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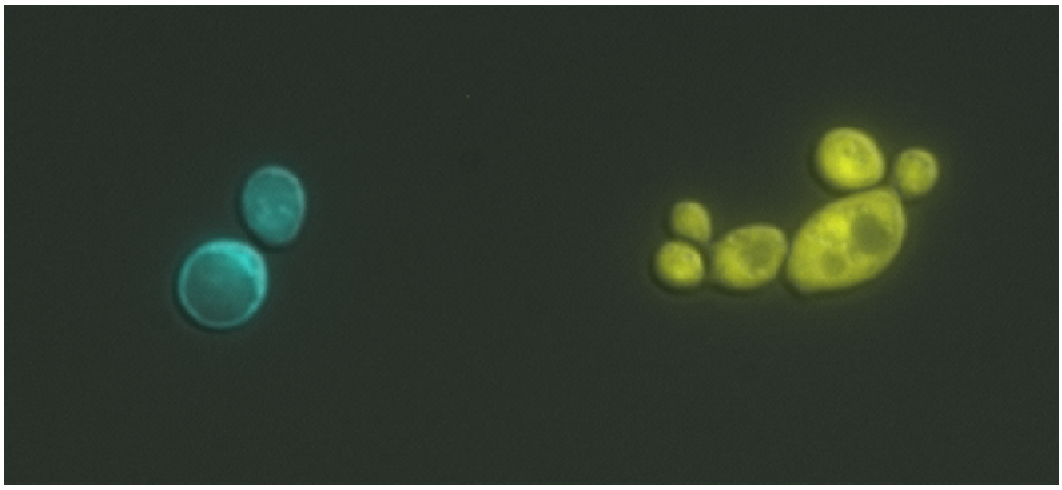

# Resource utilization and competition among yeasts

Master of Science Thesis

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**Abstract**

One of the postulates of evolutionary theory is competition for limited resources. Previous studies show that there is a trade-off between yield and rate in resource utilization and ATP production pathways, implying that different metabolic strategies will result in different competition outcomes. Two main metabolic strategies used by microorganisms are respiration and fermentation. Respiration maximizes the efficiency of ATP production, resulting in increased yields at the expense of the rate. In contrast, fermentation is an inefficient ATP production strategy, but it runs at a higher rate. Our goal was to study competition between fermentation and respiration and find conditions, such as resource availability, population and spatial structure, that favor dominance of those strategies. We used *Saccharomyces cerevisiae* and *Kluyveromyces lactis* tagged with different fluorescent proteins as models for fermentation and respiration, respectively. We experimentally determined physiological parameters and used them in dynamic models of competition for resources in spatially homogeneous (well-mixed) and in spatially structured populations. The results show competition dynamics in different conditions and we discuss implications of those results in the evolution of metabolic strategies.

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# Chapter 1

# Introduction

## 1.1 Evolution and metabolism

The theory of evolution rests on three fundamental ideas:

1. *Variability* - individuals differ in terms of their phenotypic traits
2. *Inheritance* - some of the variability is based on differences in genotypes and can be passed from parents to offspring
3. *Competition for resources* - environment contains less resources than are necessary for the survival of all offspring

It follows that variants better adapted to competition for resources, and thus having a higher probability of leaving offspring, will dominate in the population.

Resources are needed for both maintenance (survival) and reproduction. Therefore harvesting them from the environment and transforming them into biomass and biologically useful energy is a fundamental characteristic of all living things. In contrast to this universal principle, one can find individuals living in all kinds of ecological niches. Most of these niches differ greatly with respect to environmental conditions, including types and amounts of resources available. As a consequence of different selective pressures present in those niches, individuals living in them will exhibit different modes of metabolism (Table 1.1) and will respond to changes in their environments differently. Over long enough periods of time, organisms become well adapted to their respective ecological niches.

Each individual interacts with its environment, it uptakes resources from it and it excretes metabolic by-products. This dynamically changes the abiotic factors of the environment for other individuals, whether they belong to the same species or not. Therefore, to fully understand the underlying metabolic and other adaptations, we must consider and study competition for resources in evolutionary and ecological context.

Historically, theoretical and experimental studies of interspecies interactions were focused on plant and animal life. In the first part of the 20th century, it was realized that

**Table 1.1** – Organisms by their carbon and energy source (from Bailey and Ollis (1986))

| | | Energy source | |
|---|---|---|---|
| | Carbon source | Chemical | Light |
| Heterotrophs | Organic compouds | Chemoheterotrophs | Photoheterotrophs |
| Autotrophs | $CO_2$ | Chemoautotrophs | Photoautotrophs |

microorganisms are a good model for studying competition for resources and testing ideas from theoretical ecology (Gause, 1932). Nowadays, the field of microbial ecology is expanding thanks to advances in microbiology, molecular biology, advent of metagenomics and realization that microorganisms, along with competition, engage in other types of social behavior (West et al., 2006).

## 1.2 Metabolism and metabolic strategies

*Metabolism* is defined as the set of coordinated biochemical reactions for (Lehninger et al., 2004):

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. Nevertheless, 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.

Depending on the source of carbon they use, living organisms can be divided in two big groups (Table 1.1). *Autotrophic* organisms use carbon dioxide from the atmosphere as their carbon source. *Heterotrophic* organisms depend on relatively complex organic compounds from their environment as a source of carbon. Multicellular animals and most microorganisms belong to this group. Most autotrophic organisms obtain their energy from sunlight while most heterotrophs get their energy from energy-rich organic compounds produced by autotrophs.

Many organisms use glucose as the preferred carbon and energy source. Glucose will usually be metabolized in the cytosol, in a pathway called *glycolysis*. The first reaction of glycolysis is the uptake of glucose from the extracellular medium and its immediate phosphorylation. In a series of reactions glucose-6-phosphate is partially

oxidized to a three-carbon molecule - *pyruvate*. This yields 2 molecules of ATP and 2 molecules of NADH in the process. The net reaction stoichiometry describing glycolysis is:

$$\text{Glucose} + 2\ NAD^+ + 2\ ADP + 2\ P_i \rightarrow 2\ \text{pyruvate} + 2\ NADH + 2\ H^+ + 2\ ATP + 2\ H_2O$$

To keep this process going, NADH needs to be reoxidized to $NAD^+$. There are two principal ways this can be done, by *fermentation* or *respiration*, and the choice between the two will decide the fate of pyruvate.

### 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. Organic compounds are first oxidized to $CO_2$, while $NAD^+$ is reduced to NADH. 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 gradiend 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.

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. The net stoichiometric equation for oxidative phosphorylation in terms of glucose and oxygen consumption is:

$$\text{Glucose} + 6O_2 \rightarrow 6CO_2 + 6\ H_2O$$

and in terms of ATP production is:

$$x\ ADP + x\ Pi + \tfrac{1}{2}O_2 + H^+ + NADH \rightarrow x\ ATP + H_2O + NAD^+$$

where $x$ is also known as *P/O ratio*, a number of ATP molecules synthesized per atom of oxygen consumed. This number varies between 1 and 3, depending on the organism, conditions and the cytochromes used in the respiratory chain. The maximal theoretical yield of ATP molecules per molecule of glucose in respiration will be 26-32 (Lehninger et al., 2004), but in reality these yields are lower. For *Saccharomyces cerevisiae*, the maximum is 16.5 mols of ATP formed from 1 mol of glucose, under the assumption that there is no proton leakage (Famili et al., 2003).

**Fermentation**

In fermentation, glucose is only partially catabolized. End products of microbial fermentative pathways include ethanol, lactate, butyric acid and acetone. The simplest type of fermentation is lactic fermentation, characteristic for many tumors (Diaz-Ruiz et al., 2009), muscles and lactic bacteria. Pyruvate is oxidized into lactate in a single reaction. The net reaction of lactic fermentation is

$$\text{Glucose} + 2\text{ADP} + 2\ \text{P}_i \rightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ \text{H}_2\text{O}$$

In ethanol fermentation, which is characteristic for *Saccharomyces cerevisiae*, pyruvate is first decarboxylated to acetaldehyde, and then reduced to ethanol, with simultaneous reoxidation of NADH to $\text{NAD}^+$. Starting from glucose, ethanol fermentation can be summarized as

$$\text{Glucose} + 2\text{ADP} + 2\ \text{P}_i \rightarrow 2\ \text{ethanol} + 2\ \text{ATP} + 2\ \text{CO}_2 + 2\ \text{H}_2\text{O}$$

Since ATP is needed for growth and reproduction, strategies to produce it and their characteristics (yield and rate) will have large evolutionary consequences for the organism (discussed in section 1.5).

## 1.3 *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* (figure 1.1) is a widely used and well-studied eukaryotic model organism in molecular biology. This is due to its historical importance as an industrial organism in brewing industries as well as convenience of using it in laboratory settings, because of its:

1. rapid growth
2. ease of genetic manipulation
3. availability of peer-reviewed literature, kinetic data, sequences.

The genome of *Saccharomyces cerevisiae* was the first eukaryotic genome to be fully sequenced. It contains a set of sixteen chromosomes with the total genome size of 12.052 Mb. More than 80% of its about 5780 protein-coding genes have been functionally characterized (Dujon, 2010). In addition, a lot of microarray-based transcriptomic data, genome-wide function data, proteomic, interactomic and metabolomic data are available. Since a lot of related yeast genomes have been sequenced, *Saccharomyces cerevisiae* has become an important organisms for comparative genomics and for the study of genomic evolution.

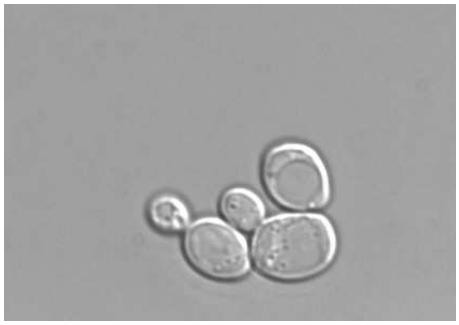


**Figure 1.1** – *Saccharomyces cerevisiae* cells

### Physiology of *Saccharomyces cerevisiae*

*S. cerevisiae* can grow on different carbon sources (glucose, fructose, sucrose, maltose, galactose, ethanol...), but glucose is a preferential carbon source. The most striking feature of its physiology is the preference for fermentative metabolism under fully aerobic conditions (Crabtree, 1928, De Deken, 1966), termed the *Crabtree effect* (table 1.2, figure 1.3). As a consequence of this, ethanol formation and low biomass yields are the characteristic of *Saccharomyces cerevisiae* batch cultivations on glucose.

Aerobic growth of *Saccharomyces cerevisiae* can be established in carbon-limited chemostat cultivations at low dilution rates. In this settings, the specific oxygen uptake rate increases linearly with the dilution rate up to a critical value (around D=0.3 $h^{-1}$). At this rate the respiratory capacity becomes saturated and further increase in dilution rate results in an onset of fermentation rate and ethanol formation.

Second, but related, phenomena in glucose metabolism of *Saccharomyces cerevisiae* is *glucose repression* or *glucose control* in media containing high glucose concentrations. Instead of utilizing different carbon sources from media simultaneously, yeast cells do it sequentially starting with glucose. The switch from utilization of glucose to other carbon sources is followed by a lag phase, resulting in *diauxic shift*.

Glucose repression acts on different levels of metabolism simultaneously. The glucose response influences:

1. transcription of different genes (repression of genes needed for metabolism of other carbon sources and genes involved in respiratory metabolism)
2. concentrations of intracellular metabolites
3. modification and degradation of enzymes
4. the stability of various mRNAs

The most studied effects of glucose control are those on the transcriptional level. Glucose influences (represses) the rate of transcription for a number of genes like SUC, GAL, MAL that are needed for metabolism of sucrose, galactose and maltose, respectively (Gancedo, 2008). It also causes accelerated proteolytic degradation of carrier

proteins like maltose permease and galactose permease. Since the induction of MAL and GAL genes requires the presence of maltose and galactose in the cytosol, this represents an additional level of repression for those pathways (Klein et al., 1998). Another mechanism by which glucose exhibits its effects is directly influencing enzymatic activities. It has been found that it acts as a direct inhibitor for maltase and melibiase (Klein et al., 1998).

**Table 1.2** – Regulatory phenomena in yeast sugar metabolism (Pronk et al., 1996)

| Regulatory phenomenon | Definition |
|---|---|
| Crabtree effect | Aerobic alcoholic fermentation |
| Pasteur effect | Suppression of alcoholic fermentation in aerobic conditions |
| Kluyver effect | Absence of ethanol fermentation in oxygen-limited conditions |
| Custers effect | Oxygen requirement for alcoholic fermentation |

## 1.4 *Kluyveromyces lactis*

Yeast *Kluyveromyces lactis* (figure 1.2) is one of the six species from the *Kluyveromyces* genus. It is an ascomycetous budding yeast, just like *Saccharomyces cerevisiae*. It can be found in many diverse habitats, but many strains were originally isolated from dairy products. Being a dairy yeast, it is able to grow on lactose as a sole carbon source (Snoek and Steensma, 2006), unlike most other yeasts. *Kluyveromyces lactis* is becoming an increasingly popular yeast with molecular biologists and in biotechnological applications. Some of the reasons for this include:

1. Availability of plasmids and other tools for genetic manipulations (Schaffrath and Breunig, 2000, Zenke et al., 1993)
2. Finished nuclear and mitochondrial genome sequencing projects (Dujon et al., 2004, Sherman et al., 2009, Zivanovic et al., 2005)
3. Close evolutionary relationship with *Saccharomyces cerevisiae* that enabled functional annotation of *Kluyveromyces lactis* genes
4. Specific physiology that results in high biomass and protein yields in fully aerobic cultivations
5. Interest in genetics and physiology *Kluyveromyces lactis* strains that express proteins toxic to other yeast species (Breunig and Steensma, 2003)

*Kluyveromyces lactis* genome, which is about 10.6 Mb in size, is organized in six chromosomes. It has approximately 5300 coding sequences (Sherman et al., 2009). Strains having the killer phenotype have cytoplasmic linear plasmids that carry a gene

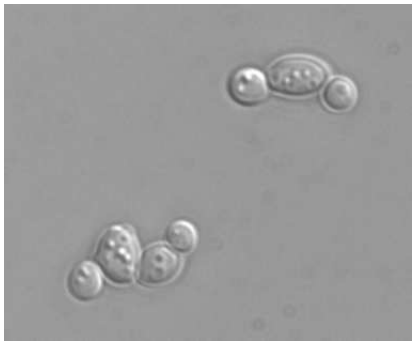


**Figure 1.2** – *Kluyveromyces lactis* cells

for zymocin, a glycoprotein toxic to a number of other yeast species (Breunig and Steensma, 2003).

### Physiology of *Kluyveromyces lactis*

*Kluyveromyces lactis* is a Crabtree-negative aerobic respiro-fermentative yeast. It is a "petite-negative" yeast and an *obligate aerobe*, meaning that, while it can ferment certain sugars, it is unable to grow in fully anaerobic conditions. So far, 20 genes were identified as required for anaerobic growth in *Saccharomyces cerevisiae*, but their homologues have not been found in the *Kluyveromyces lactis* genome (Snoek and Steensma, 2006).

Compared to *Saccharomyces cerevisiae*, batch fermentations on glucose result in much higher yields of biomass and little or no ethanol produced. This is mostly because of non-fermentative growth and partially due to an inducible alcohol dehydrogenase that uptakes any ethanol that might be produced (Schaffrath and Breunig, 2000). In carbon-limited chemostats, an increase in dilution rate results in a linear increase of specific oxygen uptake rate. In contrast to *Saccharomyces cerevisiae*, there is no shift to respiro-fermentation at some critical dilution rate.

In wild-type *K. lactis*, and non-oxygen-limited conditions, all of pyruvate is channeled into the TCA cycle. This happens even in media with high concentrations of glucose (Schaffrath and Breunig, 2000). The glycolytic flux is tightly regulated by *RAG* genes. *rag* mutants do not show an impaired growth on glucose, indicating that pentose phosphate pathway can bypass the block in glycolysis and that biosynthetic activity can be highly efficient even at low glycolytic fluxes (Breunig et al., 2000). Restriction of glucose uptake and low glycolytic fluxes may also explain why *Kluyveromyces lactis* is less sensitive to glucose repression than *Saccharomyces cerevisiae*. Comparison of preferred pathways in glucose metabolism between *S. cerevisiae* and *K. lactis* is shown in figure 1.3.

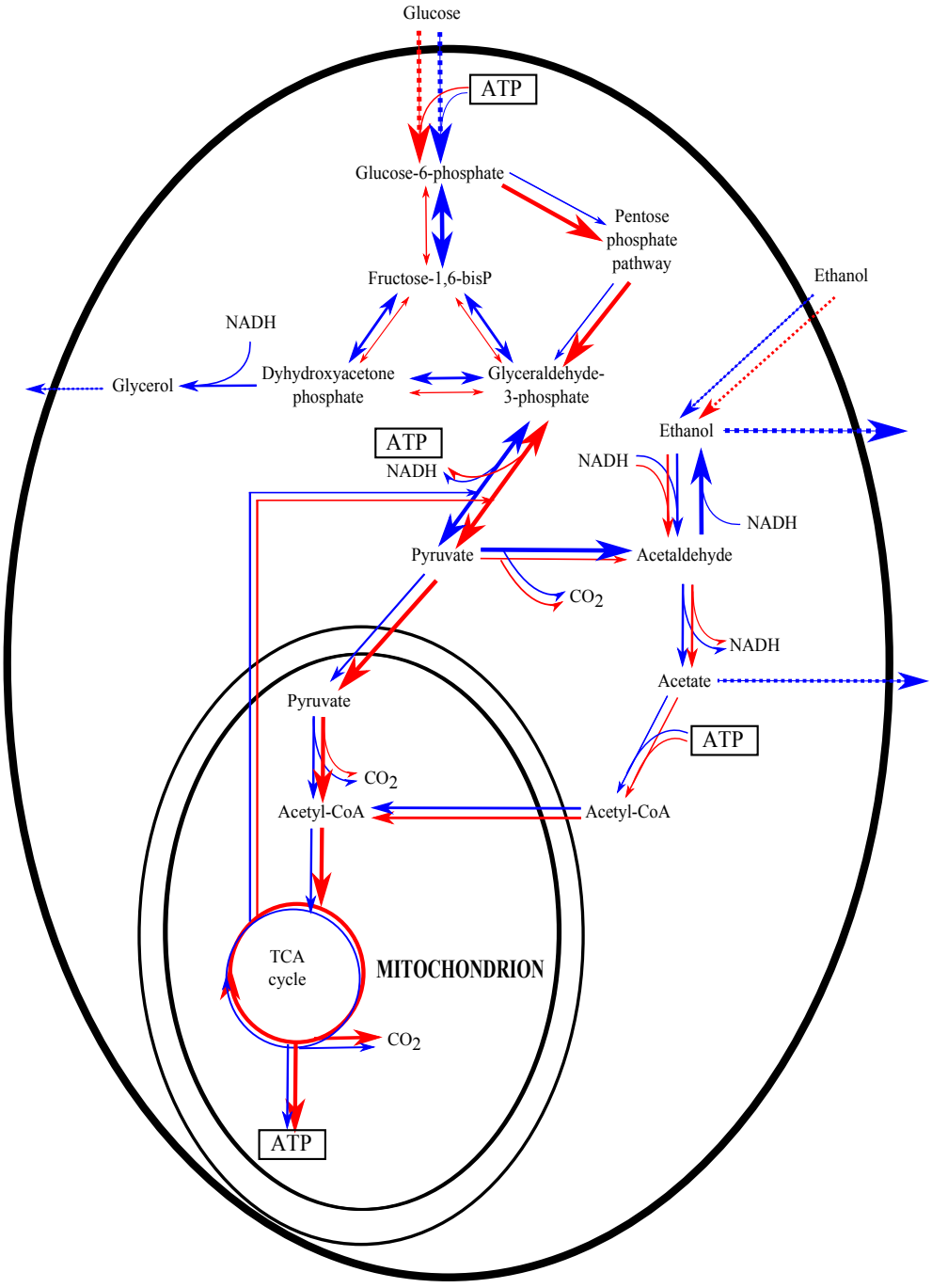



**Figure 1.3** – Comparison of primary carbon metabolism in *Saccharomyces cerevisiae* (blue) and *Kluyveromyces lactis* (red). Thickness of arrows indicates preferred pathways when utilizing glucose. *Saccharomyces cerevisiae* preferably uses the glycolysis and conversion of pyruvate to ethanol. *Kluyveromyces lactis* preferably uses the pentose phosphate pathway and oxidizes pyruvate in the TCA cycle.

