1. Introduction

1.1 Yeast cells

It doesn't exist an univocal and accurate definition of yeast. In fact, the typical information used to define them (eukaryotic unicellular microorganism that divides by gemmation and are able to do alcoholic fermentation) do not characterized a definite group of species [1].However, we can say that yeasts are ubiquitous unicellular fungi widespread in natural environments and their successful colonization is intimately related to their physiological adaptability to a highly variable environment [2].

Macromolecular constituents of yeast comprise proteins (structural protein, hormones, enzymes), glycoproteins (mannoproteins, invertases), polysaccharides (glucan, mannan, chitin), polyphosphates, lipids (free sterols in membranes, sterol esters, triglycerides, free fatty acids) and nucleic acids [3].
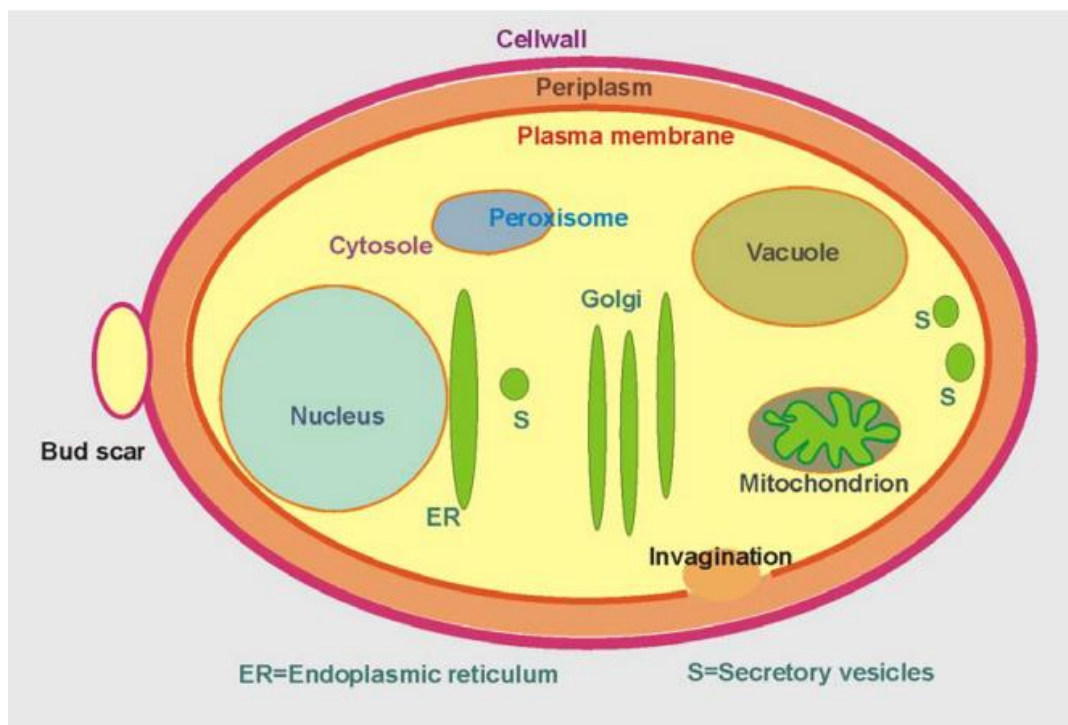

Figure 1.1: Scheme of organelles and compartments in a yeast cell.

In contrast to mammalian cells, peculiarities of yeast cells are that they are surrounded by a rigid cell wall and develop birth scars during cell division. The yeast cell envelope has a major role in controlling the osmotic properties of the cell. Thus its primary functions is to provide selective permeability: most important is the role of membrane proteins in regulating yeast nutrition, such as uptake of carbohydrates, nitrogenous compounds or ions and the extrusion of molecules hazardous to the cell [3]. Like other membranes, it is a lipid bilayer with proteins inserted into this layer or traversing it as trans-membrane proteins of various functions. The lipid composition comprises mainly phosphatidylcholine and phosphatidylethanolamine, as well as sterols, mainly ergosterol.

The yeast cytosol is an acidic (pH 5.25) colloidal fluid, mainly containing ions and low or intermediate molecular weight organic compounds (metabolites generated by cellular metabolism), and soluble macromolecules (e.g. enzyme proteins, factors, glycogen).

Nucleus is coated by the nuclear membrane and contains DNA.

The endoplasmic reticulum (ER) is the site of biosynthesis and modification (glycosylation) of proteins that must be exported.

From the ER, proteins are directed to the Golgi apparatus by vesicles. In the Golgi further modifications of the proteins by carbohydrate side chains may take place (mannosylation) [3].

The key organelle in yeast involved in intracellular trafficking of proteins is the vacuole. The main role of this lysosome-like compartment is the non-specific proteolytic cleavage of proteins, which involves a variety of intravacuolar lytic enzymes (endopeptidases, aminopeptidases and carboxypeptidases). Further physiological functions of yeast vacuoles include storage of basic amino acids, polyphosphates and certain metal ions, homeostasis of cytoplasmic ion concentrations and osmoregulation.

Yeast mitochondria exhibit a variety of important features which are absent from their counterparts in higher organisms. General structural characteristics of mitochondria include:

- an outer membrane – containing enzymes involved in lipid metabolism;

- the inter-membrane space;

- an inner membrane – containing the components of the respiratory chain and the ATP synthase;

- the mitochondrial matrix – containing enzymes of the citric acid cycle and the multienzyme pyruvate dehydrogenase (PDH) complex.

So, mitochondria perform a lot of functions in yeast cell physiology, implicating that they are relevant to intact cell metabolism [3]: they can serve as the major site of ATP production of the cell and they contain enzymes that are able to connect different metabolic pathways (PDH). In addition, there are present enzymes for the synthesis of particular amino acids, dicarboxylic acids, flavor components, pyrimidine and purine bases. Mitochondria are also involved in stress responses and adaptation to stresses and in the utilization of alternative carbon sources.
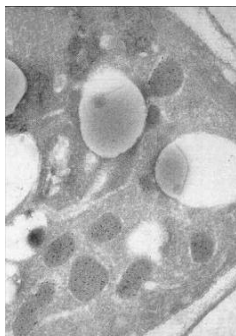

Figure 1.2: Micrograph of yeast peroxisomes.

Peroxisomes in yeasts (Figure 1.2) contain several oxidoreductase enzymes (catalase and alcohol oxidase), which have a detoxification function against reactive oxygen species. Peroxisomes are also the sites of many other metabolic pathways, as the glyoxylate cycle and the fatty acid degradation, since yeasts mitochondria lack β-oxidation.

