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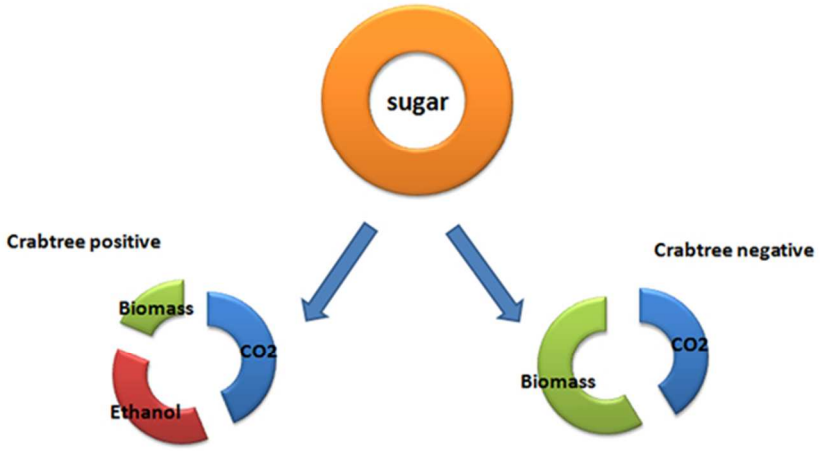

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Opinion / Perspective / Mini-Review

Why, when and how did yeast evolve alcoholic fermentation?

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Abstract

The origin of modern fruits brought to microbial communities an abundant source of rich food based on simple sugars. Yeasts, especially *Saccharomyces cerevisiae*, usually become the predominant group in these niches. One of the most prominent and unique features and likely a winning trait of these yeasts is their ability to rapidly convert sugars to ethanol at both anaerobic and aerobic conditions. Why, when and how did yeast remodel their carbon metabolism to be able to accumulate ethanol under aerobic conditions and at the expense of decreasing biomass production? We hereby review the recent data on the carbon metabolism in *Saccharomycetaceae* species, and attempt to reconstruct the ancient environment, which could promote the evolution of alcoholic fermentation. We speculate that the first step towards the so-called alcoholic fermentation lifestyle was the exploration of anaerobic niches resulting in an increased metabolic capacity to degrade sugar to ethanol. The strengthened glycolytic flow had in parallel a beneficial effect on the microbial competition outcome, and later evolved as a "new" tool promoting the yeast competition ability under aerobic conditions. The basic aerobic alcoholic fermentation ability was subsequently "upgraded" in several lineages by evolving additional regulatory steps, like glucose repression in the *S. cerevisiae* clade, to achieve a more precise metabolic control.

209 words

Introduction

Yeast fermentation of different plant carbohydrate sources is one of the oldest human technologies and its origins date back to the Neolithic period. Even nowadays, yeasts are essential for many biotechnological processes, like beer, wine and biofuel fermentations. However, the complexity of gene expression regulatory networks behind the alcoholic fermentation is still far from being completely understood (reviewed in Compagno *et al*., 2014). Similarly, the origin and the driving forces in nature determining the path and outcomes of the yeast evolutionary history, and the present day evolutionary trends, are still rather unclear. It is the main aim of this paper to speculate on and propose evolutionary pathways, trends and driving forces, which operated during yeast evolutionary history and resulted in the present aerobic fermentative capacity of *Saccharomyces* yeasts and a new lifestyle of these yeasts.

Crabtree effect

One of the most prominent features of the baker's yeast *Saccharomyces cerevisiae* is its ability to rapidly convert sugars to ethanol and carbon dioxide at both anaerobic and aerobic conditions. Under aerobic conditions, respiration is possible with oxygen as the final electron acceptor, but *S. cerevisiae* exhibits alcoholic fermentation until the sugar reaches a low level. This phenomenon is called the Crabtree effect (De Deken, 1966) and the yeasts expressing this trait called Crabtree-positive yeasts. In contrast, "Crabtree-negative" yeasts lack fermentative products and under aerobic conditions biomass and carbon dioxide are the sole products. However, it is possible to obtain pure respiratory utilization of glucose by *S. cerevisiae* under aerobic conditions if the glucose concentration is kept very low e.g. by using a glucose-limited continuous culture operating below a certain strain-specific threshold value (called "critical" dilution rate) or by using fed-batch cultivations (Postma *et al*., 1989). This glucose repression phenomenon in *S. cerevisiae* involves different signal transduction pathways activated by extracellular and intracellular levels of glucose and its related metabolites and/or their fluxes through the involved metabolic pathways (reviewed in Johnston, 1999; Westergaard *et al*., 2006). However, the complexity of glucose repression regulatory networks is still far from being completely understood. Some of the regulatory activities operate at the transcriptional level and some others operate directly on the involved enzymes. Important to note, so far it is not clear yet if the glucose repression mechanism was the original step to promote evolution of the Crabtree effect, or it has been "added" later during the evolution of some yeast lineages.

