

Heterologous products from the yeast *Kluyveromyces lactis*: exploitation of *KIPDC1*, a single-gene based system

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Heterologous products are frequent applications of microbial technology. Depending on the product, the host-vector system has to be chosen in order to fulfill process requirements. This short review summarizes published and unpublished results obtained for the production of heterologous metabolites and proteins from a single host, the yeast *Kluyveromyces lactis*, and by means of tools – promoter, mutant strains, regulators – uniquely from the single gene *KIPDC1*. We will describe the production of lactic acid and various bacterial, viral, fungal and mammal proteins or enzymes: β -galactosidase, Hepatitis C viral protein c-33, laccase, glucoamylase and interleukine-1 β .

Keywords recombinant proteins; lactic acid; fermentation, pyruvate decarboxylase, expression vectors

1. Introduction

A typical application in microbial technology is the heterologous production of metabolites or proteins. This procedure implies that a host strain is genetically modified in order to synthesize a protein or enzyme that is not encoded by its genome and the final goal of such manipulation might be the heterologous protein itself or a new metabolite that is produced by the activity of the heterologous enzyme on an available cellular substrate. Usually the choice of the hosts for the heterologous protein production is done with the aim to increase the amount and purity of the protein obtained and to reduce process operations and costs of possible industrial productions. These advantageous perspectives make heterologous protein production a common practice of applied research. However, drawbacks are often encountered in developing new production systems, depending on a large variety of problems and including specific characteristics of the protein to be produced and the host chosen for the production. These facts indicate that there is not a host universally suited to the production of any heterologous protein. Usually, first attempts for heterologous productions are made with organisms currently regarded as model organisms, like *Escherichia coli* among prokaryotes, and *Saccharomyces cerevisiae* among lower eukaryotes.

There are many factors influencing the final result of a genetically modified microorganism in producing a heterologous protein, in terms of product amount and quality, *i.e.* purity and/or biological activity. They are: dosage of the heterologous gene and stability of the exogenous DNA; level of transcription of the heterologous gene and stability and efficiency of translation of the corresponding mRNA; folding, post-translational modifications and turnover of the recombinant protein; efficiency of the leader peptide sequence that drives the protein through the secretion pathway and protein glycosylation.

Although a large amount of basic knowledge, molecular tools and protocols are available for model organisms, they may not fulfill all the requirements for a scalable process of heterologous protein production. This is particularly true for the production of biologically active eukaryotic proteins from *E. coli*, because they require specific post-translational modifications. The production from *S. cerevisiae* has drawbacks too, such as low biomass yield on glucose medium – that is the common carbon source in industrial media – because of its fermentative metabolism, and very poor secretion efficiency of proteins. For these reasons, in the past two decades, alternative yeast species with attractive properties for

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heterologous protein production have been developed [1]. Among them, *Pichia pastoris* is the most widely used [2]. However, also *Kluyveromyces lactis* has characteristics of outstanding interest [3].

2. The yeast *Kluyveromyces lactis* as a host for heterologous products

K. lactis is one of the few yeasts able to grow on lactose as sole carbon source and has been cultivated in industrial plants for lactase (β -galactosidase) production, thus allowing the development of basic knowledge about the lactose metabolism and about large scale cultivation of this organism. In the 90's, heterologous proteins started to be synthesized with this yeast [4-11] and now more than 40 examples of lab-scale productions are available. Calf prochymosin is currently produced from *K. lactis* at the industrial scale for cheese manufacturing [12]. Molecular tools (vectors) for the introduction by transformation of exogenous DNA in *K. lactis* cells have been constructed. To perform transformation, it's necessary to have vectors harboring genetic markers for the selection of transformed cells, suitable host strains to be transformed, protocols for efficient transformation and for analysis of the transformed clones.

Vectors are of basic importance for the introduction and maintenance of the heterologous gene in the host cells. They can be propagated in the progeny by integration in the cell chromosome or by autonomous replication. Both kind of vectors are available for *K. lactis*. Integrative vectors are very stable, which is an important requirement for scalable production processes, but their copy number (dosage) is usually low. Integrative vectors for the expression of genes in *K. lactis* are now on the market [3]. On the contrary, replicative vectors are maintained at high dosage in the cells, but they are unstable and need additional genetic functions for stability. These functions are chromosomal centromeric sequences (CEN vectors), which increase stability but reduce copy number, or episomal functions from the naturally occurring yeast circular plasmids [13]. Episomal vectors are stable and high copy number. The natural circular plasmid of *K. lactis* is pKD1 [14]. Many heterologous proteins from *K. lactis* have been produced from pKD1-based vectors [3].

