification of cells is often essential for studies and further improvement of production strains in order to achieve an efficient system for the overproduction of heterologous proteins. Gene deletion or substitution by a mutated gene, modulation of gene expression in a particular metabolic pathway, or controlled expression of heterologous proteins for gain of new functions, are also key to the improvement of strains used for metabolites production. An attractive feature of *K. lactis* as a host system is that such genetic modifications can be performed using a number of standard approaches and mutagenesis techniques.

#### 1.3.2.1. Selectable markers

Common auxotrophic backgrounds (e.g. ura3, leu2, trp1) that are often found in *S. cerevisiae* strains have been widely used in *K. lactis* strains. Such markers permit a wide range of genetic and molecular techniques for manipulating yeast but it has been observed, for example in *S. cerevisiae* [82], that they can also have a detrimental effect on industrial production yields. Such effect has also been observed in *K. lactis* where introduction of a uracil auxotrophy (ura3) into a strain resulted in a reduced yield of a heterologous protein over 10-fold even when grown in the presence of exogenous uracil [38]. It is likely that cells that are already burdened by abundant production of a heterologous protein are further stressed by having to scavenge the auxotrophic nucleotide from the growth medium. It is also possible that a change in a regulatory interplay of connected pathways or mechanisms could result in the effect observed. Thus, while auxotrophic markers can be helpful for initial strain development and tests, prototrophic backgrounds are often preferred for large industrial production

fermentations to achieve maximum cell growth and ultimately maximum protein yield. The same applies to dominant markers conferring resistance to antibiotics, which can be extremely useful for small-scale research applications but are normally not practical in the industrial production setting due to high costs or undesirable regulatory effects of cells. Dominant bacterial selectable marker genes that confer resistance to the antibiotics geneticin (G418) [83] or hygromycin B have also been widely used in *K. lactis*. An interesting advance in the area of selectable markers is an antibiotic-free method that enables selection by acetamide utilization [84]. The commercial kit for heterologous protein expression mentioned previously is successfully making use of this strategy.

#### 1.3.2.2. Episomal and integrative vectors

Episomal and integrative vectors have been used to direct expression of heterologous proteins in *K. lactis* and both have their inherent advantages and disadvantages. Currently, there are several plasmid vector systems available to transform *K. lactis* cells and the most widely used are derived from the pKD1-plasmid of *K. lactis* var. *drosophilarum* [15, 85]. Thus, depending on the type of genetic manipulation, one can choose from a variety of vectors and two examples of these are a useful set of cloning vectors with different selectable markers, including triple *K. lactis* / *S. cerevisiae* / *E. coli* shuttle vectors [86] and template vectors for PCR-based gene deletion markers [87]. The pGKL1 and pGKL2 linear cytoplasmic killer plasmids replicate in *K. lactis* but the manipulation of this system is difficult due to their poor stability and because these plasmids cannot recognize nuclear promoters, which makes them only suitable for basic research. Episomal vectors derived from pKD1-plasmid can provide high copy numbers of expression constructs but can also pose problems, especially in large industrial applications, due to their instability when subjected to prolonged growth in the absence of selection [38].

While several advances were made to enable broader use of plasmids in *K. lactis*, genomic integration, despite the lower copy numbers, is still preferred for industrial strain engineering due to their higher stability. In many cases, it is desirable to target the transforming DNA, such as integrative expression vectors or gene

deletion cassettes, to integrate at a specific chromosomal locus. A drawback of gene targeting by homologous recombination in *K. lactis* is its low efficiency, in contrast to the high fidelity of gene replacement in *S. cerevisiae*. The rate of off-target integration (ectopic integration) varies from organism to organism. For example, targeted integration into the *S. cerevisiae* genome is virtually 100% efficient, with as little as 40 base pairs of flanking homologous DNA in the integration cassettes. However, *K. lactis* has a tendency for non-homologous recombination, and targeted integration at many loci has often been difficult, with as little as 1–2% efficiency in some cases [56, 88-90] and at least 500 base pairs of flanking homology may be required to isolate a transformant harboring the desired gene replacement. To overcome this problem, a recent approach has been taken involving the deletion of the *KlKU80* recombination gene, which resulted in a drastic improvement in targeted integration using DNA constructs having only 50–100 base pairs of homologous flanking sequences [91]. Regarding production of heterologous proteins, a common expression method utilizes the strong *KlLAC4* promoter to drive gene expression and to direct insertion of the vector into the genome at the *KlLAC4* locus [41, 53]. A variant of this method was applied to the most frequently used integrative expression vector, pKLAC2 (allows for acetamide selection in place of G418), which is provided with the *K. lactis* expression commercial kit.

### 1.4. *K. lactis* pyrimidines pathway

Studies on pyrimidines synthesis in *K. lactis* are scarce but it is well characterized in *S. cerevisiae* (Figure 1.2). In the first two steps of pyrimidine *de novo* biosynthesis, Ura2p (carbamoylphosphate synthetase / aspartate transcarbamylase) catalyses the formation of carbamoyl-aspartic acid from bicarbonate, ATP, glutamine and aspartic acid. Carbamoyl-aspartic acid is then used by Ura4p (dihydroorotase) to produce dihydroorotic acid, which subsequently undergoes an oxidation catalyzed by Ura1p (dihydroorotate dehydrogenase, DHODase) to form orotic acid. A 5-phosphoribosyl group is then added to orotic acid by two isoenzymes, Ura5p and Ura10p (orotate phosphoribosyltransferase) leading to the formation of orotidine-5'-monophosphate (OMP). Ura3p then

mediates the decarboxylation of OMP to uridine-5'-monophosphate (UMP), which is subsequently used to produce all other pyrimidines. Besides *de novo* biosynthetic pathway, yeast cells use a pyrimidines salvage pathway to recycle pyrimidines nucleosides and bases internalized from the culture medium or generated intracellularly during nucleotide turnover [92] (Figure 1.2).

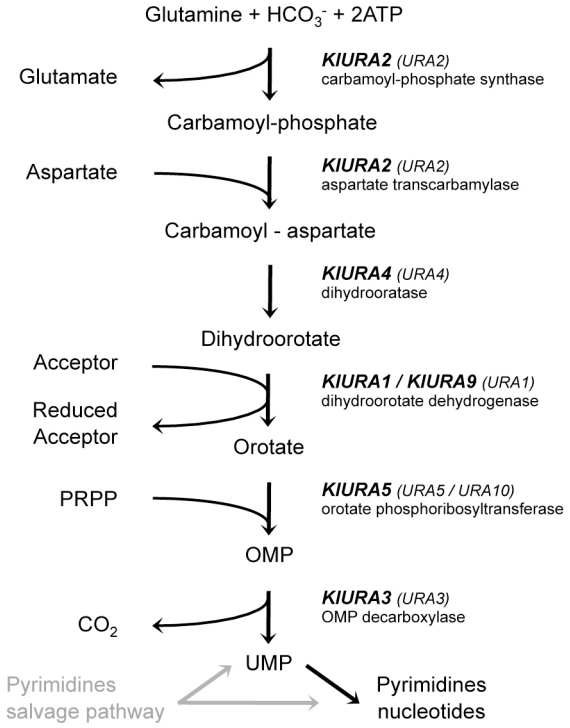


**Figure 1.2** Pyrimidine biosynthetic pathway. Six enzymatic steps lead to the formation of UMP (uridine-5'-phosphate) in *de novo* pyrimidines biosynthesis from which all pyrimidines nucleotides are further synthesized (*black*). Pyrimidines salvage pathway rely on a set of reactions in order to reutilize pyrimidines nucleosides and bases, either intracellular or internalized from the culture media, which can enter the pathway directly at UMP or at later steps (*grey*). OMP, orotidine-5'-phosphate. *S. cerevisiae* and *K. lactis* genes are represented in *brackets* and *bold*, respectively.

The enzymatic steps of this pathway are regulated by feedback inhibition and repression and/or attenuation of its genes. The *S. cerevisiae URA2* locus encodes a large 240-kDa multifunctional protein that possesses the carbamylphosphate

synthetase (CPSase) and aspartate transcarbamylase (ATCase) activities. Both enzymatic subunits are feedback inhibited by UTP [93]. In addition, UTP exerts a repression on the *URA2* transcription but negative feedback control must be considered to be the most effective regulatory process [94]. When uracil, a common and naturally occurring pyrimidines precursor, is present in the growth media, it is imported into the cells by a specific permease [95] and transformed into UMP by the enzymes of the salvage pathway [96]. This will lead to an increase of the UTP pool and consequently to the inhibition of *de novo* synthesis. The utilization of external uracil and related pyrimidines are therefore favored over the *de novo* biosynthesis [94]. The *de novo* pathway is also regulated by the weak transcriptional activator Ppr1p [97] that senses the levels of dihydroorotic acid and orotic acid [98] and upon activation under pyrimidines starvation conditions promotes the transcription of several *URA* genes [99].

Analysis of both *S. cerevisiae* and *K. lactis* genomes led to the identification of the *K. lactis* genes involved in the *de novo* pyrimidines biosynthesis [80] (Figure 1.2) and differences were recognized in the fourth and fifth steps. While *K. lactis* has one gene (*KlURA5*) coding for a orotate phosphoribosyltransferase, *S. cerevisiae* presents two isoenzymes, Ura5p and Ura10p, responsible for the formation of OMP, which can be explained by the genomic duplication undergone by *S. cerevisiae* [100]. The major difference concerns the step leading to the formation of orotic acid, which is the single redox reaction in the *de novo* pyrimidines biosynthesis pathway and is catalyzed by DHODases. *S. cerevisiae* has only one gene for a DHODase (*URA1*) but the presence in *K. lactis* of two genes (*KlURA1* and *KlURA9*) coding for DHODases that belong to two distinct families is rather unexpected [101]. Similar to its *S. cerevisiae* homologue, KlUra1p seems to belong to the DHODase family 1A, which includes cytosolic enzymes that use fumarate as electron acceptor. On the other hand, KlUra9p seems to belong to the DHODase family 2, which includes enzymes that are localized in the inner mitochondrial membrane and deliver electrons to quinone [101, 102]. Although no functional or localization studies have been made in *K. lactis*, this suggests that its *de novo* pyrimidine biosynthesis pathway may be coupled to the mitochondrial respiratory chain via the KlUra9p DHODase.

## 1.5. Orotic acid production and applications

### 1.5.1. Orotic acid production in microorganisms

Mutants blocked in one of the last two steps of UMP biosynthesis (Figure 1.2) are prone to accumulate the intermediate orotic acid (Figure 1.3) and cannot grow unless pyrimidines or its precursors that could be salvaged, such as uracil, are available [103]. Several of these pyrimidine-requiring mutant microorganisms have been described to accumulate and secrete orotic acid. This phenomena has been first described in the late 40s with a pyrimidine-requiring mutant of the filamentous fungus *Neurospora crassa* [104]. Since then, the secretion and accumulation of orotic acid in the growth media was reported for other microorganisms bearing one of the pyrimidines-requiring mutation. That was the case for the bacteria *Enterobacter aerogenes* (formerly *Aerobacter aerogenes*) [105, 106], *E. coli* [103, 107, 108], *Corynebacterium glutamicum* (formerly *Micrococcus glutamicus*) [109, 110] and *Corynebacterium ammoniagenes* (formerly *Brevibacterium ammoniagenes*) [111, 112]. A pyrimidine-requiring mutant of *Candida tropicalis* [113] and, more recently, of *Candida albicans* [114] were the only yeasts reported to accumulate orotic acid in culture media. Since similar pyrimidines-requiring mutants of other microorganisms do not accumulate orotic acid, other factors must contribute to this phenotype. For example, *C. albicans Caura3*Δ mutants are able

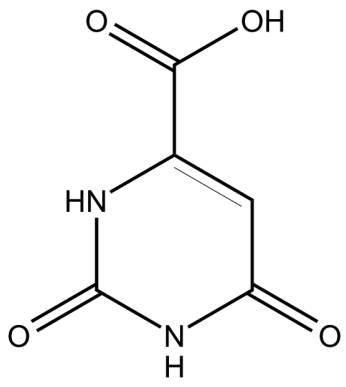


**Figure 1.3** Orotic acid molecule. Salts of orotic acid, orotates, can be formed by the deprotonation of the carboxyl group.

to produce orotic acid in yeast extract - peptone - glucose (YPD) media only if it is supplemented with acetate and *S. cerevisiae ura3*Δ mutants do not present evidences for orotic acid production in either conditions.

The reports of orotic acid producing microorganisms encouraged studies for the production of this molecule mainly driven by the interest in industrial applications. Success has been achieved for some species of the genus *Corynebacterium*, in particular for *C. glutamicum* [110]. For the other microbial cells mentioned, concentrations of orotic acid in the media were found to range from 1 – 3 g/L and no commercial production was accomplished. A process for the chemical production of orotic acid from trichloroacetyl chloride, ketene and urea was patented [115] but is much less efficient regarding its costs [116] when compared to the biological system described in the following section

### 1.5.2. Orotic acid production using *C. glutamicum*

*C. glutamicum* is an aerobic, gram-positive, nonsporulating, nonpathogenic and mycolic acid-containing bacterium with enormous importance in the biotechnology industry. Since 1957, glutamate has been industrially produced by fermentation using *C. glutamicum* and several other amino acids are now produced with this system. The excellent production capabilities of *C. glutamicum* were further exploited for the production of orotic acid. A first patent was issued in 1963 describing a method for the production of orotic acid by a fermentation process using a pyrimidine-requiring *C. glutamicum* mutant (ATCC 14275) [109]. A second patent was granted several years later in 1991 [110]. In this patent, the original mutant ATCC 14275 was further optimized by screening randomly mutagenized cells for resistance to a pyrimidine analogue or to both a pyrimidines analogue and a sulfanamide drug. In result, the new mutant strains selected significantly increased the efficiency of orotic acid production. The composition of production media mentioned in the patent and the claimed concentrations of orotic acid obtained in flask and bioreactor cultures, are presented in Table 1.1.

**Table 1.1** Composition of the production media (**A**) and orotic acid production in flask and bioreactor cultures (**B**) using pyrimidines-requiring mutants of *C. glutamicum*. Strains T-26, T-29 and T-30 were obtained by screening randomly mutagenized cells of ATCC 14275 for resistance to a pyrimidine analogue or to both a pyrimidines analogue and a sulfa drug. Examples of the pyrimidine analogue are 5-fluorouracil, 6-azauracil, 2-thiouracil, 5-hydroxyuracil, and trimethoprim. Examples of the sulfanamide drug are sulfaguanidine, sulfathiazole, sulfapyridine and sulfamerazine. Strains resistance is the following: T-26 - 5-fluorouracil resistant; T-29 - 5-fluorouracil and trimethoprim resistant; T-30 - 5-fluorouracil and sulfaguanidine resistant. Data presented in this table originates from the patent issued in 1991 [110].

**A**

| **Production media** | |
|---|---|
| Blackstrap molasses * | 20% |
| Ammonium sulfate | 0.5% |
| Urea | 1.0% |
| Potassium dihydrogenphosphate | 0.05% |
| Dipotassium hydrogenphosphate | 0.05% |
| Magnesium sulfate | 0.05% |
| Calcium carbonate | 2.0 % |
| Uracil | 50mg/L |

* 500ml 50% blackstrap molasses added to bioreactor cultures 24h after start

**B**

| **Strain** | **Orotic Acid (g/L)** | |
|---|---|---|
| | Batch (120 hr) | Bioreactor * (84 hr) |
| ATCC 14275 | 8.2 | 28.5 |
| T-26 | 13.0 | 50.3 |
| T-29 | 14.0 | 53.2 |
| T-30 | 14.5 | 56.0 |

Blackstrap molasses is the dark, viscous molasses that remain at the end of the extraction process of sugar from raw sugarcane and it has been long used as a carbon source material in the biotechnology industry. The amount of sugar of the molasses used in the patent was not disclosed but, taking into account the average and common concentration found in most blackstrap molasses, its concentration should be about 50% sugar. Therefore, in a production media containing, in general, 50 mg/L of uracil to support growth, 10% sugars and 1% urea plus 0.5% ammonium sulfate as nitrogen source, the original pyrimidine-requiring mutant was able to produce 8.2 g/L of orotic acid after 120 h in flask cultures. It is important to mention the use of 2% calcium carbonate that, besides acting as a pH stabilizer mainly in the flask cultures, may also serve as an important raw material for orotic acid production as it constitutes one of the initial substrate of the *de novo* biosynthetic pathway (Figure 1.2). The most efficient of the improved mutant strains (T-30) was able to generate a concentration of

14.5 g/L of orotic acid under the same conditions in flask cultures. In a 5 L bioreactor with 2 L of production media, the original mutant produced 28.5 g/L of orotic acid only after 84 h whereas the concentration reached with the T-30 mutant strain was 56.0 g/L. Beside the presumably fully optimized and controlled system, the production in the bioreactor was considerably higher when compared to the production in flasks also due to the addition of 500 ml of 50% blackstrap molasses into the production media after 24 h of growth.

Undoubtedly, the efficiency of *C. glutamicum* mutants in the production of orotic acid is extremely relevant and for that reason those strains are the mostly used for its production. However, to our knowledge, the 1991 patent was the last on this matter, raising the question why no further significant improvements were achieved with this bacteria and opening the way to other microorganisms that may pose as a valid and more cost-effective alternative.