Different physiological and molecular approaches have been used as the background for the current definition of the Crabtree effect. The most accepted definition explains the long-term Crabtree effect as aerobic alcoholic fermentation under steady-state conditions at high growth rates. When *S. cerevisiae* is cultivated under glucose-limited conditions, the long-term effect appears when the dilution rate (or in other words: the glucose uptake rate) exceeds the strain specific threshold value. The same effect is observed also when yeast cells are cultivated in batch cultivations. The molecular background for the long-term Crabtree effect has been explained as a limited respiratory capacity due to the repression of the corresponding respiration associated genes (Postma *et al*., 1989; Alexander and Jeffries, 1990). On the other hand, the short-term Crabtree effect is the immediate appearance of aerobic alcoholic fermentation upon addition of excess sugar to sugar-limited and respiratory cultures. This effect has been explained as an overflow in the sugar metabolism, and could be associated directly with the biochemical properties of some of the respiration-associated enzymes and their regulators (Pronk *et al*., 1996; Vemuri *et al*., 2007). However, it is still unclear if the regulatory molecular mechanisms operating during the long-term and short-term Crabtree effect are indeed different from each other. A very interesting aspect is also the evolutionary and ecological background for the development of these regulatory mechanisms (Piskur *et al*., 2006; Rozpedowska *et al*., 2011).

Ecology perspective and yeast life-style

Every autumn, when fruits ripen, a fierce competition for the fruit sugars starts within microbial communities. Yeasts, especially *Saccharomyces cerevisiae* and its close relatives, usually become the predominant group in niches with freely available mono- and oligosaccharides. The fast sugar consumption, ethanol production and tolerance, and the ability to propagate without oxygen, are likely some of the "winning" traits responsible for the competition outcome (Piskur *et al*., 2006). However, a great majority of yeasts, which we find in nature, has been only poorly studied in laboratory so far or even in their environmental context.

At least three lineages (Figure 1), including budding and fission (*Schizosaccharoymces pombe*) yeasts, have apparently independently evolved the metabolic ability to produce ethanol in the presence of oxygen and excess of glucose (reviewed in Piskur *et al*., 2006; Rozpedowska et al., 2011; Rhind *et al*., 2011). This metabolic »invention« (Crabtree effect), represents in nature a strong tool to outcompete other microbes. Both groups of ethanol-producing budding yeast, including *S. cerevisiae* and *D. bruxellensis*, can also efficiently catabolize ethanol, and therefore their corresponding lifestyle has

been named as the "make-accumulate-consume (ethanol)" strategy (Thomson *et al*., 2005; Piskur et *al*., 2006; Rozpedowska *et al*., 2011). On the other hand, *S. pombe* can grow only poorly on ethanol as sole carbon source. In short, this life strategy is based on that yeasts can consume very fast more sugar than other species, convert it to ethanol to inhibit the growth of other species, especially bacteria, and then consume the remaining carbon once they have established competitive dominance in the niche.

Ability to grow anaerobically

The availability of oxygen varies among different niches. One of the main problems an organism-faces under anaerobic conditions is the lack of the final electron acceptor in the respiratory chain. This reduces or completely eliminates the activity of Krebs cycle, respiratory chain and mitochondrial ATP generation. As a response to hypoxic and anaerobic conditions, organisms have developed several processes to optimize the utilization of oxygen and even reduce the dependence on the presence of oxygen. According to their dependence on oxygen during the life cycle, yeasts are classified as: (i) obligate aerobes displaying exclusively respiratory metabolism, (ii) facultative fermentatives (or facultative anaerobes), displaying both respiratory and fermentative metabolism; and (iii) obligate fermentatives (or obligate anaerobes) (Merico *et al*., 2007).

The ability of yeasts to grow under oxygen-limited conditions seems to be strictly dependent on the ability to perform alcoholic fermentation. In other words, enough ATP should be generated during glycolysis to support the yeast growth, and NADH generated during glycolysis gets re-oxidized. Apart from the energy and NADH/NAD redox problems, under anaerobic conditions yeasts must also find a way to run various reactions independent of the respiratory chain and a normal Krebs cycle. In other words, substrates (intermediates) for *de novo* reactions, for example for the amino acid synthetic pathways, need to originate from a modified metabolic network. On the other hand, in yeast some compounds, like unsaturated fatty acids and sterols, cannot be synthesized in the cell under anaerobiosis and must originate from the medium or from previous aerobic growth.