1.2 *Kluyveromyces lactis*

Yeasts, that belong to the fungal kingdom, have been used for fermentation of food and beverages since ancient times and are today widely used for industrial production of chemicals, pharmaceuticals and proteins. In terms of biotechnological application yeasts have the advantage of being relatively easy to grow in fermenters and they are therefore well-suited for large-scale industrial production [4].

Kluyveromyces lactis is one of the six yeast species from the *Kluyveromyces* genus. It is both scientifically and biotechnologically one of the most important non-*Saccharomyces* yeasts, commonly used for genetic studies and industrial applications. Its name comes from the ability to grow on lactose as a sole carbon source [5], unlike most other yeasts. Since the 1950s, in fact, *K. lactis* has been used as a source of lactase (β-galactosidase), an enzyme that degrades milk sugar (lactose) and is necessary for production of lactose-free dairy products [6].

K. lactis biotechnological significance builds on its well-known use in commercial production on an industrial scale of the enzyme bovine chymosin [7]. This protein was the first heterologous enzyme originating from a higher eukaryote that was produced at low cost in a microorganism and the process developed for its industrial-scale production was widely recognized as a major biotechnological achievement. In 1993 was first reviewed [8] the use of *K. lactis* as a host for heterologous protein expression. Today, over 40 proteins have been produced with *K. lactis*, illustrating its utility as an alternative yeast expression system. As a host for heterologous protein production, *K. lactis* has a number of advantages over other yeast expression systems, including easy genetic manipulation, ability to use both integrative and episomal expression vectors and the availability of a fully sequenced genome [9,10]. In addition, enzymes from *K. lactis* have GRAS (Generally Regarded As Safe) FDA status, permitting their use in various food and feed applications.

1.3 Yeasts metabolism

Metabolism is defined as the set of coordinated biochemical reactions for:

1. obtaining energy from the environment;

2. building precursors required for macromolecules (nucleic acids, proteins, complex carbohydrates).

3. polymerizing precursors into macromolecules.

4. synthesizing and degrading other biomolecules (membranes, signal molecules...).
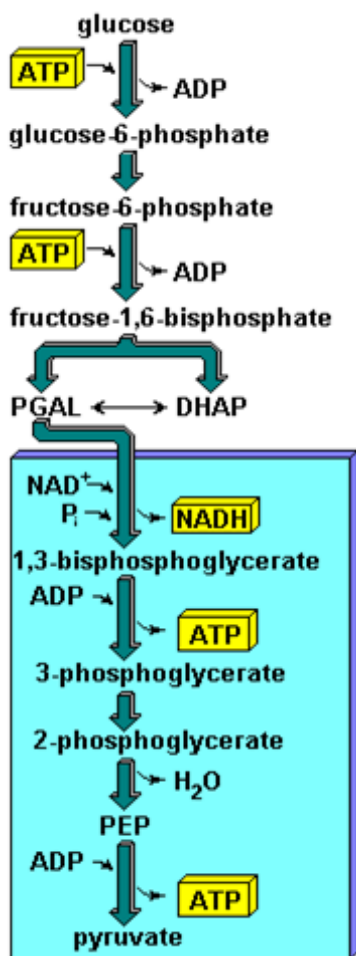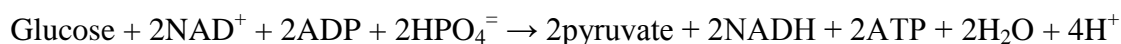
Metabolism, composed of thousands of enzymes responsible for catalyzing biochemical reactions, is the link between external resources and the inner workings of the organism. Many biochemical reactions have been studied in great detail and most of them are remarkably well conserved over a wide range of species: in fact, the metabolic pathways of the central carbon metabolism (primary metabolism) are basically identical between different yeast species, suggesting that these microorganisms might constitute, in some way, an homogenous group.

However, the same metabolic capabilities can lead to very different physiologies, depending on the way these reactions are regulated and the way enzyme synthesis is controlled. For example the number of isoenzymes and genes, the mechanisms for nutrient uptake, transport and, most importantly, the regulation of fermentation and respiration diversify yeasts substantially [2,11,12].

Although all yeasts are microorganisms that derive their chemical energy, in the form of ATP, from the breakdown of organic compounds, there is metabolic diversity in how these organisms generate and consume energy from these substrates. Knowledge of the underlying regulatory mechanisms is not only valuable in the understanding of general principles of regulation but also of great importance in biotechnology, if new metabolic capabilities of particular yeasts have to be exploited.

1.3.1 Glycolysis

Glycolysis is the process that convert glucose (carbon and energy source) into pyruvate, generating small amounts of ATP (energy) and NADH (reducing power)[2]. The net reaction stoichiometry is:

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{HPO}_4^= \rightarrow 2\text{pyruvate} + 2\text{NADH} + 2\text{ATP} + 2\text{H}_2\text{O} + 4\text{H}^+$$

The first step is the uptake of glucose from the extracellular medium and its immediate phosphorylation, by hexokinase (EC 2.7.1.1), to glucose 6-phosphate, which is then isomerized to fructose 6-phosphate, by phosphoglucose isomerase (EC 5.3.1.9). The next enzyme is phosphofructokinase (EC 2.7.1.11), that further phosphorilates fructose 6-phosphate to fructose 1,6-bisphosphate. These steps are the first part of glycolysis and requires energy in the form of ATP.

Then aldolase (EC 4.1.2.13) converts fructose 1,6-bisphosphate in glyceraldehyde 3-phosphate (GAP) and di-hydroxyacetone phosphate which is converted to another molecule of GAP by triosephosphate isomerase (EC 5.3.1.1). So 2 molecules of GAP forms to 2 molecules of pyruvate by the activity of GAP dehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3), phosphoglycerate mutase (EC 5.4.2.1), enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40).

The last block of glycolysis is also associated with a net production of energy (2ATP) and reducing equivalents (2NADH).

Glycolysis is a central pathway that produces important precursor metabolites: six-carbon compounds as glucose-6P and fructose-6P and three-carbon compounds as glycerone-P, glyceraldehyde-3P, glycerate-3P, phosphoenolpyruvate (PEP) and pyruvate.

Acetyl-CoA, another important precursor metabolite, could be produced by the pyruvate decarboxylase (PDC, EC 4.1.1.1) that catalyze the oxidative decarboxylation of pyruvate to acetyl-CoA, undergoing acetaldehyde, or by the mitochondrial pyruvate dehydrogenase complex (PDH). Glycolysis yields 2 molecules of ATP and 2 molecules of NADH; to keep this process going, NADH needs to be reoxidized to NAD^+. There are two principal ways this can be done (fermentation or respiration) and the choice between the two will decide the fate of pyruvate.