## 1.5 Trade-off between rate in yield in metabolic pathways

In the course of evolution yeasts have optimized their molecular and behavioral characteristics to adapt to the environment and maximize their fitness. Many adaptations come with a price, and as a result the design of biological systems is fraught with different trade-offs (Khersonsky et al., 2006, Lee et al., 2009, Tokuriki et al., 2008). Various trade-offs in biochemical networks have been studied intensively in the past (Maclean and Gudelj, 2006, Pfeiffer and Bonhoeffer, 2002, Pfeiffer and Schuster, 2005, Pfeiffer et al., 2001). Since reactions in metabolic networks usually involve changes in free energy between substrates and products, some of these trade-offs can be deduced from the first principles of thermodynamics (Pfeiffer and Bonhoeffer, 2002).

In heterotophic 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 (Pfeiffer and Bonhoeffer, 2002)

$$\Delta G^{NET} = \Delta G^{S \to P} - n^{ATP} \Delta G^{ATP} \tag{1.1}$$

$\Delta G^{S \to P}$ is the free energy difference between the substrate and the final product in the pathway, $\Delta G^{ATP}$ 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 can be converted to ATP. However, this results in $\Delta G^{NET} = 0$. In this case the pathway is in thermodynamic equilibrium, meaning that no more chemical work can be done by the pathway and the net reaction rate of the pathway 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, that it is impossible to maximize both rate and yield in a general ATP producing pathway.

A particular metabolic trade-off can be found when studying the difference between two specific ATP producing pathways found in nature. As described before, yeasts produce ATP from sugars, and they do so by fermentation or by respiration (section 1.2). Ethanol fermentation is a fast way of producing ATP, with a total yield of only 2 moles of ATP per mol 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. In contrast to fermentation, respiration runs at a much lower rate.

Two physical factors limit the rate of respiration. First, the dissolved oxygen concentration in the cell is what limits respiratory metabolism and, unlike intracellular glucose concentrations, it is almost impossible for cells to regulate it. Second, respiratory enzymes in yeast are an integral part of the mitochondiral membrane. Since both the number of mitochondria and the surface of the mitochondrial membrane are spatially

limited, concentrations of respiratory enzymes cannot be increased easily (Conant and Wolfe, 2007).

Switch from respiratory to fermentative metabolism usually happens in oxygen-limited condition, but some organisms, like *Saccharomyces cerevisiae*, use respiro-fermentative metabolism even in fully aerobic conditions (section 1.3). 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.

This will have an impact on population dynamics, as the rate at which ATP synthesized dictates the rate at which biomass is produced, the *specific growth rate*. Similarly, ATP yields dictate biomass yields: a reduction in ATP yield will result in a reduction of biomass yield.

## 1.6 Metabolic trade-offs and evolutionary game theory

It's natural to ask why would organisms evolve to use a less efficient way of utilizing a resource that limits their growth. When an organism has an exclusive access to the resource, it's beneficial if this resource is used in the most efficient manner. For example, individuals from species belonging to animal kingdom that ingest food, and thereby eliminate the effect of metabolic competition, use respiration as an exclusive metabolic strategy. However, resources found in nature are usually shared by communities of mixed populations and there is a direct competition for resources in the microbial world. These populations will often be composed of organisms with different types of metabolism, or *metabolic strategies*.

*Evolutionary game theory* is a framework for studying frequency-dependent selection. It considers a population of different players (phenotypes, strategies) that interact randomly among themselves. Different strategies have different pay-offs which are interpreted as fitness, and higher pay-offs mean higher reproductive success. In other words, fitness from using a certain strategy (or phenotype) will depend only on proportions of others strategies in the population (Nowak, 2006a).

The Prisoner's Dillema (PD) is a well-known game-theoretic situation that can be applied to problems in biology (Smith, 1982). Two players can choose between two strategies: cooperation and defection. This results in four different outcomes, depending on the combination that was chosen. Playing defection against cooperation results in the highest pay-off, called 'temptation'. At the same time, cooperator gets the lowest payoff from this situation, the 'sucker' pay-off. If both players cooperate, they get 'reward' and if both players defect, they get 'punishment' which is lower than the reward. This presents two problems for evolution of cooperativity. First, the stable solution (Nash equilibrium) is the defection of both players (Nowak, 2006a), and they can never increase their pay-off by switching to a cooperation. Second, a population of cooperators will be easy to invade by defectors.

Yeasts do not interact directly when competing for glucose, but the metabolic strategies they use can still be interpreted in the game-theoretic context. The trade-off between rate and yield in metabolic pathways has characteristics of PD (Pfeiffer and Schuster, 2005). The exclusive use of respiration will increase the total yield of ATP produced by the population before all the resources are consumed. Since higher ATP yield will result in high biomass yield, the whole population will benefit and will have increased its average fitness. This makes respiration a *cooperative strategy*. The population that uses fermentation will consume resources faster and produce ATP in a wasteful manner. Individual rates will be high, but the total amount of ATP produced before all the resources are consumed will be low. This makes fermentation a *selfish strategy* or defection.

Pure respiratory metabolism leads to high biomass yields. The whole population benefits from this cooperative behavior and this increases its average fitness ('reward'). However, selfish use of resources leads to high individual fitness ('temptation') through high growth rate. In a population that is predominantly fermentative, this inefficient use of resources will reduce the overall fitness of the population through lower biomass yields ('punishment'). A purely respiratory population will be easy to invade by the fast growing fermenters (defectors) and it will get outcompeted ('sucker' payoff) (Pfeiffer and Schuster, 2005). This situation is known as the *tragedy of the commons* (see figure 4.9 in section 4.2.1). In summary, direct competition for limited resources favors the selfish strategy and faster resource consumption rates.

Since cooperative strategies seem to be at a disadvantage when using shared resources, it is not trivial to explain the presence and evolution of cooperative behavior. One of the theories that tries to explain it is *kin selection theory* (Hamilton, 1963, West et al., 2006). According to this theory, altruistic behavior directed toward related individuals gives an indirect fitness, as relatives' genes are more likely to be passed on to the next generation. Mathematically, this is expressed as *Hamilton's rule*: cooperative behavior is favored when

$$rb - c > 0 \tag{1.2}$$

where $c$ is the fitness cost of the altruistic behavior, $b$ is the benefit for the recipient of the altruistic behavior and $r$ is the the genetic relatedness between the altruist and the recipient.

A possible mechanism that enables cooperation of related individuals in microbial populations is *limited dispersal* of the population. This can have great importance for microorganisms that colonize and reproduce in local areas or local population patches. In this situation, any neighboring cells are more likely to be clonal. As a consequence, populations with rigid spatial structure should be conductive to cooperative behavior (Kreft, 2004, West et al., 2006).

## 1.7 Evolutionary history of yeasts

With about 40 yeast genomes sequenced so far, the investigation of yeast evolution on the genomic level has been gaining a lot of attention in the last decade (Dujon, 2010). An interesting pattern has emerged from the study of those genomes: yeasts that are considered to be closely related (figure 1.4), even those belonging to the same genus, have genomes with surprisingly big differences. Rather than being a result of continuous and subtle change, their genomes seem to have undergone a number of abrupt changes.

Duplications have been suggested as one of the mechanisms by which genomic and physiological differences between related yeast can be explained. *Paralogous* sequences are those that appear in genomes as a result of gene duplication events. These sequences represent raw material on which natural selection can work, as one of the copies is no longer under selective pressure. There are three ways a duplicated gene can lead to new phenotypic traits: *neo-functionalization*, *sub-functionalization* and an *increase in gene dosage*. Neo-functionalization is the development of a new function and sub-functionalization is a division of labor between duplicates that leads to more specialized functions. However, it has been shown that duplications more often result in novel regulatory control than in a totally novel functions for paralogs (Conant and Wolfe, 2008).

A whole-genome duplication (WGD) has been proposed as one of the reasons for genomic differences in yeast. As polyploidy is a catastrophic event leading to reproductive isolation (Greig, 2009) and increased metabolic costs (Wagner, 2005), it would have to confer a major evolutionary advantage for the organism for it to survive natural selection. It was postulated that such an event would have to be followed by the loss of most of duplicated genes. Those that remain would diverge by neo-functionalization or sub-functionalization, or have positive fitness effects through increased gene dosage (Conant and Wolfe, 2008).

Clues for WGD event in evolution of *Saccharomyces cerevisiae* first came from studies of presence and distribution of duplicated blocks in its genome (Wolfe and Shields, 1997). Further, and definite evidence came after other yeast genomes were sequenced and the genome of *Saccharomyces cerevisiae* was compared to the genome of *Kluyveromyces waltii* (Kellis et al., 2004). It is currently unknown whether the WGD event is the result of the duplication of the entire chromosome set or a hybridization event between two closely related yeast species (Conant and Wolfe, 2007).

WGD had a major impact on the transcriptional network and regulation in *Saccharomyces cerevisiae* (Ihmels et al., 2005). Outside of WGD events, duplications of single genes involved in signalling and transcriptional regulation are rare (Wapinski et al., 2007). It has also been shown that the WGD event was responsible for the characteristic physiology of yeast belonging to *Saccharomyces* genus (Conant and Wolfe, 2007, Merico et al., 2007). Duplicated genes increased the ability of post-WGD yeasts to metabolize glucose and grow anaerobically in minimal media. Crabtree-positive phenotype

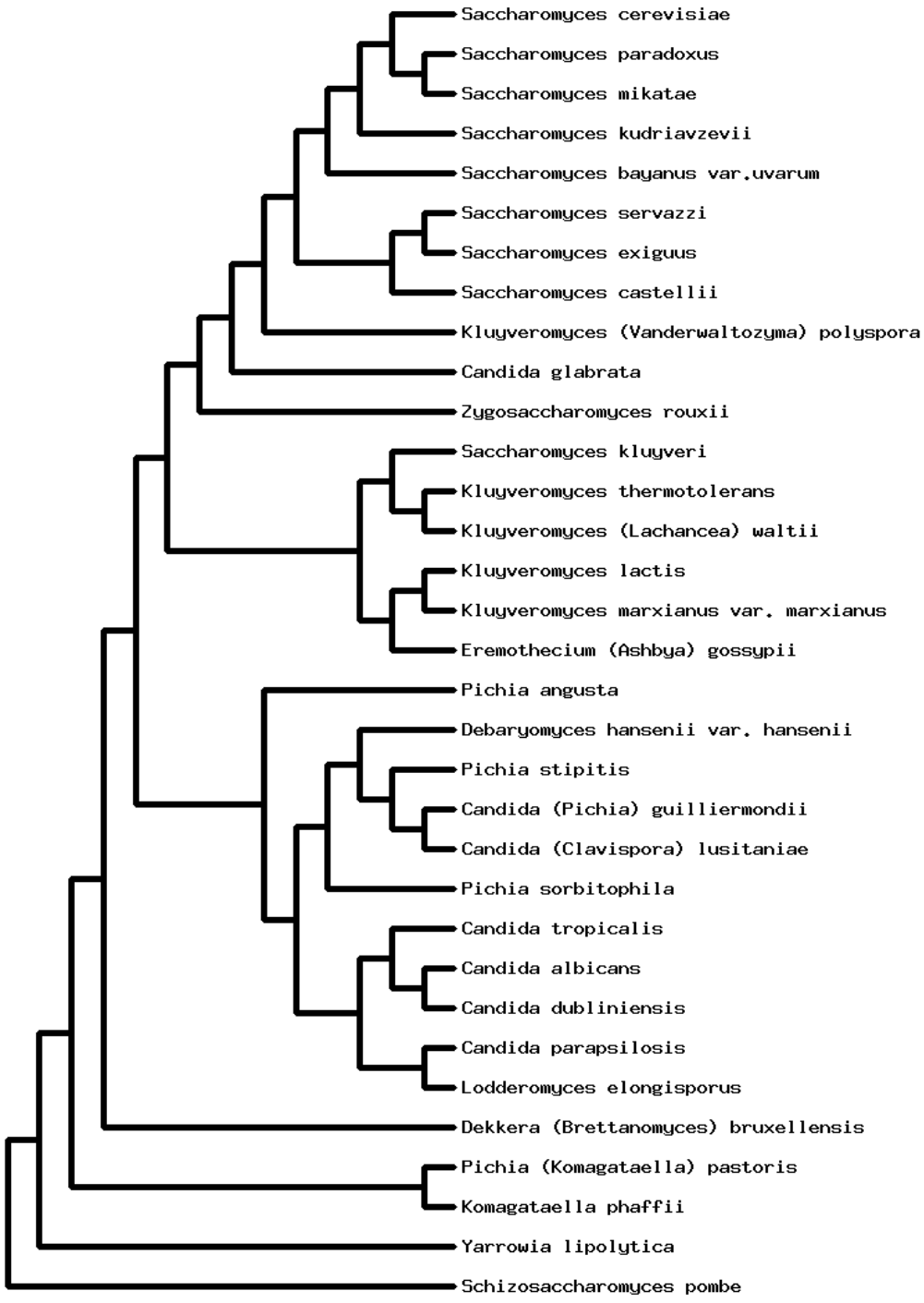



**Figure 1.4** – Phylogenetic tree of different yeast species (Sherman et al., 2009)

and the presence of petite mutants are also associated with WGD (Merico et al., 2007).

How WGD events led to a fermentative instead of a respiratory lifestyle can be explained by the following three reasons (Conant and Wolfe, 2007):

1. After WGD, the loss of other duplicated genes increased the dosage of glycolytic enzymes, thereby increasing the glycolytic flux
2. Respiration scales differently from fermentation and is less likely to be affected by WGD. This is due to its limitation by oxygen concentration and spatial factors. In addition, some respiratory enzymes are coded in the mitochondiral genome which wasn't duplicated
3. WGD happened when first fleshy fruits appeared, 65-144 million years ago (Goddard, 2008). Since fruits are rich in glucose (150-250 g/L), this meant the establishment of a new ecological niche where species with the ability to rapidly consume glucose would have an advantage

## 1.8 Theory of niche construction

Through its metabolic activities organisms change their respective environments, and directly or indirectly affect other members of the local population. This is called *niche construction* or *ecosystems engineering* (Goddard, 2008, Hastings et al., 2007, Jones et al., 1994, 1997). For any trait involved in niche construction to have a role in evolution, it must be heritable and must have an influence on reproductive success or fitness.

Ethanol fermentation by *Saccharomyces cerevisiae* is a typical example of niche construction. At the beginning of traditional wine fermentations, fruits contain a variety of yeast species, with *Saccharomyces cerevisiae* having low abundance. As the fermentation proceeds, *S. cerevisiae* increases in frequency while other yeasts decline (Goddard, 2008). This occurs in spite of the fact that fermentation is costly and inefficient way of consuming glucose in the presence of oxygen.

One hypothesis is that ethanol itself is the crucial factor behind the niche construction and the dominance of *Saccharomyces cerevisiae*. Ethanol is a general microbicidal agent and may be related to reduction of frequencies of non-*Saccharomyces* species. However, it has been shown that non-*Saccharomyces* species are tolerant to high concentrations of ethanol (Pérez-Nevado et al., 2006), higher than they produce themselves. This indicates that the invasion of fruit-niche and niche construction by *Saccharomyces cerevisiae* may have influenced their evolution as well, by selection for higher ethanol tolerance.

Another hypothesis suggests that the high glycolytic flux and the related high ATP production yield related to fermentation by itself causes dominance of *Saccharomyces cerevisiae* (Conant and Wolfe, 2007). The study of alcohol dehydrogenases suggested that ancient yeast used ethanol production in order to recycle NADH produced in glycolysis thereby increasing glycolytic rate (figure 1.3). Modern yeasts started consuming

accumulated alcohol when they acquired a duplicated copy of ADH. This is estimated to have happened in Cretaceous period, 65-144 million years ago (section 1.7), at the same time when first fleshy fruits appeared (Thomson et al., 2005).

The most startling realization is that niche construction by *Saccharomyces cerevisiae* may have influenced human metabolic and cultural evolution as well. Consumption of ethanol may have resulted in increased resistance to its toxicity, and production of bread and alcoholic beverages was a factor in the transformation from nomadic to stationary lifestyle of humans (Goddard, 2008). To fully understand the evolution genomes and metabolism, it is important to study species and their physiologies in their ecological context.

# Chapter 2

# Project summary

The goal of this project is to study dynamics and outcome of competition for glucose between fermentative and respiratory metabolism by using *Saccharomyces cerevisiae* and *Kluyveromyces lactis* as models for these two strategies, respectively. The main questions of the project were:

1. How does the initial availability of resources (glucose) influence the metabolism and growth of the mixed culture, and which metabolic strategy is more dominant in environments with higher resource availability?
2. How does the spatial structure of the population influence the outcome of competition between respiratory and fermentative metabolic strategies?

To answer the first question, I performed the cultivations of both single and mixed cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Batch mixed cultures were done with different initial glucose concentrations. In addition to this, I performed carbon-limited and nitrogen-limited chemostat cultivations with mixed cultures to test the competitive exclusion principle.

To tackle the second question, I used a modelling approach. First, I formulated a dynamic model of fermentative and respiratory metabolism based on kinetic parameters obtained from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* cultivations. I used this model to study effects of resource availability, population frequency- and density-dependence on the outcome of competition. Following game-theoretic arguments that spatial structure can promote cooperation in Prisoner's dilemma situations (Nowak and May, 1992), I formulated an ecological model of competition in spatially structured settings. In this model, the meta-population is divided into local populations whose dynamics of competition for resources were based on the metabolic model. The main idea in the spatial model is to describe the population dispersal with a single parameter, and to test how changes in this parameter influence the outcome of the competition in the meta-population. The time-scales for local population dynamics and meta-population dynamics were separated by implementing a hybrid stochastic modelling approach.

# Chapter 3

# Materials And Methods

The experimental part of the project included construction of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* strains tagged with fluorescent proteins. This was the experimental strategy for distinguishing between the two metabolic strategies in batch and continuous competition experiments with mixed populations.