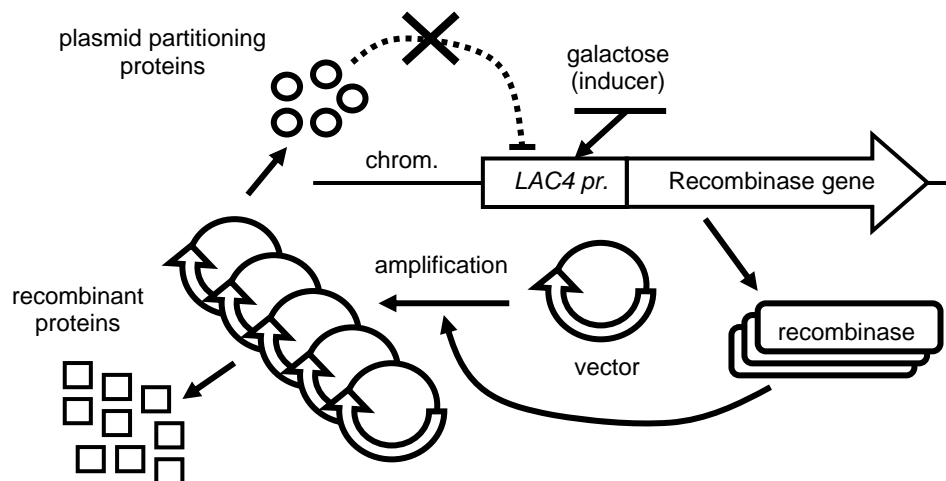


Fig. 1 An inducible system for plasmid copy number amplification in *K. lactis*.

The copy number of yeast circular plasmids is regulated by plasmid-encoded functions [15-18]. The activity on plasmid molecules of the plasmid-encoded site-specific recombinase during DNA replication induces copy number increase. The high copy number increases the cellular concentration of two plasmid-encoded proteins which, at normal dosage, are involved in the mitotic segregation of plasmid molecules. Proper segregation, also called partitioning, is a prerequisite for plasmid stability. The excess of these proteins allows them to play their secondary function: the repression of the recombinase gene

expression. This leads, in turn, to a progressive copy number decrease. The overall situation is thus a fluctuation of plasmid copy number around a physiological average value. In *K. lactis*, a host-vector system has been constructed in order to obtain a regulated system for copy number increase of expression vectors for heterologous proteins [11]. The principle was to subtract a copy of the plasmid recombinase gene from the repression by the plasmid partitioning proteins (Fig. 1). To this purpose, the recombinase gene was integrated into genome under the control of the lactose/galactose inducible promoter of the *LAC4* (β -galactosidase) gene. This new strain was transformed with pKD1-based vectors harboring the heterologous genes of glucoamylase (*GAM*) from the yeast *Arxula adenivorans* and of the human interleukin 1 β (IL-1 β). An increase of *GAM* and IL-1 β production was obtained in parallel with the vector copy number increase, upon induction of integrated recombinase expression by galactose.

Besides vector's properties, other elements have been tested and have been found to affect profoundly the production of heterologous proteins in *K. lactis*. Proteins to be secreted have been tested with leader peptides of different origins or with their own secretion signals. A comparative analysis is reported in reference [12]. Most of them have been proved to be efficient: usually, the native signal sequence of the heterologous protein is functional in driving the polypeptide through the secretion pathway, indicating the great flexibility of this mechanism in *K. lactis*.

The expression of the heterologous genes has been achieved by means of regulated or constitutive promoters from both *K. lactis* and *S. cerevisiae*. The choice of the promoter is conditioned by the necessity to improve protein production by increasing the level of transcription of the heterologous gene or by the requirement of a regulated expression. The latter might be of critical importance when the product is detrimental for cell health. In such cases, the production process is divided into two phases: an initial step in which biomass is produced and a second phase in which the heterologous gene is expressed and the protein is synthesized. Commonly used promoters are the *K. lactis LAC4* and the *S. cerevisiae PGK1* (Phospho-Glycerate Kinase gene), *PHO5* (acid PHOSphatase) and *GAPDH* (GlycerAldehyde Phosphate DeHydrogenase encoded by *TDH3* gene). *LAC4* and *PHO5* are inducible promoters by lactose/galactose and low phosphate concentration, respectively. *PGK1* and *TDH3* are glycolytic genes, usually highly expressed on glucose media.

The host strain is another important element of the production system. The intrinsic genetic differences, present among naturally occurring *K. lactis* strains and among laboratory strains deriving from mutagenesis and genetic crosses, greatly affect production [4]. In addition, specific mutant strains might be generated and selected for production efficiency. To date, mutants of the secretion pathway, of protein folding and mutants of gene expression regulation have been isolated and successfully employed in heterologous protein production [19-23]. All the above information, also reviewed in [3], confirms that there is not an universal system for this technological application: rather, elements of different origin can be put together to constitute an optimal but heterogeneous system to produce or, more often, to improve the production of an individual protein.

3. Properties of the fermentative metabolism of *K. lactis*

K. lactis is able to use lactose as carbon source thanks to a lactose permease, encoded by the gene *LAC12*, which allows the uptake of the disaccharide, and a β -galactosidase, encoded by the gene *LAC4*, which hydrolyzes lactose into glucose and galactose. Galactose is then metabolized similarly to *S. cerevisiae* [24]. *K. lactis* and *S. cerevisiae* use differently glucose of standard media (2% glucose) in growth conditions commonly used in laboratory (Erlenmeyer flask or tube cultures, without aeration devices and oxygen control) which limit oxygen availability. In fact, *K. lactis* uses glucose through the respiratory metabolism and very little ethanol is produced by fermentation. On the contrary, *S. cerevisiae* is a typical 'fermentative' yeast and it is subjected to the Crabtree effect, also called glucose repression, that represses respiration, gluconeogenesis and the use of alternative carbon sources. The physiology, metabolic regulation and enzymatic content of *S. cerevisiae* are especially suited to the fermentative metabolism. During fermentation, this yeast uptakes and metabolizes glucose at high rate through glycolysis, producing biomass at low yield and large amounts of ethanol. Coherently to this, it has at

least two genes coding for low-affinity glucose permeases, which are those expressed at high glucose concentrations and specifically involved in the fermentative metabolism. Glycolytic genes are also duplicated, to ensure metabolic flux. For this reason, the growth of *S. cerevisiae* on glucose media in the presence of mitochondrial drugs which block respiration, like Antimycin A, is always possible, even when low-affinity permeases or glycolytic genes are mutated, because of the presence of second structural genes allowing fermentation. This behavior is called RAG (Resistance to Antimycin A on high Glucose concentration) and *S. cerevisiae* is a Rag⁺ organism, because of its genetic redundancy.