1.3.2 Fermentation

In fermentation, glucose is only partially catabolized. End products of microbial fermentative pathways may include ethanol, lactate, butyric acid and acetone [11].

In ethanol fermentation pyruvate is first decarboxylated to acetaldehyde by pyruvate decarboxylase (EC 4.1.1.1), and then reduced to ethanol by alcohol dehydrogenase (NADH or NADPH dependent, EC 1.1.1.1 and EC 1.1.1.2 respectively), with simultaneous re-oxidation of NADH/NADPH to NAD^+/$NADP^+$. Concomitantly, glycerol is generated from dihydroxyacetone phosphate to ensure production of this important compound. Starting from glucose, ethanol fermentation can be summarized as:

$$Glucose + 2ADP + 2P_i \rightarrow 2ethanol + 2ATP + 2CO_2 + 2H_2O$$

1.3.3 Respiration

Respiration or oxidative phosphorylation is a process in which organic or reduced inorganic compounds are oxidized by inorganic compounds. In eukaryotes, molecular oxygen is used as an oxidant. Respiration is biochemically more complex than fermentation [11]. In the presence of oxygen and absence of repression, pyruvate enters the mitochondrial matrix where it is oxidatively decarboxylated to acetyl-CoA by the pyruvate dehydrogenase multi-enzyme complex; this reaction links glycolysis to the Krebs cycle (TCA) [2].

Tricarboxylic acid pathway (TCA)

The main catalytic function of the TCA cycle is to provide reducing equivalents to the respiratory chain through the oxidative decarboxylation of acetyl–CoA.
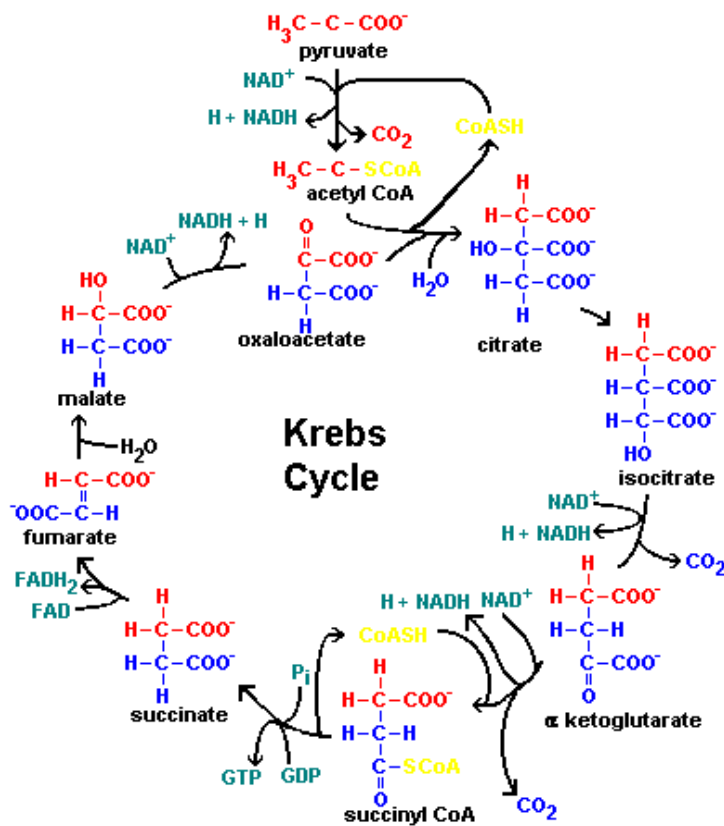
The first reaction of TCA is catalyzed by citrate synthase (EC 2.3.3.1) and it is the irreversible condensation of acetyl–CoA and oxaloacetate resulting in the formation of citrate. The next step is the stereospecific isomerization of citrate to isocitrate reversibly catalyzed by aconitase (EC 4.2.1.3); then occurs the oxidative decarboxylation of isocitrate to α-ketoglutarate thank to isocitrate dehydrogenase, NAD^+ or $NADP^+$ dependent (EC 1.1.1.41 and EC 1.1.1.42, respectively).

The irreversible formation of succinate is catalyzed by α-ketoglutarate dehydrogenase NAD^+ dependent (EC 1.2.4.2), which promotes the oxidative decarboxylation of α-ketoglutarate, via succinil–CoA, to succinate. Succinate is then converted to fumarate by succinate dehydrogenase (EC 1.3.5.1), using FAD as a prosthetic group. Then fumarate hydratase (EC 4.2.1.2) catalyzed the formation of malate. Finally, malate dehydrogenase NAD^+ dependent (EC 1.1.1.37) catalyzes the last step of the TCA cycle and leads to the oxidation of malate to oxaloacetate. There are three isoenzymes of malate dehydrogenase: a cytosolic, a mitochondrial and a peroxisomial one.

Summarizing, acetyl-CoA is completely oxidized to give two molecules of CO_2 and reductive equivalents in form of NADH and $FADH_2$.

NADH is then used as a source of electrons for reactions involving the respiratory chain. Proteins in the respiratory chain are membrane-bound and use the reductive potential of NADH to pump proton ions across the membrane. As protons are concentrated on one side of the membrane, this creates an electrochemical concentration gradient and results in a proton-motive force (PMF). Finally, PMF is used to drive the reaction in which ATP synthase creates ATP from ADP and inorganic phosphate.

The TCA cycle works in a two-minicycles model interconnecting oxaloacetate and α-ketoglutarate and their transamination products (glutamate and aspartate). So TCA cycle flux appears also to be constricted at these two steps on the basis of the limited availability of these two substrates. Replenishment of compounds necessary to drive the citric acid cycle, such as oxaloacetate and α-ketoglutarate, are:

- fixation of CO_2 to pyruvate by the actions of the enzymes pyruvate carboxylase ATP-dependent (EC 6.4.1.1) and phosphoenolpyruvate carboxykinase;
- glyoxylate cycle (see paragraph 1.3.4).

With the exception of isocitrate, every TCA cycle intermediate is commonly used by other metabolic reactions. In fact, the citric acid cycle is an amphibolic pathway, since it combines both catabolic and anabolic functions. The latter results, for example, from the production of intermediates for the synthesis of amino acids and nucleotides. All of these pathways and their regulation in yeast have been studied in great detail and they are available on line [13]. Figure 1.3 just summarizes the major reactions. Because of their complexity, we will describe only the pathways we will be interested with. For example, a simple transamination, catalyzed by aminotransferase (EC 1.4.1.13, $NADP^+$ dependent or EC 1.4.1.14, NAD^+ dependent) converts α-ketoglutarate to glutamate.