Apparently, the progenitor of *Saccharomycetaceae* was an aerobic organism, strictly dependent on oxygen. It seems that later several yeast lineages (Figure 1) have evolved the ability to grow anaerobically, or at least can grow partially independently of oxygen. *S. cerevisae* and a majority of post-WGD yeasts, as well as some lower *Saccharomycetaceae* branches, like the *Lachancea* yeasts,

show a clear ability to proliferate without oxygen. Interestingly, two other lineages, *D. bruxellensis* (Rozpedowska *et al*., 2011) and *S. pombe* (Visser *et al*., 1990) have apparently also evolved the ability to propagate under anaerobic conditions. However, they need some extra supplements in the medium to be able to propagate without oxygen. It is interesting to point out, that the same three lineages, which can perform alcoholic fermentation under aerobic conditions can also proliferate in the absence of oxygen.

How to deduce the yeast evolutionary history

The onset of yeast genomics (Goffeau *et al*., 1996) has provided a tool to reconstruct several molecular events, which have reshaped the budding yeasts during their evolutionary history (reviewed in Dujon, 2010). Several molecular events have left a clear fingerprint in the modern genomes (Figure 2), while the origin of more complex traits, like the Crabtree effect, is often not easy to determine using only a genome analysis approach.

The *Saccharomycetaceae* family covers over 200 million years of the yeast evolutionary history, and includes six post-whole genome duplication (post-WGD) genera, *Saccharomyces*, *Kazachstania*, *Naumovia*, *Nakseomyces*, *Tetrapisispora* and *Vanderwaltozyma*, and six non-WGD genera, *Zygosaccharomyces*, *Zygotorulaspora*, *Torulaspora*, *Lachancea*, *Kluyveromyces* and *Eremothecium* (Kurtzman and Robnett 2003; Casaregola *et al*. 2011) (Figure 2). The phylogenetic relationship among these genera is now relatively well understood. However, only a very few species are reported in literature for their carbon metabolism (Merico *et al*., 2007).

We have recently studied over forty yeast species, which in nature occupy similar niches and rely on glucose as the »preferred« substrate (Kurtzman *et al*., 2011), and analyzed their carbon metabolism using uniform experimental conditions all along the fully controlled growth in fermentors (Hagman *et al*., 2013a).

The origin of the long-term Crabtree effect

The studied yeasts belonged to the *Saccharomycotina* family, including six WGD genera and six non-WGD genera, thus covering 200 million years of evolution (Figure 2). The observed extent of the Crabtree effect in each species corresponds to its position on the yeast phylogenetic tree. In addition, the observed Crabtree effect is much more pronounced in a majority of WGD yeasts than in the ethanol producing none-WGD species, suggesting at least a two-step »invention«. On the other hand, carbon metabolism in the »lower« branches of *Saccharomycetaceae* yeasts, belonging to modern *Kluyveromyces* and *Eremothecium*, is similar to other Saccharomycotina yeasts, like *Candida albicans*, *Yarrowia lipolytica*, *Pichia pastoris*, which are Crabtree negative yeasts. Therefore, the origin of the "make-accumulate-consume" strategy / Crabtree effect could take place within the time interval spanning the origin of the ability to grow under anaerobic conditions, and the loss of respiratory chain Complex I, after the split of the *Saccharomyces-Lachancea* and *Kluyveromyces-Eremothecium* lineages, approximately 125 mill.years ago. On the other hand, the second step, leading towards even a more pronounced Crabtree effect, occurred relatively close to the WGD event (Shields and Wolfe, 1997), the settlement of rewiring of the promoters involved in the respiratory part of the carbon metabolism (Ihmels et al., 2005), and the settlement of the petite-positive character (Merico *et al*., 2007). There are also some other possible scenarios, which can be "deduced" from the Hagman *et al*., (2013a) results. The origin of the long-term Crabtree effect could took place much before, coinciding with the loss of respiratory chain Complex I, but this trait was later lost in some lineages, like *Kluyveromyces-Eremothecium*. The long-term effect may have even originated independently in several *Saccharomycetaceae* lineages. To clarify this point, in the near future one would need to focus on some of the "early Crabtree positive" branches, like *Lachancea*, and perform detailed carbon metabolism studies on these yeasts, including gene expression profiling, to deduce which regulatory circuits are already present in *Lachancea*, and which ones only in *Saccharomyces*.