### 1.5.3. Market application of orotic acid

Mineral salts of orotic acid, orotates, have been used in industry for several applications. There is a lifetime study on orotate by the German physician Hans Nieper [117, 118] who used orotates clinically to treat thousands of patients for nearly every ailment imaginable. Along with its important role as precursors of pyrimidines nucleotides, Nieper claimed that orotate salts, being neutrally charged, could easily cross cell membranes, effectively delivering the minerals. Unfortunately, Nieper tried to explain his clinical results by invoking nonsensical physical theories. Consequently, his contributions to medicine were ignored by the medical world, and most of his medical claims were never rigorously tested. In recent years, a few of his orotate applications have received scientific scrutiny and have been found to work. Regarding its properties, the coordination chemistry of orotate has been studied in detail. Research in this area ranges from bioinorganic to pharmaceutical and materials chemistry [119, 120].

In the pharmaceutical industry, orotate is included in a group of compounds considered to be “pharmaceutically acceptable carriers”. The recognition of its biological carrier function led to the use of orotate salts to cure syndromes associated with deficiency of metal ions, such as calcium, magnesium, zinc, or

iron [121-124]. In addition, platinum, palladium and nickel orotate complexes have been screened as anticancer agents [125, 126]. As an active ingredient, orotic acid has been associated to the treatment of heart diseases due to its cardioprotective effects since it was shown to improve the energy status of injured myocardium by stimulating, among others, the synthesis of glycogen and ATP [127-130].

The fast growing industry of nutraceutics also takes advantage of mineral salts of orotate and many products are easily available in the market for a wide variety of applications. Lithium orotate, for instance, is a highly bioavailable form of lithium that is available without a prescription to treat bipolar disorder. Initially based on Nieper studies, such products claim that lithium orotate has a higher bioavailability (it is 20 times more bio-active than other lithium salts, such as lithium carbonate or lithium citrate). Thus, lower doses of lithium orotate may be used to achieve therapeutic brain lithium concentrations and relatively stable serum concentrations while greatly reducing the likelihood and severity of potential side effects. Moreover, potassium and magnesium orotates are used by athletes to increase endurance and can be found in some mineral supplemented beverages [131].

Orotic acid is also known as vitamin $B_{13}$. Although orotic acid is not officially considered a vitamin these days, over 40 years ago it was found to have growth-promoting, vitamin-like properties when added to the diets of laboratory animals. Therefore, it has been commercially used in the feed industry for that purpose in the composition of feed additives for ruminants and poultry [132].

Applications in the chemical and biotechnological industry are of major importance since it has been used as the starting material to produce pyrimidines, pyrimidines analogues and several other related molecules with higher market value [133-135]. The most significant use of orotic acid for such purposes comes from the Japanese pharmaceutical and biotechnology company Kyowa Hakko Kirin Co., Ltd. This company is among the 40 largest in the world by revenue and was established after the merger of Kyowa Hakko Kogyo Co., Ltd. with Kirin Pharma Co., Ltd. in 2008. The patents regarding the orotic acid production by *C. glutamicum* were assigned precisely to Kyowa Hakko Kogyo Co., Ltd., which has been since then its leading manufacturer. They have further developed a strong and ingenious biotechnology to produce other valuable molecules from orotic acid now available from the Kyowa Hakko Bio Co., Ltd (succeeded

Bio-Chemical business from Kyowa Hakko Kogyo Co., Ltd). Among others is the artificial biosynthesis of sugar nucleotides (ABS system) and the artificial biosynthesis of carbohydrates (ABC system) (Figure 1.4), which are combined systems that have as the first step a long used biotechnology to produce nucleotides (UTP, GTP, CTP, etc) from orotic acid or guanosine monophosphate (GMP) [134]. This ABS system offers a simple and effective production process of sugar nucleotides and basically makes use of the combination and reaction coupling between *C. ammoniagenes*, which has a strong activity to produce nucleotides, and *E. coli*, which is metabolically engineered and expresses the genes of sugar nucleotide biosynthesis. For the ABC system a third step is combined using an engineered *E. coli* that possesses a specific glycosyltransferase gene. With this system, various complex oligosaccharide are synthesized directly from a specific sugar (basically corresponding to a sugar nucleotide) and acceptor sugar. Therefore, originally from orotic acid as a major substrate, the pyrimidines nucleotides, sugar nucleotides and complex oligosaccharides can be obtained at any step depending on the combined systems used. Having a significant higher market value, these compounds are mostly used in the pharmaceutical and other biotechnology industries.

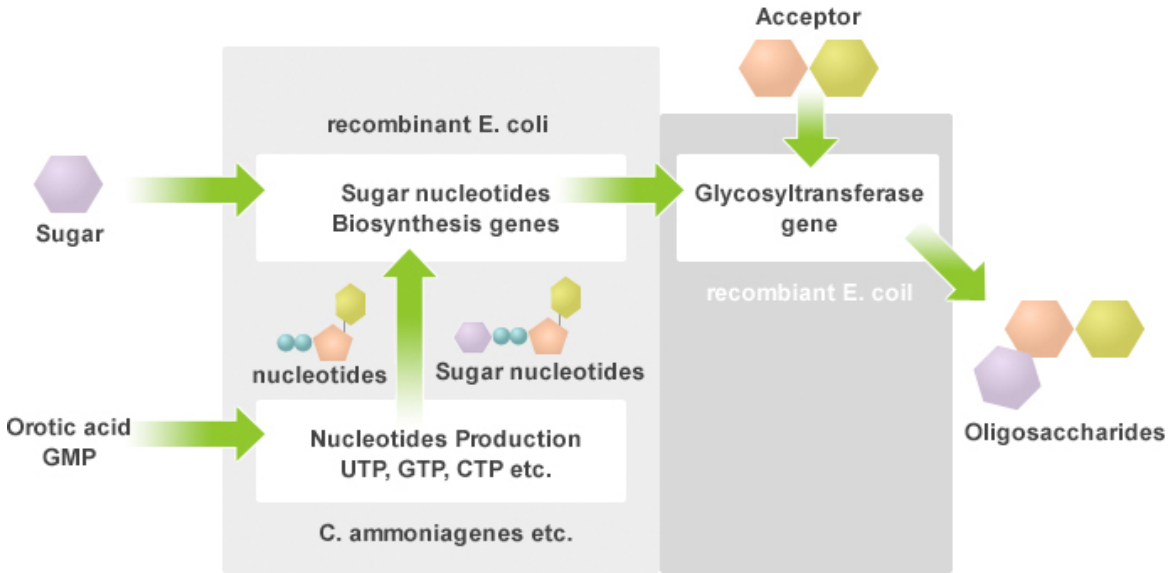


**Figure 1.4** Combined biological systems used by Kyowa Hakko Bio Co. Ltd. for the production of nucleotides, sugar nucleotides and complex oligosaccharides from orotic acid or guanosine monophosphate (GMP) and sugars. Image modified from original in www.kyowahakko-bio.co.jp.

## 1.6. Oxidative stress response in yeast

Unlike *S. cerevisiae, K. lactis* possesses two distinct DHODases that catalyze the fourth step of *de novo* pyrimidines biosynthetic pathway. According to the homology with DHODases from other species, KlUra9p seems to be linked to respiration by delivering electrons to oxygen through the mitochondrial electron transport chain [101]. This raises the hypothesis of a possible connection between *de novo* pyrimidines biosynthetic pathway and oxidative stress regulation in *K. lactis*.

The oxidative stress response in *K. lactis* is a mostly unexplored field. However, *K. lactis* has been proposed as a suitable model for the study of cellular responses to hypoxia [30] and these conditions are known to induce genes related to the oxidative stress response in *S. cerevisiae* [136, 137]. Several studies based on comparative genomics combining *in silico* and experimental approaches have been performed in *K. lactis* [138-140]. Regarding oxidative stress response, the search in the *K. lactis* genome of putative *S. cerevisiae* orthologs related to the oxidative stress response has suggested that pathways and proteins are generally conserved. The players and mechanisms involved in the oxidative response in *S. cerevisiae* have been extensively reviewed [141-143] and are briefly summarized in the next sections.

### 1.6.1. Reactive oxygen species

Oxygen is essential to all aerobic living organisms as it is used as the final electron acceptor in the mitochondrial respiratory chain. This is determinant for the efficient production of energy but, on the other hand, the reactive oxygen species (ROS) produced can oxidize biomolecules resulting in cellular damage. To cope with the toxicity of ROS, all organisms possess enzymatic and nonenzymatic antioxidant defenses [144-146]. Under normal physiological conditions, the primary antioxidant defenses, which neutralize ROS, and secondary defenses, which repair or degrade oxidized biomolecules, are able to regulate and maintain redox homeostasis and ultimately, prevent oxidative damage of the cells. An oxidative stress occurs when the levels of ROS exceed the antioxidant capacity of the cells.

Mitochondrial respiration is the major source of ROS but cells can also generate these oxidizing compounds from other sources [147-149]. The superoxide anion ($\bullet O_2-$) is one of the main ROS and is derived from leakage of electrons from the respiratory chain. Hydrogen peroxide ($H_2O_2$) is generated from several metabolic steps, including dismutation of $\bullet O_2^-$ by superoxide dismutases, oxidation of fatty acids, and from protein-folding reactions involving disulphide bond formation. A variety of organic hydroperoxides result from the oxidation of unsaturated lipids and amino acyl side chains. Of the many free radicals generated in cells, the most reactive is the hydroxyl radical ($\bullet OH$) formed by reduction of $H_2O_2$ catalyzed by a transition metal ion such as $Fe^{2+}$ (Fenton reaction). The simultaneous presence of $H_2O_2$ and a reductant (such as $\bullet O_2^-$) capable of reducing and recycling the $Fe^{3+}$ produced in the Fenton reaction is very detrimental because it exacerbates the formation of $\bullet OH$. During the oxidation of unsaturated lipids, a variety of reactive lipid radicals are also formed [150].

### 1.6.2. Oxidative damage in biomolecules

ROS are toxic agents that can damage a wide variety of cellular components resulting in protein oxidation, lipid peroxidation, and DNA damage. Protein oxidation is directly promoted by ROS but can also be modified by reaction with products of the lipid peroxidation [151]. The thiol groups of certain amino acids are especially sensitive to oxidation, resulting in increasingly oxidized forms (e.g., the sulphydryl group of cysteine can be oxidized to sulphenic, sulphinic and sulphonic acid derivatives). While some oxidized forms of cysteine are reversible modifications, the formation of sulphonic derivatives is an irreversible process that cannot be repaired. ROS can also lead to protein carbonylation, which is an irreversible modification characterized by the formation of primary or secondary protein carbonyls on the side chains of certain amino acids. Protein carbonylation is commonly used to evaluate protein oxidation [152]. Some protein co-factors can also be oxidized. For example, oxidation of the iron-sulfur centers of several proteins result in the inactivation of enzyme and the release of iron, which by promoting the formation of other ROS leads to further cellular damages [153, 154]. Depending on the type of ROS that cells are exposed to, oxidation of proteins can

result in processes of fragmentation, conformational changes, aggregation, or inactivation of amino acids in the active center [152]. When proteins cannot be repaired due to irreversible modifications, they must be marked for proteolytic pathways [155].

The damage to lipids consist in the oxidation of polyunsaturated fatty acid by an autocatalytic process triggered by the •OH [156]. Some products of lipid peroxidation are also highly reactive, increasing the levels of free radicals that can further damage other biomolecules [150]. In yeasts, lipid peroxidation is limited since they cannot synthesize polyunsaturated fatty acids, which are only integrated in the membrane when present in the growth medium.

Amongst other effects, oxidative DNA damage can alter purine and pyrimidine bases as well as cleave the phosphodiester DNA backbone [157]. Base modification is an important class of lesions due to its lethal or mutagenic effect and, once oxidized, bases have to be replaced by an enzymatic system. In *S. cerevisiae*, three DNA glycosylases are involved in the excision repair of oxidized bases [158, 159].

### 1.6.3. Oxidant defense systems

Non-enzymatic defenses typically consist of small molecules that can act as free radical scavengers, of which glutathione (GSH) is the best-known example. GSH is a tripeptide (L-γ-glutamyl-cysteinyl-glycine) that acts as a radical scavenger with the redox-active thiol group reacting with oxidants to produce glutathione disulfide (GSSG). However, glutathione is predominantly present in its reduced GSH form in *S. cerevisiae* and other eukaryotes due to the constitutive action of glutathione reductase. Glutathione reductase is an NADPH-dependent oxidoreductase that converts GSSG to GSH using reducing power generated by the pentose phosphate pathway [160]. Glutathione is possibly the most abundant redox scavenging molecule in cells and, consequently, the glutathione system plays an important role in maintaining cellular redox homeostasis. The ratio GSSG:GSH, therefore provides a good indication of the redox environment of cells and most cell compartments [161].

Cellular antioxidant defenses also include a number of protective enzymes that are present in different subcellular compartments and can be upregulated in response to oxidative stress. Several peroxidases, including catalases, glutathione peroxidases and peroxiredoxins, keep endogenous levels of $H_2O_2$ in the submicromolar range. Catalases are ubiquitous heme-containing enzymes that catalyze the decomposition of $H_2O_2$ to water and oxygen. *S. cerevisiae* has two catalases: the peroxisosomal catalase A, encoded by *CTA1*, and the cytosolic catalase T, encoded by *CTT1* [162, 163]. *CTA1* expression is coordinated with peroxisomal fatty acid metabolism, suggesting the main physiological role of Cta1p is the detoxification of $H_2O_2$ generated from fatty acid β-oxidation [164]. Ctt1p is thought to play a more general role as an antioxidant during exposure to oxidative stress, since *CTT1* expression is induced by various stress conditions including heat, osmotic, starvation, and hydrogen peroxide stress [165]. The elimination of the superoxide anion is carried out by superoxide dismutase (SOD). This enzyme catalyzes the dismutation of superoxide anion to $H_2O_2$, which can then be reduced to water by catalases or peroxidases. *S. cerevisiae* contains a cytoplasmic Cu,Zn-SOD (Sod1p) and a mitochondrial matrix Mn-SOD (Sod2p), which appear to play distinct roles during oxidative stress conditions [166].

### 1.6.4. Regulation of the response to oxidative stress

Under normal physiological conditions the cellular antioxidant defenses have a limited capacity to face a sudden oxidative stress. Several conditions, such as an increase of mitochondrial respiratory chain or exposure to environmental oxidants, can induce an adaptive response that is essential for cell viability [167-169]. *S. cerevisiae* cells respond to ROS by altering the expression of genes encoding antioxidant defenses and enzymes that repair and detoxify the resulting cellular damage [142]. The main transcription factors involved in the regulation of oxidative stress responses are Yap1p and Skn7p [170, 171] although other transcription factors are also implicated, such as Msn2p and Msn4p [165, 172]. In *S. cerevisiae*, adaptive concentrations of $H_2O_2$ increase the translation of several antioxidants and stress protective molecules. These include catalase (Ctt1p) and glutathione peroxidase (Gpx2p) that can reduce $H_2O_2$ directly [173]. There is also increasing

evidence that ROS play important regulatory functions in cell signaling and thiol groups are key mediators of oxidative signal transduction [174]. Signal transduction pathways generally involve specific protein–protein interactions and increasing evidence indicates that oxidizing agents can control these interactions by altering the redox state of available thiols in protein and low molecular weight compounds to bring about controlled signaling events [175-177].

Along with the alteration of global gene expression patterns, post-translational changes are also important regulators of oxidative stress response. In fact, metabolic changes are detected within seconds of an oxidative stress, before slower (within minutes) changes in gene expression are measured [178, 179]. Key to these metabolic changes appears to be the reprogramming of carbohydrate metabolism, which is essential to maintain the redox balance of the cell during oxidative conditions. For example, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has frequently been identified as a target of oxidative modification in diverse cellular systems [180]. In *S. cerevisiae*, a reduced activity of GAPDH redirects glucose equivalents to the pentose phosphate pathway, which is the source of cellular reducing power in the form of NADPH. NADPH is particularly important during exposure to oxidants, since it provides the reducing potential for most antioxidant and redox regulatory enzymes including the glutathione system. It has therefore been suggested that GAPDH may serve a regulatory role as a sensor of oxidative stress conditions [180] by shifting the $NADPH/NADP^+$ ratio to a more reduced state [181].

## 1.7. Scope of the thesis

### 1.7.1. Pyrimidines mutants of *K. lactis* as orotic acid producing microorganisms

Preliminary studies have shown that a pyrimidine-requiring mutant of *K. lactis* lacking the *KlURA3* gene, which encodes the enzyme orotidine-5'-phosphate decarboxylase involved in the synthesis de novo of pyrimidines, was able to accumulate a significant amount of orotic acid crystals in YPD agar media. The initial goal of this work was to assess and validate the potential of the *K. lactis* mutant *Klura3*Δ, and the similar *Klura5*Δ mutant, to produce orotic acid using YPD based media in both flask and bioreactor cultures. Supported by the concentration of orotic acid reached under best growth conditions we further aimed to optimize the production media using low cost industrial by-products, such as sugarcane molasse, cheese whey powder and corn steep liquor, as substrate.

### 1.7.2. Relation of the *de novo* pyrimidines biosynthetic pathway with oxidative stress response in *K. lactis*

DHODases catalyze the fourth step of *de novo* pyrimidines biosynthetic pathway. The presence in *K. lactis* of a DHODases that may be linked to mitochondrial respiration raised the hypothesis of a possible connection of *de novo* pyrimidines biosynthetic pathway with oxidative stress regulation in *K. lactis*. We also hypothesized that pyrimidines mutants of *K. lactis* able to produce high amounts of orotic acid may exhibit an elevated activity of KlUra9p that would in turn promote an oxidative unbalance. Thus, this work also aimed to characterize changes in the redox status and antioxidant defenses in the orotic acid producing mutants of *K. lactis*, as well as the mechanisms underlying the putative interplay of both pathways. The end goal was to obtain relevant information that could be applied in the first part of the work and further improve the production system.