Another aminotransferase (EC 2.6.1.2) catalyzes the transfer of a $-NH_2$ group to pyruvate, generating alanine; the amino group arise from another aminoacid as glutamate (EC 2.6.1.2) or glycine (EC 2.6.1.44), generating also α-ketoglutarate or glyoxylate, respectively.
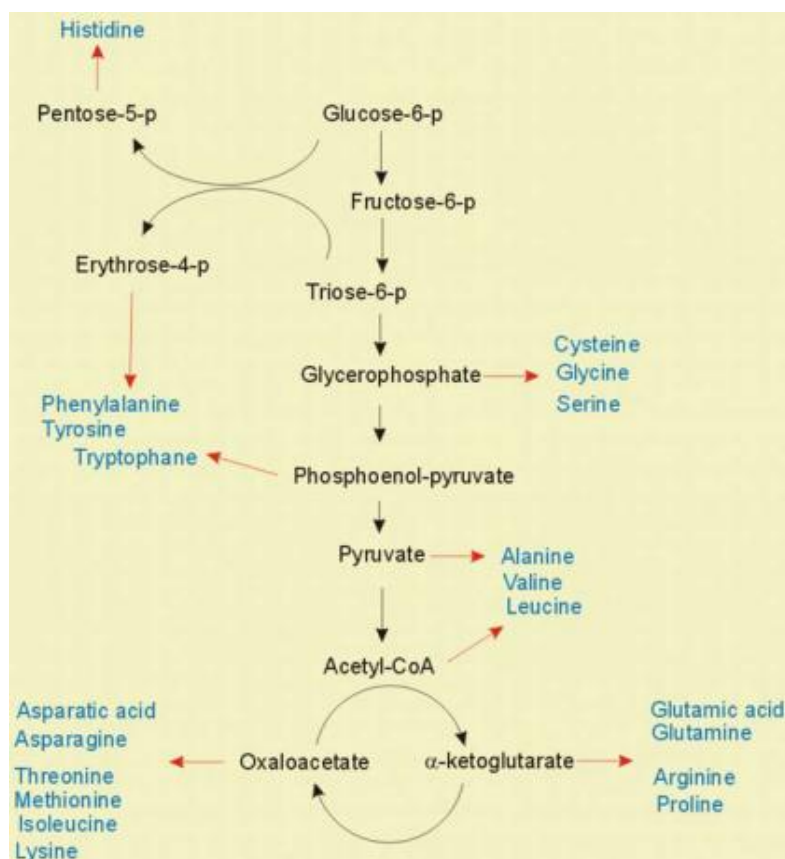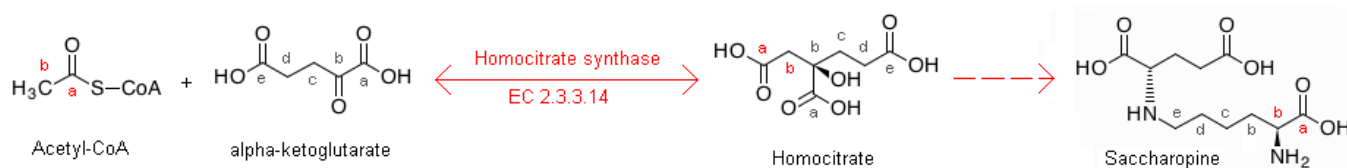

Figure 1.3: Scheme of amino acid biosynthesis in yeasts.

Lysine is synthesized via the homocitrate-α-aminoadipate pathway, instead. In the first step a synthase (EC 2.3.3.14) catalyzes the condensation of acetylcoenzyme A with α-ketoglutarate to form homocitrate which, after a series of reaction involving α-aminoadipate, is converted to saccharopine.


Finally, a dehydrogenase (EC 1.5.1.7) NAD^+ dependent generate lysine, regiving α-ketoglutarate.

1.3.4 Glyoxylate cycle

The glyoxylate cycle, also called the glyoxylate shunt, is an anabolic pathway occurring in fungi, plants and bacteria, but not in mammals [14]. Although several of the reactions of the glyoxylate and TCA cycle are identical, many of them are catalyzed by different isoezymes in different cellular compartments. The glyoxylate cycle occurs in the peroxisomes, while the TCA cycle occurs in mitochondria.

The two initial steps of the glyoxylate cycle are identical to those in the citric acid cycle: *acetyl-CoA → citrate → isocitrate*. The two cycles vary in the next step when, in the glyoxylate cycle, isocitrate lyase (EC 4.1.3.1) converts isocitrate into glyoxylate and succinate, instead of α-ketoglutarate as seen in the TCA cycle (succinate can enter into the citric acid cycle to eventually form oxaloacetate). This bypasses the two decarboxylation steps (where two carbons are lost in the form of CO_2) that take place in the TCA cycle, instead.
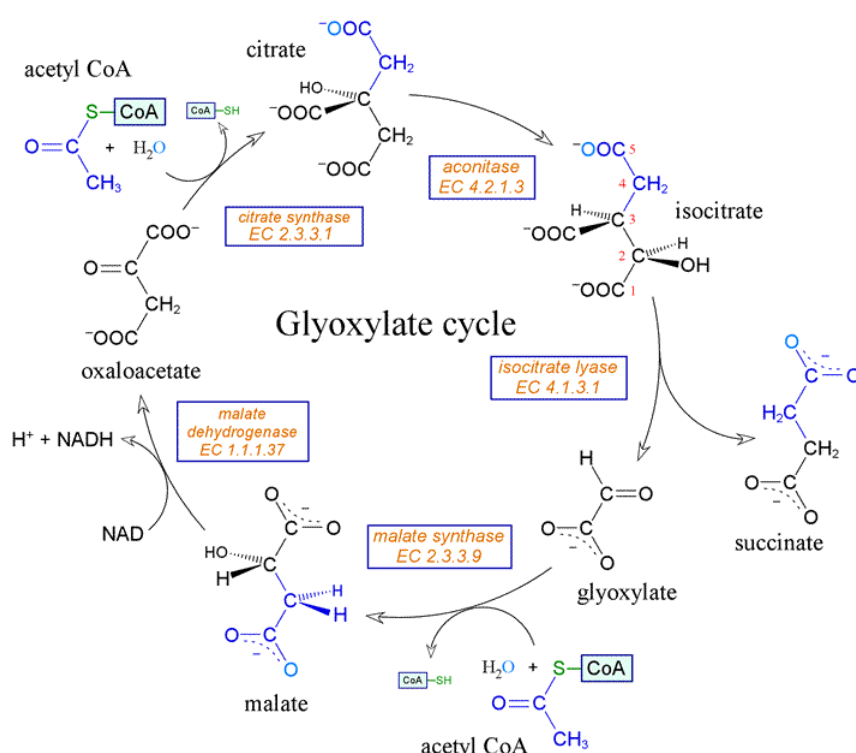
The glyoxylate cycle then continues: malate synthase (EC 2.3.3.9), catalysing the condensation of glyoxylate with acetyl-CoA, yields malate.