The origin of modern plants with fruits, at the end of the Cretaceous age, more than 125 mya (Sun *et al*., 2011), brought to microbial communities a new larger and increasingly abundant source of food based on simple sugars. On the other hand, ancient yeasts could hardly produce the same amount of new biomass as bacteria during the same time interval, and could therefore be out-competed. We speculate that slower growth rate could in principle be counter-acted by production of compounds that could inhibit the growth rate of bacteria, like ethanol and acetate. However, what were the initial molecular mechanisms that promoted the evolution of the new "lifestyle" and rewiring of the carbon metabolism? Was competition between yeast and bacteria indeed the original driving force to promote evolution of the aerobic alcoholic fermentation?

The Crabtree effect, which is the background for the yeast »make-accumulate-consume« strategy, results in a lower biomass production because a fraction of sugar is converted into ethanol. This means that more glucose has to be consumed to achieve the same yield of cells (Figure 3). Because only a fraction of sugar is used for the biomass and energy production this could theoretically result in a lower growth rate in Crabtree positive yeasts. In nature, a lower growth rate would have a negative effect for the yeast during the competition with different yeasts species and between yeasts and bacteria. However, an increased glycolytic flow (achieved by elevated uptake of glucose and its faster conversion to pyruvate and final fermentation products) could in principle compensate for the Crabtree effect and balance the growth rate providing the same number of cells during the same time interval. Just much more glucose would be consumed in this case (Figure 3). What could be the original driving force that increased the flow through the glycolytic pathway?

Short-term Crabtree effect: a strengthened glycolytic flow

The short-term Crabtree effect is defined as the immediate appearance of aerobic alcoholic fermentation upon a pulse of excess sugar to sugar-limited yeast cultures. In a recent follow-up (Hagman, 2013b; Hagman *et al*., 2014) of the above study, ten different yeast species, having a clearly defined phylogenetic relationship, have been characterized for short-term Crabtree effect. These species very roughly cover the phylogenetic span of yeasts, which have been studied in the long-term experiments. Yeasts have been cultivated as continuous cultures under glucose-limited conditions, and upon a glucose pulse their general carbon metabolism analyzed (Hagman *et al*., 2013b). In pulse experiments, yeasts belonging to *Pichia*, *Debaryomyces*, *Eremothecium* and *Kluyveromyces marxianus* have not exhibited any significant ethanol formation (just like they also do not show long-term Crabtree effect), while *Kluyveromyces lactis* behaved, surprisingly, as intermediate yeast. The *Lachancea*, *Torulaspora*, *Vandervaltozyma* and *Saccharomyces* yeasts have, upon a glucose pulse, exhibited rapid ethanol accumulation. These yeasts are also long-term positive species (Hagman *et al*., 2013a).

Roughly, long-term positive species in glucose pulse experiments behaved also as short-term positive. However, the results suggest that *Kluyveromyces* yeasts can be considered as intermediate in both phylogenetic position and their carbon metabolism. *K. lactis* is in fact on one hand a short-term Crabtree positive, but on the other hand a long-term Crabtree negative. Even if the number of studied yeasts is limited (only ten), one could still speculate that the time of origin of the short-term Crabtree effect and the time of origin of the long-term Crabtree effect seem to be very close to each other and

may even overlap, coinciding with the horizontal transfer of *URA1* and the ability to proliferate anaerobically (Merico *et al*., 2007).

However, one of the most surprising observations has been that when *S. cerevisiae* and its Crabtree-positive relatives grow in continuous culture below a sugar threshold with a respiratory metabolism, their fermentative pathways are fully expressed, whereas the respiration-associated parts are repressed when the sugar level overcomes a certain level. In other words, it seems that these yeast cells have all the time the capacity to ferment "switched-on", while the respiration ability is strictly related to the amount of sugar availability. On the other hand, in several Crabtree negative yeasts it has been demonstrated that the fermentative pathway is "switched-on" only when oxygen becomes limiting (Kiers *et al*., 1998; Jeffries, 2006; Baumann *et al*., 2010). Interestingly, in Crabtree negative yeasts the flow through glycolysis matches the respiration associated one, whereas in Crabtree positive yeasts the glycolytic flow is apparently "over-dimensioned".