# CHAPTER II

## Materials and Methods

## 2.1. *K. lactis* strains

The mutants *Klura3*Δ, *Klura5*Δ, *Klura1*Δ, *Klura9*Δ, *Klura2*Δ, *Klcta1*Δ and *Klctt1*Δ were generated from *K. lactis* DSM 70799 (CBS 2359) wild type strain (DSMZ, Braunschweig, Germany). *Klura3*Δ mutant was used to generate *Klura1*Δ*Klura3*Δ, *Klura9*Δ*Klura3*Δ, *Klcta*1Δ*Klura3*Δ and *Klctt*1Δ*Klura3*Δ mutants. *Klura1*Δ and *Klura1*Δ*Klura3*Δ mutants were used to generate, respectively, *Klura1*Δ*Klura9*Δ and *Klura1*Δ*Klura9*Δ*Klura3*Δ. *Klcta1*Δ and *Klcta*1Δ*Klura3*Δ mutants were used to generate, respectively, *Klcta1*Δ*Klctt1*Δ and *Klcta1*Δ*Klctt1*Δ*Klura3*Δ. Full length of the open reading frame (ORF) of *KlURA3*, *KlURA5*, *KlURA1*, *KlURA9, KlURA2, KlCTA1 and KlCTT1* genes were removed to generate the various deletion mutants. All primers used in this study (A1/A2 and B1/B2 sets for each gene) are listed in Table 2.1. Each ORF with flanking regions were amplified by PCR from *K. lactis* DSM 70799 genomic DNA using A1/A2 set of primers and then cloned into pGEM-T Easy (Promega, Madison, USA). A fragment of this construct containing only the flanking regions of each ORF was amplified by PCR using the B1/B2 set of primers and a *Pvu*II/*Eco*RV fragment containing *loxP–kanMX4–loxP* from pUG6 (Euroscarf, Frankfurt, Germany) was ligated into the open vector. Each deletion cassette was then amplified with A1/A2 primers and used to transform the parental strain by electroporation. The mutants were selected in YPD [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose] supplemented with geneticin (200 mg/L) and correct deletion was confirmed by PCR using a set of primers 400 bp upstream A1/A2. Cre/loxP-mediated marker removal procedure [182] was used to excise *KanMX4*.

## 2.2. Media, inoculation and growth

For the orotic acid production experiments, YPD [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose] and YPD 5% [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 5 % (w/v) glucose] were initially used as growth media. Sugarcane molasse, cheese whey powder (CWP) and corn steep liquor (CSL), were further evaluated as alternative substrates for orotic acid production. Sugarcane molasse was kindly provided by RAR - Refinarias de Açúcar Reunidas,

**Table 2.1** Primers used in this study.

| Primer name | Sequence (5'- 3') |
|---|---|
| A1-Ura3 | CACTGTCTCTTCCCCTTAATGA |
| A2-Ura3 | TGTGTGCTTGCTTCTTTTCTTATC |
| B1-Ura3 | GTGCAACTAATTGACGGGAGT |
| B2-Ura3 | CAGGAAACTTAATAGAACAAATCACA |
| A1-Ura5 | GCCTTATCAGGATCAGATGAAG |
| A2-Ura5 | CAAAGACACATCCACAAGATTTTG |
| B1-Ura5 | TGAACAGGTGATTAATGGCGGA |
| B2-Ura5 | TATCACCCTCGAATCTATCTTAAC |
| A1-Ura1 | CCGAATAGTATCTGTGACTAAGA |
| A2-Ura1 | CTACTTCTTCAGTATTAGCCTTTC |
| B1-Ura1 | TGTTCAGTCTTACCTTGAATGTTTAT |
| B2-Ura1 | TTTTTCATATAGCGGTGTTTAGTATAT |
| A1-Ura9 | AATATTGATTCGGCTTCTCGTGA |
| A2-Ura9 | TCCAACAGAATCCCAAACCCA |
| B1-Ura9 | AGTTGATAAAAGCAAAATACGCGG |
| B2-Ura9 | GCTGTTACCTAATTGAAGTAAGG |
| A1-Ura2 | TCCTCTAAGTCCCACCTTTGA |
| A2-Ura2 | ACCACATTTCAAGGCAATTCTATC |
| B1-Ura2 | CGGAAGTTTGTATTTTCTTAAGCG |
| B2-Ura2 | AGGAGCTAAACAGATTCGCGTT |
| A1-Cta1 | CTTAAAACCTTAAGCGGTCGCT |
| A2-Cta1 | AGCCACCTCTTGATAGTTGCA |
| B1-Cta1 | GCTTATGTGTTGAATGCTATGTTC |
| B2-Cta1 | GAGAATTAGCTGTGCTTTCTTTCT |
| A1-Ctt1 | CATTTTAGACCTCTTCACCTTACA |
| A2-Ctt1 | AGATCAAATGAACCAGAATCAAAATC |
| B1-Ctt1 | TGTAGGCAGACTGTCTTGACTT |
| B2-Ctt1 | GACGATATTAGGAAATTGTTCAAAGT |

S.A. (Portugal), and contained 49% (w/v) carbohydrates and 0.06% (w/v) protein [183]. CWP was kindly provided by Lactogal Produtos Alimentares, S.A. (Portugal), and contained 73% (w/w) lactose and 12% (w/w) proteins [184]. CSL was kindly provided by COPAM - Companhia Portuguesa de Amidos, S.A. (Portugal) and contained 7.5% (w/v) carbohydrates and 0.5% (w/v) protein [183].

The set-up for inoculation of all cultures was as follows: a) 4 ºC stock culture – a YPD culture grown to 2.5 g/L d.w. was centrifuged at 3000 g for 5 min and ressuspended in 1/10 of the same culture medium, stored at 4 ºC and used for

preparing every pre-culture; this stock culture was substituted every month but could be used longer without any effect on the pre-culture growth behavior or orotic acid production; b) pre-culture – an appropriate volume from the 4ºC stock culture (20 μl per ml pre-culture) was used to inoculate YPD medium and cells were grown for 24 h at 26 ºC and 180 rpm to a biomass concentration of 4.0 g/L d.w.; c) culture – irrespective of the growth parameter tested, culture medium composition and flask or bioreactor experiments, the equivalent to 1/10 of culture medium volume from the pre-culture was used for inoculation.

Several growth conditions and media were tested in flask and bioreactor and are described in the results section and figure legends. Potassium phthalate and MOPS were obtained from Sigma-Aldrich (St. Louis, USA). Bioreactor experiments were performed in a Biostat B2 (Sartorius, Goettingen, Germany) equipped with 405-DPAS-SC-K8S/200 pH probe (Mettler-Toledo, Columbus, USA) and InPro 6800 $pO_2$ sensor (Mettler-Toledo, Columbus, USA). Solutions used for pH correction were 2 M NaOH and 2 M HCl. Air supply was controlled by an MC-10SLPM mass flow controller (Alicat Scientific, Tucson, USA). Aeration, agitation, temperature and pH were automatically controlled and maintained constant for every culture.

### 2.3. Crystals characterization

Diffraction data from X-ray crystallography were collected at 293 K with a Gemini PX Ultra equipped with $CuK_\alpha$ radiation (λ = 1.54184 Å). The structure was solved by direct methods using SHELXS-97 and refined with SHELXL-97 [185]. Carbon, oxygen, nitrogen and potassium atoms were refined anisotropically. Hydrogen atoms were refined freely with isotropic displacement parameters. The refinement converged to $R$ (all data) = 5.55% and $wR_2$ (all data) = 13.09%.

The scanning electron microscopy with X-ray microanalysis (SEM / EDS) exam was performed using a high resolution scanning electron microscope with X-Ray Microanalysis, JEOL JSM 6301F/ Oxford INCA Energy 350. Samples were coated with an Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment.

## 2.4. HPLC quantification of orotic acid and glucose

For orotic acid quantification, an Hitachi HPLC system was used and consisted of the following components: L-7100 pump, L-7250 autosampler, PCR7250 peltier cooling rack, L-7300 Plus column oven, L-7400 UV detector and D-7000 interface module. The column was a XBridge $C_{18}$ (3.5 µm; 4.6 mm x 150 mm) from Waters (Milford, USA) and was kept at 22 ºC. Mobile phase consisted of 25 mM $K_2HPO_4/KH_2PO_4$ at pH 7.0 and flow rate was 0.5 ml/min for a total run time of 40 min. Column effluent was monitored at 280 nm. Orotic acid standard was obtained from Sigma-Aldrich (St. Louis, USA).

Glucose concentrations were determined by HPLC in a Jasco chromatograph equipped with refractive index (RI) detector (Jasco 830-RI) and a Chrompack (300 mm x 6.5 mm) column at 60 ºC, using 5 mM $H_2SO_4$ as eluent at a flow rate of 0.5 ml/min.

## 2.5. Oxidative stress resistance

Cells were grown overnight in YPD medium, diluted in YPD and allowed to grow until early exponential phase ($OD_{600}$ = 0.6; corresponds to 0.14 g/ml of dry weight) or later phases of growth, at 26ºC in flasks agitated at 180 rpm with a culture volume / flask volume ratio of 1 : 5. Cells grown to $OD_{600}$ = 0.6 were exposed to 10 mM $H_2O_2$ for 30 min under same growth conditions. Samples were taken before and after addition of $H_2O_2$ and briefly sonicated (10 sec, output 1, 10% duty cycle) to eliminate cell clumps. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % (w/v) agar and expressed as the percentage of the colony-forming units of treated *versus* untreated cells. For viability tests at later growth stages, cells were treated with increasing concentrations of $H_2O_2$ for 30 min and the same procedure was used to determine the concentration of $H_2O_2$ required to produce 50% cell death ($LC_{50}$).

## 2.6. Oxidative stress markers

Protein oxidation was determined by immunodetection of protein carbonyls, as previous described [186]. Control and $H_2O_2$-treated cells were centrifuged at 5,000 rpm, and protein extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Roche Applied Science), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Protein content was estimated by the Lowry method, using bovine serum albumin as a standard. Protein carbonylation assays were performed by slot-blot analysis using rabbit IgG anti-dinitrophenyl (DNP) (Sigma, St. Louis, USA) at a 1 : 5,000 dilution as the primary antibody and goat anti-rabbit IgG-peroxidase (Sigma, St. Louis, USA) at 1 : 5,000 as the secondary antibody. Immunodetection was performed by chemiluminescence, with a kit from GE Healthcare (RPN 2109). Quantification of bands was performed by densitometry.

For lipid peroxidation analysis, yeast cells were pelleted, resuspended in 20 mM sodium phosphate (pH 7.2) 10% (w/v) trichloroacetic acid and lysed as described above, prior to protein content determination. Lipid peroxidation was assayed using 600 µL of 1% (w/v) thiobarbituric acid, 0.05 M NaOH, 0.025% (w/v) butylated hydroxytoluene, 100 µL of 0.1 M EDTA, and 50 µg of total proteins. Malondialdehyde (MDA) concentration was determined spectrophotometrically at 532 nm [187].

The oxidant-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) was used to measure the levels of ROS [188]. Prior to $H_2O_2$ exposure, $H_2$DCF-DA (10 µM) was added to the culture and incubated for 15 min to allow uptake of the probe. Yeast extracts were prepared as described above for the protein carbonylation assay, except for the buffer pH, which was pH 6.0. Samples were diluted to 5 µg/mL protein and fluorescence was measured using a spectrofluorometer set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm. Autofluorescence was measured using unlabeled controls and subtracted.

## 2.7. Catalase activity and glutathione levels

For the analysis of catalase activity, yeast extracts were prepared as described for protein carbonylation and enzyme activity was determined spectrophotometrically at 240 nm, following $H_2O_2$ decomposition [189].

For the determination of glutathione levels, yeast extracts were prepared by combining equal volumes of 2 M perchloric acid and a cell suspension in 100 mM potassium phosphate buffer (pH 7.0) 2 mM EDTA. The mixture was vigorously shaken in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Cell debris was removed by centrifugation at 5,000 rpm for 5 min. The supernatant was neutralized to pH 7.0 with 2 M KOH and 0.3 M MOPS. Protein content was estimated as described above. For oxidized glutathione (GSSG) determination, the samples were treated with 2% (v/v) 2-vinylpyridine and incubated for 1 h at 4 °C with agitation. Glutathione was assayed by the method of Tietze [190]. The rate of color development was monitored at 405 nm and the concentration was determined by reference to a GSSG standard added to the assay cuvette (internal standard).

## 2.8. Oxygen consumption

Oxygen consumption rate was measured for $7.5 \times 10^8$ cells in the culture media using an oxygen electrode (Oxygraph - Hansatech, Pentney, UK). Data was analyzed using the Oxyg32 V2.25 software (Oxygraph - Hansatech, Pentney, UK).

# CHAPTER III

## Results

## 3.1. Orotic acid production by *K. lactis* mutants

This section includes results published in the following research paper:

**Carvalho N, Coelho E, Gales L, Costa V, Teixeira JA, Moradas-Ferreira P** (2016) Production of orotic acid by a *Klura3*Δ mutant of *Kluyveromyces lactis*. *J Biosci Bioeng.* **121**(6):625-30

### 3.1.1. *K. lactis Klura3*Δ and *Klura5*Δ mutants as orotic acid producing microorganisms.

YPD agar plates with fully grown *Klura3*Δ mutant cells of *K. lactis* started to display two types of crystal structures after about 1 week at 4ºC. One type was rather large presenting a well-defined star-like structure (Figure 3.1A) while the other crystals were smaller and abundant (Figure 3.1B). X-ray crystallography analysis of both type of crystals demonstrated that its constituent matched the structure of orotate, which was present as a monohydrated salt (Figure 3.1C and Figure 3.1D). Orotate counter-ion was identified as potassium by SEM / EDS examination (Figure 3.2).

The presence of two morphological structures is likely a result of distinct crystal nucleation and growth when kept at 4ºC. For the matter of simplicity, despite these crystals were composed of potassium orotate monohydrate, the molecule produced by this mutant, either in crystal structures or dissolved, will be further referred as orotic acid.

This phenotype was not observed in the wild type strain or in the triple mutant *Klura1*Δ*Klura9*Δ*Klura3*Δ, in which the step prior to the formation of orotic acid is interrupted along with *KlURA3* deletion (Figure 1.1). Also, orotic acid was not detected in the medium of wild type and *Klura1*Δ*Klura9*Δ*Klura3*Δ cells grown in liquid cultures, in contrast to what was observed with *Klura3*Δ mutant, as shown in the following sections. These evidences demonstrated that the interruption of *de novo* pyrimidines biosynthetic pathway at the Klura3p step led to the accumulation of the orotic acid generated by the irreversible oxidative reaction catalyzed by the

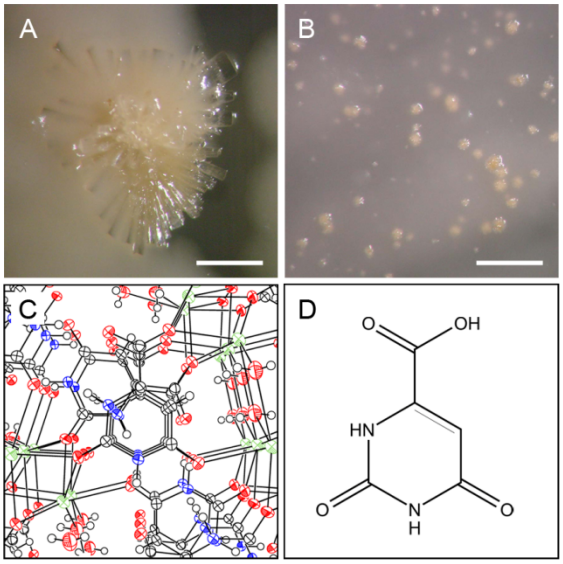


**Figure 3.1** Large (**A**) and small (**B**) crystal structures. Scale bars represent 1 mm. **C** Crystal network of potassium orotate monohydrate. In the center of the figure is a set of overlapping orotate molecules in the same disposition as the molecular structure depicted on the right. K in green, O in red, N in blue, C in black and H in open circles. **D** Molecular structure of orotic acid.

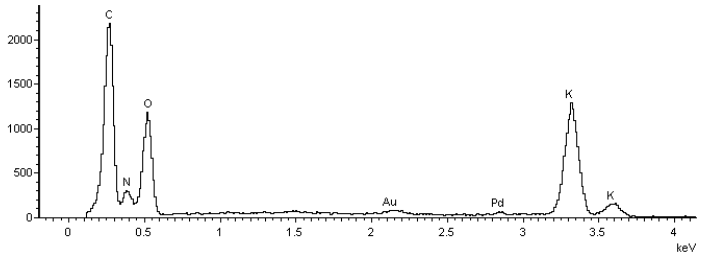


**Figure 3.2** SEM / EDS examination of crystals produced by *K. lactis Klura3*Δ. Potassium was the single atomic element present beside carbon, nitrogen and oxygen (hydrogen not detected by this method).

DHODases (KlUra1p and KlUra9p). Due to the existence of an intermediate step catalyzed by orotate phosphoribosyltransferase (KlUra5p) (Figure 1.1) the mutant *Klura5*Δ was also generated and tested. This mutant exhibited a phenotype similar to the observed with *Klura3*Δ cells, producing equivalent amounts of orotic acid in liquid cultures under any of the conditions tested. Data presented in the following

section refers to *Klura3*Δ cells since initial experiments were performed using this mutant. Data from *Klura5*Δ cells will be shown only for selected conditions to allow comparison and demonstrate their similar phenotype.