The modelling part consisted of estimating parameters for the metabolic model and using them to formulate and implement an ecological model where the effects of spatial structure on competition between the two metabolic types can be tested.

## 3.1 Strains

### *Escherichia coli*

*E. coli* was used for amplification of all plasmids. This was done by transforming competent DH5α *Escherichia coli* cells (Taylor et al., 1993). This strain has chromosomal genotype *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44* Φ*80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*. Competent cells were prepared using Inoue method (Inoue et al., 1990) and stored at -80°C for further use.

### *Saccharomyces cerevisiae*

The original *Saccharomyces cerevisiae* strain used in construction of fluorescent-tagged *Saccharomyces cerevisiae* was CEN.PK113-5D (van Dijken JP et al., 2000). The relevant genotype is MATa SUC2 MAL2-8c ura3-52. The phenotype of this strain is uracil auxotrophy ($Ura^-$ phenotype), meaning that CEN.PK113-5D is not able to grow in minimal media without uracil supplementation. Plasmids carrying a *URA3* gene can complement the uracil auxotrophy so transformants can be easily selected for on agar plates with minimal media. Fluorescently tagged *Saccharomyces cerevisiae* strain used

in all of the experiments in this project carried the p416-TEF-CFP plasmid (Figure 3.1).

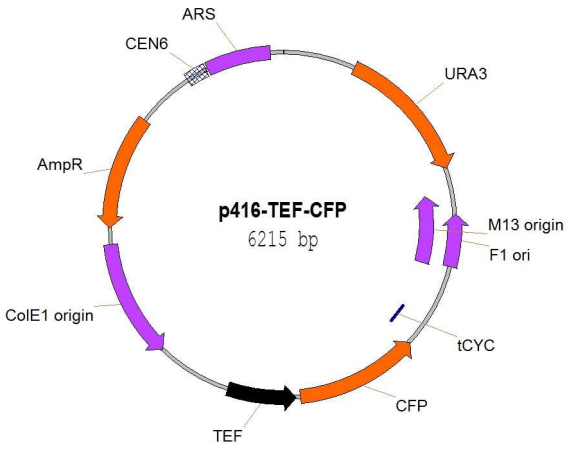



**Figure 3.1** – p416-TEF-CFP, plasmid used to transform *Saccharomyces cerevisiae*

### *Kluyveromyces lactis*

The original *Kluyveromyces lactis* strain used, RUL1888 (kindly provided by prof. K. Breunig, The Martin Luther University of Halle-Wittenberg), was also phenotypically Ura$^-$. The relevant genotype was MAT$\alpha$ ura3 lac4::ura3 (Naumov, 2005). RUL1888 strain was originally derived from NRRL Y-1140, one of the widely used *Kluyveromyces lactis* strains, and the only one whose genome has been sequenced thus far (Sherman et al., 2009). Fluorescently tagged *Kluyveromyces lactis* strain used for all the cultivations carried the plasmid pKATUC41-TEF-YFP (Figure 3.2).

### Strain maintenance

All yeast strains were grown in an incubator at 30°C, and *Escherichia coli* strains were grown at 37°C. For short term storage (up to 4 weeks), strains were kept at 4°C on agar plates. For long term storage, overnight cultures were mixed with sterile glycerol (final glycerol concentration was 15% (v/v)) and stored in -80°C. Pre-cultures for all batch and chemostat cultivations were prepared from freshly streaked cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

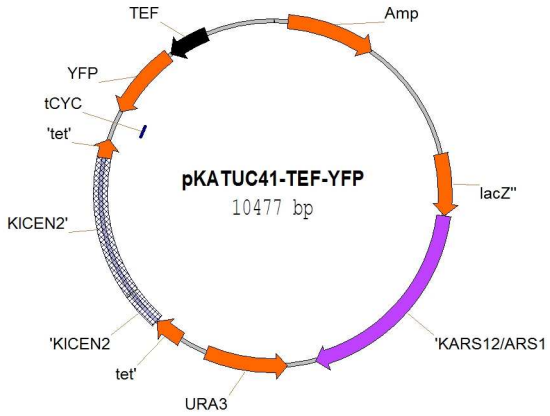



**Figure 3.2** – pKATUC41-TEF-YFP, plasmid used to transform *Kluyveromyces lactis*

## 3.2 Media

### *E. coli* media

Lysogeny broth (**LB**) media (Bertani, 1951, 2004) was prepared by dissolving the LB broth (Novagen, Madison, WI) in water according to the instructions given by the supplier. This media was sterilized by autoclaving. LB media was used to grow and prepare competent *E. coli* cells. LB agar plates were prepared by adding 15 g/L agar (Merck, Darmstadt, Germany) into LB mixture. This mixture was sterilized in the autoclave and poured into plates. Plates were left to solidify for 24 h.

LB-Amp media was prepared by adding 100 mg/mL ampicillin (AppliChem GmbH, Darmstadt, Germany) solution to the cold sterile LB media. The final concentrations of ampicillin in LB-Amp media was 100 µL/mL. LB-Amp plates were prepared in the same way as LB agar plates, except that ampicilin was added. The mixture was poured into plates immediately after adding ampicilin.

### Yeast media

Complex **YPD** media was used for growing auxotrophic mutants CEN.PK113-5D and RUL1888 (Section 3.1). It was prepared by mixing 10 g/L yeast extract (Sigma Aldrich, St Louis, MO) and 20 g/L peptone (Merck, Darmstadt, Germany) in water. This mixture was sterilized in the autoclave. Glucose solution of 200 g/L was prepared and autoclaved separately to avoid Maillard reactions. The two solutions were mixed after they cooled down and the final glucose concentration was 20 g/L.

YPD agar plates were prepared by adding 15 g/L agar to peptone-yeast extract solution. Sterile glucose solution was added for the final glucose concentration of 20 g/L when the temperature was about 60°C. Plates were poured out immediately after adding

glucose and left to solidify for 24 hours.

Synthetic minimal media without uracil (**SD-ura**) was used for selecting, maintaining and growing yeast strains transformed with plasmids carrying the *URA3* selection marker. It was prepared by mixing CSM-Ura (Formedium, Norfolk, UK) with 6.7 g/L of yeast nitrogen base without amino acids (Formedium). Sterile glucose solution was added for the final glucose concentrations of 20 g/L. SD-ura plates were prepared by adding 15 g/L agar to the SD-ura solution. Sterile glucose solution was added for the final glucose concentration of 20 g/L when the temperature was about 60°C. Plates were poured out immediately after adding glucose and left to solidify for 24 hours.

Composition of minimal defined media (Verduyn et al., 1992) that was used for all batch cultivations is listed in tables 3.1, 3.2 and 3.3.

**Table 3.1** – Composition of the minimal defined media

| Compound | Concentration |
|---|---|
| $(NH_4)_2SO_4$ | 5 g/l |
| $KH_2PO_4$ | 3 g/l |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g/l |
| trace element solution (Table 3.2) | 1 mL/L |
| antifoam 204 (Sigma-Aldrich) | 50 µL/L |
| vitamin solution (Table 3.3) | 1 mL/L |
| glucose | 20 g/l |

**Table 3.2** – Composition of the trace element solution

| Compound | Concentration (g/L) |
|---|---|
| EDTA | 15 |
| $ZnSO_4 \cdot 7H_2O$ | 4.5 |
| $MnCl_2 \cdot 4H_2O$ | 0.8 |
| $CoCl_2 \cdot 6H_2O$ | 0.3 |
| $CuSO_4 \cdot 5H_2O$ | 0.3 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.4 |
| $CaCl_2 \cdot 2H_2O$ | 4.5 |
| $FeSO_4 \cdot 7H_2O$ | 3 |
| $H_3BO_3$ | 1 |
| KI | 0.1 |

**Table 3.3** – Composition of the vitamin solution.

| Compound | Concentration (g/L) |
|---|---|
| d-Biotin | 0.05 |
| Calcium D(+)panothenate | 1 |
| Nicotinic acid | 1 |
| Myo-inositol | 25 |
| Thiamine HCl | 1 |
| Pyridoxine HCl | 1 |
| Para-amino benzoic acid | 0.2 |

## 3.3 Strain Construction

### 3.3.1 Cloning strategy

Strain construction was done by transforming yeasts with a single copy plasmid carrying fluorescent proteins under the constitutive promoter. Plasmid construction strategy was to use the polymerase chain reaction (PCR) to amplify genes for fluorescent proteins and insert those genes into plasmids for *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

The plasmid for *Saccharomyces cerevisiae* was derived from pRS416-TEF-CYC (Mumberg et al., 1995). This is an autonomously replicating centromeric plasmid. The centromeric sequence ensures that yeast cells keep it at a low copy number. It is a shuttle vector, meaning that it can be replicated and amplified in *E. coli* cells. The selection marker for *E. coli* is *Amp*. *Amp* gene codes for β-lactamase, an enzyme that degrades antibiotics belonging to the lactame group. This allows selection of transformed *E. coli* cells on LB-plates containing high concentrations of ampicilin. The genetic marker used for selecting yeast transformants is *URA3*. Since yeast host cells are uracil auxotrophs, only transformed cells can give rise to colonies on plates containing minimal synthetic media without uracil (SD-ura).

The pRS416-TEF-CYC plasmid carries a TEF promoter and CYC terminator flanking a multiple cloning site (MCS). Any gene cloned into the MSC will be under the control of the TEF promoter (Steiner and Philippsen, 1994). This promoter is a constitutive promoter, meaning that it is unregulated and that it confers continuous expression of the gene. Cloned and purified CFP and YFP PCR products have been cloned into pRS416-TEF-CYC plasmid downstream of the TEF promoter.

PCR primers were designed to have restriction sites (sequences highlighted in yellow in table 3.4) that correspond to the restriction sites in the multiple cloning site on the carrier plasmid. The same restriction enzymes were used to digestion both the insert (PCR product with restriction sites) and the plasmid. This generated products with compatible sticky ends which were then ligated to create a final circular plasmid (figures 3.1

and 3.2).

### 3.3.2 *Saccharomyces cerevisiae* plasmid construction

Vector NTI software was used for design and analysis of the primers. Primers were ordered from Sigma-Aldrich (table 3.4). PCR was used to amplify genes for fluorescent proteins CFP, YFP and RFP from a pRK-2 plasmid (kindly provided by Dr. Verena Siewers from Chalmers University of Technology (Klica, 2008)).

**Table 3.4** – Primer sequences and restriction enzymes used in plasmid construction. Sequences recognized by restriction enzymes are marked in yellow.

| Targeted gene | Primer sequence (5'→ 3') | Restriction site |
|---|---|---|
| CFP and YFP | AGACTA **GGATCC** ATGAGTAAAGGAGAAGAACTTTTCACTGG | BamHI |
| | TTAGTG **ATCGAT** TTATTTGTATAGTTCATCCATGCCATGTG | ClaI |
| RFP | AGACTA **GGATCC** ATGGCCTCCTCCGAGGACG | BamHI |
| | GTCATT **GAATTC** TTAGGCGCCGGTGGAGTGG | EcoRI |

PCR was run with a Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to the protocol supplied by the manufacturer. The table 3.5 displays concentrations of the components used for PCR reaction.

**Table 3.5** – PCR reagents

| Component | Final concentration |
|---|---|
| Template DNA (pRK-2) | 1 ng |
| dNTP mix(200 mM of each dNTP) | 10 mM |
| forward primer | 0.5 µM |
| reverse primer | 0.5 µM |
| 5x Phusion HF buffer | 1x |
| Phusion polymerase | 0.02 UI |

The PCR reactions were run on a MJ Mini thermocycler (Bio-Rad, Hercules, CA, U.S.) with the total reaction volume of 50 µL. The thermal profile for the reaction is shown in table 3.6.

PCR products were checked for correct sizes by agarose gel electrophoresis. Bands with correct sizes were cut from the gel and purified using GFX DNA gel extraction kit (GE Healthcare, Buckinghamshire, UK). Purified PCR products and pRS416-TEF plasmid were double digested using restriction enzymes. BamHI and ClaI were used for CFP and YFP constructs, while BamHI and EcoRI were used for RFP construct. FastDigest restriction enzymes (Fermentas, Burlington, Canada) were used for all restriction reactions according to the protocol supplied by the manufacturer. Digestion products

**Table 3.6** – Thermal profile for the PCR reaction

| Cycles | Temperature(°C) | Duration |
|---|---|---|
| 1 | 98 | 30 s |
| 32 | 98 (denaturation) | 10 s |
| | 60 (annealing) | 30 s |
| | 72 (elongation) | 30 s |
| 1 | 72 | 7 min |
| 1 | 4 | hold |

were purified by agarose gel electrophoresis and DNA gel extraction. Digested and purified PCR product and linearized plasmid were ligated using T4 DNA ligase (Fermentas). Ligation mixture was used to transform competent *E.coli* cells according to the Inoue method (Inoue et al., 1990). The plasmid was amplified by growing colonies of transformants in LB-Amp media overnight and purified from the bacterial culture with GeneJET Plasmid miniprep kit (Fermentas).

The final products were p41-6TEF-CFP, p416-TEF-YFP and p416-TEF-RFP plasmids. p416-TEF-CFP is shown in figure 3.1, the other plasmids differ only in the sequence of the gene for the fluorescent protein.

Purified plasmids were used for transformation of CEN.PK113-5D cells. The transformation was done using the standard LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Yeast transformants were grown in SD-ura media overnight. Successful cloning and expression of fluorescent proteins was confirmed DMI 4000B fluorescent microscope (Leica, Wetzlar, Germany) (figure 3.3).

### 3.3.3 *Kluyveromyces lactis* plasmid construction

Sequences on centromeric regions of *Kluyveromyces lactis* chromosomes are different from those found in *Saccharomyces cerevisiae*. To ensure transformation and stable maintenance of the plasmids in *Kluyveromyces lactis* cells, a separate plasmid with centromeric region characteristic of *K. lactis* was constructed. The original plasmid pKATUC41 (Zenke et al., 1993) was kindly provided by prof. Karin Breuning. This plasmid has a selection marker for ampicilin resistance enabling amplification in *E. coli*. The other marker on the plasmid is *URA3*, used for complementing uracil auxotrophy in RUL1888 cells (section 3.1).

Restriction enzymes SacI (Fermentas) and EagI (Fermentas) were used to cut the TEF-YFP-CYC cassette from the p416-TEF-YFP plasmid. The same enzymes were used to linearize pKATUC41 and create sticky ends compatible with TEF-YFP-CYC cassette. Products of the digestion reaction were purified by agarose gel electrophoresis and then ligated by T4 DNA ligase (Fermentas).

The ligation mixture was used to transform competent *E. coli* cells (section 3.1) by using the Inoue method. Transformants were selected on LB-Amp plates and then cultured in liquid LB-Amp media overnight for plasmid amplification. Plasmid was purified from the culture the next day using GeneJET Plasmid miniprep kit (Fermentas). Amplified plasmid pKATUC41-TEF-YFP (figure 3.2) was then used to transform RUL1888 strain. Transformation protocol was the same as for *Saccharomyces cerevisiae*. Transformants were selected on SD-ura agar plates. Successful transformation and gene expression were verified using the fluorescent microscope (figure 3.3) .

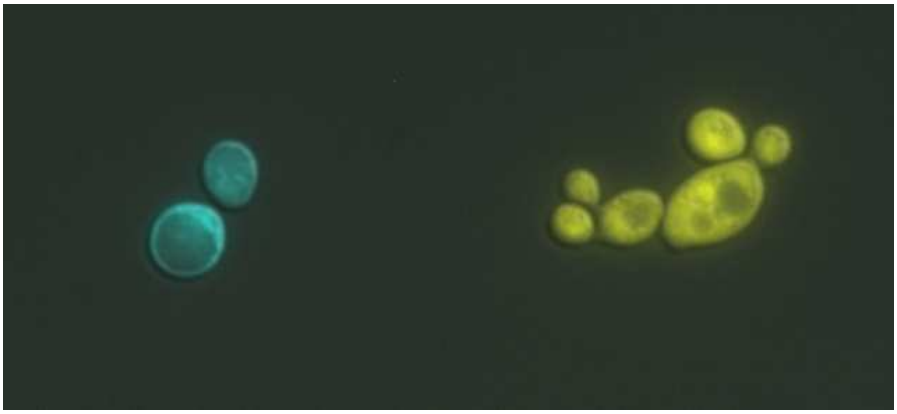


**Figure 3.3** – *Saccharomyces cerevisiae* (cyan) and *Kluyveromyces lactis* (yellow) as seen under the fluorescent microscope. CFP and YFP channels are overlayed

## 3.4 Cultivations

### 3.4.1 Batch cultivations - single culture

All batch cultivations were done in duplicates in 2.5 L glass bioreactor vessels (Applikon Biotechnology, Schiedam, The Netherlands)) with the working volume of 2 L. Minimal defined media with 20 g/L glucose, whose composition is shown in table 3.1, was used in pure culture cultivations. Media was prepared without glucose and vitamins and poured into vessels. Bioreactors were then sterilized in the autoclave, and glucose solution was autoclaved separately to avoid Maillard reactions. When vessels and solutions cooled down, glucose was added along with the vitamin solution.

Cultivation parameters were monitored with pH probes (pH was maintained at 5 throughout the cultivation by adding 2 M KOH solution), dissolved oxygen (DO) probes and temperature sensors. The temperature was kept constant at 30°C. Inlet air was sterilized by filtration. Aeration rate was set to 120 sL/h of air, which is the equivalent of 1 unit of gas volume flow per one unit of liquid volume per minute (vvm). Offgas

passed through a condenser, and offgas $CO_2$ and $O_2$ concentrations were monitored by the GA4 gas analyzer (DasGip, Jühlich, Germany). Bioreactors were equipped with two disk-turbine impellers and the agitation rate was set to 600 rpm.

Inoculum was prepared by picking colonies from the fresh (no more than 3 days old) SD-ura agar plates. Those colonies were transfered to 500 mL baffled shake flasks containing 100 mL of the same media as bioreactors. Pre-cultures were grown overnight at 30 °C on a shaker set to 150 rpm. Volume for inoculation was set so that the final optical density at 600 nm ($OD_{600}$) in the bioreactor is 0.01.

#### Sampling

The time of inoculation was set to be the zero time point of the cultivation. The sampling procedure started after the initial lag phase (about 12 h after the inoculation). Sampling was done in intervals of about 1.5 to 2 hours during the exponential phase for both *Saccharomyces cerevisiae* and *Kluyveromyces lactis* and 2-3 h during the ethanol phase for *Saccharomyces cerevisiae*. At every time-point, a sample was taken for cellular dry-weight measurement (3-7 mL, depending of the total cell density). At the same time, another sample was taken for measurement of extracellular metabolites (500 µL). Cellular dry-weight was measured by filtering the culture through a polyethersulfone membrane filters (Sartorius Stedim Biotech S.A., Aubagne, France) with pore size of 0.45 µm. Pellets were washed with distilled water twice. Filters with the pellet were first dried in a microwave oven and then stored in the desiccator for 48 hours before weighing then.