Hypothesis on the original driving forces

Why would the progenitor yeast initially benefit from the strengthened glycolytic and fermentative flow? It is apparent from previous studies that the ability to proliferate under anaerobic conditions originated at approximately the same time as the origin of the first modern fruits and aerobic alcoholic fermentation, upon the split of the *Kluyveromyces-Eremothecium* and *Lachancea-Saccharomyces* lineages (Hagman *et al*., 2013a). It could be that regular exposure to poorly aerobic niches represented a selection pressure which promoted yeast "mutant" lineages with strengthen glycolytic and fermentation pathways, as well as having improved resistance towards ethanol. In these cells, up-regulation of the glycolytic-fermentative capacity should have provided sufficient carbon flow and energy yield even in the absence of oxygen. In other words, exploration of anaerobic niches could be a driving force to build up a carbon metabolism network, which is better adapted to ferment. However, in principle these yeasts could still alternate between aerobic and anaerobic niches.

At the same time, the yeast progenitor could also get re-modeled several general metabolic pathways, not only the energy yielding ones, to be able to proliferate also under anaerobic conditions. For example, the fourth step of the de novo pyrimidine synthesis became, upon horizontal transfer of the *URA1* gene, less dependent on the functional respiratory chain (Gojkovic *et al*., 2004). Note, that this step towards independence of oxygen occurred before the separation of the *Kluyveromyces* and

Lachancea-Saccharomyces lineages, and therefore likely before the origin of the aerobic fermentative-respiratory life style.

Fast consumption of glucose through an elevated uptake would simply "starve-out" other microbial competitors. The duplication of glucose transporter genes in the progenitor of the *Lachancea-Saccharomyces* lineages could represent one of the molecular backgrounds for the initial increased ability to consume glucose. However, the increased carbon flow through glycolysis generated an overflow and resulted in synthesis of fermentation products. When these metabolites, especially ethanol, were accumulated at high concentrations, they could impair the growth of other competing microbes. The fermentation products could thus become a new weapon to out-compete other microbes. At this point the driving biological force for optimizing the ethanol fermentation pathway, even in presence of oxygen, could be "to kill" other competitors. In parallel, yeasts had also to evolve the ability to better tolerate ethanol. Further on, the origin of glucose repression of the respiratory pathways under fully aerobic conditions, in the *S. cerevisiae* lineage, could represent fine tuning mechanism, which increased the efficiency of ethanol production.

Conclusions

During the recent years there has been more and more research focus on non-conventional yeasts, especially many of these yeasts got their genomes sequenced. These sequence data now help to deduce a reliable phylogenetic relationship among yeasts, and provides us with a possibility to reveal evolution fingerprints, which have remained preserved in the genome. These data should now be complemented with physiology and molecular genetic studies on a variety of yeast species. This will open additional avenues in biotechnology and evolution research. In this paper we attempted to analyze the most recent results on yeast carbon metabolism and develop a hypothesis on the evolution of alcoholic fermentation. We speculate that the exploration of anaerobic niches and later on the competition with other microbes were the driving forces behind the remodeling of the yeast carbon metabolism.

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Figure 1

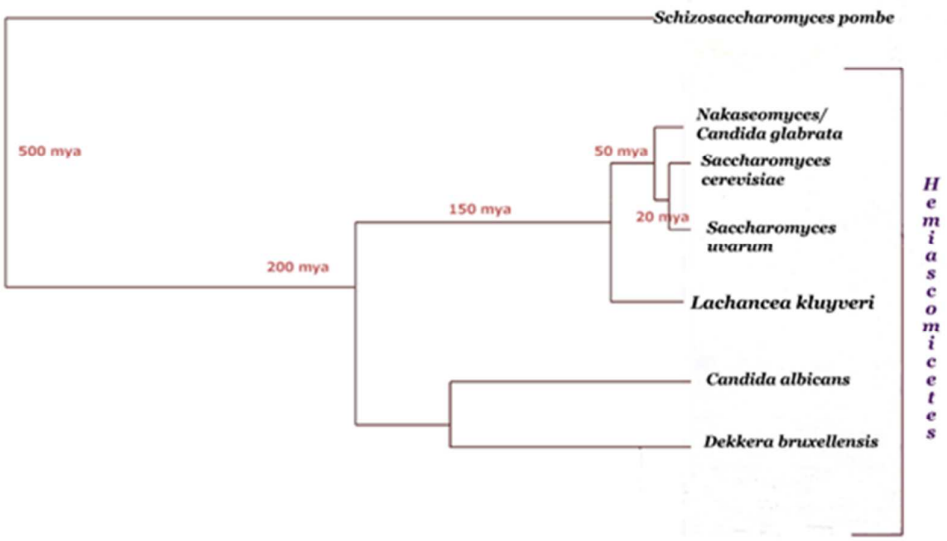
Phylogenetic relationship among some yeasts. Note that some of the shown yeast lineages separated from each other many million years ago and have therefore accumulated several molecular and physiological changes regarding their carbon metabolism. However, during the evolutionary history, there have also been parallel events. Apparently, at least three lineages, *Saccharomyces*, *Dekkera* and *Schizosaccharomyces*, have evolved (i) the ability to ferment in the presence of oxygen and (ii) to proliferate under anaerobic conditions. This figure was adopted from Compagno *et al* (2014).