### 3.1.2. Production of orotic acid in flask and bioreactor using YPD based media

To assess production yields of orotic acid by *K. lactis Klura3*Δ mutant, it was important to monitor its accumulation in liquid cultures. As the phenomenon of orotic acid crystal formation in agar plates was observed using the rich and commonly used YPD growth media, the same was considered for growth in liquid cultures. Growth parameters were tested in flask cultures to determine their influence in orotic acid production and further evaluation in a bioreactor was required to confirm the potential value of this *K. lactis* mutant strain for the biotechnology industry.

#### 3.1.2.1. Production of orotic acid in flask cultures

Oxygen requirements of *Klura3*Δ mutant and its interference in orotic acid production were tested in different culture : flask volume ratio conditions using 250 ml flat bottom flasks agitated at 180 rpm, this way providing different levels of culture aeration (Figure 3.3). Although the different ratios tested did not affect cell growth, major differences were observed in the rate of orotic acid production. Production rate was negatively affected below 1 : 8.3 ratio (30 ml : 250 ml) and was maximal with at least a 1 : 12.5 ratio (20 ml : 250 ml) that led to a concentration of about 2.2 g/L after 48 h. We also evaluated the production at higher aerations using 20 ml cultures in 250 ml baffled flasks agitated at 180 rpm and 250 rpm but no further improvement was observed (Figure 3.4). Therefore, aeration conditions for further experiments were set to 20 ml culture in 250 ml flat bottom flasks agitated at 180 rpm.

The results shown in Figure 3.3 also indicate that an additional oxygenation, above the levels that already allow normal cell growth, is required for optimal

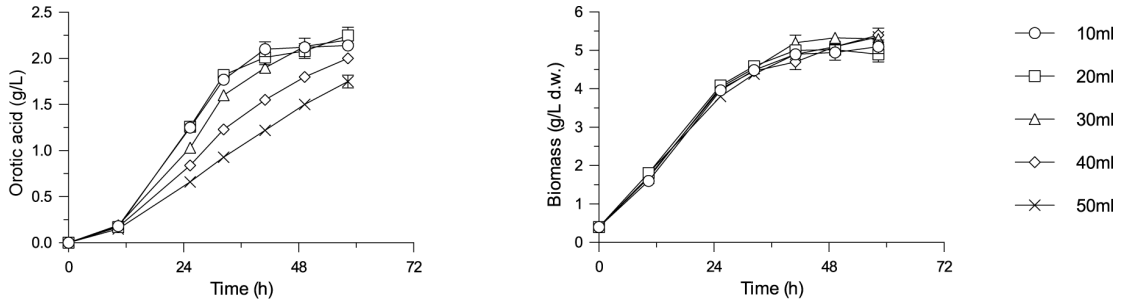


**Figure 3.3** Effect of aeration on orotic acid production and cell growth in flask cultures. Increase in aeration was achieved by incrementally decreasing volumes of YPD growth medium (50 ml to 10 ml) in flat bottom 250 ml flasks agitated at 180 rpm. Growth was performed at 26ºC.

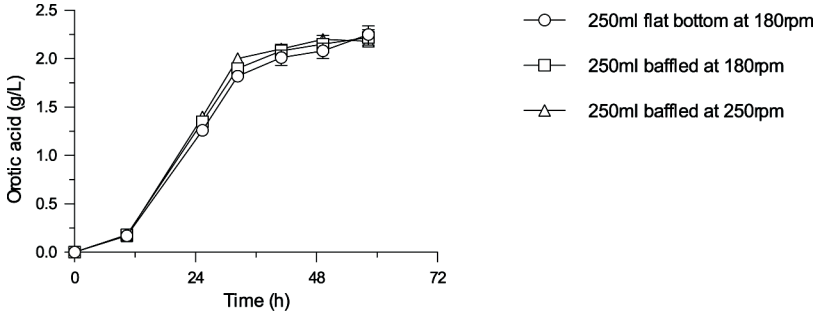


**Figure 3.4** Effect of strong aeration / agitation on orotic acid production in flask cultures. Growth performed at 26ºC using 20ml YPD in 250ml flasks. Increase in aeration was achieved by changing flat bottom flasks to baffled flasks and from an agitation of 180 rpm to 250 rpm.

production rate. This is consistent with the presence of a putative mitochondrial Klura9p that links pyrimidines pathway to the respiratory chain and, in contrast to the other DHODase KlUra1p, is most likely playing a major role in the enzymatic step leading to the formation of orotic acid. This hypothesis was confirmed by the analysis of orotic acid production in the double mutants *Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ. These mutants exhibited similar growth rates but, whereas orotic acid production was similar using *Klura3*Δ and *Klura1*Δ*Klura3*Δ mutants, it was strongly and negatively affected using *Klura9*Δ*Klura3*Δ mutant (Figure 3.5). This mutant was only able to produce 0.7 g/L of orotic acid after 48 h. Therefore,

oxygen seems to be directly linked to orotic acid production due to the predominant or more efficient activity of KlUra9p DHODase.

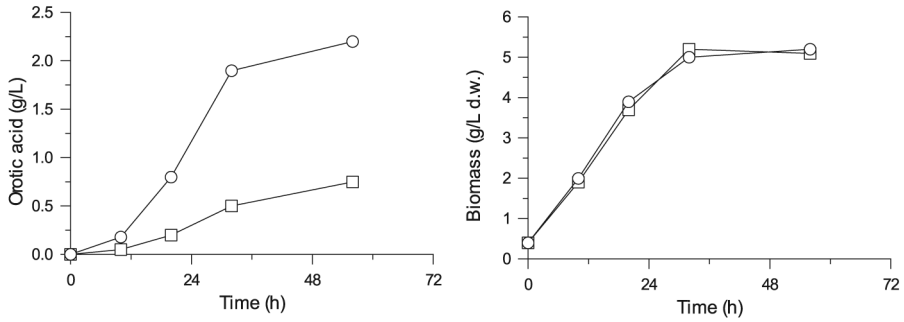


**Figure 3.5** Orotic acid production and cell growth of *K. lactis Klura1*Δ*Klura3*Δ (*circle*) and *Klura9*Δ*Klura3*Δ (*square*). Growth performed at 26ºC using 20ml YPD in 250 ml flat bottom flasks at 180 rpm.

Using the aeration conditions already defined, different growth temperatures from 24 ºC to 32 ºC were tested (Figure 3.6). Although cell growth was lower at 24 ºC and 32 ºC, orotic acid production at 24 ºC was only delayed during the early stage, while at 32 ºC the maximum amount of orotic acid produced was decreased. At 24 ºC specific productivity was therefore higher than at temperatures from 26 ºC to 30 ºC. The lower biomass and orotic acid production at 32 ºC is probably associated with temperature sensitivity of *Klura3*Δ mutant at a particular growth stage (after 12 h) or with deviation of energy or resources (glucose or other nutrients) to other mechanisms or metabolic pathways required for growth at this temperature. Since the best results were obtained at 26 ºC - 30 ºC with minor differences in both growth and orotic acid production, 28 ºC was chosen as the growth temperature for further optimization.

Under these best conditions, the initial pH (ipH) of the culture medium dropped from 6.6 (pH of YPD medium) to 4.4 during the first 36 h and then remained rather constant (Figure 3.7). To evaluate the effects of pH in the production process, different buffering systems were tested. For these experiments, glucose

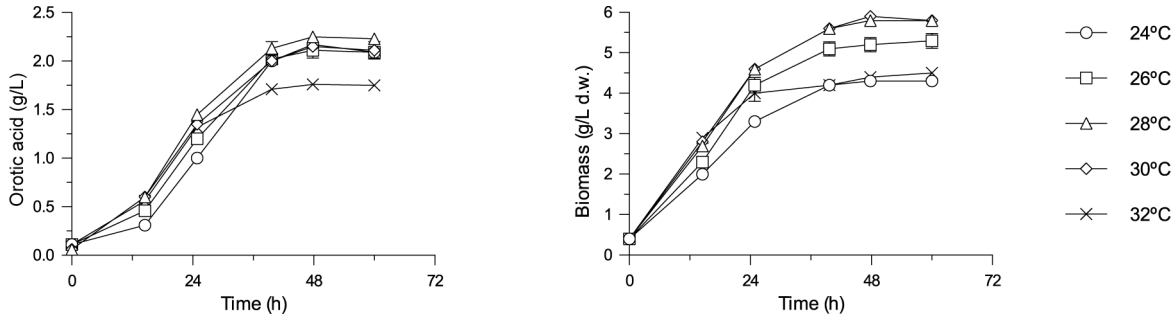


**Figure 3.6** Effect of temperature on orotic acid production and growth in flask cultures. Cells in 20 ml YPD were grown at different temperatures (24 ºC to 32 ºC) in 250 ml flat bottom flasks agitated at 180 rpm.

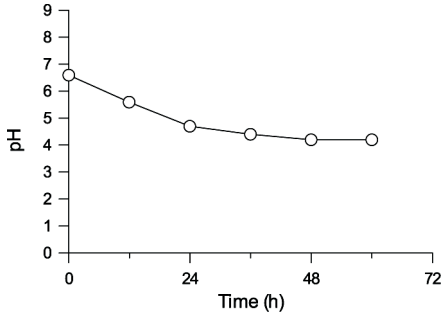


**Figure 3.7** pH behavior of cultures grown at 28ºC using 20ml YPD in 250 ml flat bottom flasks at 180 rpm.

concentration was increased from the 20 g/L used in standard YPD to 50 g/L (YPD 5% glucose) not only to show that the culture media could be improved and a glucose increment alone was sufficient to significantly increase orotic acid production but also because it would better demonstrate the efficacy of the buffering system under conditions of stronger acidification. Data regarding pH behavior of the cultures and its effect on orotic acid production and cell growth are summarized in Figure 3.8. Cells grown in unbuffered YPD 5% glucose with an ipH 6.6 as in previous experiments, produced 2.9 g/L after 48 h and 3.9 g/L after 72 h and culture pH dropped from 6.6 to 3.4. Optimum pH for growth of *K. lactis* wild type strains is known to be 4.5 - 5.0 and for that reason phthalate ($pK_a$ 5.5) was used to buffer the cultures and maintain pH values close to that range. The

concentration of phthalate used (75 mM) did not affect the growth of *Klura3*Δ cells and was sufficient to prevent a drop over one pH value from an ipHs of 5.5. However, stabilizing pH with phthalate for most of the culture period at about 4.7 did not improve orotic acid production.

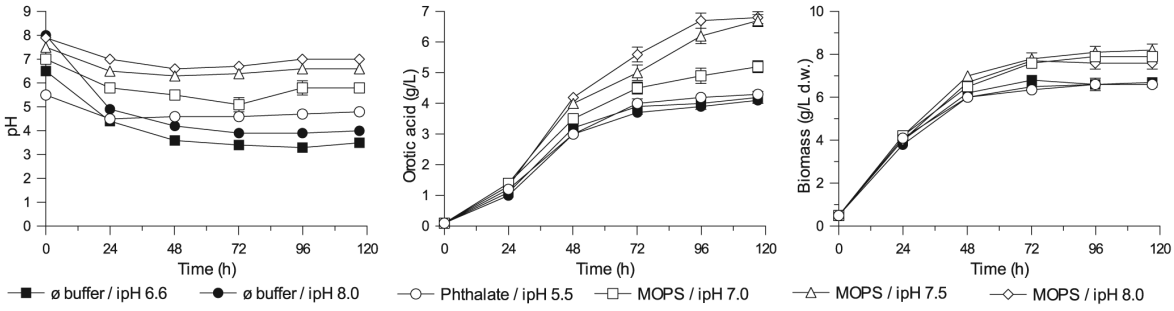


**Figure 3.8** pH behavior of unbuffered and buffered cultures and its effect on orotic acid production and growth in flask cultures. 75 mM phthalate at ipH 5.5 and 200 mM MOPS at ipH 7.0, 7.5 and 8.0 were used to stabilize culture pH above the values obtained with unbuffered cultures at ipH 6.6 and 8.0. Growth was performed using 20 ml YPD 5 % glucose in 250 ml flat bottom flasks at 28 ºC and 180 rpm.

To test orotic acid production in cultures with pH stabilized at higher values, MOPS buffer ($pK_a$ 7.2) was used. MOPS has lower buffering capacity than phthalate and a concentration of 200 mM was required to prevent a drop of pH higher than 1 -1.5 in the cultures tested without having a negative effect on growth (Figure 3.8). In fact, some increase in final biomass was observed in cultures buffered with MOPS. Under these conditions, orotic acid concentration far exceeded its solubility (1.8 g/L in water at 18 ºC) and microscopic spike like crystals were already visible at 48 h. Therefore, for orotic acid quantification, concentrated NaOH was added to a final concentration of 250 mM to dissolve orotic acid crystals in the samples (solubility in 1 M NaOH is 50 g/L). The results shown in Figure 3.8 clearly show that stabilizing culture pH with MOPS at values closer to 7 strongly increased orotic acid production. Both unbuffered cultures with ipH 6.6 and ipH 8.0 acidified to pHs below 4. On the other hand, MOPS cultures that started at ipH 8.0

maintained its pH close to 7. Whereas unbuffered cultures only produced about 3.9 g/L after 72 h without significant increase up to 96 h, MOPS cultures with ipH 8.0 were able to produce 5.6 g/L after 72 h and 6.7 g/L after 96 h. Culture pH is therefore critical for orotic acid production and, due to the strong tendency of the culture to acidify, the use of this buffer system is essential to sustain pH at optimal values. It was not possible to test orotic acid production in buffered cultures with pH stabilized at even higher values, either with MOPS or other buffer with higher $pK_a$, since ipH of the culture would necessarily have to be increased and cell growth is affected above pH 8. Nevertheless, taking into account the differences observed between MOPS cultures at ipH 8.0 and ipH 7.5, a significant improvement in production would not be expected if pH could be stabilized above 7.0. Moreover, as shown in the following section, maintaining culture pH at 8.0 in a bioreactor had a negative effect on the production.

The results obtained from cultures buffered with phthalate and MOPS demonstrated that *Klura3*Δ cells can grow well at any pH from about 4.5 to 7 and that pH 7 is probably the most favorable for specific mechanisms involved in the production and/or secretion of orotic acid. With respect to glucose concentration, we observed that the increase of orotic acid production was only relevant if glucose was increased up to 50 g/L. In a buffered culture at ipH 8.0, an additional 25 g/L of glucose (YPD 7.5% glucose) only generated a 0.6 g/L increment of orotic acid (Figure 3.9). In conclusion, under the conditions tested, 200 mM MOPS in YPD 5% glucose with ipH 8.0 was considered as the most adequate buffering system for orotic acid production in flask cultures.

Available data on orotic acid producing strains with industrial value [110] show that a similar mutant of *C. glutamicum* (ATCC 14275), with pyrimidines pathway interrupted and no further genetic optimization, is able to produce in flask cultures 8.2 g/L of orotic acid after 120 h (Table 1.1). However, this production was achieved using optimized and cheap industrial media. Having reached an orotic acid concentration of 6.7 g/L at 96 h in flasks, our results demonstrated the high potential of *K. lactis Klura3*Δ as an orotic acid producing microorganism. They strongly suggest that with an optimized industrial media and an additional 24 h production time, final amounts of orotic acid above 8.2 g/L could be reached at 120 h.

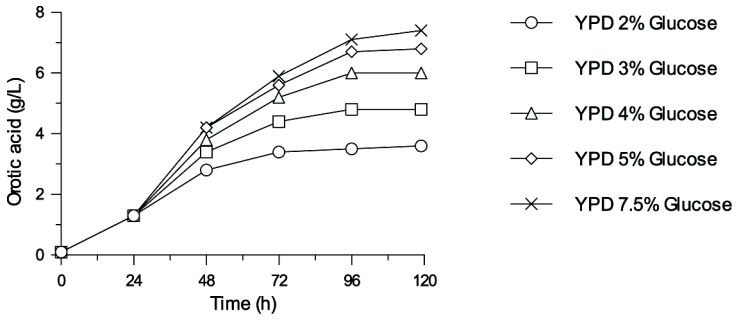


**Figure 3.9** Orotic acid production in buffered YPD based media with glucose concentration from 2 % to 7.5 %. Cultures started at ipH 8.0 and were buffered with 200 mM MOPS. Growth performed at 28ºC using 20 ml growth media in 250 ml flat bottom flasks at 180 rpm.

### 3.1.2.2. Production of orotic acid in bioreactor

To further assess the potential of *K. lactis Klura3*Δ strain for the biotechnology industry, the production process was tested in a bioreactor system. Using the information obtained from flask experiments and YPD 5% glucose as production medium (1 L), several conditions of agitation / aeration, pH and temperature were tested in a 2 L bioreactor (Figure 3.10). As for flask cultures with MOPS buffer, NaOH was added to all samples removed from cultures after 24 h to dissolve orotic acid crystals and allow its quantification by HPLC.