The samples for extracellular metabolites were immediately filtered through a nitrocellulose filter with pore size of 0.45 µm and the filtrate was kept at -20°C until the time of analysis. Rapid filtration was necessary to avoid changed in metabolite concentrations after sampling. Analysis of extracellular metabolites, glucose and ethanol, was done with Dionex Ultimate3000 HPLC (Dionex, Sunnyvale, CA, USA) on Aminex HPX-87H Ion Exchange Column (Bio-Rad, Hercules, CA, USA) at 45°C.

### 3.4.2 Batch cultivations - competition experiments

Batch cultivations with mixed cultures were prepared in the same way and performed with the same operating parameters (gassing, agitation, pH, temperature, offgas monitoring) as single cultivations. Media for cultivations with 20 and 40 g/L glucose was minimal defined media with composition shown in table 3.1. To avoid limitation for other nutrients, cultivations with 150 g/L initial glucose were done in 4x concentrated minimal defined media (table 3.7).

Pre-cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were prepared separately. Single colonies were picked up from SD-agar plates and inoculated in 500 mL baffled shake flasks containing 100 mL of the same media as bioreactors and grown

overnight at 30 °C on a shaker set to 150 rpm. Each of the strains was inoculated in the bioreactor to the final $OD_{600}$=0.005 and the total $OD_{600}$ of the mixed culture was 0.01.

Sampling was done as described in section 3.4.1.

**Table 3.7** – x4 concentrated minimal defined medium

| Compound | Concentration |
|---|---|
| $(NH_4)_2SO_4$ | 20 g/L |
| $KH_2PO_4$ | 12 g/L |
| $MgSO_4 \cdot 7H_2O$ | 2 g/L |
| trace element solution (table 3.2) | 4 mL/L |
| antifoam 204 (Sigma-Aldrich) | 50 µL/L |
| vitamin solution (table 3.3) | 4 mL/L |
| glucose | 150 g/L |

### 3.4.3 Continuous cultivations - mixed cultures

Continuous cultivations were done in two conditions: carbon-limited and nitrogen-limited. Four cultivations were performed for each condition. All chemostat cultivations were run in 0.7 L bioreactor vessels (DasGip) with working volumes of 0.5 L. Bioreactors and media were prepared in the same way as described in section 3.4.1. Bottles with feed media were prepared separately and feed was sterilized by filtration. Tables 3.8 and 3.9 show the initial and feed media composition. Aeration was set to 1 vvm by using the gas flow of 30 sL/h, and all other conditions were the same as with single cultures. Inoculum was prepared like described in section 3.4.2. Cultivations were run with all the parameters the same as for batch cultivation. When the offgas CO2 profiles showed diauxic shift, the initial batch phase was over. After this initial batch phase, feeding pumps were turned on and this was marked as the beginning of the chemostat cultivation.

**Table 3.8** – Carbon-limited media for continuous cultivation

| Compound | Feed | Initial media |
|---|---|---|
| $(NH_4)_2SO_4$ | 5 g/L | 5 g/L |
| $KH_2PO_4$ | 3 g/L | 3 g/L |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g/L | 0.5 g/L |
| trace element solution (table 3.2) | 1 mL/L | 1 mL/L |
| antifoam 204 (Sigma-Aldrich) | 50 µL /L | 50 µL /L |
| vitamin solution (table 3.3) | 1 mL/L | 1 mL/L |
| glucose | 10 g/L | 20 g/L |

**Table 3.9** – Nitrogen-limited media for continuous cultivation

| Compound | Feed | Initial media |
|---|---|---|
| $(NH_4)_2SO_4$ | 1 g/L | 1 g/L |
| $KH_2PO_4$ | 3 g/L | 3 g/L |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g/L | 0.5 g/L |
| $K_2SO_4$ | 5.3 g/L | 5.3 g/L |
| trace element solution (table 3.2) | 1 mL/L | 1 mL/L |
| antifoam 204 (Sigma-Aldrich) | 50 µL /L | 50 µL /L |
| vitamin solution (table 3.3) | 1 mL/L | 1 mL/L |
| glucose | 80 g/L | 20 g/L |

**Sampling and fluorescence measurements**

Sampling for extracellular metabolites and dry-cell biomass was done as described in section 3.4.1. Residual ammonia concentrations were measured in nitrogen-limited chemostats by using ammonia assay kit (Sigma-Aldrich) according the protocol supplied by the manufacturer.

Population dynamics of the two yeast populations were monitored by using fluorescence measurements. Samples were taken from the bioreactor and were transfered to into opaque 96-well plates (Nunc). When needed, samples were diluted with distilled water to $OD_{600}$ between 0.1 and 1.5 to avoid light scattering and non-specific fluorescence due to high cell densities. Samples were prepared in quadruplicates in volumes of 150 µL. Fluorescence was measured on the TECAN Safire2 plate reader with excitation/emission wavelengths of 433/475 nm for YFP and 525/538 for CFP. At the end of the cultivation, wash-out of one species was confirmed with the fluorescent microscope.

### 3.4.4 Analysis of physiological data and parameter estimation

All physiological parameters were calculated separately for each cultivation and expressed as the means over the duplicate experiments. Biomass growth in the exponential phase was assumed to follow the expression:

$$X(t) = X_0 e^{\mu_{max} t} \tag{3.1}$$

where $X(t)$ is the biomass as a function of time, $X_0$ biomass at the start of exponential phase, $\mu_{max}$ is the maximal specific growth rates and $t$ is time. Maximal specific growth rates, were calculated by fitting the model 3.1 to the biomass data from the exponential phase of growth. Biomass yields on glucose, $Y_{x/s}$ were calculated by plotting the glucose concentration as a function of biomass concentration. A line was obtained by linear regression and the negative inverse of the slope was taken to be $Y_{x/s}$. Maximal specific

glucose uptake rates, $r_{GLC}$ were calculated as

$$r_{GLC} = \frac{\mu_{max}}{Y_{x/s}}$$

Volumetric oxygen uptake rate for each sampling point, $q_{O_2}$ was calculated as:

$$q_{O_2} = \frac{\mathrm{O_2\%(inlet)} - \mathrm{O_2\%(offgas)}}{100} \frac{V_{O_2}}{TR}$$

where $V_{O_2}$ was the aeration rate in L/h, $T$ was the temperature ($T = 303$ K for all cultivations) and $R$ is the ideal gas constant ($R = 0.08257$ L amt $\mathrm{K}^{-1}$ $\mathrm{mol}^{-1}$). The specific oxygen uptake rate, $r_{O_2}$, was estimated by plotting the $q_{O_2}$ against total dry-cell weight and using the slope of the line fitted to the data points. Specific oxygen requirement, $Y_{o/x}$ was calculated from

$$Y_{o/x} = \frac{r_{O_2}}{\mu_{max}}$$

Similarly, the volumetric $CO_2$ production rate for each sampling point, $q_{O_2}$ was calculated as:

$$q_{CO_2} = \frac{\mathrm{CO_2\%(inlet)} - \mathrm{CO_2\%(offgas)}}{100} \frac{V_{CO_2}}{TR}.$$

Using data from oxygen uptake and $CO_2$ production rate is a reliable way to asses the difference between the fermentative and respiratory metabolism. Respiratory quotient (RQ) in the glucose phase was calculated according to the equation:

$$RQ = \frac{q_{CO_2}}{q_{O_2}} \tag{3.2}$$

and expressed as an average for pure and mixed cultures. Fermentation is characterized by higher rates of $CO_2$ production and this is indicated by an increase of the RQ compared to respiratory metabolism.

In mixed cultures, the relative success of the two strategies was expressed as fractional contribution of fermentative and respiratory metabolism to the growth of the total population biomass (equation 3.11 in section 3.5.2).

## 3.5 Mathematical models

### 3.5.1 Monod model

The simplest way of modelling the microbial population dynamics is to use the “black-box” representation of the cell. In this view, the substrate is taken up and converted into biomass, with all the details of the metabolism combined in just a small number of parameters. In one of the simplest models, the population growth is assumed to be

a function of the available substrate and to follow the Monod equation (Nielsen et al., 2003):

$$\mu(S) = \mu_{max}\frac{S}{S+K_s} \tag{3.3}$$

$\mu$ is the growth rate at a specific substrate (glucose) concentration $S$, and $\mu_{max}$ is the maximal growth rate of the population when substrate is not limiting. $K_s$ is the concentration of glucose at which the population grows at half the maximal growth rate.

The dynamic balances for the biomass and substrate are described with the system of ordinary differential equations:

$$\frac{dX}{dt} = \mu X \tag{3.4}$$

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{x/s}} \tag{3.5}$$

In the equation for the balance on substrate, $Y_{x/s}$ denotes biomass yield on substrate, expressed in grams of dry-cell weight of biomass per gram of substrate.

This model doesn't capture any of the regulatory phenomena in yeast. However, it is possible to use it to model the population dynamics for balanced growth on glucose for both *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

### 3.5.2 Monod model and competition for resources

Monod model can be used to model the competition between micro-organisms for a single limiting substrate. The way to describe competition between $n$ species is to use $n$ equations of the form 3.3, each having its own set of kinetic parameters $\mu_{max}$, $Ks$ and $Y_{x/s}$. Growth rate for species $i$ is:

$$\mu_i(S) = \mu_{max,i}\frac{S}{S+K_{s,i}} \tag{3.6}$$

The system describing mass balance then becomes:

$$\frac{dX_i}{dt} = \mu_i X_i \tag{3.7}$$

$$\frac{dS}{dt} = -\sum_{i=1}^{n}\frac{\mu_i X}{Y_{x/s,i}} \tag{3.8}$$

Biomass yield of the total population can be expressed as a sum of contributions from the two metabolic strategies or strains:

$$Y_{x/s,Tot} = f_F Y_{x/s,Sc} + f_R Y_{x/s,Kl} \tag{3.9}$$

where $f_F$ and $f_R$ are contributions or *fractions of fermentative* and *respiratory growth* to the growth of the total biomass, respectively. $Y_{x/s,Sc}$ and $Y_{x/s,Kl}$ are biomass yields on glucose for *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, respectively. $f_F$ can be interpreted as the relative success of the fermentative metabolism in mixed culture. Because

$$f_R = (1 - f_F) \tag{3.10}$$

equation 3.9 can be rearranged to give an expression for calculating $f_F$:

$$f_F = \frac{Y_{x/s,Tot} - Y_{x/s,Kl}}{Y_{x/s,Sc} - Y_{x/s,Kl}} \tag{3.11}$$

To see how the two metabolic strategies perform with different resource availability, the Monod model of competition of resources was simulated for different initial glucose concentrations. In this scenario, fractional contributions of metabolic strategies were used as measures of their relative success because the same measure was used to estimate their relative success in the experimental part.

Another measure of fitness is the *Malthusian parameter*. For a single strain, this is $w = \ln \frac{N(final)}{N(initial)}$, with $N$ being the cell number at the start (initial) and at the end (final) of the cultivation. The ratio of Malthusian parameters for two strains is the measure of their competitive fitness. If $N(0)$ and $N(1)$ denote initial and final population densities, relative competitive fitness for strain $i$ when competing against strain $j$ can be expressed as (De Visser et al., 2002)

$$w_{i,j} = \frac{\ln \frac{N(1)_i}{N(0)_i}}{\ln \frac{N(1)_j}{N(0)_j}}$$

This is the ratio of realized growth potentials from the start of the competition to the point where all the glucose was consumed. Assuming that the biomass composition stays the same over the course of competition for both competing strains, biomass concentrations can be used instead of population counts:

$$w_{i,j} = \frac{\ln \frac{X(1)_i}{X(0)_i}}{\ln \frac{X(1)_j}{X(0)_j}} \tag{3.12}$$

To test frequency- and density-dependence of the two strategies, Monod model of competition was simulated for different initial densities (total population biomass) and different initial frequencies (fraction of the two strains in the total populations). In this scenario, the competitive fitness was used as a measure of their success.

### 3.5.3 Modelling growth in oxygen-limited conditions

While the Monod equation captures the population dynamics in mixed and pure cultures, it doesn't account for the fact that there might be limiting factors other than glucose. Monod model for single limiting substrate (equation 3.3) can be extended to *n* limiting substrates:

$$\mu = \mu_{max} \prod_{i=1}^{n} \frac{S_i}{K_{s,i} + S_i} \tag{3.13}$$

Oxygen is needed for the respiratory metabolism of glucose, and can be considered as an additional substrate needed for the biomass growth. Since the solubility of oxygen in most liquids is actually very low, oxygen availability may become a limiting factor for growth. This happens in cultures with very high cell densities. For this, an oxygen concentration term is used in the equation 3.13 :

$$\mu(S) = \mu_{max} \frac{S}{S + K_s} \frac{O}{O + K_o} \tag{3.14}$$

In this model, $K_o$ denotes the Monod constant for oxygen. For a complete description of the system, equation for dynamic balance on oxygen is added to equations for balances for biomass growth and substrate:

$$\frac{dX}{dt} = \mu X \tag{3.15}$$

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{x/s}} \tag{3.16}$$

$$\frac{dO}{dt} = k_L a * (O^* - O) - Y_{o/x} \mu X \tag{3.17}$$

Parameters $k_L a$ and $Y_{o/x}$ are the volumetric oxygen mass transfer coefficient and specific oxygen requirement for biomass. Note that $Y_{o/x}$ is the inverse of $Y_{x/o}$, the yield of biomass on oxygen. $O^*$ is the maximal concentration of dissolved oxygen in the medium, 8 mg/L (Nielsen et al., 2003), while $O$ is the actual dissolved oxygen concentration.

### 3.5.4 Model for spatially structured meta-population

It was hypothesized that spatial structure might favor cooperative metabolic strategy (section 1.5). Competition for resources between respirators and fermenters in spatial settings has been studied using individual-based models, including and lattice-based models (Pfeiffer and Bonhoeffer, 2003) and three-dimensional biofilm models (Kreft, 2004, Kreft et al., 2001). In contrast, the spatial model described here is population-based, i.e. population is assumed to be a continuous variable.

In this spatial model, global or the *meta-population* is divided into a number of local populations. Local populations are structured into a grid of patches. Each patch contains resources (glucose), and is populated by *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, or both at the same time. Dynamics of the local populations dynamics and local resource utilization are modeled using the model described in section 3.5.1. Spatial dynamics are modeled with stochastic events acting on local populations: *migration*, *substrate influx* and local population *extinction*. These stochastic events are modeled as Poisson processes. The waiting time for each type of an event has its own characteristic exponential distribution. The consequence of this type of model is the separation of timescales in the system: local dynamics are based on short timescales related to metabolic rates, while global dynamics are governed by events happening on longer timescales. This is idea is schematically shown in figure 3.4.

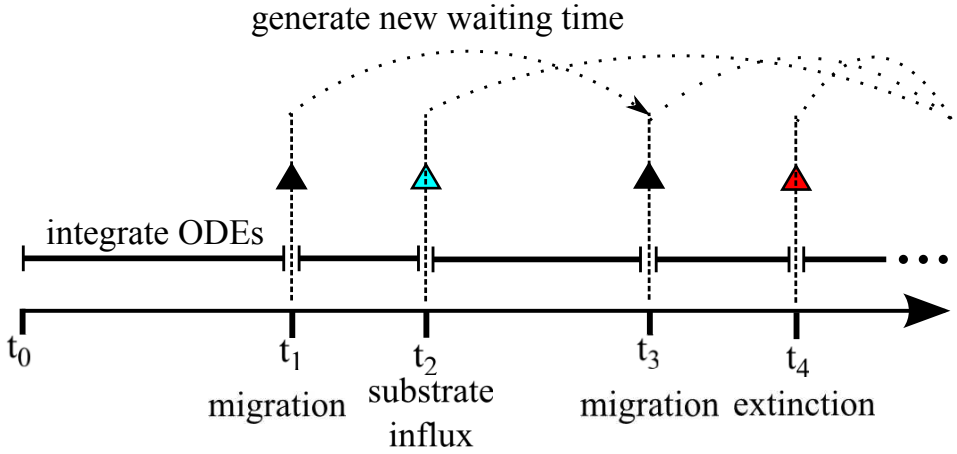



**Figure 3.4** – Timescales in the hybrid stochastic spatial model. Migration (black triangle), substrate influx (cyan triangle), and local population extinction (red triangle) are discrete events modeled as Poisson processes. Waiting times between them are exponentially distributed random variables. Local population and metabolic dynamics are modeled by a set of ordinary differential equations, essentially operating in continuous time intervals determined by two subsequent stochastic events.

### Migration

For each migration event, a random patch is selected from the grid. This is the donor patch. If the selected patch is populated, a fraction of the population is moved to one of the neighboring patches. Each of the four neighboring patches has an equal probability to be chosen as the destination patch. A periodic boundary condition applies to the migration of patches on the edges of the grid. In other words, the grid is topologically a torus with Euclidean distances between patches. If the population in the donor patch

is mixed, only one of the strains is migrated to the neighboring patch. Probability of a particular strain being migrated is proportional to the frequency of that strain in the donor patch. For the patch with coordinates $(i, j)$ this is

$$pMigrate_{i,j}^{Kl} = \frac{X_{i,j}^{Kl}}{X_{i,j}^{Kl} + X_{i,j}^{Sc}} \tag{3.18}$$

$$pMigrate_{i,j}^{Sc} = 1 - pMigrate_{i,j}^{Kl} \tag{3.19}$$

where indices $X_{i,j}^{Sc}$ and $X_{i,j}^{Kl}$ denote local *Saccharomyces cerevisiae* and *Kluyveromyces lactis* populations, respectively.

### Substrate influx

For substrate influx event, a random patch is chosen from the grid. The substrate concentration in this is increased by:

$$S(i, j) = S_0(i, j) + S_{Influx} \tag{3.20}$$

where $S_0(i, j)$ is the substrate concentration in the patch before the stochastic event occurred and $S_{Influx}$ is the amount of glucose (in g/L) added to the patch.

### Local extinction

Local extinction event is realized by randomly choosing one of the patches in the grid. If that patch is populated, the probability that the local population will survive an extinction event will depend on the local population density. Specifically, probability of surviving depends on the negative exponent of the local biomass:

$$pSurvive_{i,j} = 1 - e^{-(X_{i,j}^{Kl} + X_{i,j}^{Sc})} \tag{3.21}$$

where *pSurvive* is the probability of surviving. $X_{i,j}^{Kl}$ and $X_{i,j}^{Sc}$ are the local population densities of *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *pSurvive* as a function of the local population density is shown in figure 3.5.