Figure 2

The *Saccharomycetaceae* family covers over 200 million years of the yeast evolutionary history, and includes six post-whole genome duplication (post-WGD) genera, *Saccharomyces*, *Kazachstania*, *Naumovia*, *Nakaseomyces*, *Tetrapisispora* and *Vanderwaltozyma*, and six non-WGD genera, *Zygosaccharomyces*, *Zygotorulaspora*, *Torulaspora*, *Lachancea*, *Kluyveromyces* and *Eremothecium*. Hereby we show a rough phylogenetic relationship among these genera. Two evolutionary events are shown, WGD, which took place app. 100 mya and the loss of Respiratory Complex I (which took place app. 150 million years ago). This figure was adopted from Hagman *et al* (2013a).

Figure 3

Crabtree effect results in lower biomass production because a fraction of sugar is converted into ethanol. This means that more glucose has to be consumed to achieve the same yield of cells if comparing with Crabtree negative yeasts. Because only a fraction of sugar is used for the biomass and energy production this could theoretically result in lower growth rate in Crabtree positive yeasts and these could then simply be out-competed by Crabtree negative yeasts and other microbes. However, ethanol could be used as a tool to slow down and control the proliferation of other competitive microbes.



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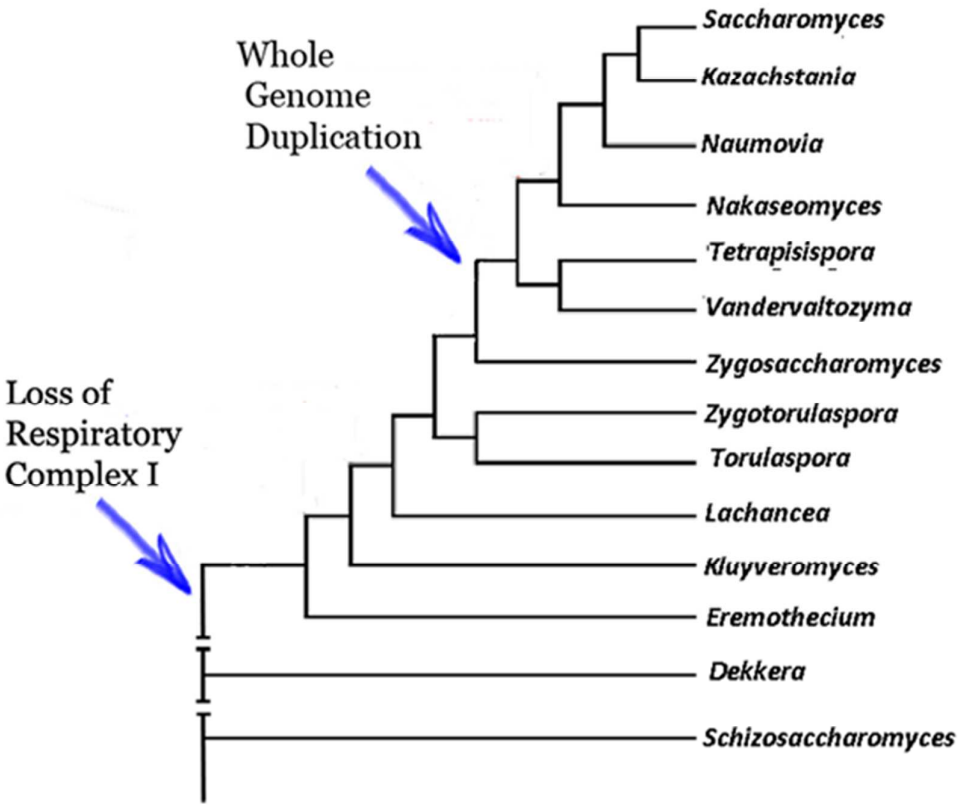
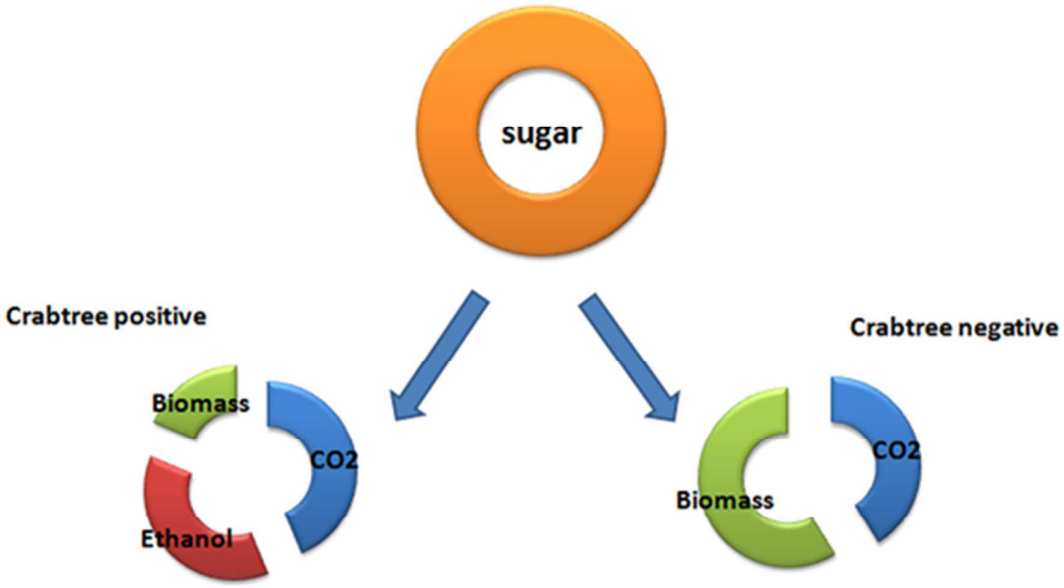