On the contrary, in *K. lactis* mutations of the low-affinity permease or of glycolytic genes lead to the Rag⁻ phenotype [25], because these genes are in single copy and their mutation completely blocks fermentation. The Rag⁻ phenotype is a powerful tool for the isolation of structural and regulatory genes of glucose metabolism. Among the RAG genes, *RAG1* is the one encoding the low-affinity glucose permease: *rag1* mutants are naturally occurring strains of *K. lactis* [26, 27]. On the other hand, induced mutagenesis [25] allowed the isolation and characterization of many other RAG genes, which code for glycolytic enzymes, proteins involved in glucose transport or sensing, fermentative enzymes and transcription regulators.

4. The Pyruvate Decarboxylase gene of *K. lactis*

In *K. lactis* there is a single gene, named *KIPDC1*, coding for pyruvate decarboxylase (PDC), the first enzyme of the ethanologenic pathway [28]. In the following sections, we will survey the regulatory characteristics of *KIPDC1*, the physiology of strains deleted for this gene, and we will describe how these peculiarities could be exploited for the synthesis of heterologous products from *K. lactis*.

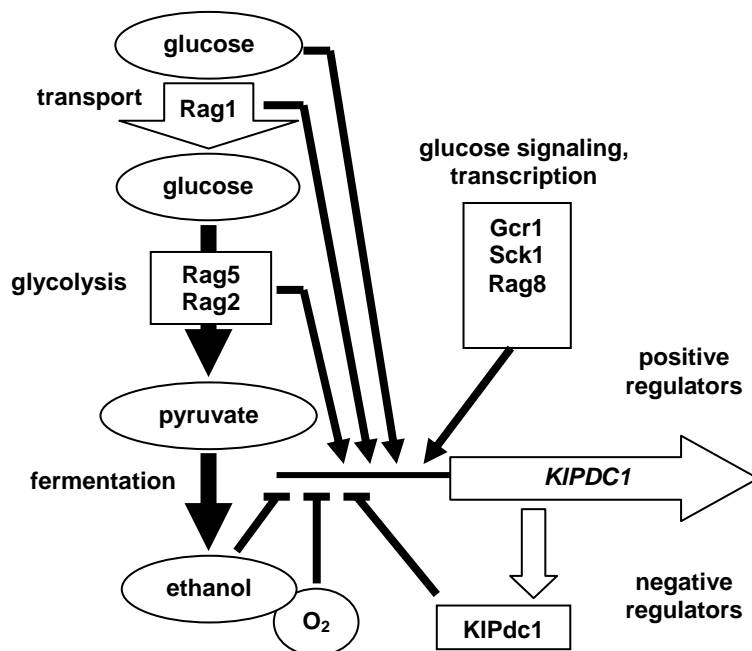


Fig. 2 Transcription regulation of the *KIPDC1* gene. Rag1: low-affinity glucose permease; Rag2: phospho-glucosomerase; Rag3: regulator of *KIPDC1* (see text); Rag5: hexokinase; Rag8: casein kinase (transducer of the glucose signal); Sck1: a suppressor of casein kinase mutations (transducer of the glucose signal); Gcr1: transcription factor of glycolytic genes.

Mutations in *KIPDC1* are allelic to the *rag6* complementation group. In *S. cerevisiae*, the PDC enzymes are encoded by two functional structural genes, *PDC1* and *PDC5*, and a gene, *PDC6*, that can be activated in some mutant strains [29]. PDC genes are present in many yeasts, in plants and in some biotechnologically important bacteria, like the ethanol producing *Zymomonas mobilis*. PDC enzymes are highly conserved among organisms in primary structure and functional domains. They require thiamine diphosphate (ThDP) and Magnesium (Mg^{2+}) for decarboxylase activity.

The expression of *KIPDC1* is regulated at the level of transcription by various gene products and metabolites (Fig. 2). They are positive or negative regulators of transcription. Glucose is the main inducer of *KIPDC1* transcription: addition of 2% glucose to *K. lactis* cell cultures increases at least 20 fold the level of *KIPDC1* mRNA within some minutes [28], making this gene one of the more expressed on glucose and its promoter one of the more powerful inducible element in fermentative conditions. Similarly to glucose, oxygen depletion (hypoxia) is able to induce many fold *KIPDC1* transcription [30]. Abundance of fermentable sugars like glucose, and oxygen shortage are typical environmental conditions favorable to the fermentative metabolism and, consequently, to the production of ethanol from pyruvate by the activity of PDC and alcohol dehydrogenase (ADH) enzymes. In *S. cerevisiae*, PDC genes are induced by glucose but, differently from *K. lactis*, they are not induced by hypoxia [31]. Ethanol, the final product of fermentation, acts as a repressor of *KIPDC1* transcription [32].