### Simulation of the spatial model

The simulation of the spatial model can be summarized by the following steps:

1. Initialize the world grid
   (a) Randomly select patches to be initially populated
   (b) All patches in the grid are initialized with the same amount of substrate

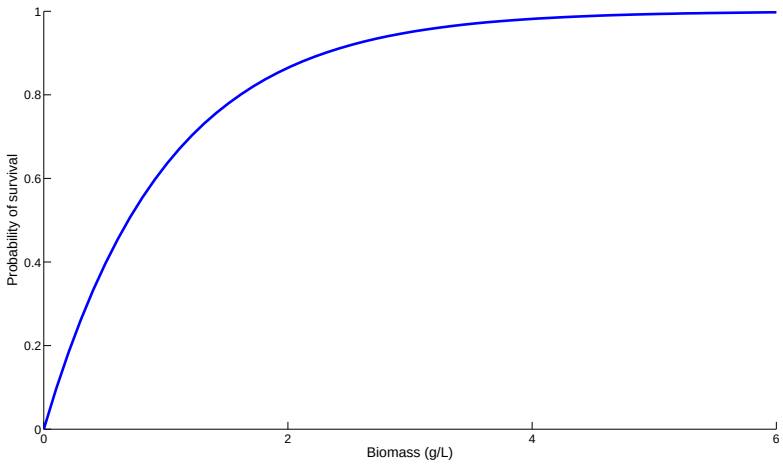



**Figure 3.5** – Probability of surviving the local extinction event as a function of local population density

2. Randomly generate exponentially distributed waiting times for the three stochastic events (migration, substrate influx, extinction)
3. Repeat until one of the species is wiped out from the grid or until total simulation time is exceeded
   (a) Assign the shortest of the three waiting times to be the waiting time until the next stochastic event.
   (b) Integrate the systems of ODEs describing local dynamics for all populated patches in the time interval until the next stochastic event. Update local population densities and substrate concentrations with the outputs from the ODE solver
   (c) Perform the stochastic event with the shortest waiting time, update population and substrate grids
   (d) Reduce the waiting times for stochastic events by the elapsed time. Generate new waiting time for the stochastic event performed in this iteration

The model was simulated for a range of parameters determining the migration waiting time. This was done to see how different population dispersal values affect the outcome of competition in meta-populations. The model was written and simulated in MATLAB® (7.7.0, The MathWorks, 2008, Natick, MA). Variable order ODE solver *ode15s* was used for integration of ODEs describing local dynamics. The model was simulated 100 times for each parameter value.

Parameters for the metabolic model were experimentally determined and can be found in the table 4.1. Death rate kinetics are often assumed to be negligible in models

for homogeneous populations that are simulated for shorter times, e.g. when modelling a typical batch cultivation on glucose. However, the spatial model is run over an longer time-scale where population are faced with starvation periods and where death rate can influence the outcome of the model. This is why cell death was included in the spatial model. It was assumed that both species have the same specific death rate, $k_d = 0.01\ \mathrm{h}^{-1}$.

Experimental values for parameters specific for the spatial model (migration, substrate and extinction waiting times) were not available. For that reason, the values were arbitrarily assigned, and are listed table 3.10. All parameter values, except for the mean migration waiting time, were kept constant for all simulations.

**Table 3.10** – Parameters for the spatial model

| Symbol | Description | Values |
|---|---|---|
| | Grid size | $10 \times 10$ |
| $totalTime$ | Total simulation time | 15000 (h) |
| $klaInitialPatches$ | Number of patches initially populated by *Kluyveromyces lactis* | 10 |
| $sceInitialPatches$ | Number of patches initially populated by *Saccharomyces cerevisiae* | 10 |
| $klaInitalBiomass$ | Initial biomass for *Kluyveromyces lactis* per patch | 0.01 (g/L) |
| $sceInitalBiomass$ | Initial biomass for *Saccharomyces cerevisiae* per patch | 0.01 (g/L) |
| $initalSubstrate$ | Initial glucose concentration per patch | 20 g/L |
| $t_{mig}$ | Mean waiting time for the migration event | 0.5-10 (h) |
| $t_{sInflux}$ | Mean waiting time for the substrate influx event | 10 (h) |
| $t_{er}$ | Mean waiting time for the extinction of the local patch | 20(h) |
| $sInflux$ | Substrate added to a patch during substrate influx event | 20 (g/L) |

The results of simulation were analyzed to give average time-courses for *Saccharomyces cerevisiae* and *Kluyveromyces lactis* biomasses and frequencies in the metapopulation. The frequency of *Kluyveromyces lactis* is

$$\phi_{Kla} = \frac{X_{Kl,Tot}}{X_{Kl,Tot} + X_{Sc,Tot}} \tag{3.22}$$

and the frequency of *Saccharomyces cerevisiae* is

$$\phi_{Sce} = 1 - \phi_{Kla} \tag{3.23}$$

Spatial spread was calculated by counting the number of patches occupied by one of the strategies and expressing them as ratio to total number occupied patches. Ratio of patches occupied by *Kluyveromyces lactis* is:

$$RP_{Kl} = \frac{\#K.\ lactis \text{ patches}}{\#K.\ lactis \text{ patches} + \#S.\ cerevisiae \text{ patches}} \tag{3.24}$$

Ratio of patches occupied by *Saccharomyces cerevisiae* is:

$$RP_{Sc} = 1 - RP_{Kl} \tag{3.25}$$

The pseudo code can be found in the table 3.11. The complete code for MATLAB scripts can be found in the appendix.

**Table 3.11** – Pseudo code for simulation of the spatial model

```
SET random pathes in populationGrid to initialPopulation
SET all patches in substrateGrid to initialSubstrate
GENERATE tMigration, tSInflux, tEradication from exponential distribution
SET interval to 20, totalTime to 15000
GENERATE time as a linearly spaced vector from 0 to totalTime
FOR i = 2 to length(time)
  SET timeSlice to time(i) - time(i-1)
  WHILE timeSlice > 0
    SET timeToNextEvent to min(tMigration, tSInfulx, tEradication)
    IF timeSlice < timeToNextEvent
      SET timeSpan to [0 timeSlice]
      INTEGRATE localDynamics for patches in populationGrid>0 with timeSpan
      UPDATE populationGrid, substrateGrid
      DECREASE tMigration, tSInflux, tEradication by timeSlice
      SET timeSlice to 0
    ELSE
     SET timeSpan to [0 timeToNextEvent]
     INTEGRATE localDynamics for patches in populationGrid>0 with timeSpan
     UPDATE populationGrid, substrateGrid
     DECREASE tMigration, tSInflux, tEradication by timeToNextEvent
     IF tSInflux is zero
      ADD substrate to random patch in the substrateGrid
      GENERATE tSInflux from exponential distribution
     ELSE IF tMigration is zero
      CALL migration script
      GENERATE tMigration from exponential distribution
     ELSE IF tEradication is zero
      GENERATE randomPatchIndex
      CALCULATE probability of survival for randomPatchIndex
      CALCULATE probability of extinction
      IF probability of extinction > probability of survival
        SET populationGrid (randomPatchIndex) to zero
      END IF
      GENERATE tEradication from exponential distribution
     END IF
     DECREASE timeSlice by timeToNextEvent
   END IF
  END WHILE
 RECORD population values and number of occupied patches
END FOR
```

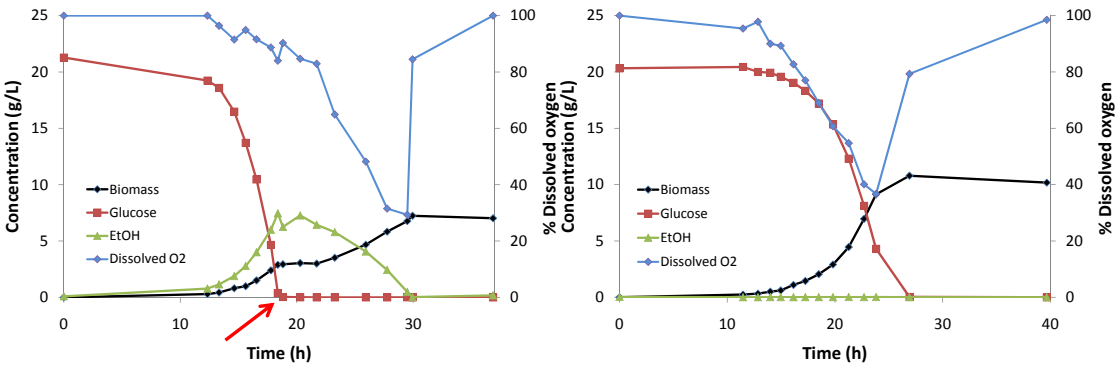
# Chapter 4

# Results

## 4.1 Experimental results

### 4.1.1 Batch cultivations - single cultures

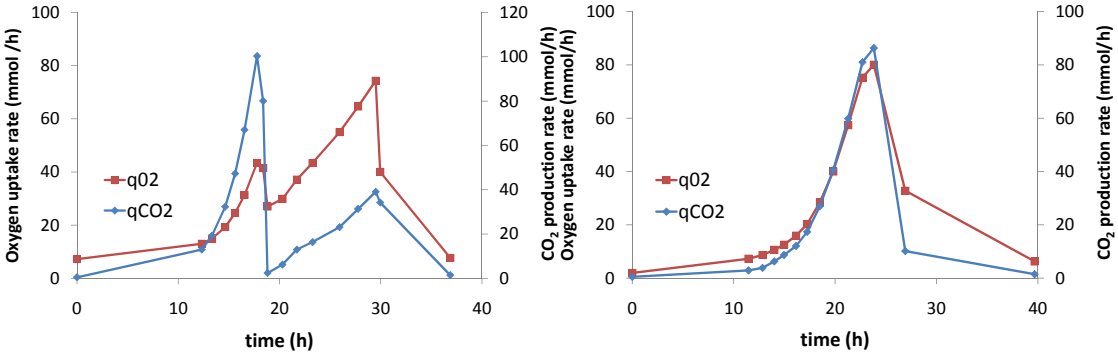
Pure cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* were grown in batch fermentations in order to obtain physiological parameters for growth on glucose. Figures 4.1a and 4.1b show the typical batch fermentation profiles on glucose for *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.




**(a)** *S. cerevisiae* **(b)** *K. lactis*

**Figure 4.1** – Fermentation profiles for aerobic batch cultivations of pure *Saccharomyces cerevisiae* and *Kluyveromyces lactis* cultures. (a) Representative *Saccharomyces cerevisiae* single culture fermentation profile with initial glucose concentration 21 g/L. The red arrow indicates the time point where glucose is depleted and diauxic shift (lag phase preceding growth on ethanol) starts. (b) Representative *Kluyveromyces lactis* single culture fermentation profile with initial glucose concentration 20 g/L. Ethanol is not accumulated during the cultivation indicating respiratory growth.

Figures 4.2a and 4.2b show they oxygen uptake and $CO_2$ production rate profiles for single *Saccharomyces cerevisiae* and *Kluyveromyces lactis* cultivations.




**(a)** *S. cerevisiae* **(b)** *K. lactis*

**Figure 4.2** – $O_2$ uptake and $CO_2$ production rate profiles for aerobic batch cultivations of pure *Saccharomyces cerevisiae* and *Kluyveromyces lactis* cultures. (a) Representative *Saccharomyces cerevisiae* single culture profile with initial glucose concentration 21 g/L. (b) Representative *Kluyveromyces lactis* single culture profile with initial glucose concentration 20 g/L.

The most striking difference between the physiology of the two strains is the presence of diauxic shift in *Saccharomyces cerevisiae* growth, which is absent in the case of *Kluyveromyces lactis* cultivation. Growth up to that point (indicated with the red arrow in figure 4.1) is characterized by accumulation of ethanol. After that point, glucose is depleted from the medium. There is a second lag phase followed by continued growth and ethanol assimilation.

The length of the glucose phase is longer for *Kluyveromyces lactis*, but the total biomass yield at the end is higher than for *Saccharomyces cerevisiae*, even when biomass yield at the end of the ethanol phase is taken into account.

**Table 4.1** – Comparison of experimentally determined kinetic parameters from single cultures

| Parameter | *Saccharomyces cerevisiae* | *Kluyveromyces lactis* |
|---|---|---|
| $\mu_{max}$ | $0.369 \pm 0.020\ \mathrm{h}^{-1}$ | $0.309 \pm 0.060\ \mathrm{h}^{-1}$ |
| $Y_{x/s}$ | $0.133 \pm 0.008 \frac{\text{g glc}}{\text{g DCW}}$ | $0.515 \pm 0.007 \frac{\text{g glc}}{\text{g DCW}}$ |
| $r_{GLC}$ | $15.38 \pm 1.24 \frac{\text{mmol glc}}{\text{g DCW h}}$ | $3.33 \pm 0.74 \frac{\text{mmol glc}}{\text{g DCW h}}$ |
| $K_s$[1] | 0.357 g/L | 0.558 g/L |

[1] Estimated by fitting the Monod model to the experimental data

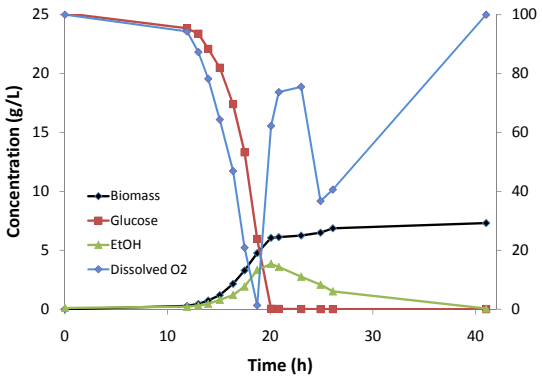
Table 4.1 summarizes growth, biomass yield and glucose uptake parameters calculated from fermentation data. Oxygen requirement for growth of *Kluyveromyces lactis* was calculated to be $Y_{o/x} = 289.54 \pm 32.61$ mg/g. $K_o$ was estimated to be 0.99 mg/L by fitting the Monod model with oxygen limitation to the experimental data. These values were used as parameters for the metabolic models.

### 4.1.2 Batch cultivations - mixed cultures

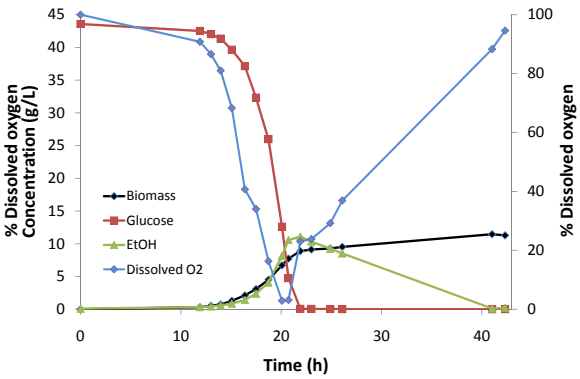
The competition experiments between *Saccharomyces cerevisiae* and *Kluyveromyces lactis* with different initial glucose concentrations were done with mixed batch cultivations.

Figures 4.3a, 4.3b and 4.3c show typical batch fermentation profiles for mixed cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* at different initial glucose concentrations. There is a significant amount of ethanol produced in all three conditions, which shows the fermentative character of the mixed cultures. On the other hand, dissolved oxygen concentrations drop rapidly in the glucose phase due to respiratory growth on *Kluyveromyces lactis*.

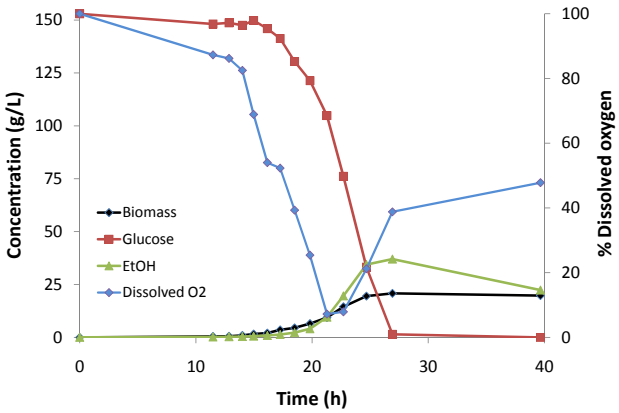
Table 4.2 summarizes growth, biomass yield and glucose uptake parameters calculated from fermentation data of mixed cultures.




**(a)** 20 g/L initial glucose




**(b)** 40 g/L initial glucose




**(c)** 150 g/L initial glucose

**Figure 4.3** – Profiles of mixed culture batch fermentations with different initial glucose concentrations. Plotted biomass profiles correspond to the total biomass of the mixed population

**Table 4.2** – Comparison of experimentally determined kinetic parameters for mixed cultures for different initial glucose concentrations

| | **Initial glucose concentration** | | |
|---|---|---|---|
| | 20 g/L | 40 g/L | 150 g/L |
| $\mu_{max}$ | $0.399 \pm 0.012\ \mathrm{h}^{-1}$ | $0.351 \pm 0.023\ \mathrm{h}^{-1}$ | $0.330 \pm 0.008\ \mathrm{h}^{-1}$ |
| $Y_{x/s}$ | $0.253 \pm 0.009 \frac{\text{g glc}}{\text{g DCW}}$ | $0.207 \pm 0.009 \frac{\text{g glc}}{\text{g DCW}}$ | $0.155 \pm 0.005 \frac{\text{g glc}}{\text{g DCW}}$ |
| $r_{GLC}$ | $8.77 \pm 0.43 \frac{\text{mmol glc}}{\text{g DCW h}}$ | $9.45 \pm 0.75 \frac{\text{mmol glc}}{\text{g DCW h}}$ | $11.81 \pm 0.46 \frac{\text{mmol glc}}{\text{g DCW h}}$ |

Figure 4.4 is based on combined data from tables 4.1 and 4.2. It shows the biomass yield as a function of specific glucose uptake rates for single and mixed cultures. Overall, in mixed cultures, increase of initial glucose availability was accompanied by an increase of glucose uptake rate and a decrease in total biomass yields.

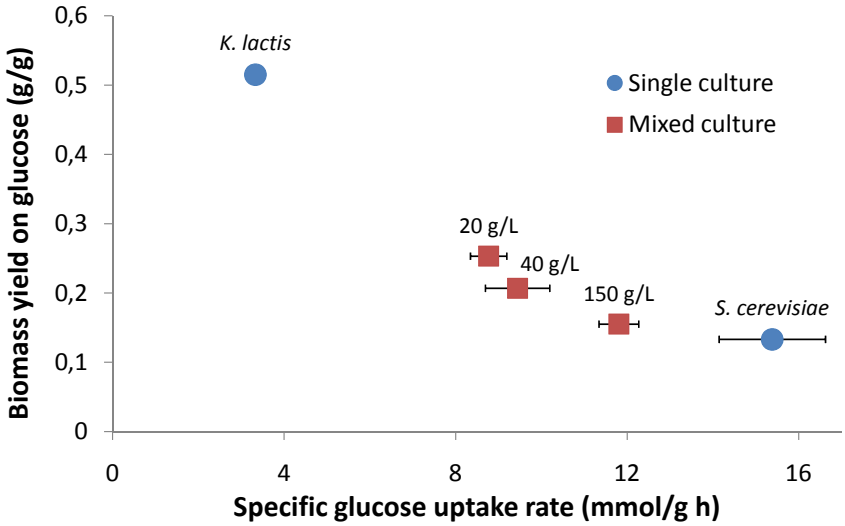
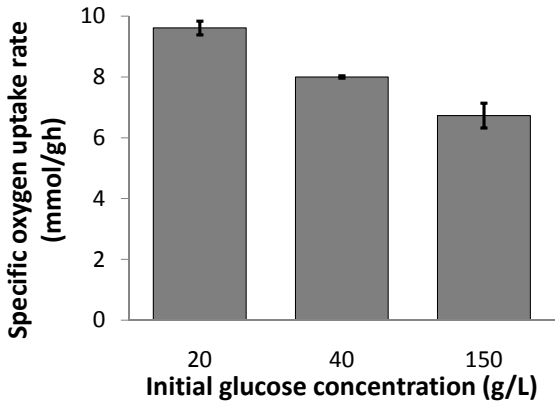
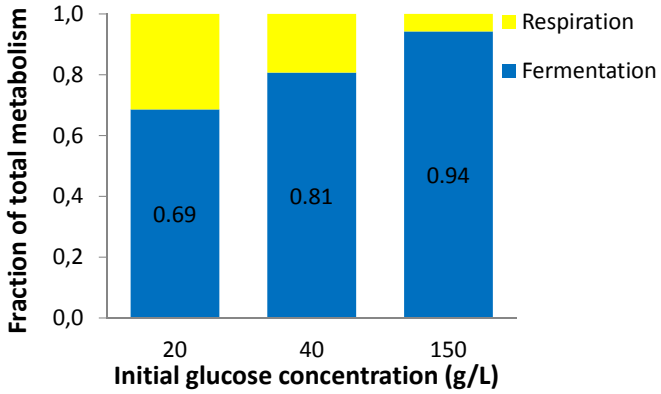



**Figure 4.4** – Rate versus yield trade-off in single and mixed cultures. Result from single *Saccharomyces cerevisiae* culture represents fermentative metabolism and results from *Kluyveromyces lactis* represent respiratory metabolism. Mixed populations show a trend of increasing fermentative character with an increase of initial glucose concentrations.