Fig. 2



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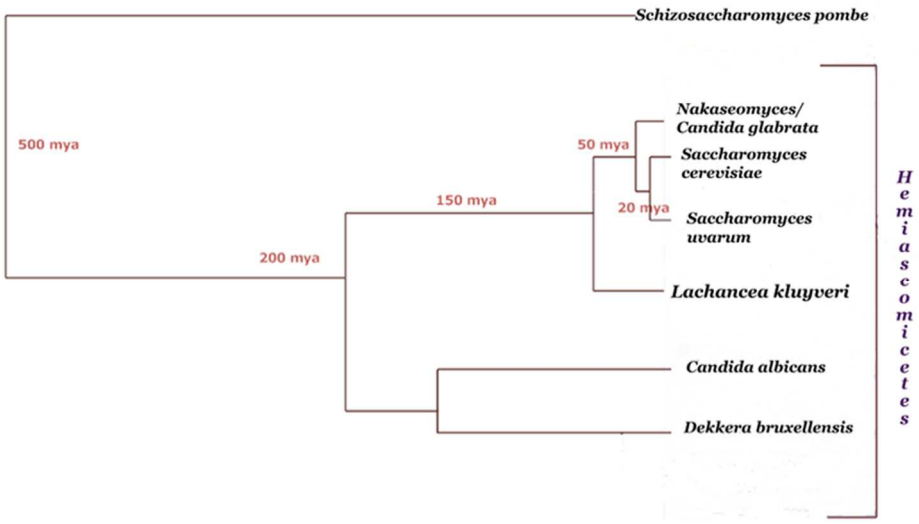

