Analysis of the Role of Mitochondria of Sake Yeast during Sake Brewing and Its Applications in Fermentation Technologies

Hiroshi Kitagaki1,2*, Haidong Tan3 and Lahiru Niroshan Jayakody1,2

1Department of Environmental Sciences, Faculty of Agriculture
Saga University
1 Honjo-cho, Saga 840-8502, Japan
2Department of Biochemistry and Applied Biosciences
United Graduate School of Agricultural Sciences
Kagoshima University
Korimoto 1-21-24, Kagoshima 890-8580, Japan
3Biotechnology Department
Dalian Institute of Chemical Physics
CAS. Dalian 116023, China
*e-mail: ktgkhrs@cc.saga-u.ac.jp

Abstract
Mitochondrion is an organelle necessary for oxidative respiration. During industrial fermentation, brewery yeasts are exposed to long periods of hypoxia; however, the structure, role, and metabolism of mitochondria of brewery yeast during hypoxia have not been studied in detail. Our recent studies, for the first time, elucidated that the mitochondrial structure of brewery yeast can be observed throughout the brewing of sake, the Japanese traditional rice wine. As sake brewing proceeded, mitochondria of sake yeast change their morphology, which is coupled with an increase in malate production. On the basis of these insights, new fermentation technologies were developed. They include (1) breeding of low pyruvate-producing sake yeast by isolation of a mutant resistant to an inhibitor of mitochondrial pyruvate transport; and (2) modification of malate and succinate production by manipulating mitochondrial activity. These approaches provide new and practical methods to improve industrial fermentation technologies of sake brewing.

1. Sake brewing process
Sake is the Japanese traditional rice wine going back more than 1300 years. First, polished and steamed rice is inoculated with mold Aspergillus oryzae. The fermented rice is called “koji”, and used as a source of glycolytic, proteolytic and lipolytic enzymes. Koji is pitched into the mash tank together with steamed rice and yeast to form moto, the starter culture of yeast. In a traditional brewing process, lactic acid bacteria are allowed to proliferate to decrease pH of moto. In a modern brewing process from the early 1900s, lactic acid is added to moto prior to yeast addition, which is not accompanied by proliferation of lactic acid bacteria. The formed moto, koji and rice are pitched into the mash tank in a three-step amplification process to form moromi. Moromi is allowed to cause simultaneous saccharification of starch by enzymes produced by A. oryzae and ethanol fermentation by sake yeast in the mash tank.

2. Sake yeast: A facultative anaerobe
Since sake yeast belongs to Saccharomyces cerevisiae (Akao et al., 2011), it is a facultative anaerobe, just as S. cerevisiae and can grow both with and without molecular oxygen. During respiration, namely, in the presence of oxygen and in the absence of fermentable sugars, sake yeast uses mitochondria to obtain energy through oxidative phosphorylation. In contrast, during fermentation, namely, in the absence of molecular oxygen and in the presence of fermentable sugars, sake yeast mainly obtains energy by substrate-level phosphorylation. This is accomplished by re-
pressed gene expression under high concentrations of fermentable sugars (Gascón and Lampen 1968) and in the absence of molecular oxygen (Plattner et al. 1970). Since S. cerevisiae is Crabtree-effect positive, it produces ethanol even in the presence of oxygen and glucose rather than the tricarboxylic acid (TCA) cycle-related constituents (González Siso et al. 2012).