Comparison of biomass yields on glucose between single and mixed cultures was used for calculating the fraction of fermentative and respiratory metabolism in the biomass growth for the mixed cultures (equation 3.11 in section 3.5.2). The results are shown in figure 4.5b. Contribution of the fermentative growth the to growth of the mixed culture increases as glucose levels are increased.




**(a)** Specific oxygen uptake




**(b)** Fraction of fermentative metabolism

**Figure 4.5** – Fermentation and respiration in mixed cultures. (a) Specific oxygen uptake rates of the mixed culture in the glucose phase. Rates decrease with increasing glucose concentrations indicating a reduction of the respiratory metabolism in the mixed culture. (b) Fractional contribution of fermentation and respiration to the biomass growth of the mixed culture. Increased initial glucose concentrations result in an increase of fermentative metabolism.

Since oxygen is needed for respiratory metabolism, specific oxygen uptake rates can show the how the mixed culture's metabolism responds to changes in glucose concentrations. Figure 4.5a shows that specific oxygen uptake rates decrease when glucose levels are increased.

Additional results showing the increase in fermentative metabolism in mixed cultures come from measured RQ values in single and mixed cultures displayed in figure 4.6.

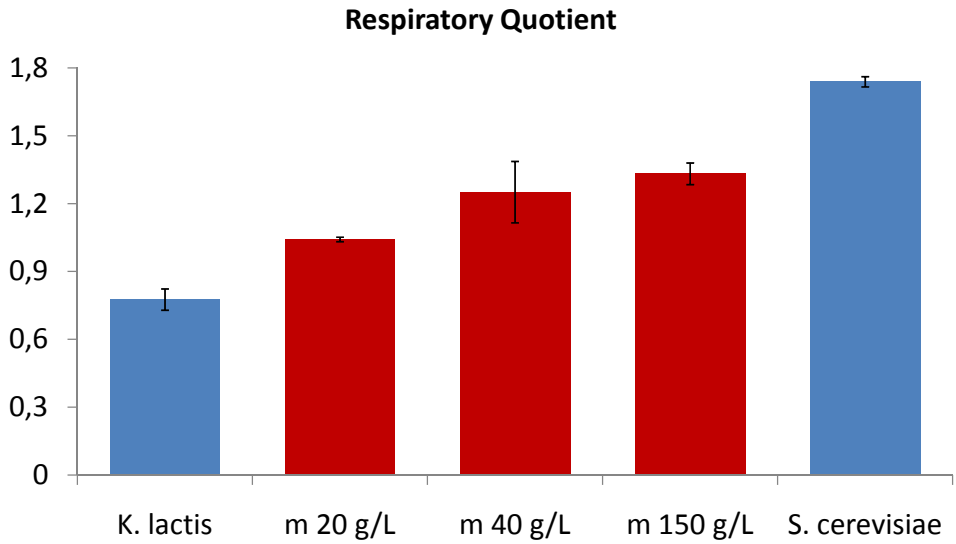


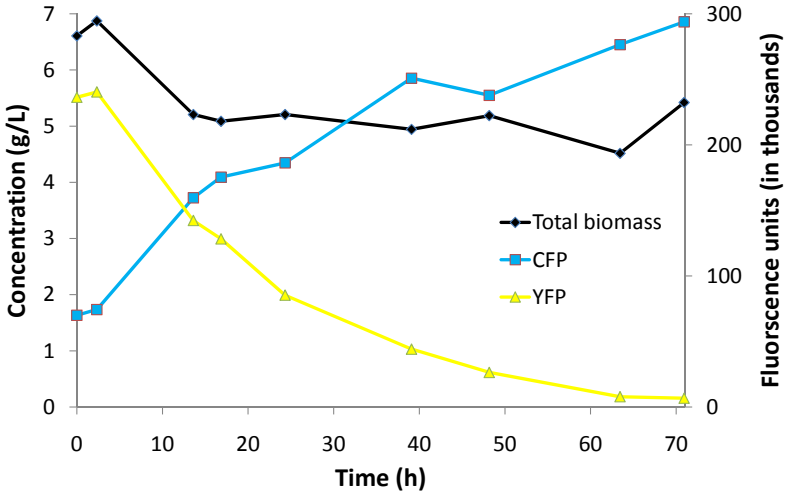

**Figure 4.6** – Average respiratory quotient (RQ) in single (blue bars) and mixed cultures (red bars) for growth on glucose. Result from single *Saccharomyces cerevisiae* culture represents RQ characteristic of fermentative metabolism and results from *Kluyveromyces lactis* represent RQ for respiratory metabolism. Mixed populations show a trend of increasing RQ with an increase of initial glucose concentrations.

### 4.1.3 Continuous cultivations

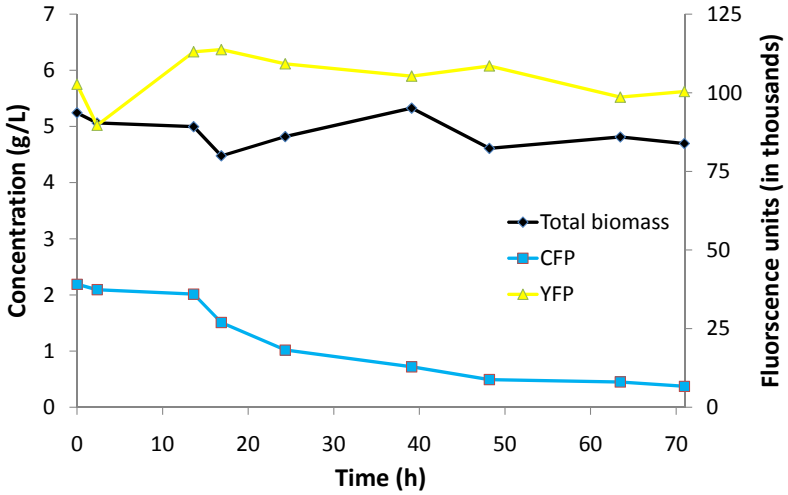
Competition in mixed continuous cultivation was done in carbon-limited and nitrogen-limited conditions. Results of competition for resources in a continuous culture are shown in figure 4.7. In both cases, the total biomass density stays roughly the same while the composition of the population changes. At the end of the cultivation, both cultures result in competitive exclusion of one of the strains.

In carbon-limited conditions (figure 4.7a), where glucose is the growth-limiting substrate, the decrease in YFP and simultaneous rise in CFP fluorescence shows that *Kluyveromyces lactis* gets washed out of the culture. Residual concentration of glucose in the media was $0.0228 \pm 0.0005$ g/L.

Nitrogen-limited conditions (figure 4.7b), where ammonium is growth-limiting substrate, led to an opposite outcome. CFP fluorescence intensity dropped indicating wash-out of *Saccharomyces cerevisiae* from the culture. Since the wash-out of the CFP-tagged cells was confirmed by fluorescence microscopy, residual CFP fluorescence at the end of the cultivation can be explained by unspecific background fluorescence from *Kluyveromyces lactis* cells. The residual concentration of ammonia in the culture was $75.3 \pm 31.8$µM.




**(a)** Carbon-limited chemostat




**(b)** Nitrogen-limited chemostat

**Figure 4.7** – Competition in chemostats leads to competitive exclusion, D=0.1 $h^{-1}$ for both conditions. Figure shows the profile of the continuous cultivation from the point where feed pumps are turned on (marked as 0 h). (a) Competition in carbon-limited conditions leads to wash-out of *Kluyveromyces lactis* (YFP) and dominance of *Saccharomyces cerevisiae* (CFP). (b) Competition in nitrogen-limited conditions leads to wash-out of *Saccharomyces cerevisiae* (CFP) and dominance of *Kluyveromyces lactis* (YFP).

## 4.2 Simulation results

### 4.2.1 Competition in homogeneous environment

Simulation of the Monod model (section 3.5.1) based on experimentally determined parameters from the table 4.1 showed that *Saccharomyces cerevisiae* has a faster growth rate than *Kluyveromyces lactis* for all glucose concentrations (figure 4.8).

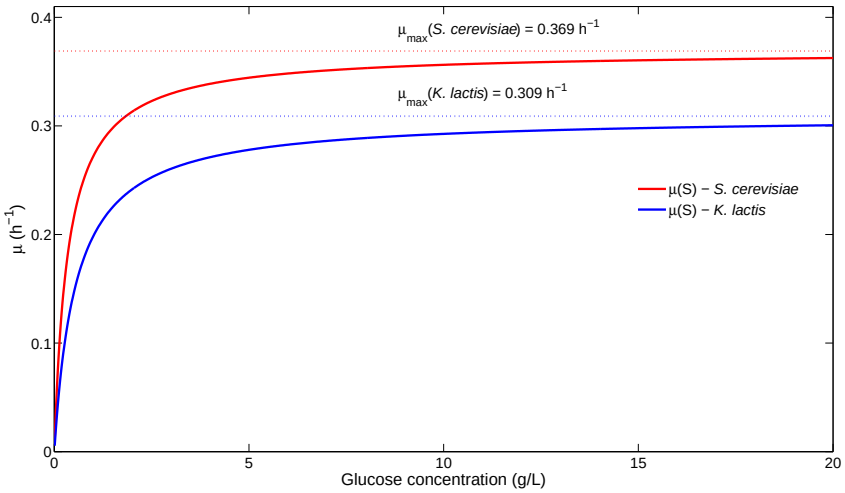



**Figure 4.8** – Comparison of growth rates for *Saccharomyces cerevisiae* and *Kluyveromyces lactis* at different glucose concentrations. *Saccharomyces cerevisiae*has higher growth rate than *Kluyveromyces lactis*at all glucose concentrations. Simulations based on parameters from the table 4.1

Figure 4.9 shows the results of simulations of the Monod model for single (section 3.5.1) and mixed (section 3.5.2) populations. Single *Kluyveromyces lactis* population shows slower growth and slower sugar consumption, but a higher biomass yield on glucose than the pure *Saccharomyces cerevisiae* population. In mixed population the situation is reversed. At the point when all the glucose is consumed, biomass yield of *Saccharomyces cerevisiae* is higher than the biomass yield of *Kluyveromyces lactis*. The figure shows only the phase where biomass grows on glucose, while the diauxic shift is omitted.

Figure 4.10 shows the how the choice of metabolic model for *Kluyveromyces lactis* influences the outcome of competition at different initial glucose concentrations. When oxygen is treated as a limiting substrate, fermentation becomes even more dominant in the mixed culture than in the case where only glucose is considered.

Another simulation was run to test how the frequency of strains and the total population density influence the competition. The simulation was done with three different

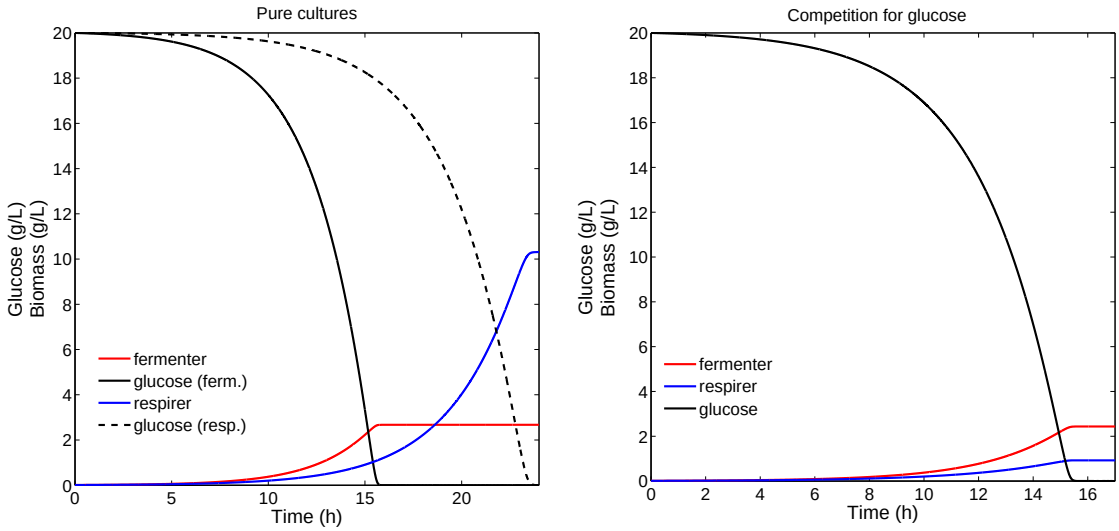



**Figure 4.9** – Simulation of Monod model for growth on glucose and glucose consumption dynamics of fermentative and respiratory strategy in single and mixed cultures. (Left) In pure culture, the high-yielding respiratory strategy (*Kluyveromyces lactis*) is dominant. (Right) The situation is reversed when respiration and fermentation compete for the same resource pool - in mixed culture, fast growing fermenter (*Saccharomyces cerevisiae* )becomes dominant and outgrows the respirer. This situation is called “the tragedy of the commons”

population compositions (1:100, 1:1, and 100:1) and with total population densities ranging over four orders of magnitude. Figure 4.11 shows how the fitness of the fermenter depends on frequency and density in the two metabolic models. With both models, *Saccharomyces cerevisiae* competitive fitness is positive density-dependent. When only glucose is limiting the growth of *Kluyveromyces lactis*, competitive fitness of *Saccharomyces cerevisiae* is positive frequency-dependent. When the metabolic model for *Kluyveromyces lactis* includes oxygen limitation term, the competitive fitness of *Saccharomyces cerevisiae* is negative frequency-dependent.

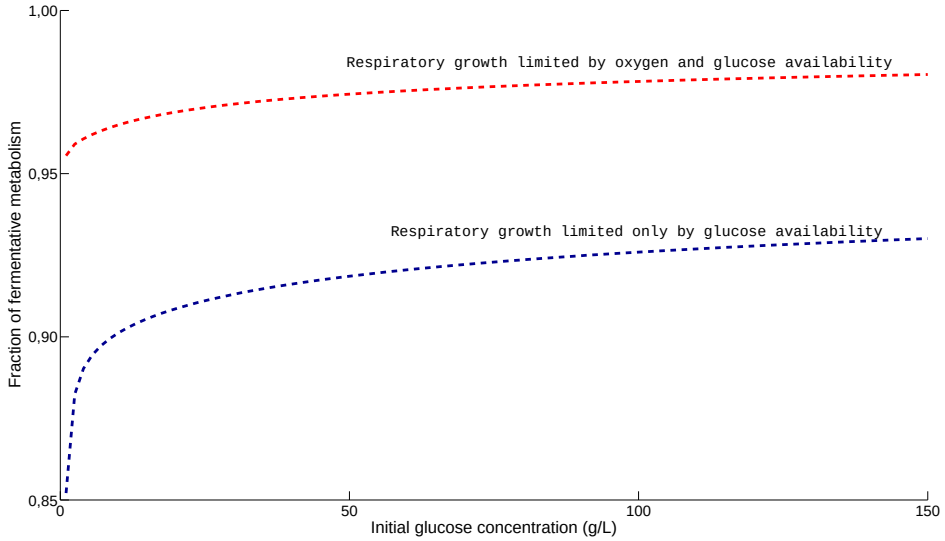



**Figure 4.10** – Fraction of fermentative growth in the growth of the total mixed biomass as predicted by the two *Kluyveromyces lactis* metabolic models. Both models predict dominance of the fermentative metabolism, but the dominance is more pronounced if oxygen is a limiting substrate. Red line shows the outcome of competition when growth of *Kluyveromyces lactis* is limited only by available glucose and oxygen: $\mu_{Kl} = \mu_{max,Kl} \frac{S}{Ks+S} \frac{O}{Ko+O}$. Blue line shows the outome of competition when growth on *Kluyveromyces lactis* is limited only by glucose: $\mu_{Kl} = \mu_{max,Kl} \frac{S}{Ks+S}$

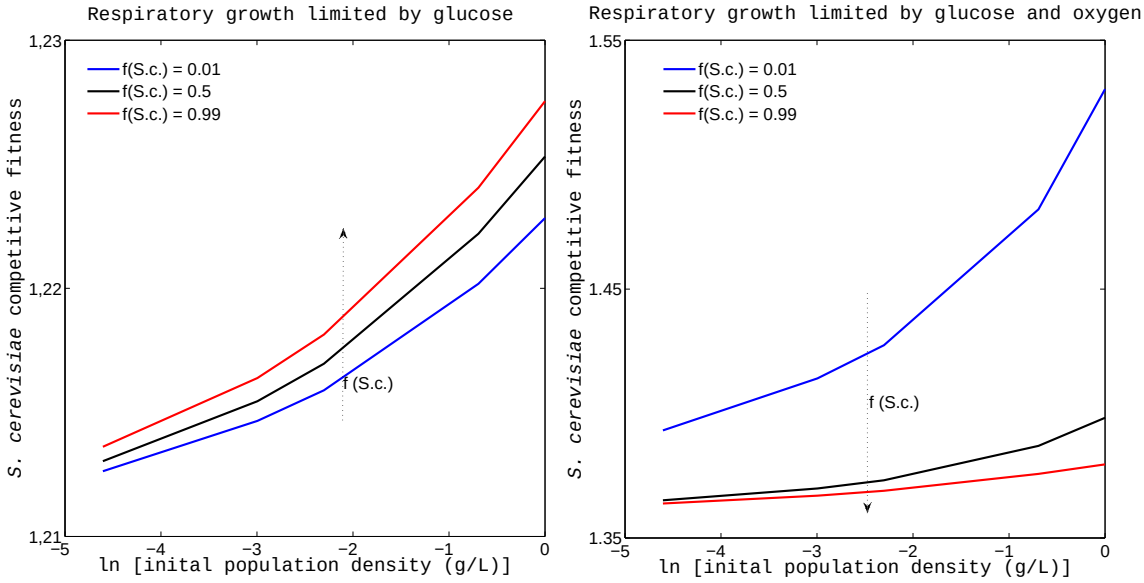



**Figure 4.11** – Competitive fitness of *Saccharomyces cerevisiae* as a function of initial frequency and population density as a result of two models. Left plot shows the dependence for the model where *Kluyveromyces lactis* growth is limited only by glucose concentration. Right plot shows the simulation results for competition where *Kluyveromyces lactis* growth is limited by both glucose and oxygen concentration.