All results obtained in flask cultures regarding optimum conditions for orotic acid production were confirmed in the bioreactor. First, with temperature and pH maintained constant at 28 ºC and pH 7.0, a minimum aeration / agitation (500 rpm / 1 vvm) above the one required for optimal growth of this mutant strain, is essential and sufficient for best orotic acid production rate and yield (Figure 3.10A). For each aeration conditions we observed that $pO_2$ dropped to about 80% (800 rpm / 2 vvm), 40% (500 rpm / 1 vvm) and 10% (400 rpm / 1 vvm) [data not shown], thus indicating culture $pO_2$ should not drop below 40%. Second, at pH 7.0 and optimum aeration / agitation conditions, orotic acid production was higher at 26 ºC and 28 ºC, being significantly affected at 30 ºC (Figure 3.10B). Third, at 28 ºC and optimum aeration / agitation conditions, best orotic acid production was obtained at neutral pH (Figure 3.10C) indicating that optimum pH

for orotic acid production would be very close or at pH 7.0. Regarding glucose consumption, despite a minor difference observed at 30 ºC (Figure 3.10B), the results were similar for all other conditions tested (Figure 3.10A and 3.10C). Strikingly, the *Klura3*Δ mutant strain was still able to accumulate in the growth media large amounts of orotic acid after glucose has been depleted. Also, differences in orotic acid production under the different conditions tested were mostly observed after that point.

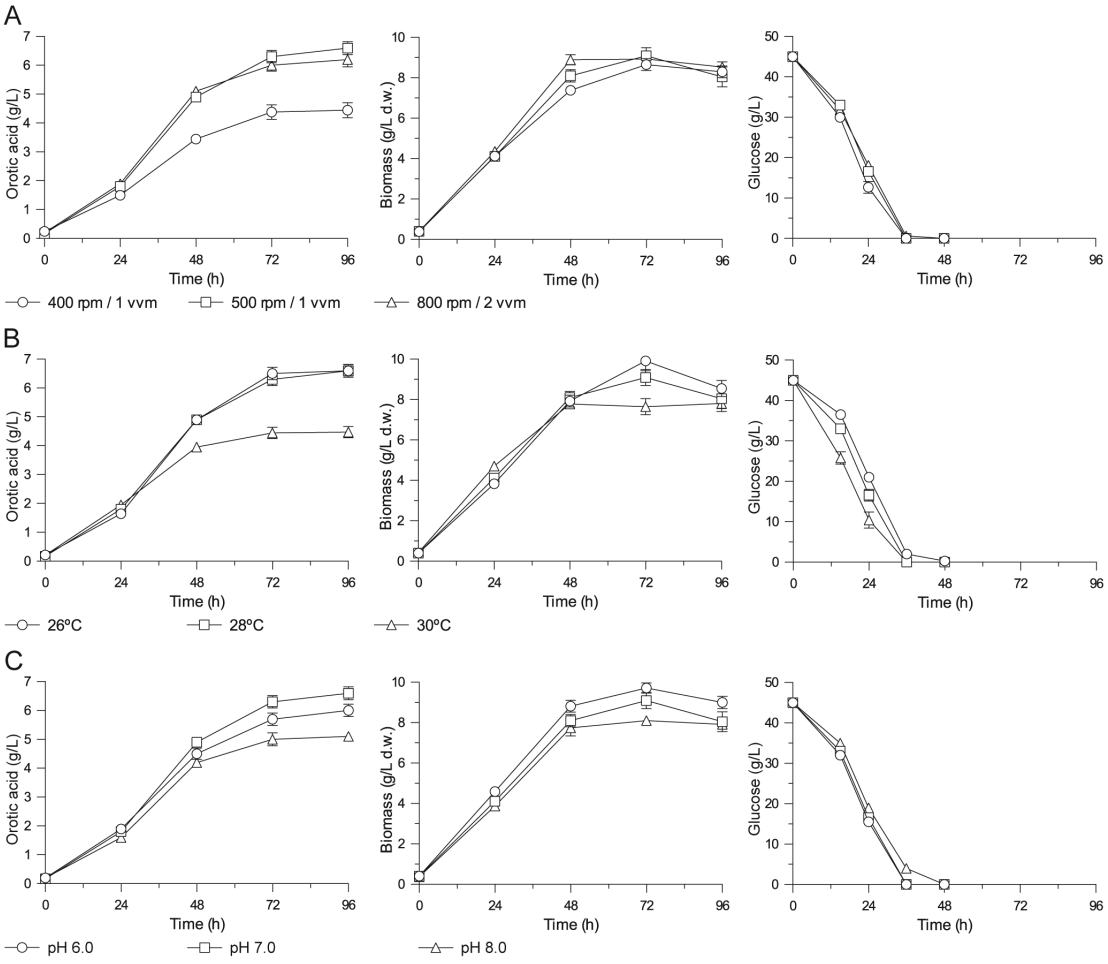


**Figure 3.10** Effect of agitation (rpm) / aeration (vvm) (**A**), temperature (**B**) and pH (**C**) on orotic acid production, growth and glucose consumption in bioreactor cultures. For each set of experiments the following conditions were maintained constant: A - temperature and pH respectively at 28.0 ºC and pH 7.0; B - agitation, aeration and pH respectively at 500 rpm, 1 vvm and pH 7.0; C - agitation, aeration and temperature respectively at 500 rpm, 1 vvm and 28.0 ºC.

Production in bioreactor was optimal with growth parameters set to 28 ºC, 500 rpm, 1 vvm and pH 7.0 and the amount of orotic acid obtained after 96 h was 6.7 g/L (Figure 3.10A-C). Analyzing these results together with the production obtained in flasks under the optimized conditions already defined, it is evident that the rates of production are similar and the same maximum amount of orotic acid is reached at 96 h (Figure 3.11). Using same flask and bioreactor optimized conditions, it is clear that similar production was obtained with *Klura5*Δ mutant (Figure 3.11). Equivalent orotic acid production from *Klura3*Δ and *Klura5*Δ mutants is most likely due to a limiting phosphoribosyl pyrophosphate (PRPP) availability (Figure 1.1) that, together with the reversible nature of the enzymatic step catalyzed by KlUra5p, favors orotic acid accumulation upon *KlURA3* deletion. This can be further favored considering the possibility that transport across the membrane is only possible for orotic acid and not for OMP. However, none of these hypotheses have been experimentally confirmed.

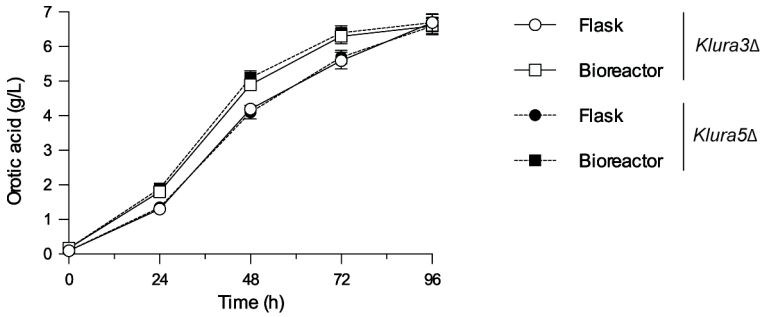


**Figure 3.11** Orotic acid production under best conditions obtained for flask and bioreactor cultures using *K. lactis Klura3*Δ and *K. lactis Klura5*Δ. Flask production performed at 28ºC and 180 rpm in 250 ml flat bottom flasks with 20 ml YPD 5% glucose media with ipH 8.0 containing 200mM MOPS. Bioreactor production performed at 28 ºC, 500 rpm, 1 vvm and pH 7.0 in YPD 5% glucose media.

The high reproducibility of orotic acid production in flask and bioreactor is very relevant regarding experimental optimization of the industrial production process. It is not uncommon to encounter biological systems that behave considerably

different after scaling up production to a bioreactor. Inversely, flask systems are often not able to provide full optimum conditions, which can only be achieved in a highly controlled bioreactor system. In this case, experimental results obtained in flasks regarding industrial media optimization or *K. lactis* mutants screening, are expected to be replicated in bioreactor at any stage of the development process.

### 3.1.3. Assessment of production using low cost industrial media

Production of orotic acid by *K. lactis Klura3*Δ or *Klura5*Δ cells using YPD based media would not be used in industry due to its costs. Thus, full media optimization should be performed using available and cheap media containing nutrients identified as relevant for orotic acid production. For that purpose, sugarcane molasse, cheese whey powder (CWP) and corn steep liquor (CSL) were evaluated as cheap nutrient sources. Sugarcane molasse is a concentrated by-product of the manufacture or refining of sucrose from sugar cane, CWP is the dried and concentrated form of the liquid cheese whey that is generated as a waste stream from cheese production facilities and CSL is a liquid by-product of wet milling process of maize-starch industry. Due to their high sugars content they have been used in various biotechnological applications as a carbon source. The sugar content of the raw materials obtained was 49% (w/v), 73% (w/w), and 7.5% (w/v) for molasse, CWP and CSL, respectively.

The orotic acid producing mutant chosen for these tests was again *Klura3*Δ, and its ability to grow solely on molasse, CWP or CSL, was assessed. Even though the types of sugars present in each industrial growth media were different they could all be metabolized by *K. lactis*. Hence, the initial criteria used for comparison of the performance of each industrial media, as well as with previous YPD media, was their total sugar content. The appropriate amount of each raw material was used to obtain a growth media with final sugar content of 20 g/L, as in regular YPD (2% glucose). Initial pH of each medium solution was set to 6.5 and no buffer was used to stabilize pH. With respect to molasse and CWP, a minor growth was observed only when uracil was added to a concentration normally used for growth of ura3 auxotrophic yeast mutants in minimal media (50 mg/L) but orotic acid was not detected (Figure 3.12). This indicates that, despite sugar availability, these

media alone do not provide all sufficient nutrients for the growth of *K. lactis Klura3*Δ. Supplementation of uracil is essential and, among others, addition of a suitable nitrogen source would most certainly allow better growth. On the other hand, CSL alone was not only sufficient to allow growth but significantly surpassed the rich YPD media, considering both had the same amount of sugars (Figure 3.12). More importantly, there was production of orotic acid. The lower amount obtained (0.33 g/L) when compared to YPD suggests that under these conditions its production was not significantly promoted or was subject to strong inhibition.

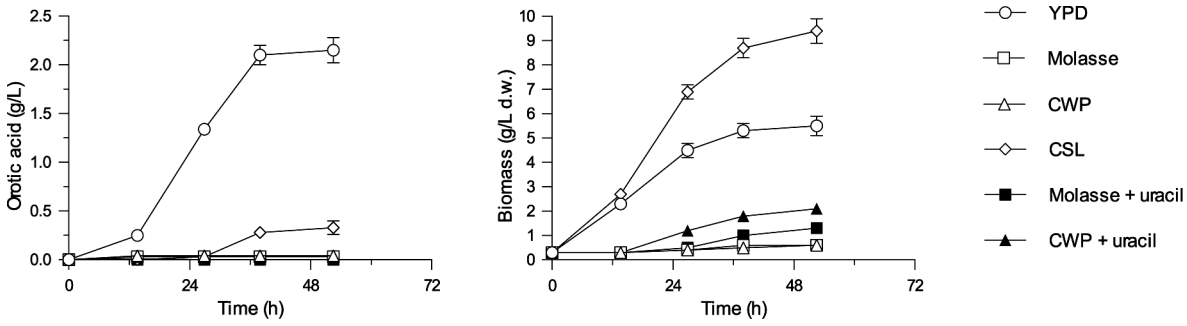


**Figure 3.12** Orotic acid and biomass production using cheap industrial media. Each media was prepared from industrial raw material to allow a final sugar concentration of 20 g/L. Concentration of raw material was as followed: molasse - 4% (v/v); CWP - 2.75% (w/v); CSL - 26.7% (v/v). Uracil concentration used was 50 mg/ml. Initial pH was 6.5 and growth was performed at 28ºC using 20 ml media in 250 ml flat bottom flasks at 180 rpm.

Analysis of data from Figure 3.13 suggests that an inhibitory effect is indeed interfering with the production of orotic acid by *K. lactis Klura3*Δ in CSL. Decreasing the concentration of CSL from 26.7% (v/v) to 6% (v/v), corresponding respectively to a concentration of sugars from 2% (w/v) to 0.45% (w/v), cell growth decreased and less biomass was produced. However, not only the final amount of orotic acid reached was incrementally higher but also the rate and onset of production was improved. With 4% (v/v) CSL, the rate of production was further improved but the final amount of orotic acid produced was lower, probably as a consequence of less nutrients available when compared to 6% (v/v) CSL. Cell

growth at 2% (v/v) CSL was minor and consequently the level of production was very low.

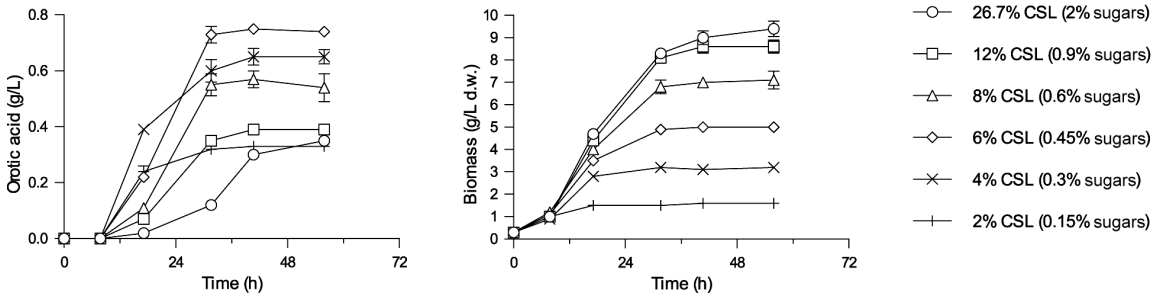


**Figure 3.13** Orotic acid and biomass production using different concentrations of CSL. Concentration of raw CSL (v/v) is depicted for each symbol followed by the resulting sugar concentration (w/v). Initial pH was 6.5 and growth was performed at 28ºC using 20 ml media in 250 ml flat bottom flasks at 180 rpm.

The growth observed by the pyrimidines-requiring *Klura3*Δ mutant in CSL supports the idea that it is probably rich in uracil and derivatives, which are internalized and used by the pyrimidines salvage pathway. Those uracil concentrations most certainly led to the inhibition of orotic acid production as a result of the natural system involved in the regulation of *de novo* pyrimidines biosynthetic pathway in *K. lactis*. A concentration of 4-6% (v/v) seems to be optimum regarding orotic acid production as a result of the levels of inhibition *versus* cell growth achieved.

The potential of CSL as a raw material for orotic acid production by *K. lactis Klura3*Δ is extremely significant. Its nutritional value as a cheap supplement to other raw materials, such as molasse and CWP, should be considered assuming it could provide all the remaining necessary nutrients for an optimal cell growth with minimal effect on the inhibition of orotic acid production. Thus, production media containing CSL and molasse or CWP with a combined concentration of 2% (w/v) sugars were tested (Figure 3.14). In both media, an increment of CSL up to 12% (v/v) always led to an increase in biomass production and its supplementation to molasse resulted in a slightly better growth when compared to CWP. Regarding

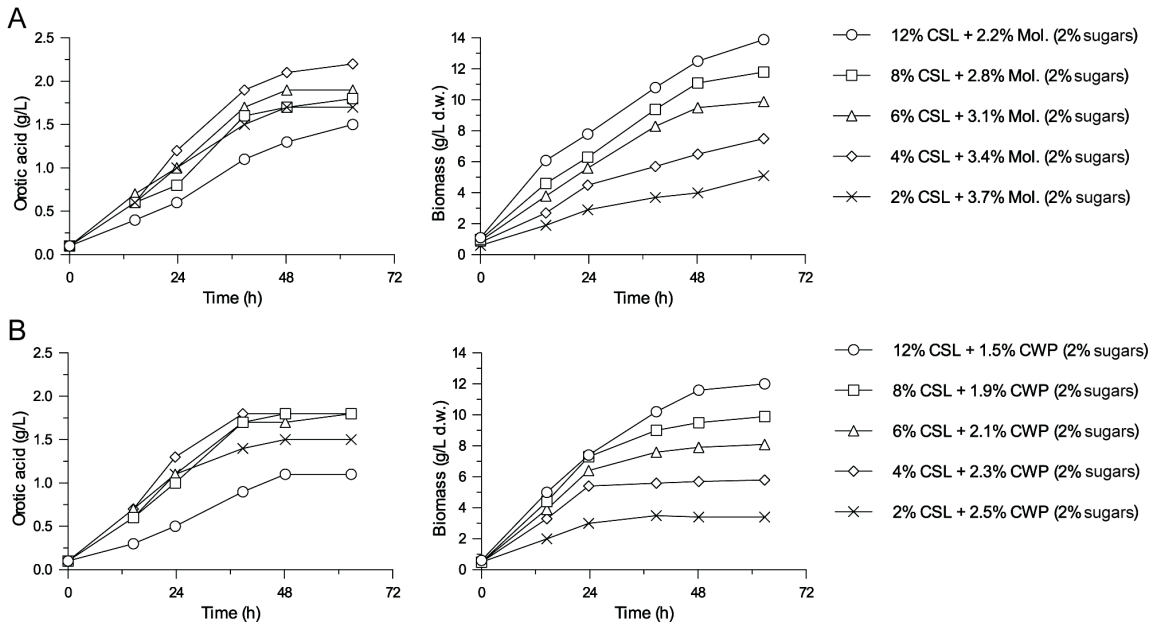


**Figure 3.14** Orotic acid and biomass production using CSL + Molasse (**A**) and CSL + CWP (**B**) as production media. Concentration of raw CSL (v/v), Molasse (Mol.; (v/v)) and CWP (w/v) is depicted for each symbol followed by the respective sugar concentration, which resulted in 2% (w/v) for all media. Initial pH was 6.5 and growth was performed at 28ºC using 20 ml media in 250 ml flat bottom flasks at 180 rpm.

orotic acid production, optimal concentration of CSL for each combined media (Figure 3.14) fall within a range [4 – 8% (v/v)] similar to the one already observed when CSL was used as the sole nutrient source (Figure 3.13). However, orotic acid production was better when CSL was supplemented to molasse. Whereas a concentration of 2.2 g/L of orotic acid was achieved with 4% (v/v) CSL + 3.4% (v/v) molasse, a maximum amount close to 1.8 g/L was obtained in the CWP media supplemented with 4, 6 or 8% (v/v) CSL. Actually, having the same amount of sugars, orotic acid production with CSL + CWP was always lower than with CSL + molasse when comparing media with same CSL concentrations. The possibility that CWP contains higher amounts of uracil and derivatives than molasse, which would add to the effect already induced by CSL, is not supported by the fact that addition of uracil to CWP induced higher growth than molasse (Figure 3.12). The difference observed could simply be the result of less overall nutritive value of CSL + CWP after the adjustment of both combined media with respect to sugar

content. The types of sugar that are present in molasse and CWP may also play a significant role in the difference observed. Due to the distinctive advantage of *K. lactis* to utilize lactose, the use of CWP should not be completely discarded. It should always be accounted as a potential substitute of molasse and market availability together with costs of CWP compared to molasses should be evaluated for the validation of the production system for full scale-up. However, taking these results into consideration, a production media containing molasse and CSL was chosen for further tests. Under similar growth conditions and amount of sugar (2% (w/v)), orotic acid production with cheap media composed by 4% (v/v) CSL and 3.4% (v/v) molasse (Figure 3.14A) was the same 2.2 g/L obtained with the rich and expensive YPD media (Figure 3.6; 28ºC).