The transcription of *KIPDC1* is negatively regulated also by the KIPdc1 protein [32]. This mechanism is called autoregulation and has been first described in *S. cerevisiae*, especially in mutant strains with a PDC enzyme that has lost its metabolic activity but has maintained its regulatory property [33]. In *K. lactis* autoregulation has been demonstrated with *KIPDC1* deleted strains (*Klpdc1Δ* strains) transformed with vectors harboring the reporter gene *LacZ* (the *E. coli* β -galactosidase gene) under the control of *KIPDC1* promoter sequence. In the *Klpdc1Δ* transformants the β -galactosidase activity is 10 fold higher than in the wild type transformants [32].

The Rag3 protein is a positive regulator of *KIPDC1* transcription [34]. The functional homologue of *RAG3* in *S. cerevisiae* is *PDC2*. In both yeasts, these genes regulate PDC expression and the expression of genes involved in thiamine biosynthesis. The *rag3* mutants have two phenotypes: they are unable to grow on glucose plus Antimycin A (Rag⁻) and are auxotrophic for thiamine (Thi⁻) [35]. These phenotypes are not necessarily linked. In fact it has been possible to select, by UV mutagenesis of a *rag3* strain, a second mutation able to suppress the Rag⁻ phenotype but not the Thi⁻ phenotype. This reversion to the Rag⁺ phenotype is due to the mutant allele *Klgcr1-1* of the glycolytic transcription factor KIGcr1. The *Klgcr1-1* mutation is recessive, but is able to restore transcription of *KIPDC1* to the wild type level [36].

Transcription of *KIPDC1* also requires elements of the cellular pathway that sense the presence of glucose and transmit the glucose signal to the transcription machinery of the cell. Among them, we find the signal transducers Rag8 and Sck1 (Fig. 2). The low-affinity glucose transporter, the hexose kinase and the phosphoglucose isomerase - Rag1, Rag5 and Rag2, respectively - are also required for full expression of *KIPDC1*.

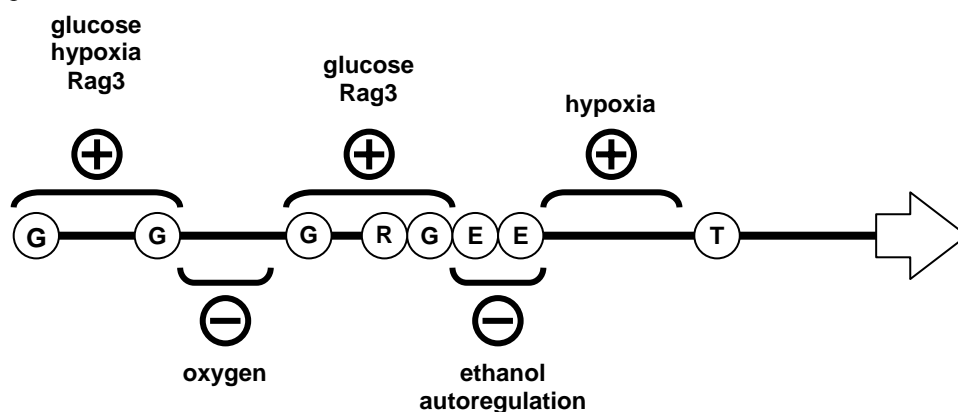


Fig. 3 Regulatory elements present in the *KIPDC1* promoter. (+) and (-) indicate sequences of the promoter involved in positive and negative regulation, respectively. The white arrow is the start of KIPdc1 coding region. G, R, E and T indicate the positions of recognition sites of the transcription factors Gcr1 (G) and Rap1 (R), the *ERA*-like sequences (E) and the TATA box (T).

The *KIPDC1* entire promoter and parts of it, fused to the reporter gene *LacZ*, have been used to identify

sequences involved in the transcriptional response to activators and repressors. This kind of analysis is useful to the expression of heterologous gene, in order to optimize transcription during the production process. The comparison of the activity of the reporter gene between fusions with the entire promoter sequence and with deleted forms of the promoter, in various conditions of transcriptional activation or repression, allowed the identification of promoter sequences required for glucose and hypoxic induction, ethanol repression, autoregulation and Rag3 induction (Fig. 3). This analysis is substantiated by the presence in the *KIPDC1* promoter of recognition sequences for general and glycolytic transcription factors – Rap1 and Gcr1 - and sequences similar to those found in the promoters of *PDC1* and *PDC5*, like the *ERA* sequences involved in Ethanol Repression and Autoregulation [37].

Glycolysis produces ATP, NADH/H⁺ and pyruvate. ATP is required for biosynthesis, NADH has to be re-oxidized. Pyruvate decarboxylation does not only provide acetaldehyde as the substrate for NAD⁺ regeneration and ethanol production. In fact, acetaldehyde is also oxidized to acetate and acetate converted to acetyl-coenzyme A, which is the biosynthetic basic component of many pathways. The second source of acetyl-CoA in the cell is the mitochondrial pyruvate dehydrogenase (PDH) complex, that oxidatively decarboxylates pyruvate to acetyl-CoA. *S. cerevisiae* strains with dramatically reduced PDC activity, like *pdc1-pdc5* double mutants and *pdc2* mutants [29, 38], are not able to grow on glucose as unique carbon source, because cytoplasmic acetyl-CoA is not available from pyruvate decarboxylation and glucose represses mitochondrial activities, including PDH. They can grow only if a C2 compound – ethanol or acetate – is added to the medium. The C2 pathway that furnishes acetyl-CoA is called pyruvate by-pass. *K. lactis* has a single PDC gene: differently from *S. cerevisiae*, *Klpdc1Δ* strains can grow on glucose approximately at the same rate as the wild type strains [28]. This is possible because in *K. lactis* glucose does not repress respiration (it is a Crabtree-negative yeast) and mitochondrial acetyl-CoA can be transported to the cytoplasm.