### 4.2.2 Competition in a spatially structured population

The stochastic model was used to study the effect of spatial population structure and population mixing on the outcome of the competition. Figure 4.12 shows the typical output of the single simulation run of the model. It compares the two levels of competition in the spatial model, the meta-population level and the local level.

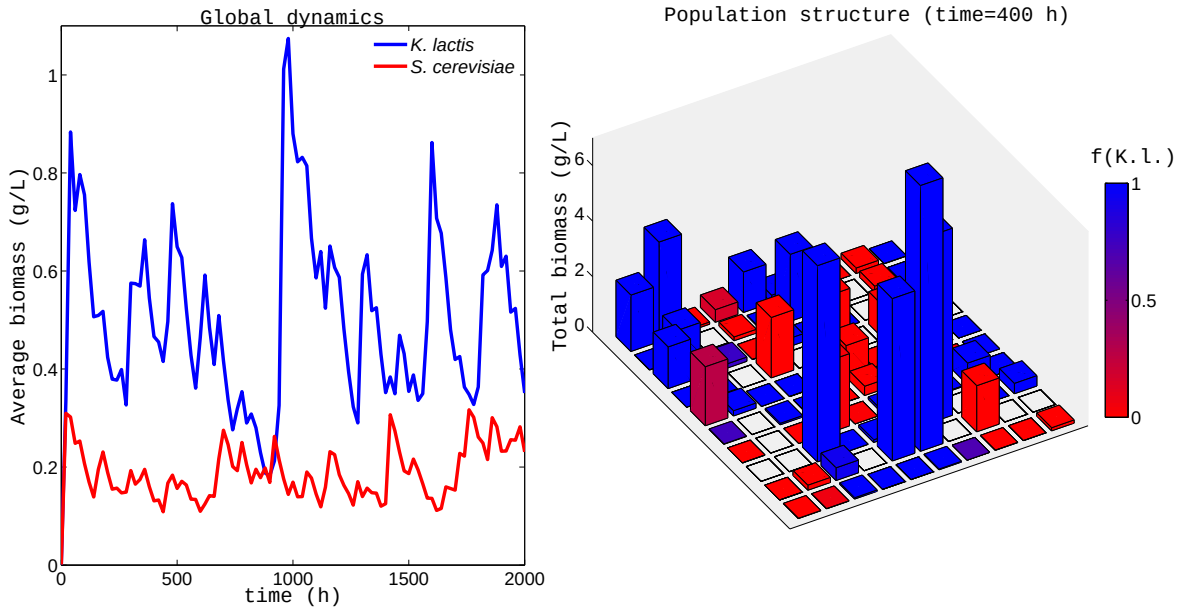



**Figure 4.12** – Sample plots showing the behavior of the spatial model. Left plot shows a part of the time-course of average biomasses of *Kluyveromyces lactis* (blue) and *Saccharomyces cerevisiae* (red) in the meta-population for one simulation run. Right plot shows the spatial structure of the meta-population for one time point. Each bar represents one local population. Height of a bar is the local population density in that patch and the color of the bar indicates the population composition for that patch. Patches dominated by *Saccharomyces cerevisiae* (purple to red coded) tend to have a lower population density or biomass than patches populated by pure *Kluyveromyces lactis* populations (blue coded).

Outcome of the competition in the spatially structured population depended on the dispersal or rigidity of the population, which is in this case modeled by the mean time between stochastic migration events. This is shown in the figure 4.13, where average biomass time courses are shown for three different migration waiting time values. With shorter times between migration events, *Saccharomyces cerevisiae* dominated in the meta-population, both measured by average biomass and spread (occupancy) of the world grid. As waiting times increased, *Kluyveromyces lactis* became dominant in biomass and spread (figure 4.15).

Spread of *Kluyveromyces lactis* is the ratio between patches occupied by *K. lactis* and patches occupied by *Saccharomyces cerevisiae*. Comparison of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* spread in the meta-population at different migration rates is show in the figure 4.14. Details of calculations for frequencies and spatial spread (ratio of occupied patches) are explained in section 3.5.4.

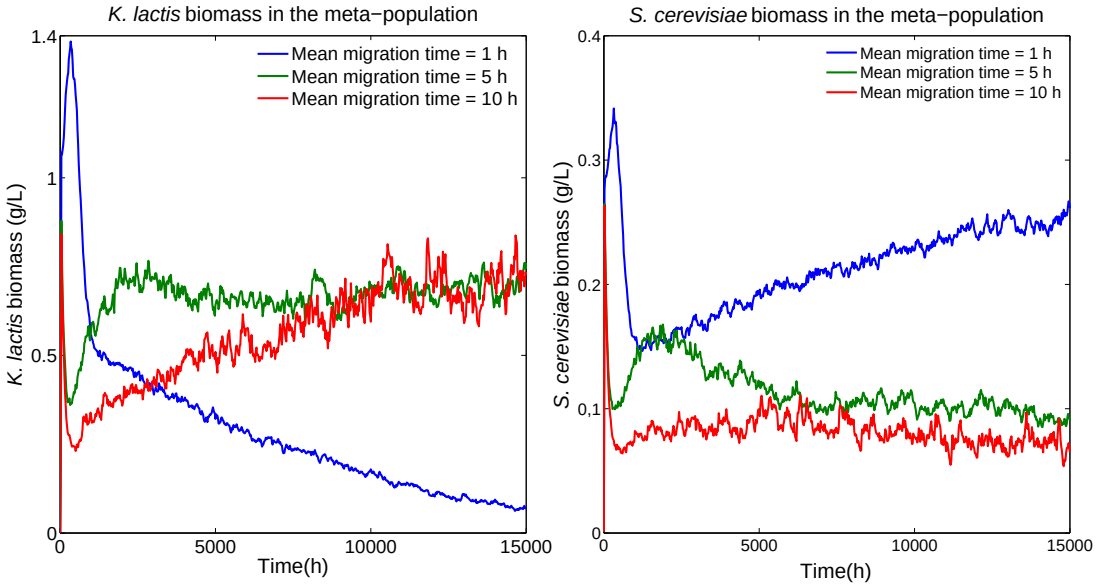



**Figure 4.13** – Comparison of average *Saccharomyces cerevisiae* and *Kluyveromyces lactis* biomasses in the meta-population - results for the simulation of the spatial model with different average migration times. Each trajectory is calculated as the mean of 100 simulations.

More comprehensive results of the competition in the spatially structured population are shown in figure 4.16. The exploration of the mean migration waiting time parameter space shows a trend of *Saccharomyces cerevisiae*dominance at shorter waiting times, co-existence at intermediate waiting times, and *Kluyveromyces lactis* domination at longer waiting times.

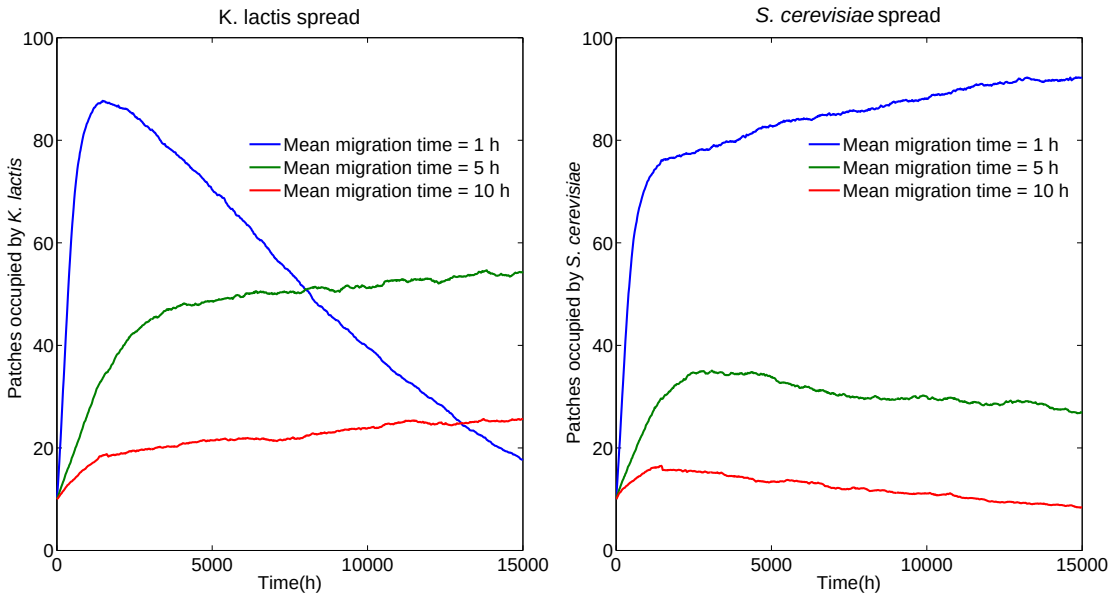



**Figure 4.14** – Comparison of number of patches occupied by *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Results for the simulation of the spatial model with different average migration times. Each trajectory is calculated as the mean of 100 simulations.

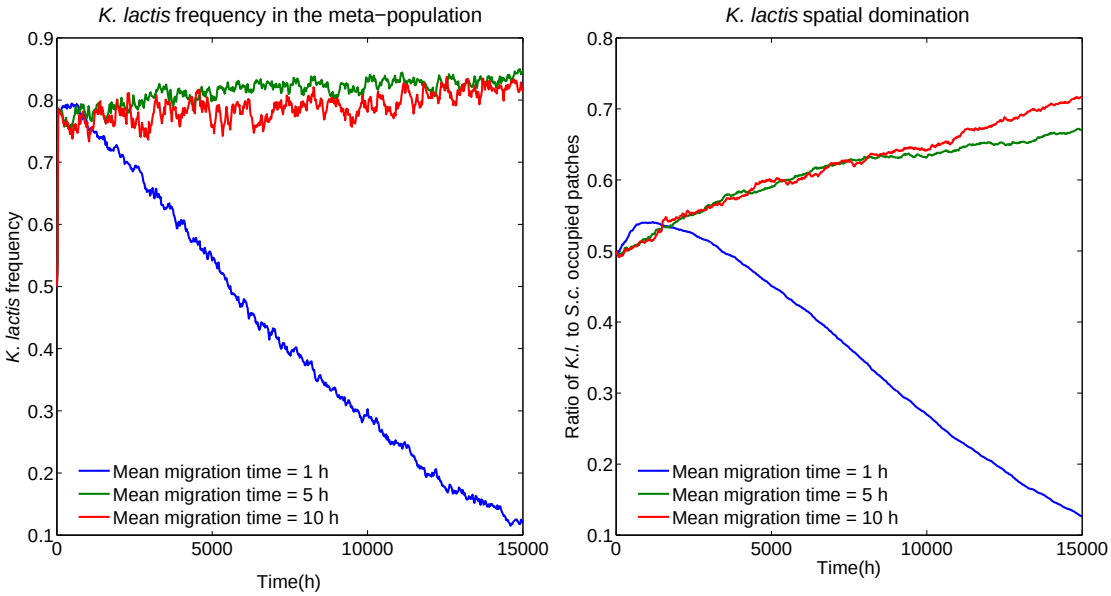



**Figure 4.15** – Results show *Kluyveromyces lactis* frequencies in the meta-population and the ratio of patches occupied. Results for the simulation of the spatial model with different average migration times. Each trajectory is calculated as the mean of 100 simulations. Calculation was done according to the equations 3.22 and 3.24

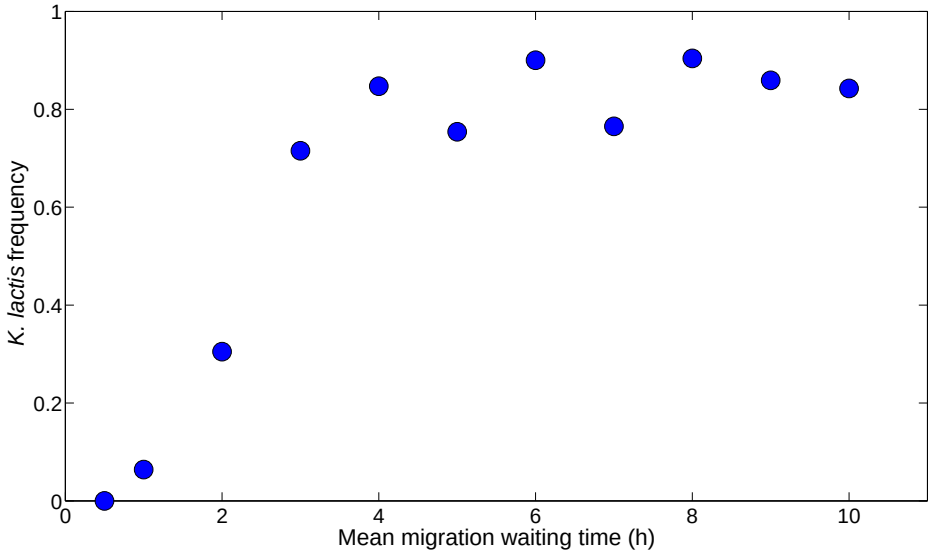



**Figure 4.16** – Exploration of the migration waiting time parameter space. Each point corresponds to an average *Kluyveromyces lactis* frequency in the meta-population at the end of the simulation resulting from the corresponding waiting times between migration events. Increase of waiting time leads to an increase in *Kluyveromyces lactis* frequencies.

# Chapter 5

# Discussion

## 5.1 Fermentation is dominant in homogeneous populations

The experimental part of this study shows that the fermentative metabolism is a dominant metabolic strategy for competition for glucose in well-mixed population. High glucose availability make this dominance is even more pronounced.

### 5.1.1 Batch cultivations - single cultures

The comparison of physiological profiles of *S. cerevisiae* and *K. lactis* from pure batch cultivations (figure 4.1) shows a typical pattern of growth on glucose for Crabtree-positive (figure 4.1a) and Crabtree-negative (figure 4.1b) yeasts. This difference can also be seen by comparing oxygen uptake and $CO_2$ production rate profiles in figure 4.2.

Although cultivation conditions are aerobic in both cases, the distinction between the two modes of metabolism is clear from ethanol concentration curves and dissolved oxygen measurements. *Kluyveromyces lactis* requires more oxygen during the glucose phase because it is using respiratory mode of metabolism. On the other hand, growth of *Saccharomyces cerevisiae* is characterized by accumulation of ethanol. This is the hallmark of fermentative growth. *S. cerevisiae* only uses fully respiratory growth only after all the glucose has been consumed and when ethanol becomes the main carbon source. A major metabolic change is needed for the switch from fermentative to respiratory metabolism and ethanol uptake. A second lag phase occurs during which *Saccharomyces cerevisiae* synthesizes all the necessary enzymes (diauxic shift). This second exponential growth phase explains the second peak oxygen uptake and $CO_2$ profiles in figure 4.2a. In the pure *Kluyveromyces lactis* cultivation, there are only single peaks in $CO_2$ production and oxygen uptake rates, and they show a sharp drop once all the glucose in consumed (figure 4.2b).

As expected, *Saccharomyces cerevisiae* shows higher maximal growth rate on glucose while *Kluyveromyces lactis* has a higher biomass yield (table 4.1). $K_s$ value for *Saccharomyces cerevisiae* displayed in the table 4.1 is higher that what is reported for wild-type *S. cereviaise* (0.357 g/L compared to 0.180 g/L reported in Nielsen et al. (2003)). This value might indeed be higher for the strain used in the experiments. Alternative explanation comes from studies of identifiability of Monod parameters from batch cultivation data: different sets of parameters show equally good fit to the data, and parameters estimated from the data coming from similar conditions often show large variability (Holmberg, 1982, Holmberg and Ranta, 1982). Regardless of the cause of this discrepancy, it should be pointed out that experiments and simulations in this study occur at glucose concentrations that are much higher than the $K_s$ constant for glucose. Growth rates are close to the saturation part of the $\mu(S)$ curve (figure 4.8), and the actual value of $K_s$ should not influence the outcome of competition in a qualitative manner.

### 5.1.2 Batch cultivations - competition experiments

Physiological profiles of mixed cultures show mixed characteristics of both types of metabolism. In the glucose phase the ethanol accumulates, indicating fermentation. At the same time, the dissolved oxygen levels drop faster than they do in purely fermentative growth. The dissolved oxygen in cultivations with 40 g/L and 150 g/L of glucose drops to levels where cells are in oxygen-limited conditions. This happens even though cultivation conditions are carefully monitored, and air is sparged through the medium.

The dominance of fermentative type of metabolism can be inferred by just considering the growth rate parameters from the table 4.1. In addition, oxygen limitation presents an additional problem for strains using respiratory growth. It is impossible to sustain maximal purely respiratory growth once the population density reaches a certain threshold and oxygen demand surpasses oxygen supply. This is in agreement with the negative frequency- and negative density-dependence of the respiratory growth in simulations that include oxygen limitation of *Kluyveromyces lactis* growth (section 5.1.4). Oxygen-limitation due to high cell densities also explains the absence of the pattern characteristic for diauxic shift in profiles of mixed cultures with 40 and 150 g/L glucose.

Table 4.2 and figures 4.4 and 4.5b quantitatively show how mixed cultures compare to pure cultures. Even at lower glucose concentrations (20 g/L), fermentation is a dominant strategy. It is important to note that *Kluyveromyces lactis* has an alcohol dehydrogenase enzyme, *KlADH4*, that is induced by ethanol and is insensitive to glucose repression (Breunig et al., 2000). This means that it is able to uptake ethanol even when the glucose is present in the media. In other words, in addition to using glucose from the media, *Kluyveromyces lactis* uptakes and metabolizes ethanol produced by *Saccharomyces cerevisiae*. Experimentally obtained values for biomass yields on glucose in table 4.2 include the *Kluyveromyces lactis* biomass produced by growth on ethanol.

This means that the true *Kluyveromyces lactis* biomass yields, and consequently the total mixed biomass yields on glucose, are lower than those observed experimentally in mixed culture. Hence, considering just competition for glucose, the true fraction of fermentative growth should actually be higher than what is shown in figure 4.5b.

The decrease in respiratory metabolism in mixed cultures can also be seen by comparing specific oxygen uptake rates in figure 4.5a. The specific oxygen uptake rate (sOUR) is expressed as the rate of oxygen in mmoles consumed per gram of dry cell weight per hour. Decrease in this number shows that the fraction of the biomass that uses respiratory metabolism decreases as glucose levels are increased. Furthermore, the sOUR values for mixed cultures in 40 g/L and 150 g/L are calculated only for the part of glucose phase before the onset of oxygen limitation, meaning that the dominance of fermentation is larger still.

An additional evidence for dominance of fermentative metabolism in competition for glucose comes from comparison of RQ values of pure and mixed cultures. This is shown in figure 4.6. The higher the RQ value, the higher the $CO_2$ production rate compared to oxygen uptake rate. This is a clear indication of fermentative metabolism. The figure shows that mixed cultures have RQ values higher than pure respiratory (*Kluyveromyces lactis*) culture and that these values approach that of pure fermentative culture (*Saccharomyces cerevisiae*).