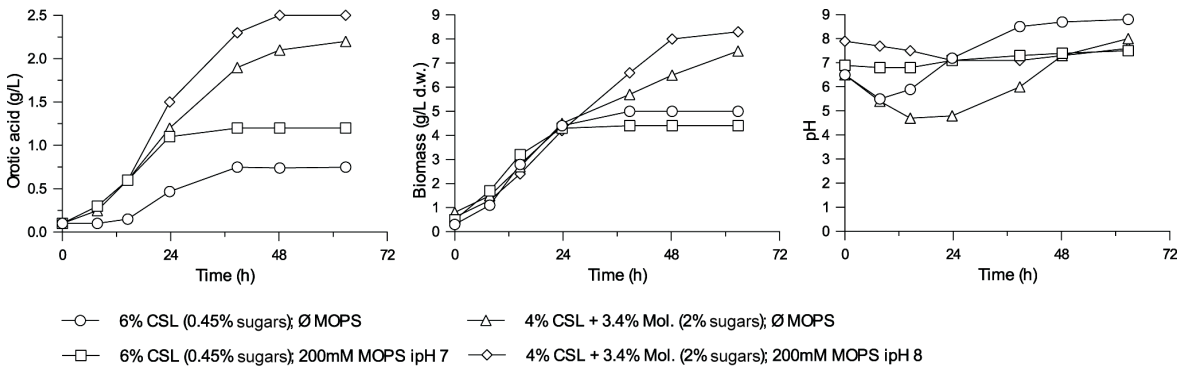


**Figure 3.15** Effect of pH on orotic acid production and growth using CSL and CSL + Molasse media. Concentration of raw CSL (v/v) and Molasse (Mol.; v/v) is depicted for each symbol followed by the resulting sugar concentration (w/v). Initial pH of the cultures: unbuffered – ipH 6.5; buffered CSL – ipH 7; CSL + Molasse – ipH 8. Growth was performed at 28ºC using 20 ml media in 250 ml flat bottom flasks at 180 rpm.

Culture pH in CSL containing media (6% CSL (v/v) or 4% (v/v) CSL + 3.4% (v/v) molasse) did not show the behavior previously observed for YPD unbuffered cultures (Figure 3.15). In unbuffered 6% (v/v) CSL cultures, the pH dropped initially as in YPD but, instead of staying at acidic pH for the remaining culture period, it increased considerably up to pH 8.8. A rather delayed but similar pH

behavior was observed in the unbuffered culture containing 4% (v/v) CSL + 3.4% (v/v) molasse. CSL appears to be the inducing factor of such effect in the combined media as well but the underlying *K. lactis Klura3*Δ metabolic mechanisms and CSL constituents involved in this behavior were not determined. To test pH stabilization of these cultures at neutral values, MOPS buffer was added to a concentration of 200mM MOPS. An ipH 7 was sufficient to maintain culture pH close to 7 in buffered 6% (v/v) CSL culture whereas an ipH 8 was required for the cultures containing 4% (v/v) CSL + 3.4% (v/v) molasse. As observed for YPD buffered cultures, pH stabilization at values close to pH 7 increased orotic acid production (Figure 3.15). A more marked positive effect was observed for the culture containing CSL alone, since the final amount of orotic acid produced was higher and the onset of production was significantly reduced. For the culture containing CSL and molasse, beside an increased final amount of orotic acid obtained, pH stabilization at pH 7 also resulted in a positive effect on the rate of production.

**Table 3.1** Orotic acid production after 80 h using 4% (v/v) CSL+ 3.4% (v/v) Molasse production media supplemented with molasse (Mol.), and therefore sugars, nitrogen sources ($(NH_4)_2SO_4$ or urea), phosphate ($K_2HPO_4$) or magnesium ($MgSO_4$). Each culture contained 200mM MOPS for pH stabilization and ipH was set to 8. Growth was performed at 28ºC using 20ml media in 250 ml flat bottom flasks at 180 rpm.

| Orotic acid production after 80 h (g/L) | | | | | |
|---|---|---|---|---|---|
| 4% (v/v) CSL + 3.4% (v/v) Molasse | | | | | |
| + Ø | + 3.4% (v/v) Mol. | + 0.25% $(NH_4)_2SO_4$ | + 0.25% Urea | + 0.05% $KH_2PO_4$ | + 0.025% $MgSO_4$ |
| 2.4 | 2.6 | 3.5 | 4.5 | 2.5 | 2.5 |

Using the buffered 4% (v/v) CSL + 3.4% (v/v) molasse media as the minimal composition, we incremented the carbon source (molasse) or added defined sources of nitrogen ($(NH_4)_2SO_4$ and urea), phosphate ($KH_2PO_4$) and magnesium

($MgSO_4$) (Table 3.1). Besides the carbon source, the compounds $(NH_4)_2SO_4$, $KH_2PO_4$ and $MgSO_4$ are the major and most common constituents used for yeast growth in minimal synthetic media. Urea is often used as a complement or alternative to $(NH_4)_2SO_4$ as nitrogen source. After 80 h growth it was clear that the addition of sugar (molasse) did not improve orotic acid production. This was likely due to the fact that nitrogen sources were a limiting factor, as the supplementation of $(NH_4)_2SO_4$ or urea resulted in an improvement of final amount of orotic acid produced. It is evident that the composition of the production media can be further and significantly improved by optimizing the amount and ratio of molasse *versus* nitrogen source. The increase in orotic acid production was considerably higher when urea was used as nitrogen source, indicating that it should be considered as the best option for that purpose. Addition of phosphate and magnesium did not improve production suggesting that at these production levels CSL is providing sufficient amounts of these elements. Flask cultures of *K. lactis Klura3*Δ grown in a buffered production media containing 4% (v/v) CSL, 3.4% (v/v) molasse and 0.25% (w/v) urea, which provided 2% (w/v) sugars, were able to produce 4.5 g/L of orotic acid after 80 h. For the same culture time, the production in buffered YPD (2% glucose) under similar growth conditions was shown to be 3.4 g/L (Figure 3.9). This indicates that the cheap production media described can provide better results regarding orotic acid production.

The production of 4.5 g/L of orotic acid after 80 h by *K. lactis Klura3*Δ with industrial production media is still far from the 8.2 g/L obtained after 120 h in flask cultures with the original ATCC 14275 *C. glutamicum* mutant strain. However, when the production media used with *C. glutamicum* (Table 1.1) is compared with the one we used, the yield obtained with *K. lactis* is higher than *C. glutamicum* and the potential of production improvement is convincingly strong. The production media used by *C. glutamicum* contained the same type of molasses used in this work but it was not disclosed the amount of sugars. Assuming sugar content of those molasses used would be 50% (w/v) (average found in molasses), final amount of sugars in the production media would be 10% (w/v). Using the amount of sugar to compare the yield of the orotic acid obtained by both production media we verify that *C. glutamicum* mutant required 100 g/L of sugars to achieve 8.2 g/L of orotic acid whereas *Klura3*Δ produced 4.5 g/L with only a combined 20 g/L of sugars. The productivity of *Klura3*Δ (0.225) is almost 3 times higher than the one

obtained for *C. glutamicum* mutant (0.082). As observed on the flask studies of buffered YPD based media an increment in glucose could improve orotic acid production since its composition must provide other required nutrients in excess. An increment from 2% glucose used in regular YPD to 5% led to an increase of orotic acid from 3.6 g/L to 6.7 g/L (Figure 3.9). Taking all together, the final amount obtained and yield of orotic acid production at this stage is already promising and further optimization of the production media would certainly allow higher production levels.

The knowledge obtained from the tests on the production media base on cheap industrial by-products should next be used in the development of experimental matrixes to determine best combinatorial composition of these constituents and to identify other nutrient requirements at higher production levels of orotic acid. However, the inhibition of production by CSL constituents should be first addressed to confirm it results from the action of uracil but mostly, to evaluate the possibility of reducing or even eliminate such effect. In that case, the amount of CSL used in the production media could be increased, which would significantly alter the characteristics of the media, and therefore, its development strategy.

## 3.2. Relation of the *de novo* pyrimidines biosynthetic pathway with oxidative stress response in *K. lactis*.

### 3.2.1. Studies at early exponential phase

The *de novo* pyrimidine biosynthetic pathway includes a redox step catalyzed by DHODases, including in *K. lactis* a KlUra9p possibly localized in mitochondria [101]. Redox homeostasis and mitochondrial function are critical for oxidative stress resistance. This led us to assess the role of the pyrimidine pathway in $H_2O_2$ resistance, using wild type (WT) cells and mutants with the pathway interrupted at distinct steps, namely *Klura1*Δ*Klura9*Δ (lacks both DHODases), *Klura5*Δ and *Klura3*Δ cells. Cells were grown overnight in YPD to early exponential phase ($OD_{600}$ = 0.6) and then exposed to 10 mM $H_2O_2$ for 30 min. Under these conditions, the viability of WT cells was 27% but decreased to 14% in *Klura3*Δ mutants (Figure 3.16). However, the viability of mutants with the pyrimidines pathway interrupted in the previous steps, *Klura5*Δ and *Klura1*Δ*Klura9*Δ, was similar to the observed in the WT strain. At this stage of cell growth, it is not expected a fully active *de novo* synthesis of pyrimidines since YPD media pyrimidines derivatives and salvage pathway are most likely providing sufficient

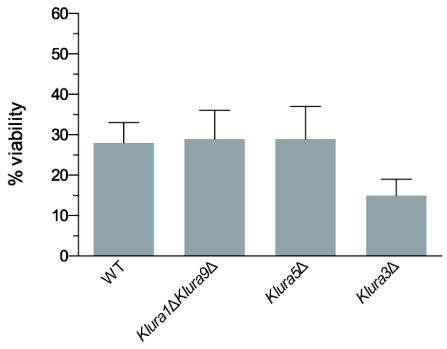


**Figure 3.16** Oxidative stress resistance of *K. lactis* WT, *Klura1*Δ*Klura9*Δ, *Klura5*Δ and *Klura3*Δ cells. Cells were grown in YPD to $OD_{600}$ = 0.6 and exposed to 10 mM $H_2O_2$ for 30 min. Cell viability was determined as described in methods. Values are mean ± SD of at least three independent experiments.

pyrimidines to the pathway and inhibiting synthesis *de novo*. This could explain the similar phenotypes of WT, *Klura1*Δ*Klura9*Δ and *Klura5*Δ cells but not the reduced viability of *Klura3*Δ mutants. In addition, since both *Klura5*Δ and *Klura3*Δ cells have the pathway interrupted in steps following the redox reaction catalyzed by DHODases, it was expected that these mutants would have similar phenotypes. However, the deletion of the *KlURA3* but not of the *KlURA5* gene decreased the ability of the cells to cope with a similar external oxidative stress.

Several biomarkers for oxidative damage and oxidative stress response were then evaluated in WT and *Klura3*Δ cells before and after exposure to $H_2O_2$ (Figure 3.17). No differences were observed in protein carbonylation, lipid peroxidation and ROS levels, suggesting that WT and *Klura3*Δ cells are subjected to identical levels of oxidative damage. The analysis of oxidative stress defenses revealed that *KlURA3* deletion did not affect catalase activity or total glutathione levels. However, $H_2O_2$-induced glutathione oxidation was lower in *Klura3*Δ cells, despite the higher sensitivity of this mutant to $H_2O_2$.

The oxidative nature of the DHODase reaction in the *de novo* pyrimidines biosynthesis urged us to investigate the potential role of this enzymatic step in *Klura3*Δ phenotype. The *K. lactis* mutants *Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ, previously generated to demonstrate that the mitochondrial KlUra9p is more relevant to the pathway than KlUra1p under higher aerations conditions (Figure 3.5), were used in this study. Mutants bearing the single deletion of each of the DHODase genes were also produced (*Klura1*Δ and *Klura9*Δ) and analyzed together with the double mutants. Cell viability after $H_2O_2$ exposure was determined on these mutants, and remarkably, clear differences to WT and *Klura3*Δ were observed (Figure 3.18). Both *Klura1*Δ and *Klura9*Δ mutants had a significant decrease in viability compared to WT or the double mutant *Klura1*Δ*Klura9*Δ (see Figure 3.16) despite a more pronounced effect on *Klura1*Δ. The viability of this last mutant was even inferior to the one observed for *Klura3*Δ but similar in the case of *Klura9*Δ. On the other hand, in *Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ mutants, the viability significantly increased to levels even superior to WT or *Klura1*Δ*Klura9*Δ. Opposite to the single mutants *Klura1*Δ and *Klura9*, the double mutant *Klura1*Δ*Klura3*Δ showed a more pronounced effect than *Klura9*Δ*Klura3*Δ. It is not possible to correlate the results of these mutants and explain the phenotypes observed based solely on the viability tests outcome.

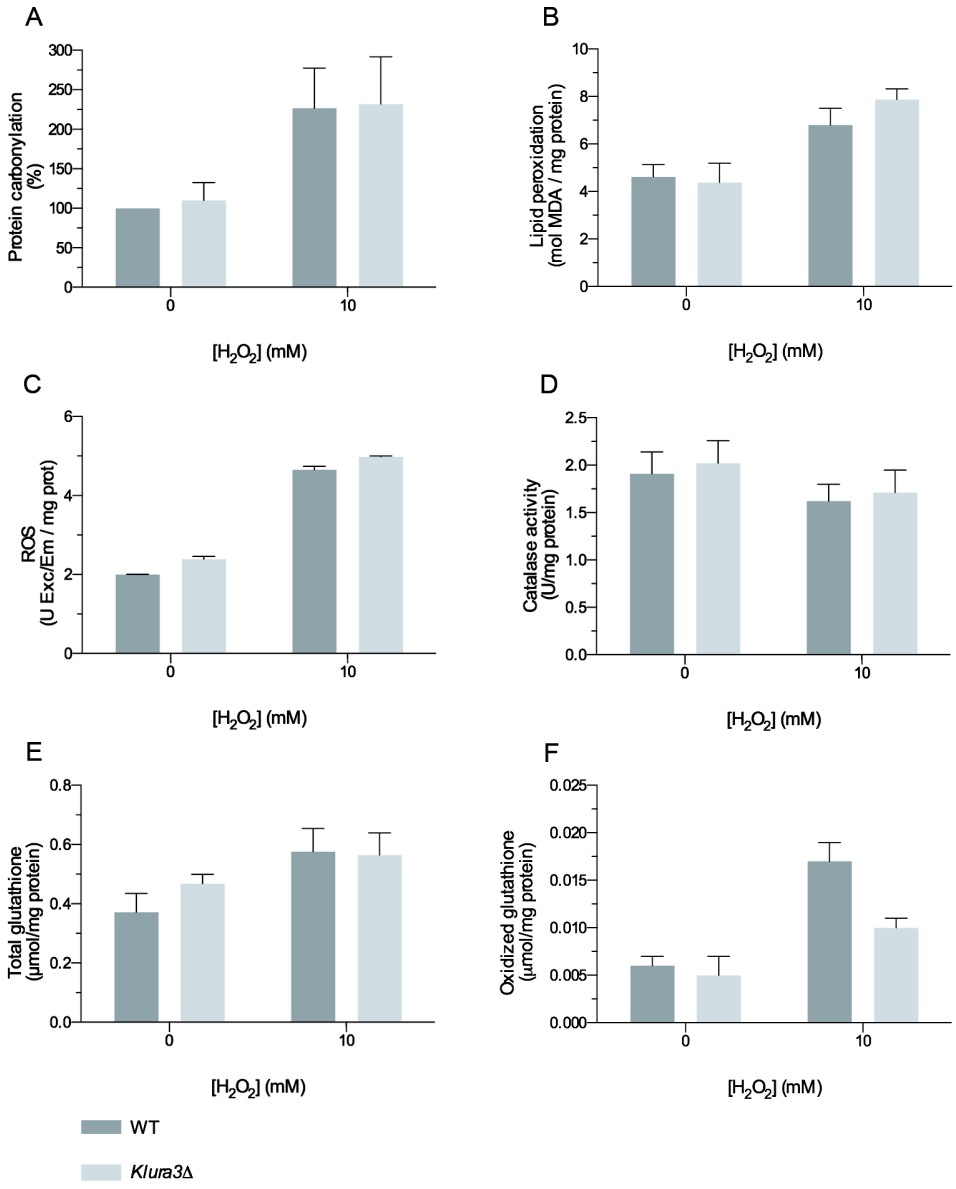


**Figure 3.17** Assessment of oxidative stress markers and antioxidant defenses in WT and *Klura3*Δ cells. Cells were grown in YPD to $OD_{600}$ = 0.6 and exposed to 10 mM $H_2O_2$ for 30 min. For evaluation of oxidative stress markers, the levels of protein carbonylation (**A**), lipid peroxidation (**B**) and ROS (**C**) were measured. Values for protein carbonylation are expressed as % of untreated WT. The antioxidant defenses measured were catalase activity (**D**) and total (**E**) and oxidized (**F**) glutathione levels. Values are mean ± SD of at least three independent experiments.