As a net result, two-carbon substrates, which enter the cycle as acetyl-CoA, can be converted to four-carbon compounds, which in turn can be further metabolized and being used in the later synthesis of macromolecules, sugars (including glucose) and other essential organic compounds.

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1.3.5 Pentose phosphate pathway (PPP)

An alternative mode of glucose oxidation is the pentose phosphate pathway, which provides the cell with pentose (ribose) sugars, which serve in the biosynthesis of nucleic acid precursors and nucleotide coenzymes, and cytosolic NADPH as reducing equivalents, necessary for biosynthetic reactions, such as the production of fatty acids, amino acids and sugar alcohols.

There are two different phases in the pathway:

1) irreversible oxidative phase in which glucose-6P is converted to ribulose-5P by oxidative decarboxylation, and NADPH is generated. The first step in this phase is the dehydrogenation of glucose-6P to 6-phosphogluconolactone generating one mole of NADPH (by glucose-6P dehydrogenase, EC 1.1.1.49). Subsequently 6-phosphogluconate, generated by 6phosphoglucono-lactonase (EC 3.1.1.31), is decarboxylated by the action of phosphogluconate dehydrogenase (EC 1.1.1.44), to give ribulose-5P and a second mole of NADPH.
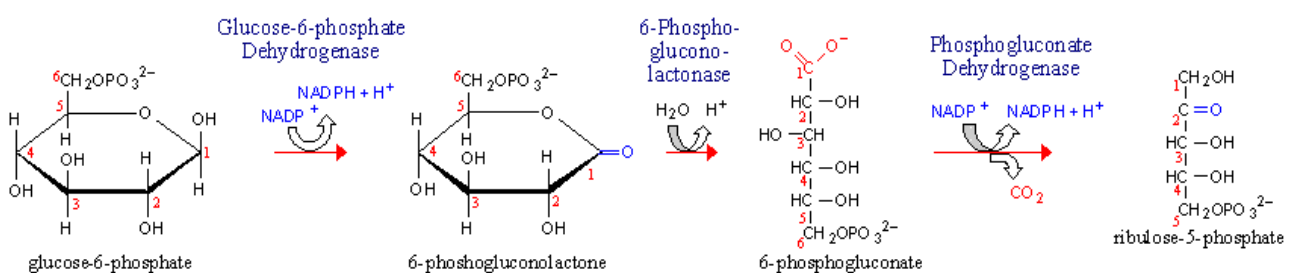
2) Reversible non-oxidative phase in which phosphorylated sugars are interconverted by isomerase (EC 5.3.1.6), epimerase (EC 5.1.3.1), transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2) enzymes to generate xylulose-5P, ribulose-5P and ribose-5P.
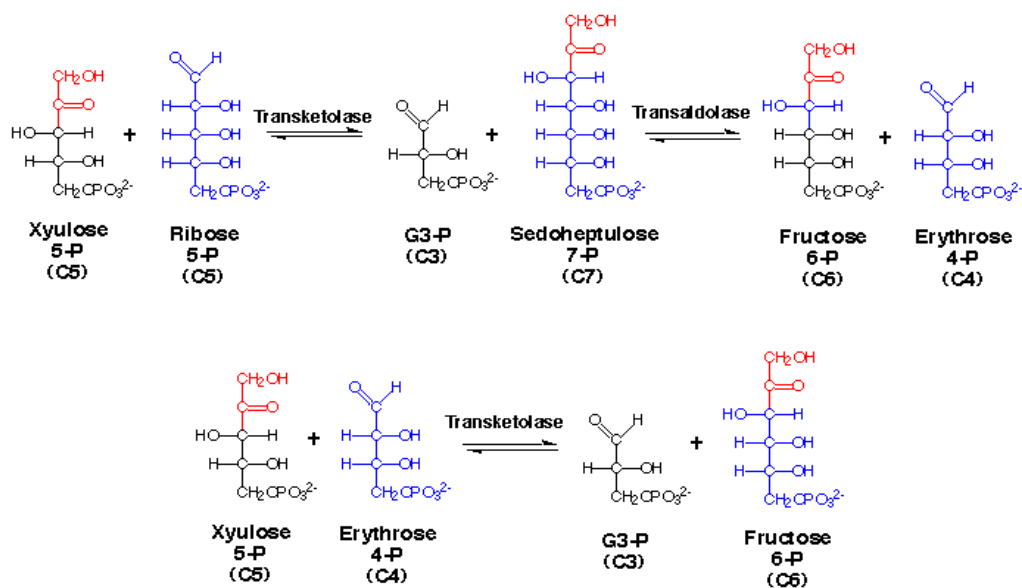
Phosphoribosyl pyrophosphate (PRPP) formed from ribose-5P is an activated compound used in the biosynthesis of histidine and purine/pyrimidine nucleotides.

1.3.6 Fatty acids (FA)

Nature utilizes the intrinsic properties of fatty acids in multiple ways [15]:

1. the hydrophobic nature of acyl chains creates the force to establish membrane bilayer structures that are the basis of sub-cellular compartmentalization.

2. Fatty acids provide an ideal form to store metabolic energy (the formation of C–C bonds is a highly energy-demanding process); the energy contained in the C–C bonds can be efficiently released by β-oxidation, a reaction formally equivalent to the reverse of fatty acid biosynthesis. Acetyl-CoA generated by fatty acid degradation is utilized as an anabolic building block, or further catabolized by the TCA cycle.

3. Specific fatty acids serve as precursors for biologically more active compounds and may thus harbor signaling functions.

4. Synthesis of fatty acids utilizes substantial amounts of metabolites, acetyl-CoA, ATP and NADPH, and may thus compete with other cellular processes dependent on these compounds.

Biosynthesis

The pathways for fatty acid synthesis in yeast have been documented but the mechanics of regulation are less well defined [16]. The series of reactions leading to the synthesis of long-chain fatty acids, starting from acetyl-CoA is achieved by a multi-enzyme complex, the fatty acid synthase. Fatty Acid Synthase (FAS) is a multi-enzyme protein that catalyzes fatty acid synthesis. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids [17].

Yeast FAS is composed of two subunits, encoded by FAS1 (β subunit) and FAS2 (α subunit) which are organized as a hexameric α6β6 complex. So it is not a single enzyme but a whole enzymatic system composed of two identical 272 kDa multifunctional polypeptides, in which substrates are handed from one functional domain to the next [17].