### 5.1.3 Competition in chemostat culture

A well-known outcome of competition for a single resource between species with different growth and resource uptake capabilities is the competitive exclusion of all species but the one that is best adapted for that particular niche. Indeed, the competition experiments between *Saccharomyces cerevisiae* and *Kluyveromyces lactis* in chemostats result in competitive exclusion.

Competition experiments in carbon-limited chemostat results in competitive exclusion of *Kluyveromyces lactis*. This can be explained by the fact that *Saccharomyces cerevisiae* grows faster than *Kluyveromyces lactis* at all glucose concentrations (figure 4.8). At dilution rate of D=0.1 $h^{-1}$, which is not enough to induce fermentation (section 1.3), the growth and metabolism of *Saccharomyces cerevisiae* are respiratory. Regardless, *S. cerevisiae* is able to grow faster than *Kluyveromyces lactis* and dominate in the carbon-limited chemostat. One of the factors influencing this outcome is the higher specific glucose uptake rate of *Saccharomyces cerevisiae* . This is not surprising, as *Saccharomyces cerevisiae* has six primary glucose transporters (Youk and van Oudenaarden, 2009), and a total of 20 hexose transporters, 17 of which have been shown to facilitate glucose uptake (Breunig et al., 2000, Wieczorke et al., 1999). Furthermore, affinities of glucose transporters in *S. cerevisiae* cover a wide range of possible glucose concentrations.. On the other hand, *Kluyveromyces lactis* is a dairy yeasts, better adapted for niches containing galactose and lactose, sugars found in dairy products

(Schaffrath and Breunig, 2000).

The nitrogen-limited chemostat resulted in the opposite outcome, *Kluyveromyces lactis* dominance and washout of *Saccharomyces cerevisiae* from the culture. Since glucose is in excess, the competitive exclusion in this condition comes from competition for ammonia, rather than glucose. Residual concentration of ammonia found in the chemostat after *Saccharomyces cerevisiae* is washed out is lower than that found in pure *Saccharomyces cerevisiae* cultivations at the same dilution rate (ter Schure et al., 1995). In other words, *Kluyveromyces lactis* is more efficient in competing for nitrogen due to its ability to uptake it even when it is scarce in the media. Respiratory metabolism results in high biomass yields and, since proteins are a major constituent of biomass (Nielsen et al., 2003), this places a high demand for an efficient nitrogen metabolism and uptake. However, it needs to be noted that nitrogen metabolism in *Kluyveromyces lactis* has not been thoroughly studied and further research is needed to fully explain this result.

### 5.1.4 Resource availability, frequency and population density determine the outcome of the competition

Two different metabolic models were used to study the effects of resource availability, population density and frequencies on the outcome of the seasonal competition in well-mixed populations. The first model represents the situation where the growth of both species is limited only by glucose availability. The second model includes the limitation of *Kluyveromyces lactis* respiratory growth by oxygen. Both models show, as experiments do, that fermentation is dominant and that the dominance is more pronounced as glucose levels are increased (figure 4.10). However, models differ with respect to density- and frequency-dependence of the relative success of the two metabolic strategies (table 5.1).

**Table 5.1** – Initial frequency and initial density dependence of competitive fitness of the two strategies

| | Glucose limitation | | Glucose and oxygen limitation | |
|---|---|---|---|---|
| Metabolism | Frequency | Density | Frequency | Density |
| Fermentative | Positive | Positive | Negative | Positive |
| Respiratory | Positive | Negative | Negative | Negative |

When *Kluyveromyces lactis* growth was limited only by available glucose (equation 3.3), the dominance of fermentation comes from the growth and glucose uptake rates. Since fermentation is a strategy that gives higher growth rate and higher glucose uptake rate, *Saccharomyces cerevisiae* dominates in the culture. This situation, where one strain outcompetes the other because of its rapid use of limiting substrate is

called *scramble competition* (Hibbing et al., 2010). In this case, fermentation is positive-frequency dependent and positive density-dependent. Negative density-dependence of respiration means that efficient use of resources is favored only when the resources are shared between a smaller number of individuals. This has also been shown in competition models based on thermodynamic arguments (Pfeiffer and Bonhoeffer, 2002).

When *Kluyveromyces lactis* growth is limited by both glucose and oxygen concentrations (equation 3.14), fermentation becomes negative-frequency dependent. In other words, fermentation is more dominant when it is a rare strategy in the population. This is a natural consequence of the oxygen requirement for respiration: higher frequencies and densities of respirers will sooner reach the critical population density where they get limited by available oxygen. Fermenters will invade and outcompete these populations of respirers easier than the less dense populations that are growing closer to their maximal growth rate.

## 5.2 Limited population dispersal favors respiration

The stochastic model described in the section 3.5.4 was used to explore the effect of population dispersal on the outcome between strain that use glucose in an efficient and slow manner (cooperative), and strains that use glucose in a wasteful but fast manner (selfish). The simulation results show that the intensity of the population dispersal, modeled by waiting times between migration events, does affect the outcome of long-term competition in the global population (figures 4.13, 4.14, 4.15 and 4.16).

The actual values of the mean migration time parameters that result in different outcomes depend on the other "ecological" parameters used in model, most of which are not know exactly. However, when all parameters all fixed and only mean waiting times between migration events are varied, simulation results still show a definite change in competition outcomes.

Frequent migration events lead to well-mixed meta-populations. As a result, the outcome of competition will be the same as in the experiments and in simulations with no spatial structure imposed on the population - the fermentative strategists become dominant and respiratory strategists get washed out of the meta-population or only make a small fraction of it (figure 4.16). In simulations where population dispersal is limited, the opposite thing happens - respiratory strains become dominant.

In a spatially structured population, respiratory populations will have a higher probability of being surrounded by other respiratory populations and will get an advantage of using resources efficiently. Low population yields of fermenters have long-term disadvantage and make them more susceptible to cell death and local population extinctions. This is a typical game-theoretic situation where spatial structure enables locally successful strategies to dominate in the meta-population in the long run (Nowak and May, 1992). However, it should be noted that this is only the case for the "Prisoner's

dilemma" games, and that in games of other types spatial structure may inhibit cooperation (Hauert and Doebeli, 2004). This result show that cooperative strategy can become dominant even in the absence of factors like rationality, memory of past interactions and punishement (Nowak, 2006b).

The positive correlation between success of respiratory strategies, and spatial structures can be observed in nature. Biofilms are typical bacterial cooperative communities, they are both the result of cooperative behavior and promote cooperative behavior (Kreft, 2004). All multicellular lifeforms use respiration as a main metabolic strategy for production of ATP from glucose. Slow but efficient use of glucose is not under immediate threat of invasion from selfish, fast-consuming individuals because foods are ingested before they are digested. It has been suggested that cooperative use of resources in spatially structured setting is the primitive form of multi-cellularity and that "true" multicellular organisms evolved from respiratory cells that lost the ability to fully detach from their parental cells (Pfeiffer and Bonhoeffer, 2003).

Two notable exceptions to the use of respiratory metabolism in the multicellular world are muscle cells and tumors. Muscle cells use fermentative metabolism, that yields lactate, whenever there is need to produce ATP at faster rates than is possible through the respiration. Tumor cells grow at faster rates and are often limited by oxygen availability. In addition to showing other selfish traits, tumors often use fermentative pathways (Vazquez et al., 2010).

# Chapter 6

# Conclusion

This study shows how different characteristics of metabolism, resource availability, population and spatial structure influence population dynamics in microbial populations and promote different metabolic strategies. In nature, these populations undergo fierce competition for resources. Specific modes of metabolic regulation and metabolism can be optimized for maximizing efficiency (yields) or rate, but never both at the same time. The choice between those two will decide the outcome of competition and will depend on the evolutionary history and the ecological niche the organism has adapted to.

Our experiments show that maximization of growth and uptake rates, as seen in fermentative lifestyle, is a strategy that will lead to dominance in well-mixed populations. Abundance of glucose will make fermentative strategy even more dominant. Increase in fraction of fermentative metabolism with increased glucose concentrations can be compared to what happens in glucose-rich fleshy fruits during ethanol fermentation and explains the dominance of *Saccharomyces cerevisiae* in this ecological niche. Although fast glucose consumption, or scramble competition, is enough for fermentation to become dominant, respiration is further limited by oxygen requirement.

The model of the spatially structured population shows that the relative success of fermentative and respiratory strategies will depend on the intensity of the population mixing or dispersal. The simulations show that, although being washed-out from the meta-population that are mixed at higher rate, respirers can become dominant in the population when the population is less dispersed. In other words, rigid spatial population structure can favor the dominance of the yield maximizing respiratory strategy and, in general, promote cooperative behavior.

# Acknowledgments

I have worked on my thesis project in the Systems Biology Group at the Department of Chemical and Biological Engineering at Chalmers University of Technology under the supervision of Goutham Vemuri.

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# Appendix A

# Appendix - MATLAB code

### Main script

This is the MATLAB code for the script that was used to explore the parameter space of the mean waiting time between migration events.

```
tMigParameterSpace=[0.5 1:10];

for p=1:length(tMigParameterSpace)
    fileName=['tMig',num2str(tMigParameterSpace(p))];
    if ~(exist(dirName,'dir'))
        mkdir(dirName);
    end;

    nSimulations=100;    % run nSimulations for each parameter value
    for r=1:nSimulations
        clearvars -except tMigParameterSpace dirName nSimulations r p;
        worldx=10;        % world dimensions
        worldy=10;
        totalTime=15000;  % time in hours for total simulation run
        snapInterval=20; % record population densities every snapInterval hours
        initialSubstrate=20;  % initial glucose concentration in each patch
        substrateInflux=20;  % amount of substrate added on sInflux event
        migrationMean=tMigParameterSpace(p);  % waiting time for migration event
        sInfluxMean=10;  % waiting time for sInfulx event
        eradicationMean=20;  % waiting time for erradication event
        nPatches=worldx*worldy;
        klaInitialPatches=floor(nPatches/10);  % number of patches to populate with kla
        sceInitialPatches=floor(nPatches/10);  % number of patches to populate with sce
        klaInitialBiomass=0.01;         % initial biomasses in g/L
        sceInitialBiomass=0.01;

        worldGrid=zeros(worldy,worldx); % grid of local populations, initialize to zero
        substrateGrid=worldGrid;
        substrateGrid(:)=initialSubstrate;
        klaGrid=worldGrid;        % grid of s.cerevisiae and k.lactis
        sceGrid=worldGrid;

        % populate k. lactis
        popPatches=randperm(worldy*worldx); % generate random indices
        klaGrid(popPatches(1:klaInitialPatches))=klaInitialBiomass;
        % populate s. cerevisiae
        popPatches=randperm(worldy*worldx); % generate random indices
```

```
sceGrid(popPatches(1:sceInitialPatches))=sceInitialBiomass;
worldGrid=klaGrid+sceGrid;  % total cell population initially

%generate the initial stochastic times
tToMigration=random('exp',migrationMean);
tToSInflux=random('exp',sInfluxMean);
tToEradication=random('exp',eradicationMean);

if ~(mod(totalTime,snapInterval))         % a time vector for taking snapshots
    time=0:snapInterval:totalTime;
else
    time=[0:snapInterval:totalTime-mod(totalTime,snapInterval) totalTime];
end;
%vectors for storing the results of populations and populated patches
avgKlaPopulation=zeros(1,length(time));
avgKlaPopulation(1)= sum(sum(klaGrid))/nPatches;
avgScePopulation=zeros(1,length(time));
avgScePopulation(1)=sum(sum(sceGrid))/nPatches;
patchesKlaPopulated=zeros(1,length(time));
patchesKlaPopulated(1)=sum(sum(klaGrid>0));
patchesScePopulated=zeros(1,length(time));
patchesScePopulated(1)=sum(sum(sceGrid>0));

for i=2:length(time)
    timeSlice=time(i)-time(i-1); % timeslice to run
    while (abs(timeSlice)>1e-8)
        timeToEvent=min([tToMigration tToSInflux tToEradication]);
        if (timeSlice<timeToEvent)
            tSpan=[0 timeSlice];  % time span for integration of local dynamics
            world_ind=(worldGrid>1e-8); % save indices of populations with nonzero population
            count=sum(sum(world_ind));  % count populated patches
            if ~(count) %stop loop if no patches are populated
                break
            end;

            initialValues=zeros(1,count*3); %create the vector of initial values for integration
            initialValues(1:3:end)=sceGrid(world_ind);
            initialValues(2:3:end)=klaGrid(world_ind);
            initialValues(3:3:end)=substrateGrid(world_ind);

            components=1:count*3;     %count total number of state variables
            options=odeset('NonNegative',components); %disallow negative states
            [t y]= ode15s(@localDynamics, tSpan, initialValues,options); %integrate local population dynamics
            %update grids after integration
            sceGrid(world_ind)=y(end,1:3:end);
            klaGrid(world_ind)=y(end,2:3:end);
            substrateGrid(world_ind)=y(end,3:3:end);
            worldGrid=klaGrid+sceGrid;
            tToMigration=tToMigration-timeSlice;  %reduce waiting time for the amount of elapsed time:
            tToSInflux=tToSInflux-timeSlice;
            tToEradication= tToEradication-timeSlice;
            timeSlice=0;
        else

            tSpan=[0 timeToEvent];        %time span for integration of local dynamics
            world_ind=(worldGrid>1e-8);   %save indices of populations with nonzero population
            count=sum(sum(world_ind));  %count populated patches
            if ~(count)                     %stop loop if no patches are populated
                break
            end;
            initialValues=zeros(1,count*3);  %create the vector of initial values for integration
            initialValues(1:3:end)=sceGrid(world_ind);
```

```
            initialValues(2:3:end)=klaGrid(world_ind);
            initialValues(3:3:end)=substrateGrid(world_ind);
            components=1:count*3;
            options=odeset('NonNegative',components); %disallow negative states
            [t y]= ode15s(@localDynamics, tSpan, initialValues,options);
            sceGrid(world_ind)=y(end,1:3:end); %update grids after integration
            klaGrid(world_ind)=y(end,2:3:end);
            substrateGrid(world_ind)=y(end,3:3:end);
            worldGrid=klaGrid+sceGrid;
            tToMigration=tToMigration-timeToEvent; %reduce waiting time for the amount of elapsed time
            tToSInflux=tToSInflux-timeToEvent;
            tToEradication= tToEradication-timeToEvent;
            %perform stochastic event (check which waiting time is zero)
            if (abs(tToSInflux)<1e-8) %add substrate to random patch
                randPatch=floor(rand()*nPatches)+1;
                substrateGrid(randPatch(1))=substrateGrid(randPatch(1))+substrateInflux;
                tToSInflux=random('exp',sInfluxMean); %regenerate time to the next substrate influx event

            elseif (abs(tToMigration)<1e-8) %migrate cells
                [klaGrid sceGrid]=migrate(klaGrid,sceGrid);
                worldGrid=klaGrid+sceGrid;
                tToMigration=random('exp',migrationMean);

            elseif (abs(tToEradication)<1e-8) %eradication event
                randPatch=floor(rand()*nPatches)+1;
                pEradicate=rand();
                pSurvive=1-exp(-worldGrid(randPatch)); %calculate the probability of surviving
                if (pEradicate>pSurvive)
                    klaGrid(randPatch)=0;
                    sceGrid(randPatch)=0;
                    worldGrid(randPatch)=0;
                end;
                tToEradication=random('exp',eradicationMean);
            end;
            timeSlice=timeSlice-timeToEvent;
        end;
    end; %end while loop (one timeSlice ended)
    %record data
    worldGrid=klaGrid+sceGrid;
    avgKlaPopulation(i)= sum(sum(klaGrid))/nPatches;
    avgScePopulation(i)=sum(sum(sceGrid))/nPatches;
    patchesKlaPopulated(i)=sum(sum(klaGrid>0));
    patchesScePopulated(i)=sum(sum(sceGrid>0));
    if ((patchesScePopulated(i)+patchesKlaPopulated(i))<1)
        %stop loop if no patches are populated
        break
    end;
end;%end big loop, next time interval

%save results in a file
saveFile=[fileName,'resultKla'];
save(saveFile, 'avgKlaPopulation', '-ASCII', '-append');
saveFile=[fileName,'resultSce'];
save(saveFile, 'avgScePopulation', '-ASCII', '-append');
saveFile=[fileName,'resultKlaPatch'];
save(saveFile, 'patchesKlaPopulated', '-ASCII', '-append');
saveFile=[fileName,'resultScePatch'];
save(saveFile, 'patchesScePopulated', '-ASCII', '-append');
if~(exist('simulationTime','file'))
    saveFile='simulationTime';
    save(saveFile,'time','-ASCII','-append');
end;
```

```
    end; %end one simulation run

end; %end for one parameter value


%% Function for calculating local dynamics
function dydt=localDynamics(t,x)
%takes a vector of cell densities and substrate concentration
%input vector: (sce, kla, substrate)

%metabolic parameters
muMax_sc=.369;
Yxs_sc=.138;
Ks_sc=.357;
muMax_kl=.309;
Yxs_kl=.536;
Ks_kl=.558;
kd=0.01;%death rate, the same for both

% state variables
dydt=zeros(length(x),1);
X_sc=x(1:3:end);
X_kl=x(2:3:end);
S=x(3:3:end);

mu_sce=muMax_sc.*(S./(Ks_sc+S)); % instantaneous growth rates according to the Monod model
mu_kla=muMax_kl.*(S./(Ks_kl+S));

% dynamic balances for biomasses and substrate
for i=0:(length(X_sc)-1)
    dydt(3*i+1)=(mu_sce(i+1)-kd)*X_sc(i+1);
    dydt(3*i+2)=(mu_kla(i+1)-kd)*X_kl(i+1);
    dydt(3*i+3)=-(mu_sce(i+1)*X_sc(i+1)/Yxs_sc + mu_kla(i+1)*X_kl(i+1)/Yxs_kl);
end;




%Function for population migration event
function [kla sce]=migrate(kla0,sce0)
kla=kla0;
sce=sce0;
fracMigrate=.1; %fraction of the donor population to migrate
donorX=floor(size(kla0,2)*rand())+1; %choose random indices
donorY=floor(size(kla0,1)*rand())+1;
randDirection=floor(rand()*4)+1; %randomly choose a direction to migrate
%1-left (x-1)
%2-right (x+1)
%3-up (y-1)
%4-down (y+1)
newX=donorX-(randDirection==1)+(randDirection==2);
newY=donorY-(randDirection==3)+(randDirection==4);

%periodic boundary conditions
newX(newX>size(kla0,2))=1;
newX(newX==0)=size(kla0,2);
newY(newY>size(kla0,1))=1;
newY(newY==0)=size(kla0,1);

%choose strain to move
klaFreq=kla0(donorY,donorX)/(kla0(donorY,donorX)+sce(donorY,donorX));
```

```
randomStrain=rand();
if (randomStrain>(1-klaFreq))     %move kla
    klaTemp=kla(donorY,donorX)*fracMigrate;
    kla(donorY,donorX)=kla(donorY,donorX)*(1-fracMigrate);
    kla(newY,newX)=kla(newY,newX)+klaTemp;
elseif (randomStrain<=(1-klaFreq))     %move sce
    sceTemp=sce(donorY,donorX)*fracMigrate;
    sce(donorY,donorX)=sce(donorY,donorX)*(1-fracMigrate);
    sce(newY,newX)=sce(newY,newX)+sceTemp;
end;
```

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