Even so, it is evident that at this growth stage and under these conditions a) the deletion of the *KlURA3* induces a distinct phenotype characterized by an

increased sensibility to $H_2O_2$ that was not observed in WT or other mutants with the pathway interrupted at the two earlier steps; b) even with the presence of the gene that codes for the other DHODase, independent deletion of *KlURA1 or KlURA9* promotes a phenotype similar to *Klura3*Δ whereas double DHODase deletion (*Klura1*Δ*Klura9*Δ) shows no difference to WT; c) an obvious relation exists between the deletion of *KlURA1 or KlURA9* along with *KlURA3* since the phenotypes of any of these individual mutants are reversed and a significant increase in the resistance to $H_2O_2$ is observed above WT values. In conclusion, these results suggest an interrelationship, which requires the three genes *KlURA1, KlURA9* and *KlURA3*, that may be involved in oxidative stress mechanisms or regulation.

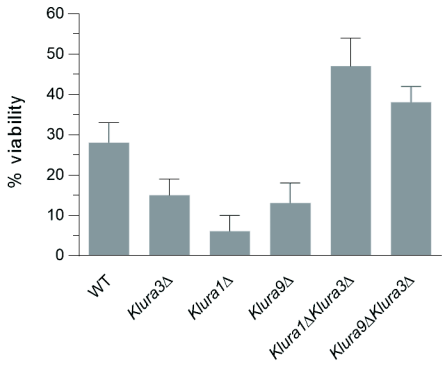


**Figure 3.18** Oxidative stress resistance of *K. lactis Klura1*Δ, *Klura9*Δ, *Klura1*Δ*Klura3*Δ e *Klura9*Δ*Klura3*Δ cells. Cells were grown in YPD to $OD_{600}$ = 0.6 and exposed to 10 mM $H_2O_2$ for 30 min. Cell viability was determined as described in methods. Results of WT and *Klura3*Δ from Figure 3.16 are included for comparison. Values are mean ± SD of at least three independent experiments.

### 3.2.2. Studies at later growth phases

Bearing in mind the possible application on orotic acid production of the new insights resulting from this part of the work, it was essential to further investigate oxidative stress resistance throughout growth. WT strain and *Klura3*Δ mutant were

then followed overtime from $OD_{600}$ = 0.6 (starting point, T0). Growth is shown in Figure 3.19A and the relevant time points at the bottom of Figure 3.19. Viability tests were performed at several time points and, since the concentration of $H_2O_2$ required to induce a visible effect increased substantially with culture growth, we chose to present data as the concentration of $H_2O_2$ required to produce 50% cell death ($LC_{50}$) after 30 min exposure (Figure 3.19B and Table 3.2). These assays were performed only up to 36h but seemed sufficient to demonstrate maximum values have been reached. Opposite to what was observed at T0, the mutant *Klura3*Δ exhibited an increased resistance to $H_2O_2$ at latter stages of growth in relation to WT. Maximum $LC_{50}$ for WT was 650 mM of $H_2O_2$, but 50% of *Klura3*Δ cells were able to survive a striking concentration of 3.6 M already at 24 h. To our knowledge, there is no other yeast with such level of resistance to $H_2O_2$. A possible biotechnological application of this attribute should be considered but we did not examine its potential since it falls out the scope of this work. Measurements of oxygen consumption of both strains were made to determine if it was elevated in *Klura3*Δ possibly due to an increased respiratory metabolism or a higher activity of KlUra9p DHODase. In that case, it would suggest an elevated ROS production and subsequent oxidative stress response that could help explain the resistance of *Klura3*Δ to $H_2O_2$. However, other factors must be associated with *Klura3*Δ phenotype since no relevant difference was observed compared to WT, and actually, a minor decrease in oxygen consumption was observed for *Klura3*Δ after 18 h (Figure 3.19C). Catalase activity was measured and a significant increase was observed for *Klura3*Δ, which at 24 h showed a catalase activity 4.5 fold higher to the observed in WT (Figure 3.19D). Catalase activity and $H_2O_2$ $LC_{50}$ throughout the growth clearly correlate. At 12 h (T1), the phenotypic divergence of *Klura3*Δ starts to be visible and maximal difference is already observed at 24 h (T2), after which, the values for both strains remain steady. These results suggest that the remarkable resistance of *Klura3*Δ to $H_2O_2$ may be attributed to the cellular catalase activity.

Cellular detoxification of $H_2O_2$ is mostly carried out by the enzymatic action of catalases and, as in *S. cerevisiae,* genes for both peroxisomal (*KlCTA1*) and cytosolic (*KlCTT1*) catalases are present in *K. lactis*. To determine the relative contribution of each of the *K. lactis* enzymes (KlCta1p and KlCtt1p) or the existence of other unknown catalase, several catalase mutants were generated

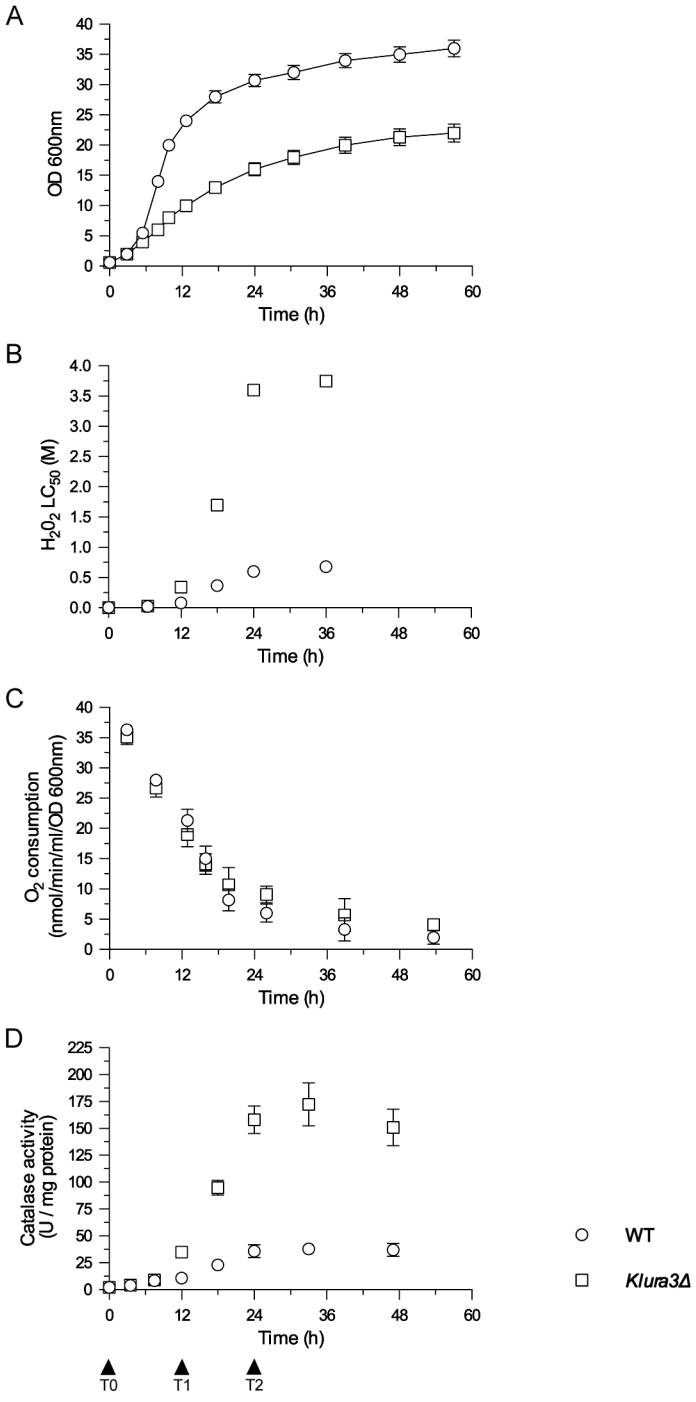


**Figure 3.19** Determination at several time points of *K. lactis* WT and *Klura3*Δ growth (**A**) of their resistance to $H_2O_2$ (**B)**, $O_2$ consumption rate (**C**) and catalase activity (**D**). Resistance to $H_2O_2$ was determined at each point by identifying the concentration required to induce 50% cell death ($LC_{50}$) after 30 min exposure (Table 3.2). Values are mean ± SD of at least three independent experiments.

**Table 3.2** Concentration of $H_2O_2$ required for 50 % cell death after 30 min exposure at several time points of *K. lactis* WT and *Klura3*Δ growth.

| | WT | | *Klura3*Δ | |
|---|---|---|---|---|
| Time (h) | $H_2O_2$ | %Viabilitity | $H_2O_2$ | %Viabilitity |
| 0 (T0) | 6 mM | 54 ± 7 | 3.5 mM | 54 ± 4 |
| 6.5 | 25 mM | 55 ± 9 | 30 mM | 54 ± 6 |
| 12 (T1) | 80 mM | 54 ± 4 | 340 mM | 48 ± 1 |
| 18 | 370 mM | 54 ± 4 | 1.7 M | 56 ± 3 |
| 24 (T2) | 600 mM | 53 ± 5 | 3.6 M | 46 ± 2 |
| 36 | 650 mM | 52 ± 6 | 3.7 M | 49 ± 3 |

and activity was measured at T1 and T2 (Figure 3.20). Single catalase mutants (*Klcta1*Δ and *Klctt1*Δ) had a cell growth similar to that of WT cells (Figure 3.20A) but a decreased catalase activity at both time points (Figure 3.20B). A very similar pattern, but at considerably higher levels of activity, was also observed for *Klcta1*Δ*Klura3*Δ and *Klctt1*Δ*Klura3*Δ when compared to *Klura3*Δ, despite no difference in growth. It seems that in WT and *Klura3*Δ, both KlCta1p and KlCtt1p are implicated in the catalase activity measured. For *Klura3*Δ in particular, the contribution of KlCtt1p seems to be greater than KlCta1p when maximum catalase activity was detected at T2 but the contrary was observed at T1 when the effect of a strong catalase activity is only starting to emerge. Catalase activity was not detected in neither of the mutants with both catalase genes deleted, *Klcta1*Δ*Klctt1*Δ and *Klcta1*Δ*Klctt1*Δ*Klura3*Δ, ruling out the existence of other catalase in *K. lactis*.

Growth of *Klcta1*Δ*Klctt1*Δ was similar to WT cells (Figure 3.20A) in spite of the absence of catalase activity. However, growth of *Klcta1*Δ*Klctt1*Δ*Klura3*Δ cells was significantly decreased when compared to that of *Klura3*Δ cells. This is probably related with the decrease of orotic acid production observed in the triple mutant, which was close to 20% [data not shown]. While in WT the catalases seem to be dispensable under the conditions used, its activity is essential to provide normal growth in *Klura3*Δ. The glutathione system also acts as a cellular defense mechanism in the elimination of $H_2O_2$ and may be sufficient to prevent its toxic effects in *Klcta1*Δ*Klctt1*Δ cells. On the other hand, it is possible that *Klura3*Δ is subjected to an abnormal amount of $H_2O_2$ and, therefore, catalase activity is

required for this mutant to cope with the oxidative stress and to efficiently produce orotic acid.

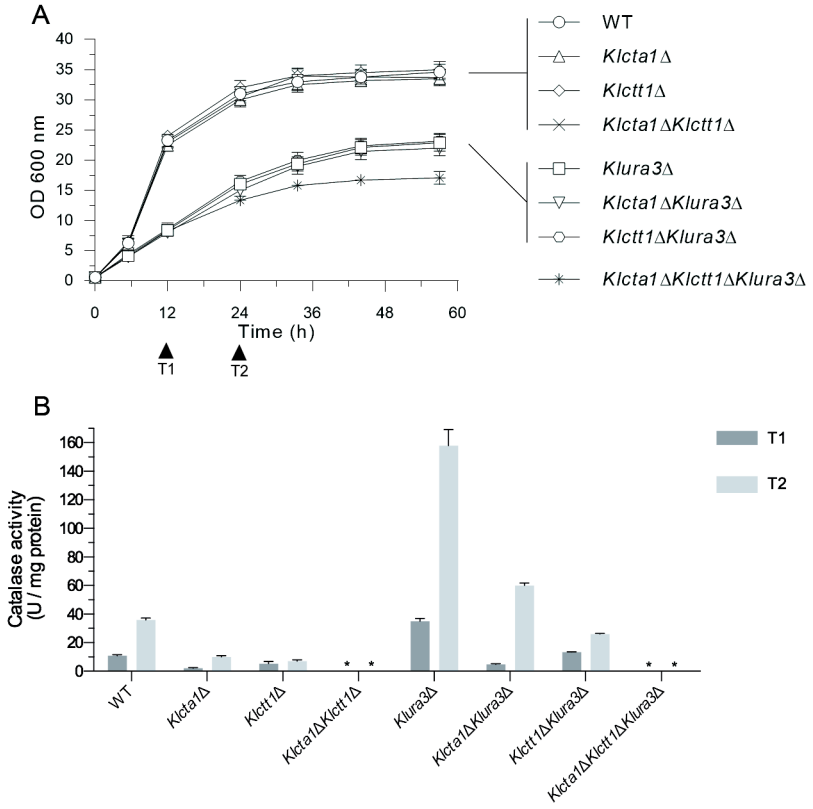


**Figure 3.20** Growth (**A**) and catalase activity at T1 and T2 (**B**) of WT and *Klura3*Δ derived mutants with single (*Klcta1*Δ or *Klctt1*Δ) or double catalase gene deletion. Symbol "*" represents no catalase activity detected. Results of WT and *Klura3*Δ from Figure 3.19 are included for comparison. Values are mean ± SD of at least three independent experiments.

To investigate if the phenotype found at later growth stages was unique to *Klura3*Δ and to evaluate if the putative link to the DHODases persisted, catalase activity was measured in other pyrimidines mutants at T1 and T2 (Figure 3.21). With respect to the pyrimidines mutants with interruption of the pathway at steps prior to that catalyzed by Klura3p, the *Klura2*Δ mutant that has the initial steps of the

pathway interrupted, was also included in this study together with *Klura1*Δ*Klura9*Δ and *Klura5*Δ. Growth of these three mutants was similar to that of *Klura3*Δ (Figure 3.21A). As observed in *Klura3*Δ cells, *Klura2*Δ, *Klura1*Δ*Klura9*Δ and *Klura5*Δ mutants showed a considerable increase in catalase activity at both T1 and T2 when compared to WT (Figure 3.21B). This increase was similar in the three mutants but they exhibited a catalase activity at T2 inferior to that of *Klura3*Δ cells. Additional deletion of any of the genes coding for the two distinct DHODases (*Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ) again altered the *Klura3*Δ phenotype (Figure 3.21B). Their effect was characterized by a drop in catalase activity that became close to values found at T2 in all other mutants besides *Klura3*Δ.

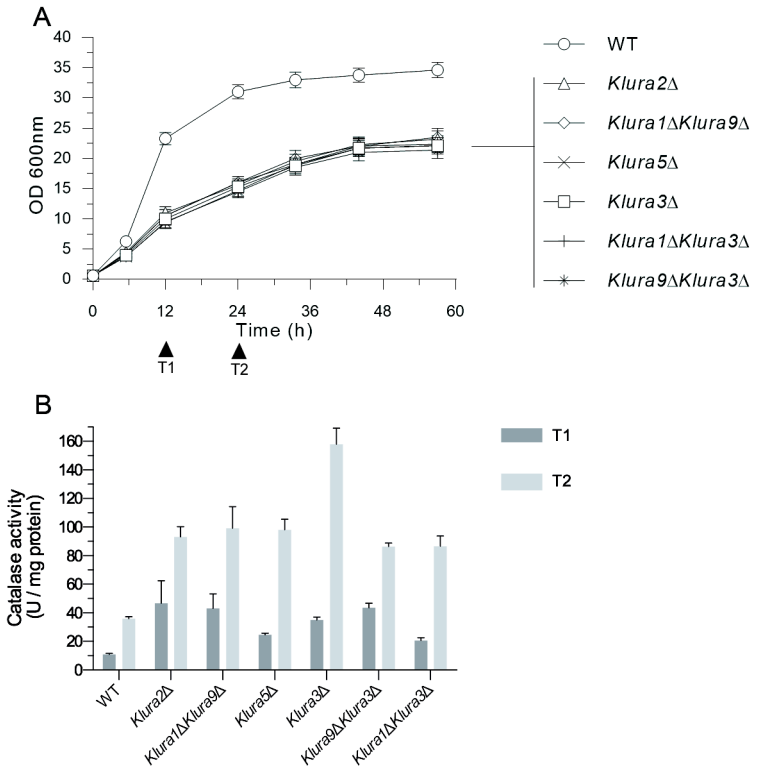


**Figure 3.21** Growth (**A**) and catalase activity at T1 and T2 (**B**) of pyrimidines mutants with interruption of the pathway at steps prior to that catalyzed by Klura3p (*Klura2*Δ, *Klura1*Δ*Klura9*Δ, *Klura5*Δ, *Klura1*Δ*Klura3*Δ and *Klura9*Δ*Klura3*Δ). Results of WT and *Klura3*Δ from Figure 3.19 are included for comparison. Values are mean ± SD of at least three independent experiments.