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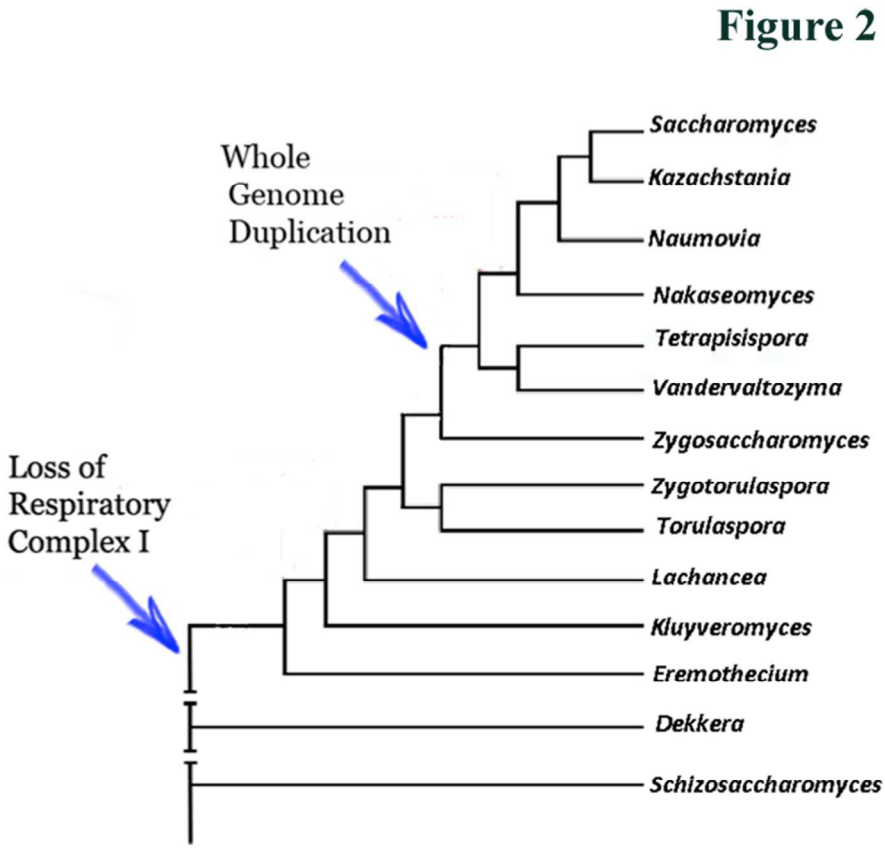

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The Saccharomycetaceae family covers over 200 million years of the yeast evolutionary history, and includes six post-whole genome duplication (post-WGD) genera, Saccharomyces, Kazachstania, Naumovia, Nakaseomyces, Tetrapisispora and Vanderwaltozyma, and six non-WGD genera, Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces and Eremothecium. Hereby we show a rough phylogenetic relationship among these genera. Two evolutionary events are shown, WGD, which took place app. 100 mya and the loss of Respiratory Complex I (which took place app. 150 million years ago). This figure was adopted from Hagman et al (2013a).

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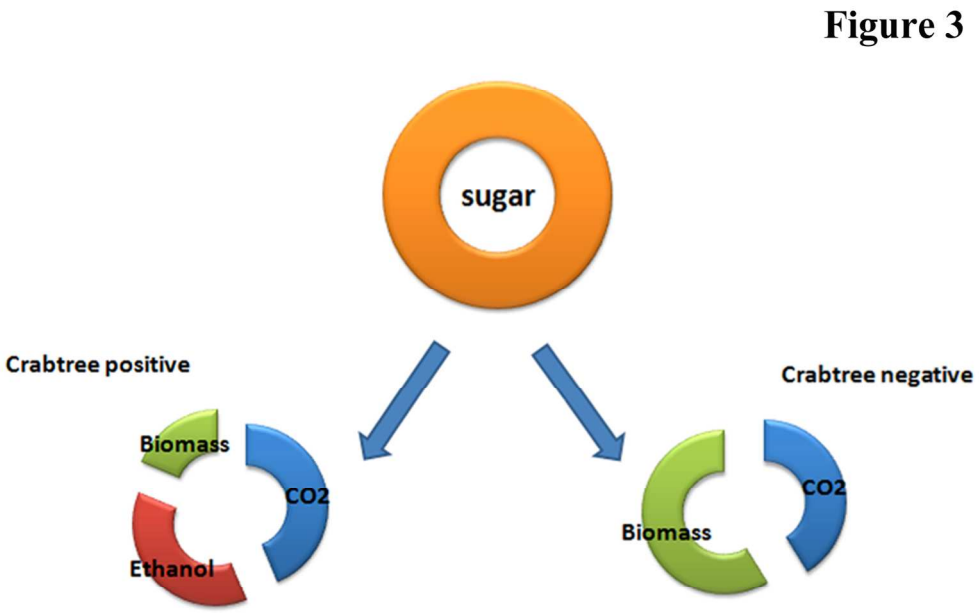

Figure 3
Crabtree effect results in lower biomass production because a fraction of sugar is converted into ethanol. This means that more glucose has to be consumed to achieve the same yield of cells if comparing with Crabtree negative yeasts. Because only a fraction of sugar is used for the biomass and energy production this could theoretically result in lower growth rate in Crabtree positive yeasts and these could then simply be out-competed by Crabtree negative yeasts and other microbes. However, ethanol could be used as a tool to slow down and control the proliferation of other competitive microbes.

160x111mm (300 x 300 DPI)