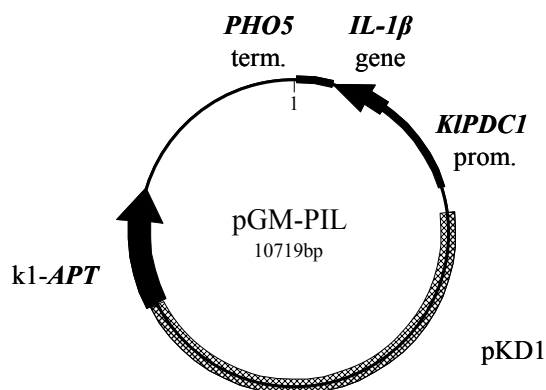


Fig. 4 Map of the expression vector pGM-PIL. This vector contains the entire sequence of pKD1, the *APT* aminoglycoside phosphotransferase gene from the transposon Tn903 as yeast selectable marker (geneticin resistance) and the *IL-1β* expression cassette with the *KIPDC1* promoter and the *S. cerevisiae* *PHO5* terminator.

5. Exploitation of *KIPDC1* for the synthesis of heterologous products

In the following section we report a list of possible applications of the *KIPDC1* gene in heterologous proteins and metabolites production. Proteins from very different organisms and with very different properties have been produced. In addition to the bacterial β -galactosidase, that is routinely tested as a quantitative reporter enzyme for transcription efficiency of the promoters, we have expressed two fungal enzymes: glucoamylase (GAM) and laccase (LCC); a viral protein: the c-33 NTPase/helicase of the hepatitis C virus (HCV) and the human interleukin 1 β (IL-1 β). Glucoamylase is used for starch saccharification in industrial production of glucose syrups. Laccase is a redox enzyme with diverse applications in paper and textile industry. In nature, it is involved in lignin and humic acids synthesis or degradation. Both GAM and LCC are secreted enzymes. The HCV C-33 protein has potential diagnostic applications. IL-1 β is a monokine with a large variety of cellular activities. We have also engineered *K. lactis* for the production of lactic acid, which is industrially produced by lactic acid bacteria and used in

food and chemical industry. In the majority of cases, these productions have been performed in laboratory scaled processes with 1-2 liters bioreactors.

5.1 Production of Glucoamylase in wild type strains

The Glucoamylase gene from *Arxula adeninivorans* has been expressed under the control of the *KIPDC1* promoter and of the *S. cerevisiae* GAPDH promoter in the wild type strain CBS2359/152F [39]. This strain is considered a standard reference strain in the *K. lactis* scientific community. The *KIPDC1* and the GAPDH expression cassettes of the *GAM* gene were cloned in identical multicopy vectors containing the entire pKD1 plasmid: pDC-GAM and pGM-GAM, respectively. Production (Units of activity per ml) was 6-7 fold higher with the *KIPDC1* promoter in shake flasks experiments. This difference in production was associated with a corresponding difference in *GAM* expression, experimentally determined as mRNA level.

The higher transcription level of the *KIPDC1* cassette was accompanied by a slight decrease of vector stability (percent of vector-containing cells) and a reduction of vector copy number (copies of the vector molecules per cell). These are frequent drawbacks of highly expressed genes on replicative vectors. Identical results were obtained with PM6-7A, another frequently used wild type strain. These experiments indicate that the glucose-inducible promoter of *KIPDC1* is suitable for high level of expression and production of heterologous genes and proteins. However, the performance of the *KIPDC1* promoter can be further improved, as reported in the following sections.

5.2 Production of Glucoamylase and Interleukin-1 β in *Klpc1 Δ strains*

The expression of *KIPDC1* is repressed by the KIPdc1 protein by a mechanism called autoregulation. The expression of a heterologous gene from the *KIPDC1* promoter could therefore be increased by escaping from autoregulation in *Klpc1 Δ strains. This trick has been exploited for the comparative production of excreted *GAM* and IL-1 β from vectors pDC-GAM and pGM-PIL (Fig. 4) in wild type and *Klpc1 Δ strains. Vectors pDC-GAM and pGM-PIL are identical, except for the heterologous gene. *GAM* production was tested in the two couples wild type and *Klpc1 Δ strains: CBS2359/152F and CBS2359/152F/2, and PM6-7A and PMI/C1 [39]. IL-1 β was assayed in the couple CBS2359/152F and CBS2359/152F/2 (Fig. 5). As expected, in all cases the production of both proteins was higher in the deleted strains. Surprisingly and differently from the expression of the reporter gene *LacZ* [32], in these cases the expression level of the heterologous gene was not higher in the deleted strains than in the wild type [39]. An explanation of these contradictory results is that activation/derepression from autoregulation might rely on some poorly expressed transcription factor that could be easily titrated by the high copy number of *KIPDC1* promoter sequences in pDC-GAM and pGM-PIL transformants (7-12 copies per cell). Actually, autoregulation has been shown with the *LacZ* reporter gene fused to the *KIPDC1* promoter on a single copy centromeric vector [32].***