Acetyl-CoA is the C2-carbon donor for fatty acid synthesis and elongation, which is also typically initiated by the attachment of acetyl-CoA to the FAS complex.

In the initial reaction of *de novo* fatty acid biosynthesis, acetyl-CoA is carboxylated by the addition of CO_2 to malonyl-CoA, by the trifunctional enzyme acetyl-CoA carboxylase (ACC, EC 6.4.1.2). Malonyl-CoA produced by ACC serves as a two- carbon donor in a cyclic series of reactions catalyzed by fatty acid synthase and elongases.

In detail, fatty acids are synthesized by a first decarboxylative Claisen condensation reaction starting from malonyl-CoA and another molecule of acetyl-CoA, catalyzed by a ketoacyl synthase (KS, FAS2) that lead to 3-ketoacyl-ACP synthesis.

Following each round of elongation the β-keto group is reduced to the fully saturated carbon chain by the sequential action of:
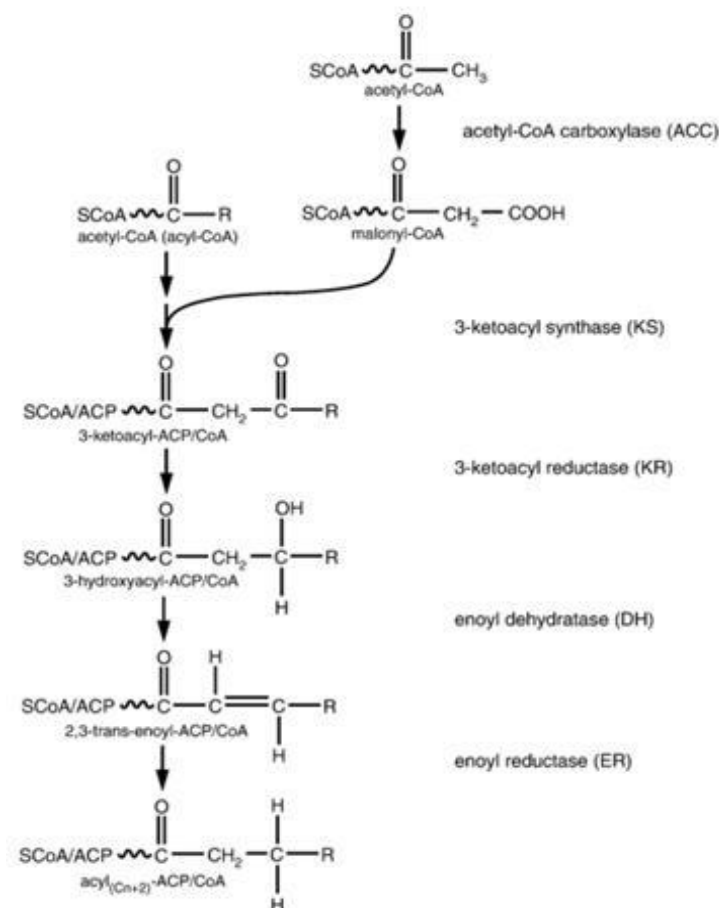
I. ketoreductase (KR, FAS2) NADPH-dependent, that catalyzed the reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP;

II. dehydratase (DH, FAS1) that generates 2,3-trans-enoyl-ACP;

III. enol reductase (ER, FAS1) NADPH-dependent, yielding saturated acyl-residues that are extended by two carbon atoms (acyl(n+2)-ACP) in each cycle.

The growing fatty acid chain is carried between these active sites while attached covalently to the phosphopantetheine prosthetic group of an acyl carrier protein (ACP), and is released by the action of a thioesterase (TE) upon reaching a carbon chain length of 16 (palmitic acid) [17].

Very long-chain fatty acids (VLCFA) of C20 − C26 carbon atoms are synthesized by chain-elongation of C16 or C18 saturated acyl-CoAs, through a cyclic series of reactions reminiscent of fatty acid de novo synthesis. This elongation follows similar reaction schemes as mitochondrial or cytosolic FAS, i.e., condensation of the acyl-chain with ACC-derived malonyl-CoA, reduction of the 3-ketoacyl-CoA to 3-hydroxyacyl-CoA, dehydratation to enoyl-CoA, and second reduction to a saturated acyl-chain that is extended by two carbon atoms.

The primary products of fatty acid biosynthesis, in most organisms, are C16 and C18 fatty acids. The relative ratio of chain lengths and degree of instauration of these fatty acids vary widely. Yeasts generally abound in unsaturated fatty acids, oleic acid (C18 monounsaturated) being the major component. The C18 polyunsaturated acids are usually associated with specific yeast strains [16]. For example, *Saccharomyces cerevisiae* primarily produces saturated and mono-unsaturated fatty acids of 16 and 18 carbon compounds. *Kluyveromyces lactis* can produce both the di-unsaturated linoleic acid (C18:2) as well as the tri-unsaturated α-linolenic acid (C18:3), instead [18].

So, fatty acid synthase produces saturated fatty acids, which are converted to unsaturated fatty acids by the introduction of double bonds in an oxidative desaturation reaction catalyzed by desaturases.

Fatty acid desaturases are enzymes that convert a single bond between two carbon atoms (C-C) to a double bond (C=C) in a fatty acyl chain. The resultant double bond is often referred to as an unsaturated bond and the reactions catalyzed by these enzymes are known as desaturation reactions. These reactions require molecular oxygen and occur under aerobic conditions [19]. There are three types of fatty acid desaturase:

1. acyl-CoA desaturases (in animal, yeast and fungal cells) introduce unsaturated bonds into fatty acids that are bound to coenzyme A (CoA);

2. acyl-ACP desaturases (in the plastids of plant cells) introduce the first double bond into fatty acids that are bound to acyl carrier protein (ACP);

3. acyl-lipid desaturases (in plants and cyanobacteria) which introduce unsaturated bonds into fatty acids that are in a lipid-bound form.

Although the desaturation of fatty acids is, in the end, an oxidation reaction, it requires two electrons in addition to one molecule of oxygen.

Ferredoxin is the electron donor in the desaturation reactions catalyzed by acyl-ACP desaturases and most of the acyl-lipid desaturases. By contrast, the acyl-CoA desaturases of yeasts use cytochrome b5 as the electron donor [19].

Each fatty acid desaturase introduces an unsaturated bond at a specific position in a fatty acyl chain: typically, monounsaturated fatty acids are synthesized via a reaction catalyzed by the ER-resident and essential Δ9-desaturase (OLE1) [15]. As far as is known, linoleic acid (18:2) comes from oleic acid (C18:1) via a Δ12 fatty acid desaturase (FAD2) and then α-linolenic acid (18:3) via a Δ15 fatty acid desaturase (FAD3) could be obtained [18].