3. Hypoxia and yeast mitochondria

As described above, genes involved in oxidative respiration are repressed in the presence of fermentable sugars and in the absence of oxygen. Similar to this regulation, several genes are known to be induced in response to hypoxia. In contrast to upregulation of the mitochondrial genes in response to molecular oxygen and low concentrations of glucose, under hypoxia, genes such as TIR1, ECM22, MGA2, COXS5, MET16, MET3, MET1, MET5, and HEM13 are induced through transcription factors such as UPC2, ROX1, TPA1, SUT1, SUT2, and IXR1 (González Siso et al. 2012). In some fungi, nitrate and nitrite are used as electron acceptors to produce mitochondrial electron potential, which leads to ATP synthesis through complex V (Takaya 2009). In Saccharomyces cerevisiae, cytochrome oxidase has been reported to reduce nitrite, which produces nitric oxide (NO) and mitochondrial electron potential (Castello et al. 2008), although the homolog of the nitrate reductase gene is not contained in the genome of S. cerevisiae. The role of these reactions during industrial fermentation is of significant interest for the development of fermentation technologies.

4. Effect of oxygen availability on yeast cell constituents

The existence of oxygen has great impact on not only the physiology of yeast cells but also the constituents of yeast cells. This is because molecular oxygen is required for the synthesis of many biologically important molecules such as unsaturated fatty acids, ergosterol, and heme in yeast (Ernst and Tielker 2009). Heme regulates the transcription of genes involved in oxidative growth through transcriptional regulator Hap1 together with the CCAAT-binding complex Hap2/3/4/5. The regulation of these transcription factors is accomplished by the transcriptional regulation of HEM13, which encodes the enzyme coproporphyrinogen III oxidase, a rate-limiting enzyme in heme synthesis. Furthermore, yeast dynamically alters their cell wall proteins according to oxygen availability. Specifically, yeast expresses a cell wall protein Tnf1 only under hypoxic conditions (Kitagaki et al. 1997), whereas Rox1 represses the hypoxic expression of TIR1. Rox1 is a specific DNA-binding protein that recognizes the promoter of a diverse number of hypoxic genes and prevents their expression in aerobic conditions together with other components, such as Mot3 and Ssn6/Tup1 (Kwast et al. 2002). Oxygen availability also affects SO2 synthesis through cardiolipin synthesis in the mitochondria (Samp 2012). These molecules are considered to have important roles under alcoholic fermentation.

5. Existence of molecular oxygen affects directions of biochemical reactions

Molecular oxygen completely changes the direction of reactions that occur in living cells. Many reactions involving oxidation and reduction occur in the cells, and these reactions use NADH/NAD+, NADPH/NADP+, and FADH2/FAD as cofactors. However, since biochemical reactions are driven by their Gibbs free energy, the direction of the biochemical reactions is determined by the concentrations of the substrates and products. Gibbs energy is given by the constant below;

\[ \Delta G = \Delta G^0 + RT \ln\left(\frac{[C]^a[D]^b}{[A]^c[B]^d}\right) \]

\[ \Delta G^0 \]

stands for the standard Gibbs energy and a, b, c, and d stand for the moles of A, B, C, and D, respectively, in the equilibrium.

In an environment with oxygen, where oxidative respiration occurs in the electron transport chain of the mitochondria, NADH, NADPH, and FADH2 are oxidized to NAD+, NADP+, and FAD, respectively, and NADH/NAD+, NADPH/NADP+, and FADH2/FAD ratios decrease. Therefore, in the presence of oxygen, biochemical reactions that use NADH, NADPH, and FADH2 as cofactors rarely occur, and those that use NAD+, NADP+, and FAD as a cofactor occur predominantly. In contrast, in the absence of oxygen, because NADH, NADPH, and FADH2 are not oxidized to NAD+, NADP+, and FAD, respectively, NADH/NAD+, NADPH/NADP+, and FADH2/FAD ratios increase. Biochemical reactions that use NAD+, NADP+, and FAD as cofactors rarely occur unless they are coupled with biochemical reactions with minus Gibbs free energy. In contrast, reactions which use NADH, NADPH, and FADH2 as cofactors occur predominantly. Therefore, the presence of molecular oxygen determines the direction of biochemical reactions by changing the cofactor balance.