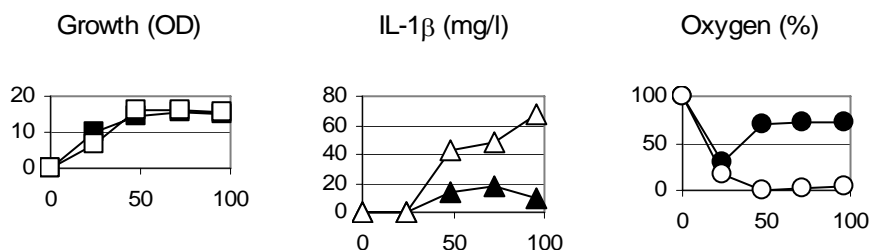


Fig. 5 Production of IL-1 β from wild type (black symbols) and *Klpc1 Δ mutant (open symbols) transformants in 100-hours fermentations in bioreactor. Cell growth (squares), product (triangles) and percent of dissolved oxygen (circles) are reported.*

Production of GAM and IL-1 β (Fig. 5) from *Klpdc1 Δ strains has been performed also in bioreactor with fermentation times up to 100-140 hours. The profiles of cell growth, heterologous protein production and oxygen consumption reveal that production from the *Klpdc1 Δ strains, in particular CBS2359/152F/2, is higher than from the wild type and, unexpectedly, continues in the stationary phase. This result indicates that *Klpdc1 Δ strains have an active metabolism also in the stationary phase, as confirmed by the expression of a housekeeping gene like the actin gene [39] and oxygen consumption (Fig. 5).***

5.3 Production of heterologous proteins under hypoxic conditions

Hypoxia is an inducer of *KIPDC1* (Fig. 6) and does not require the presence of glucose for induction [30, 32]. In industrial fermentations, fully aerated conditions are difficult to obtain and are costly. Hypoxia is thus more than a gratuitous inducer. Some cytoplasmic or secreted proteins have been produced in *K. lactis* from expression cassettes with the *KIPDC1* promoter under hypoxic induction of transcription. In these experiments, the *KIPDC1*-promoter expression cassettes of GAM, IL-1 β , β -galactosidase, LCC and c-33 were harbored by the multicopy or centromeric vectors pDC-GAM, pS13-PIL, pMD12, pLC12 and pC-3312, respectively. In all cases, an increase of heterologous protein production, from 4 to 17 fold, was observed after hypoxic induction [32]. Productions were measured by enzymatic activity (β -galactosidase, GAM and LCC) or by quantitative western-blotting analysis after hybridization with antibodies (IL-1 β , c-33). Extreme hypoxic condition (anoxia) does not allow *K. lactis* growth [40]. For this reason, a further increase of production could be obtained when hypoxic induction was followed by a production phase with low air supply.

The availability of the *LacZ* reporter gene fused to various portions of the *KIPDC1* promoter, allowed to establish the best arrangement of promoter sequences for hypoxic induction. In particular, a promoter sequence in which the internal fragments involved in glucose/Rag3 induction and ethanol/autoregulation repression were deleted, showed the highest induction ratio and a satisfactory absolute level of expression. With this modified form of the promoter, hundreds fold increase of β -galactosidase and LCC were obtained after hypoxic induction [30]. Hypoxic induction was observed also in *Klpdc1 Δ strains. However, in this context the induction ratio was low because the basal level of expression was high, as a consequence of absence of autoregulation in these strains.*

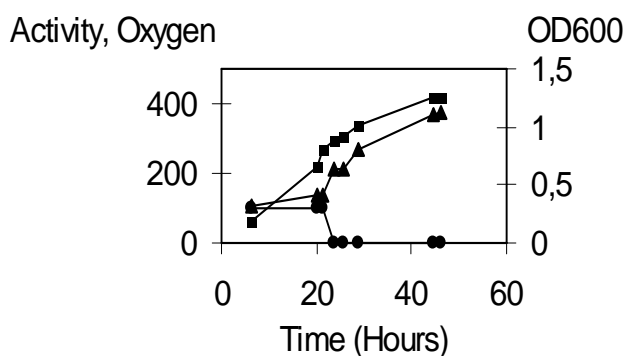


Fig. 6 Induction of β -galactosidase activity from the *KIPDC1* promoter after interruption of air feeding at 20 hours. Squares: cell density; circles: dissolved oxygen (%) and triangles: enzyme activity (mIU/ml)

5.4 Production of Glucoamylase and Laccase from *Klger1-1* mutant strains

The Rag⁻ phenotype of *rag3* Δ strains of *K. lactis* is suppressed by a mutation in the glycolytic transcription factor KlGer1 [36]. This mutation, *Klger1-1*, produces a truncated protein that is still able to bind to the *KIPDC1* promoter and shows a gain of function (fermentative growth) in the *rag3* Δ background with respect to the wild type protein. A transcriptional analysis of the reporter gene *LacZ* fused to the *KIPDC1* promoter indicates that KlGer1-1 allows transcription levels about 25% higher than in the wild type, both in the absence and presence of Rag3: *rag3* Δ and *RAG3* strains, respectively. This

finding suggests the possibility to assay heterologous gene expression and protein production in *Klgcr1-1* strains.