1.3.7 Sterols

Fungi are among the most primitive organisms that synthesize sterols [20]. Sterols are essential structural and regulatory components of eukaryotic cell membranes. Mammals, plants and fungi produce similar sterols, which differ in the number and location of double bonds and methyl side chains [21]. Most steps within the biosynthetic pathway of ergosterol are performed within the ER [22] and only a subset of enzymatic activities is found in mitochondria [23].

The principal sterol, in yeast, is ergosterol. Other sterols identified include intermediates in the pathway of ergosterol biosynthesis or those appearing upon inhibition of biosynthesis [24]. Like cholesterol in mammalian cells, ergosterol is responsible for membrane fluidity and permeability. In *S. cerevisiae* and *Candida* species, many experiments have demonstrated a role for ergosterol in physiological functions, such as membrane permeability, resistance to drugs, protein transport to the plasma membrane, sporulation and endocytosis (to respond properly to outside stimuli eukaryotic cells internalize extracellular material along with parts of their own limiting membranes, in a process termed endocytosis) [21,22].

Ergosterol synthesis

Two molecules of acetyl-CoA can condensate via Acetyl-CoA transferase (EC 2.3.1.9) to form acetoacetyl-CoA. Another acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).
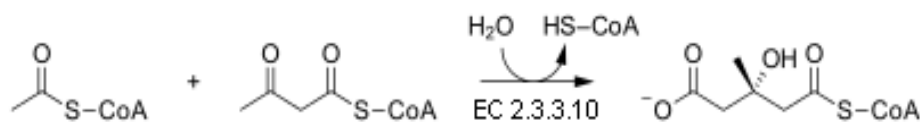
HMG-CoA is reduced to mevalonate converting 2NADPH to 2 NADP$^+$.
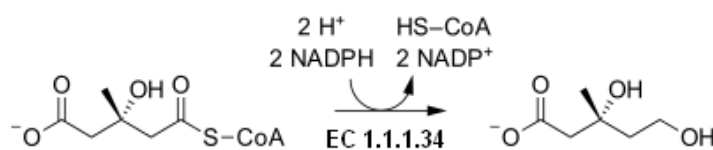
Mevalonate is doubly phosphorylated by kinase enzymes (EC 2.7.1.36 and EC 2.7.4.2) giving mevalonate-5-pyrophosphate then converted to 3-isopentenyl pyrophosphate (IPP). An isomerase (EC 5.3.3.2) convert IPP to dimethylallyl pyrophosphate that can generate geranyl pyrophosphate thanks to the same enzymes (EC 2.5.1.1) that catalyse the reaction between geranyl-PP and IPP to give farnesyl pyrophosphate (farnesyl-PP) [25].

Two molecules of farnesyl-PP formed presqualene pyrophosphate (EC 2.5.1.21) which is then converted to squalene by a transferase NADPH-dependent (EC 2.5.1.21). Another molecule of NADPH is oxidised to NADP$^+$ by squalene monooxygenase (EC 1.14.13.132) to produce 2,3-epoxysqualene [26].

A molecule of aspartic acid activates the substrate by protonating the epoxide ring, increasing its susceptibility to intramolecular attack by the nearest double bond. Lanosterol synthase (EC 5.4.99.7) catalyzes this cascade of ring forming reactions, producing lanosterol (sterols backbone).

Sterol C14 demethylase (EC 1.14.13.70) consumes 3NADPH to form 4,4-dimethyl-cholesta-8,14,24-trienol, removing the CH_3 group at the carbon atom position 14.

The double bound formed between C14 and C15 is removed by a reductase NADPH-dependent (EC 1.3.1.70) and then the methylsterol monooxygenase (EC 1.14.13.72) generate 4-methylzymosterol-carboxylate by the oxidation of 3NADPH. The COOH group inserted in this way is removed by a dehydrogenase (EC 1.1.1.170) $NADP^+$-dependent, forming 3keto-4-methyl-zymosterol. Another reductase NADPH-dependent (EC 1.1.1.270) forms 4-methylzymosterol that is then converted in zymosterol.

Sterol C24-methyl transferase (EC 2.1.1.41) converts zymosterol to fecosterol, by the insertion of a CH_3 group (position 28), involving as cosubstrate S-Adenosyl methionine (SAM). An isomerase converts fecosterol in episterol which is transformed in 5,7,24(28)-ergosta-trienol by lathosterol oxydase (EC 1.14.21.6), reducing a molecule of $NADP^+$. 5,7,22,24(28)-ergosta-tetraenol is then formed and finally converted into ergosterol by a reductase (EC 1.3.1.71) NADPH-dependent [27].

1.4 Metabolism regulation

The sugar composition of the media and oxygen availability are the two main environmental conditions that have a strong impact on yeast metabolic physiology [2].

In heterotrophic organisms, ATP is produced in pathways that degrade energy-rich substrates, into products with lower free energy. The net free energy difference of such pathway is [28]:

$$\Delta G^{NET} = \Delta G^{S \rightarrow P} - n^{ATP} \Delta G^{ATP}$$

$\Delta G^{S \rightarrow P}$ is the free energy difference between the substrate and the final product in the pathway, ΔG^{ATP} is the free energy conserved in one mol of ATP and n^{ATP} is the yield of ATP.

In order to maximize ATP yield, the entire difference in free energy between the substrate and the product should be converted to ATP. However, this results in $\Delta G^{NET} = 0$.

In this case the pathway is in thermodynamic equilibrium, meaning that the net reactions rate is zero. Consequently, the rate of ATP production is also zero. On the other hand, when n^{ATP} is not maximal, $\Delta G^{NET} < 0$ and this results in driving the reactions of the entire pathways forward. In summary, it is impossible to maximize both rate and yield in a general ATP producing pathway.

Respiration, where glucose is completely oxidized to CO_2 and H_2O, results in bigger decrease in free energy than fermentation. This results in a much higher yield of ATP per molecule of glucose. So, yeasts produce ATP from sugars and they can do it by fermentation or by respiration.

Ethanol fermentation is a fast way of producing ATP, with a total yield of only 2 moles of ATP per mole of consumed glucose. On the other hand respiration, where glucose is completely oxidized to CO_2 and H_2O, results in a theoretical total yield of 26-32 moles of ATP per mol glucose [29,30] but, in contrast to fermentation, it runs at a much lower rate.

Kluyveromyces lactis is a respiro-fermentative, facultative aerobe yeast. This means that, depending on the growth conditions, the type and concentration of sugars and/or oxygen availability, it may display either a fully respiratory or a fermentative metabolism or even both in a mixed respiro-fermentative metabolism.