We have postulated that the presence of a DHODase linked to the mitochondria together with a probable higher DHODase activity in orotic acid producing mutants could be responsible for an increased ROS production leading to the induction of an oxidative stress response. Under the conditions used, the action of DHODases enzymatic step is apparently not playing any role in that effect since the orotic acid producing mutant, *Klura5*Δ, and the mutants with no DHODase activity, *Klura2*Δ or *Klura1*Δ*Klura9*Δ, shared the same phenotype. An apparently similar phenotype was also observed when each DHODase gene was deleted in the orotic acid producing mutant, *Klura3*Δ.

These results show that a non-functional *de novo* pyrimidines synthesis pathway leads to a strong increase in catalase activity regardless of the step interrupted. However, a more marked increase of catalase activity at T2 was observed in *Klura3*Δ, suggesting that specific mechanisms may be involved in this particular mutant. Furthermore, the phenotypes of *Klura3*Δ were similarly altered, but in apparently opposite directions when comparing T0 (increased resistance to $H_2O_2$) with T2 (decreased catalase activity), by further deleting either *KlURA1* or *KlURA9*, which code for two enzymatically distinct DHODases. This indicates that further deletion of either DHODases genes may be disturbing a possible connection that requires the three genes. The apparently opposite phenotypic outcomes at T0 and T2 may be a consequence of such putative connection being played at distinct metabolic phases of cells since, with respect to pyrimidines synthesis, at T0 cells are most likely using the salvage pathway and at T2 the *de novo* pyrimidines synthesis may be fully active.

# CHAPTER IV

## General Discussion and Future Perspectives

## 4.1. Orotic acid production by *K. lactis* mutants

### 4.1.1. Production using YPD based media

Studies on flasks and bioreactor using YPD based media demonstrated that the *K. lactis Klura3*Δ and *Klura5*Δ mutants are able to accumulate in the growth medium considerable amounts of orotic acid. The concentration of orotic acid, as well as the time required to achieve it (6.7 g/L after 96 h), are comparable to the ones obtained with a similar mutant strain (ATCC 14275) of the industrially valuable *C. glutamicum* (8.2 g/L after 120 h). The studies on YPD based media were decisive for the initial validation of the biotechnological potential of the *K. lactis* pyrimidines mutants as orotic acid producing microorganisms. The amounts obtained were highly relevant and efforts should be taken for further medium and strain optimizations that may ultimately lead to an even more efficient and industrially competitive production process when compared to *C. glutamicum*. Those studies were also proven very important regarding the information attained. As discussed below, it could be used in the subsequent optimizations steps and the development of parallel lines of research to deepen the understanding of key mechanisms that could be improved for orotic acid production.

Production scale-up using *K. lactis* has been made and was proven useful for the biotechnology industry. According to the tests on the production of orotic acid in a bioreactor we foresee that the scaling-up of this process should be simple to accomplish and only general issues regarding adaptation to large tanks would be required. More importantly was the high reproducibility of orotic acid production in flask and bioreactor once the optimal conditions in both cases have been determined. Thus, flask experiments to fully elaborate the composition of a production medium are expected to be replicated in the bioreactor at any stage of the development process. This would eliminate the need for an expensive line of parallel bioreactors or minimize time-consuming experiments in a single bioreactor by simply testing several conditions in a single experiment using flask cultures.

In addition to media optimization, this feature is also an advantage for the genetic optimization of *K. lactis* mutant strains. *K. lactis* is closely related to the well-studied yeast *S. cerevisiae*, its genome has been fully sequenced and many

specific genetic tools have been developed. Identification of genetic targets and directed mutagenesis could be easily achieved and already assessed with this YPD / MOPS flask system. Differences in the efficiency of orotic acid production observed in this media should be also visible using other optimized media that could be later accomplished. Moreover, it could also be further and easily adapted for screening in high-throughput systems of mutants that have been randomly generated. We demonstrated that production in a closed and controlled 2 L bioreactor with 1 L media could be successfully downscaled to flasks containing 20 ml media. Testing if sufficient media aeration could be achieved using 24, 48 or even 96 deep well plates specifically developed for cell growth, the system could be used for screening a great number of mutants in a short period of time through a less laborious process. Instead of using the time-consuming HPLC procedure for the accurate determination of orotic acid concentration, easy and quick measurements at $OD_{278}$ of all cell free samples in a microplate reader should be sufficient to evaluate rate of production and final amounts obtained.

*K. lactis* possesses a DHODase (KlUra9p) linked to the mitochondria. We showed that best production of orotic acid occurred under higher aeration conditions above the ones that are sufficient for regular growth. Using those conditions, we demonstrated that the KlUra9p is the most relevant DHODase. It is feasible that the particular features of the step catalyzed by the DHODases in *K. lactis* are the major contributors for its exceptional ability to produce large amounts of orotic acid. Since this is the limiting step of the *de novo* pyrimidines biosynthetic pathway, genetic engineering of orotic acid producing mutants to increase expression of KlUra9p may reveal an important improvement in the production system.

Since it is very unlikely that orotic acid crosses the cell membrane of *K. lactis* by diffusion, it is probably making use of a transport system present in the membrane. The identification of the transporter involved in the secretion of orotic acid is crucial for the understanding of the entire process and may reveal another distinct attribute of *K. lactis* that is determinant for the ability of this yeast to accumulate large amounts of orotic acid in the growth media. Also, it would be an important candidate for genetic improvement of *K. lactis* strains by increasing its presence on the membrane.

Optimum pH for *K. lactis* growth is known to be pH 4.5 – 5 but the optimal pH for orotic acid production was found to be at or close to pH 7. However, growth at neutral pH by *Klura3*Δ mutant cells was not affected. Optimal production of biomass and orotic acid could be achieved at same pH conditions favoring therefore the process yields. It would be important to unravel if pH affects specific mechanisms involved in the production of orotic acid or is enhancing the production and accumulation by promoting its secretion across the membrane.

According to the studies performed in the bioreactor, *Klura3*Δ mutant cells continued to accumulate orotic acid in the media after glucose has been depleted and growth stopped. It is possible that *de novo* pyrimidines pathway in this mutant is not effectively down regulated after growth has ceased and cells continue production as long as substrates are readily available or generated from other carbon sources, such as metabolites derived from glucose degradation or other amino acids. We should also recognize that the mechanism of orotic acid secretion might be playing the major role on this effect. Transport across the membrane could be a limiting factor and once accumulated inside the cells, the orotic acid would continue to be secreted to the media long after its production has stopped or became residual.

Yet, we do not know whether it is any specific metabolic or regulatory feature of the *de novo* pyrimidines biosynthetic pathway, the presence of an unknown transporter or other distinctive characteristic of *K. lactis,* that account for the amount of orotic acid produced and accumulated in the growth media by the *Klura3*Δ and *Klura5*Δ mutants. The uncover of the exceptional feature or the possible interplay between several particular characteristics may provide a main focus for the optimization of the orotic acid production system.

### 4.1.2. Production using low cost industrial media

Studies on the production of orotic acid using YPD based media were important for the initial assessment of the potential of *K. lactis* mutants as orotic acid producing microorganisms. Growth and production on the YPD system can also provide the standards for further research in the fundamental aspects that are involved in the production and secretion of orotic acid. However, it would not be

used at an industrial production level due to its costs as a large-scale raw material. The efficiency of production using industrial by-products as low cost constituents of the production media had to be evaluated to confirm its value for the biotechnology industry.

We have chosen molasse as the main carbon source but the use of CWP must not be discarded. The unique ability of *K. lactis* to use lactose together with the local availability and price of unprocessed cheese whey or CWP may end up making it more attractive than molasse. With respect to the nitrogen source, urea was found to be the best option since it clearly led to a higher orotic acid production when compared to $(NH_4)_2SO_4$. We identified CSL as a very important constituent since its use seems to supplement all other nutrients required for the growth of *K. lactis* mutant strains. Growth of *K. lactis Klura3*Δ in a buffered media containing 4% (v/v) CSL, 3.4% (v/v) molasse and 0.25% (w/v) urea, which provided a combined total of 20 g/L sugars, led to the production of 4.5 g/L of orotic acid after 80 h. Under similar growth conditions and culture time the production in YPD media containing 20 g/L glucose was shown to be only 3.4 g/L. Regarding the *C. glutamicum* mutant strain ATCC 14275 it was claimed that it can produce in flask cultures 8.2 g/L of orotic acid using low cost production media. However, to reach such values it takes 120 h and seems to require 100 g/L of sugars. This indicates that the production obtained with *K. lactis Klura3*Δ is extremely promising taking that full media optimization has not been performed.

The results already obtained enforce the biotechnological potential of pyrimidines mutants of *K. lactis* to produce high amounts of orotic acid through a low cost industrial process. We considered they should be the starting point for a comprehensive work to design and execute experimental matrixes in order to identify and determine the optimum concentration of other general and specific nutrients relevant for orotic acid production. As previously demonstrated with YPD based medium, a multitude of media composition could be easily and exhaustively tested in flasks, which would then be closely replicated in the bioreactor expecting only minor or straightforward adaptations. For most of the compounds to be tested in flasks, such as vitamins and inorganic salts, ordinary growth conditions and equipment could be used. Bicarbonate is one of the initial substrates of the pyrimidines pathway and it is a cheap raw material, mostly as calcium carbonate (limestone powder) but also as sodium bicarbonate, which makes it one of the

most promising nutrients to be tested. Bicarbonate can be used not only as substrate but has the advantage of serving as a pH buffer at neutral pH and could therefore diminish the requirements or even completely eliminate the need to use the expensive MOPS buffer for pH stabilization in flask cultures. However, to maintain bicarbonate in solution, a specialized $CO_2$ growth chamber would be required to allow a controlled and closed environment similar to what can be achieved in a bioreactor.

However, prior the experimental work regarding optimization of the production media, *K. lactis Klura3*Δ or *Klura5*Δ should be further genetically manipulated to address the inhibition of orotic acid production. A genetically modified strain less prone to inhibition would significantly alter the limitations for CSL utilization, and consequently, the requirements for all other media constituents. Using such an optimized strain, it is reasonable to consider that higher concentrations of CSL would be allowed in solution having less interference in orotic acid production. Taking into account the strong growth achieved with this industrial by-product, we consider that addition of concentrated CSL through a fed batch system able to maintain optimum and non-inhibiting concentrations, the needs for other media constituents could be considerably diminished. Ultimately, it is possible to consider that a fed batch system with a production media composed solely of cheap CSL could be achieved.

The presence and concentration of uracil and related pyrimidines in CSL has not been determined and full analytical examination should be performed but according to the strong growth observed in CSL by the pyrimidines-requiring *K. lactis Klura3*Δ mutant, it is most likely rich in such molecules. As in *S. cerevisiae*, the pyrimidines salvage pathway is expected to use uracil and the newly formed UTP may in turn inhibit the first step of the *de novo* pathway in *K. lactis* as well. Initial studies on the mechanisms of the feedback inhibition of CPSase and ATCase activities of Ura2p were made possible by the use of the uracil analogue, 5-fluorouracil (5-FU). *S. cerevisiae* mutants generated by UV mutagenesis that have been selected according to their increased resistance to 5-FU presented a decreased sensitivity to UTP inhibition [191, 192]. Mutations at several amino acids positions in Ura2p were identified and shown to completely eliminate the inhibition of ATCase activity by UTP. Since these amino acids are conserved in *K. lactis* KlUra2p we should test the effect of their substitution on

orotic acid production. Screening of randomly generated *K. lactis* mutants for their resistance to 5-FU should also be undertaken since mechanisms of inhibition in *K. lactis* may be different. Also important, it may reveal mutations of KlUra2p that could also abolish or at least diminish the inhibition of CPSase activity as well.

## 4.2. Relation of the *de novo* pyrimidines biosynthetic pathway with oxidative stress response in *K. lactis*.

Our results show that interruption of the *de novo* pyrimidines pathway in *K. lactis* affects oxidative stress resistance and catalase activity. The increase of catalase activity in these mutants may result from a specific adaptive response or from a general oxidative stress response. For future work though, it is important to distinguish two general phenotypes. One seems to result from the interruption of the *de novo* pathway up to the fifth step (*KlURA5*), since those mutants equally evolved from $H_2O_2$ resistance similar to WT at early exponential phase (T0; $OD_{600}$=0.6) to an increased catalase activity at a later growth phase (T2; 24 h after T0). It seems reasonable to consider that interruption of the *de novo* pyrimidines pathway is merely promoting a particular metabolic mechanism or pathway containing a redox reaction that indirectly leads to an increased production of $H_2O_2$, which would in turn signal to an oxidative stress response.

The other phenotype seems to result from the specific deletion of the *KlURA3* gene (interrupts the sixth step of the pyrimidine biosynthetic pathway) and may be related with a disturbance in an existing relation that requires *KlURA3, KlURA1 and KlURA9*. The *Klura3*Δ mutant showed lower resistance to $H_2O_2$ at T0 and higher catalase activity at T2 (even when compared to that of any other of the pyrimidines mutants tested) associated with a striking increase of oxidative stress resistance. An additional deletion of any of the distinct DHODases genes led to an increase of $H_2O_2$ resistance at T0 but a lower catalase activity at T2. Moreover, single deletion of *KlURA1 or KlURA9* also induced a decrease in the resistance to $H_2O_2$ at T0, as observed in *Klura3*Δ. We should acknowledge that these effects may result from an alteration of an existing regulatory cross-talk between *de novo* pyrimidines pathway and the oxidative stress response that is dependent on the presence of the three genes. Further studies should be carried out to unravel the

nature of this putative relation and it would be important to understand if it relies on a direct or indirect involvement of the DHODases (KlUra9p and KlUra1p) and OMP decarboxilase (KlUra3p) and if other regulatory proteins or a specific effector metabolite, such as the pyrimidine intermediate OMP, are involved.

The crucial and initial steps to continue with these studies would be to perform in all of *K. lactis* mutants already produced a comprehensive and full analysis on the redox status, oxidative damage, activity and gene expression of selected antioxidant defenses at T0 and T2. It should be determined the time when the shift from a possibly inhibited to a fully active *de novo* synthesis occurs and use it for the analysis instead of T1. The generation and inclusion of the double mutants *Klura1*Δ*Klura5*Δ and *Klura9*Δ*Klura5*Δ in this investigation must be considered.

We presented the hypothesis that overproduction of orotic acid through the activity of the putatively mitochondrial KlUra9p DHODase would create additional oxidative pressure and therefore possibly compromise cell performance and production efficiency. Indeed, that seems to occur when both catalases genes are deleted in *Klura3*Δ. However, the high level of catalase activity and the cell viability at extremely high concentrations of $H_2O_2$ suggests that the *K. lactis Klura3*Δ can effectively maintain the normal cellular redox balance under high orotic acid production conditions. Also, on the contrary to *S. cerevisiae* that under oxidative stress redirects glucose equivalents from glycolysis to the pentose phosphate pathway that is normally less active, *K. lactis* preferably uses the pentose phosphate pathway to consume glucose. The NADPH generated from the pentose phosphate pathway is decisive upon cellular exposure to an oxidative stress since it provides the reducing potential for most antioxidant and redox regulatory enzymes. Together with the observations of the strong and effective response to $H_2O_2$ exposure, it is possible that these features, that are most likely related, may also contribute to the robustness of *K. lactis Klura3*Δ as an excellent biological system for the production of orotic acid.

## 4.3. Concluding remarks

Using YPD based media, growth conditions for the production in flask cultures were optimized and a high reproducibility with the bioreactor system was achieved, thus validating the use of the flask set-up for straightforward optimization of production media and strains.

CSL and urea have been identified as promising candidates for the development of a low cost production media. CSL alone was shown to sustain an excellent growth of the pyrimidines-requiring *Klura3*Δ mutant but due to the inhibitory effect on orotic acid production its use was limited to low concentrations and was regarded as an important supplement to molasse or CWP. A decreased sensitivity to inhibition of the production strains should therefore be accomplished by genetic modification to improve production yields and allow the development of a cost effective CSL based media.

We observed that the DHODase putatively linked to the respiratory chain is essential for the production of orotic acid, on the contrary to the cytosolic DHODase, and best production demands an oxygen supply above the levels that are sufficient for optimum cell growth. Also, a putative involvement of both DHODases and Klura3p with the oxidative stress response seems to occur. However, despite our results suggest that cells were indeed responding to an oxidative stress, other factors may be involved because interruption at any step of the *de novo* pyrimidines biosynthetic seems to be inducing a response. It is possible that even higher levels of orotic acid production would not pose a major problem to the cells regarding the maintenance of the redox balance.

In this study, a new feature of *K. lactis* with high biotechnological potential has been discovered. Optimization of strains and growth conditions may establish the yeast *K. lactis* as a solid alternative to the bacteria *C. glutamicum* for the production of orotic acid.

# CHAPTER V

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