Table 1 – Production of Glucoamylase in *Klgcr1-1* mutant strains transformed with pDC-GAM

Strain	Relevant genotype	CFU (10 ⁸ /ml)	Stability (%)	Activity (U/ml)	Specific activity (nU/cell)
MW346/5	<i>RAG3, KIGCR1</i>	5.55±0.39	89±7	0.92±0.07	1.8±0.09
MW109-8C	<i>RAG3, KIGCR1</i>	4.01±0.38	100±1	0.88±0.02	2.3±0.26
MWK23	<i>Δrag3, KIGCR1</i>	4.92±0.37	96±5	0.32±0.04	0.6±0.07
RVR1	<i>Δrag3, Klgcr1-1</i>	5.87±0.65	92±6	1.11±0.08	2.1±0.14
FRVR109-5C	<i>RAG3, Klgcr1-1</i>	1.96±0.95	100±1	0.92±0.08	4.6±0.42

We have tested the productions of GAM and LCC from pDC-GAM and pLC12 transformants, as reported in Tables 1 and 2. MWK23 is the *rag3Δ* deletion mutant of MW346/5. RVR1 is the Rag⁺ revertant strain obtained from MWK23 after UV mutagenesis. MW109-8C is the wild type strain used in a cross with RVR1 to obtain, after meiosis, the segregant *RAG3/Klgcr1-1* strain, named FRVR109-5C. As expected, in the *rag3Δ/KIGCR1* strain MWK23 production of GAM and LCC is reduced to one half than the wild type strains. In the *rag3Δ/Klgcr1-1* strain RVR1 production was restored to the wild type level (GAM) or more (LCC). Higher levels of production were obtained in the *RAG3/Klgcr1-1* strain FRVR109-5C for both proteins. As in the case of autoregulation, production of GAM might have suffered for titration problems and for this reason production increase was lower than LCC. However it should be considered that a two fold increase of production might still be very attractive at the industrial scale.

Table 2 - Production strains of Laccase in *Klgcr1-1* mutant strains transformed with pLC12

Strain	Relevant genotype	CFU (10 ⁸ /ml)	Stability (%)	Activity (pkat/ml)	Specific activity (10 ⁻⁸ pkat/cell)
MW346/5	<i>RAG3, KIGCR1</i>	3.83±0.23	78±10	57±14	22±7
MWK23	<i>Δrag3, KIGCR1</i>	4.51±0.56	65±11	13±2	6±2
RVR1	<i>Δrag3, Klgcr1-1</i>	3.12±0.22	87±6	165±29	65±11
FRVR109-5C	<i>RAG3, Klgcr1-1</i>	4.12±0.59	89±11	429±28	126±24

5.5 Production of lactic acid from *K. lactis*

Industrial production of lactic acid makes use of lactic acid bacteria (LAB) and first production plants were constructed at the end of XIX century. LAB are acid sensitive and the production medium has to be neutralized by alkali addition during the fermentation process. Since the final wanted product is lactic acid, the fermentation broth has to be treated with sulfuric acid in the downstream process, with the concomitant accumulation of high amounts of sulfate salt as by-product. Medium neutralization and getting rid of sulfates are costly operations. Yeasts can tolerate acidic conditions more than LAB but they do not produce lactic acid. However, they can be easily engineered for the production of lactic acid by transformation with lactic acid dehydrogenase (LDH) genes [41, 42]. One drawback of engineered yeasts, especially *S. cerevisiae*, for lactic acid production is that they produce also ethanol (heterolactic fermentation), that adds costs for purification. Ethanol cannot be avoided with *S. cerevisiae*, because only wild type strains, or single *pdcl* or *pdcs* mutants, and not mutant strains strongly impaired in fermentation (double *pdcl-pdcs* mutants or *pdcs* mutants), can be advantageously used for lactic acid fermentations [43, 44]. In fact, double *pdcl-pdcs* mutants or *pdcs* mutants do not grow fast on glucose, which is the favorite carbon source for lactic acid production, because the PDC reaction is the only metabolic step in fermentative growth that leads to acetyl-CoA, through the acetaldehyde dehydrogenase and Acetyl-CoA synthetase reactions, and acetyl-CoA is absolutely required for biosynthesis.

The yeast *K. lactis* has a single *KIPDC1* gene, that can be deleted without affecting growth on glucose, and strains deleted for *KIPDC1* do not produce ethanol. In *Klpdcl1Δ* strains, the carbon metabolic flux can be entirely redirected from the ethanologenic pathway to lactic acid, by transformation with a *LDH*

gene: pyruvate reduction by the LDH enzyme provides redox balancing and acetyl-CoA is furnished by the mitochondrial PDH. This metabolic restructuring has been assayed [41] and it was proved to be successful for homolactic fermentation of glucose in bioreactor. In order to have high LDH activity on glucose, the *LDH* gene was placed under the control of the *KIPDC1* promoter and on multicopy vectors (pEPL2 or pLAZ10). In the engineered *Klpdc1* Δ strains, LDH activity was higher than in the engineered wild type strains probably because of the additional effect of absence of autoregulation.

Table 3 – Production of lactate in *Klpdc1* and *Klpda1* mutant strains transformed with pEPL2 or pLAZ10. Data adapted from references [41] and [42].