Using both pathways at the same time results in a lower net yield of ATP per molecule of glucose than when respiration is used exclusively, corresponding to a reduction of biomass yield. This will have an impact also on population dynamics, as the rate at which ATP is synthesized dictates the rate at which biomass is produced (*specific growth rate)*.

Switch from respiratory to fermentative metabolism usually happens in oxygen-limited condition, so that *K. lactis* rely on a predominantly respiratory metabolism of glucose under aerobic conditions [11], resulting in no intracellular accumulation of ethanol; however it can also ferment but only in anaerobic condition (*Crabtree-negative)*.

Some others yeasts, like *Saccharomyces cerevisiae*, perform alcoholic fermentation also under aerobic conditions [2,12] and they are called *Crabtree-positive*, instead.

1.5 Biotechnology: genetic engineering

Yeasts represent a good model organisms for studying molecular mechanisms in eukaryotic cells. One of the major objectives of biotechnology is the improvement of the productivity of cellular systems. When desired features or organisms have been identified, it is often desirable to manipulate and/or transfer these features to other production hosts that are more suited for industrial production or change the organism to one better suited for the production environment, to increase yield [4]. This can be accomplished by optimizing the production process (i.e. bioprocess engineering) and/or by optimizing the cellular system involved (i.e. metabolic engineering).

Whereas bioprocess optimization directs metabolism towards desired activities [31], metabolic engineering, instead, is the rational redesign of cellular metabolism in order to optimize living systems for biotechnological applications [32]. In a broader sense, it can be viewed as a modification of the bioreaction network aims at a redirection of carbon and energy fluxes to achieve conversion or degradation of biotechnological target compounds. For example, increase the rate of a desired product, reduce the rate of undesired side-products [33], decompose toxic or undesired substances.

From the chemical engineer's point of view, the living system participating in a biotechnological process is the central unit that catalyses chemical reactions [32]. Biotechnologically based industrial production of high value added bio-products such as vitamins, amino acids, enzymes, bulk chemicals, antibiotics and bio-pharmaceuticals has been accomplished in the past with the use of micro-organisms.

The increase in penicillin production by strain improvement based on random mutagenesis, which was pursued from 1940 to the mid 1970s, represents an early contribution of life scientists to improve a bioprocess that is of utmost medical importance [34].

The advent of recombinant DNA technology in the late 1970s has led to specific direct introduction of specific product pathway of interest, allowing microbial mutants with improved product specific production rates compared with the wild type parents. An example is the production of insulin by *E. coli* or *S. cerevisiae* [35], or the enhancement of the flux to the products naturally produced by the organisms.

Genetic modification of the biological systems can significantly enhance the process performance, compared to the classical strain development programs based on random strain mutagenesis and selection procedures [35]. However, even when the environmental conditions are well defined, we are far from understanding how the genomic program determines the living organism [32]. So, although the complete knowledge of the genome's DNA sequence of yeast *K. lactis* [10], it will clearly not suffice to unravel all principles governing the most complex phenomenon of life.

1.6 *rag8* mutation

K. lactis is a Crabtree-negative yeast in which both respiratory and fermentative pathways can co-exist during growth on glucose [36-38], although respiration appears to be dispensable since antimycin A (AA, a mitochondrial inhibitor) does not inhibit growth on glucose (Rag$^+$ phenotype) [39,40].

A decade ago, indeed, the glycolytic pathway was studied in this yeast by screening for *rag* mutants [10] (RAG means Resistance to Antimycin on Glucose and allows mutants of the glycolytic pathways to be selected) and it was found that *K. lactis* wild type strains, except for a few variants, do not show an impaired growth on glucose in the presence of AA.

Rag mutants, instead, are enable to grow under strictly anaerobic conditions, i. e. in the absence of respiration: this is called a Rag⁻ phenotype and it is an indication of a glycolytic defect.

Moreover, it was previously reported that *rag* mutant cells displayed fatty acids membrane/cell wall defects and an enhanced sensitivity to osmotic stress conditions caused by their inability to accumulate/produce glycerol, indicating that a common pathway regulates glucose utilization and stress response mechanisms [41].

In many yeasts glucose is transported across the membrane by facilitated diffusion. Facilitated diffusion is mediated by permease, a membrane protein, down a concentration gradient without the consumption of energy, whereas energy consuming H^+ symport can regulate glucose influx in *non-Saccharomyces* yeasts [12].

Genetic studies of glucose transport in *K. lactis* have shown that this yeast has a simplified glucose uptake system based on the proteins encoded by two single genes, *HGT1* and *RAG1*. The *HGT1* gene is constitutively expressed and encodes an high-affinity glucose transporter, whereas *RAG1* is induced by glucose and encodes a membrane protein involved in the low-affinity glucose/fructose carrier system [42].

There have been identified several *RAG* genes regulating the glycolytic flux, that control the expression of the low affinity carrier gene *RAG1*. All of them are single genes encoding various proteins:

- *RAG4* (coding for the unique transmembrane glucose sensor in *K. lactis*) [43]

- *RAG5* (coding for the single glucose phosphorylating enzyme hexokinase in *K. lactis*) [44]

- *RAG8* (an essential gene coding for a casein kinase I, CKI) [45]

- the transcription activators SCK1 [42]

The large number of regulators identified suggests that several pathways control *RAG1* expression. In particular, the *RAG8* gene has been found to share a high degree of identity with the two casein kinases I of *Saccharomyces cerevisiae* (Yck1p and Yck2p) encoded by *YCK1* and *YCK2* genes [45].

So, the equivalent of the *YCK1/YCK2* pair, in *K. lactis*, is the single essential gene *RAG8*; its product (Rag8p) would be an homolog of this eukaryotic protein belonging to casein kinase I family, which are ubiquitous serine/threonine-specific kinases characterized by a preference for acidic substrates that can be phosphorylated. CKI has long been proposed to play important regulatory roles, as suggested by its widespread distribution of isoforms [46].

So *RAG8* plays a crucial role in the regulation of glucose metabolism in *K. lactis*: mutations in this *K. lactis* genes cause multiple defects, suggesting that casein kinases are involved in a wide range of cellular processes. Sensitivity to AA on glucose (Rag$^-$ phenotype) indicates that *rag8* mutant cells are deficient in either the low-affinity glucose transport system or other steps of glycolysis, and this is associated with mutations in genes encoding glycolytic enzymes, glucose transporters and sensing components, as well as their regulatory factors [43-52].

In order to analyze the metabolic changes taking place in *K. lactis rag* mutants, strains unable to grow under strictly fermentative conditions [39], we took advantage of the *rag8* mutant affected in the CKI activity [41].