Strain	Relevant genotype	Lactate yield (g/g)
PM6-7A	<i>KIPDC1</i> , <i>KIPDA1</i>	< 0.10
PM1/C1	Δ <i>Klpdc1</i> , <i>KIPDA1</i>	0.23-0.58
PM6-7A/ <i>Klpda1</i> Δ	<i>KIPDC1</i> , Δ <i>Klpda1</i>	0.35
BM3-12D	Δ <i>Klpdc1</i> , Δ <i>Klpda1</i>	0.85

The encouraging results obtained with *Klpdc1* Δ strains suggested to further improve lactic acid production by blocking also the PDH step of pyruvate utilization [42]. The PDH mutant strain, deleted for the *KIPDA1* gene that encodes the E1 α subunit of the PDH enzyme, produced lactic acid with yield comparable to that of the *Klpdc1* Δ strain (Table 3). The double deleted *Klpdc1* Δ -*Klpda1* Δ strain of course cannot grow on glucose and biomass could be only produced by the pyruvate by-pass from added ethanol. The double deleted strain engineered for lactic acid production was tested in bioreactor. Two phases of the process could be separated: in the first phase the added ethanol was completely consumed and biomass produced in association with a small consumption of glucose. In the second phase, biomass was stationary but glucose was almost entirely and rapidly transformed in lactate with yield close to the theoretical. A summary of results of lactate production from *K. lactis* strains is reported in Table 3. Interestingly, sequential feeds of glucose, at 230 and 400 hours, to the double deleted producing strain resulted in continuous accumulation of lactate (Fig. 7), without apparent loss of metabolic activity, similarly to the single deleted *Klpdc1* Δ strain [41].

6. Concluding remarks

In this short review, we have surveyed the synthesis of heterologous products, proteins and metabolites, from the respiratory yeast *K. lactis*. We have used the *KIPDC1* promoter to express heterologous genes and we took advantage of its regulation to increase product formation. Induction by glucose and by hypoxia are especially fitting to large scale processes, because glucose is the routinely used carbon source and hypoxia is the cheapest inducer one can envisage. The effectiveness of these inducers can still be improved by the analysis of *cis*-acting regulatory sequences present on the promoter and by assemble modified promoters with high induction ratios. This approach has been already started for hypoxic induction. Alternatively, one can search for regulatory mutants with higher expression levels. The isolation and exploitation of the *Klger1-1* mutation is an example of this approach. Mutants can be searched for all the regulatory mechanism: glucose and hypoxic induction, ethanol repression and autoregulation.

The use of *Klpdc1* Δ mutants to bypass repression by autoregulation was successful especially for heterologous genes on single copy vectors, while multicopy vectors masked this effect, probably by titration of regulatory factors. However, these strains revealed an intriguing characteristic in long term fermentations processes: the capability to maintain an active metabolism in the stationary phase at high cell density. This resulted in continuous production of lactate, GAM and IL-1 β and was not a prerogative of expression of genes from the *KIPDC1* promoter. A more general change in the metabolism must be involved, since oxygen consumption remains equally high and a housekeeping gene, like actin, is still expressed. The outcome is a sort of ‘continuous fermentation’ process. After these observations, one can consider to use these strains in immobilized systems with cells at high density, where culture does not

grow but production takes place. On the other hand, the physiological basis of this extended metabolic activity and the mechanism of autoregulation are still open questions for basic research.

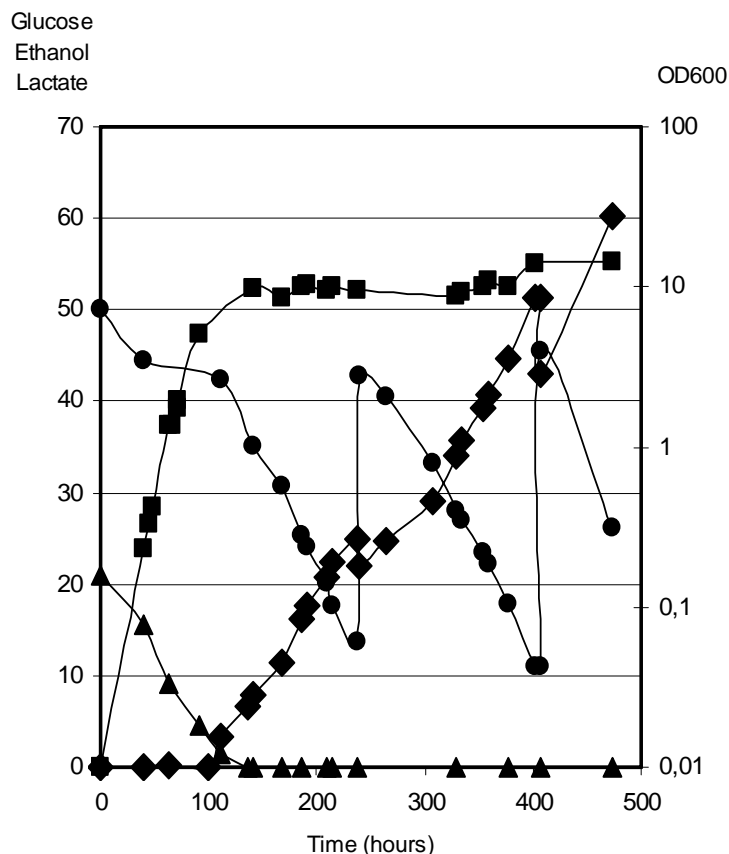


Fig. 7 Production of lactate from the double deleted *Klpc1-Klpda1* strain. Squares: cell density; triangles: ethanol (g/l); circles: glucose (g/l) and rombs: lactate (g/l). Adapted from reference [42].

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