6. Reactive oxygen species

Reactive oxygen species (ROS) are molecules derived from molecular oxygen gas. Since these molecules are radicals and only have one electron in the...
molecular orbital, they can easily remove one electron from other molecules. Most intracellular ROS are derived from superoxide anion (O$_2$•−), which is generated by one electron reduction of O$_2$ (Cadenas et al. 1977). Superoxide anion is converted into hydrogen peroxide by superoxide dismutases (SODs) (Boveris et al. 1972). Eight sites in the mitochondria are involved in the generation of superoxide anion (Boveris et al. 1972). Within these sites, site IIIQo on complex III and glycerol-3-phosphate dehydrogenase can release superoxide anion into the intermembrane space of mitochondria. Since most molecules can pass the outer membrane of mitochondria, superoxide anion is considered to diffuse into the cytosol and cause various radical reactions. During hypoxia, reactive nitrogen species are generated instead of ROS (Plattner et al. 1970).

7. Roles of yeast mitochondria during alcoholic fermentation

Most industrial alcoholic fermentations are performed in an environment with a limited concentration of molecular oxygen. For example, during sake brewing, after the initial stage, the concentration of oxygen decreases to lower than 5 ppb (Nagai et al. 1992). Therefore, during the fermentation, yeast cells are exposed to long and extreme anaerobiosis. Moreover, since sugars as the source of carbon biomass are converted into ethanol, sugars are present at high concentrations throughout the fermentation. In addition, many researchers have described that genes encoding mitochondrial proteins are subject to glucose repression (Trumbly 1992) and oxygen upregulation (Ter Linde and Steensma 2002). These regulations only...
occur when mitochondria are functional, irrespective of retrograde response (Kitagaki et al. 2009). Therefore, under the condition of low oxygen concentration and high glucose concentration, yeast cells neither respire and nor develop mitochondria.

Considering the above regulations, many studies have documented that the mitochondria do not have a significant role in alcoholic fermentation (O’Connor-Cox et al. 1996). For example, some researchers reported that rhodamine-stained mitochondria are not observed during alcoholic fermentation, and thus proposing that mitochondrial structures are not present (Lloyd et al. 1996). Based on experiments involving the addition of chloramphenicol, an inhibitor of mitochondrial protein synthesis, it appears that protein synthesis in the mitochondria does not play a major role in alcoholic fermentation (Lodolo et al. 1995). Therefore, research has focused on non-respiratory oxygen consumption pathways, which mainly consist of the sterol synthesis in yeast (Rosenfeld et al. 2003).

However, there are several studies, which imply that the mitochondria play a role during fermentation. For example, it has been reported that bongkrekic acid, an inhibitor of the ATP-ADP translocation system of the mitochondrial inner membrane, protracts fermentation (O’Connor-Cox et al. 1993). In addition, azide, an inhibitor of cytochrome c oxidase, inhibits fermentation performance (O’Connor-Cox et al. 1993; Lodolo et al. 1999). It was also shown that respiratory-deficient mutants of wine yeasts achieve about 10% and 18% improvement in their glucose-to-ethanol conversion efficiency compared to their respective parent strains (Ooi and Lankford 2009). Together, these results suggest the potential role of yeast mitochondria during alcoholic fermentation.

8. Mitochondrial structure of sake yeast during sake brewing

Despite the potential significance of mitochondria during alcoholic fermentation, mitochondrial structures of brewery yeasts during alcoholic fermentation have
not been elucidated. To investigate the structure of yeast mitochondria during alcohol brewing, mitochondrially targeted green fluorescent protein (mito-GFP), which enables observation of the dynamic structure of mitochondria, was introduced into the sake yeast. Mito-GFP contains the first 69 amino acids of subunit 9 of the F0 ATPase of Neurospora crassa matrix-targeting sequence and GFP under the triosephosphate isomerase promoter and has been confirmed to localize to the mitochondria (Okamoto and Shaw 2005). Using this method, yeast mitochondrial structures can be monitored throughout the sake brewing process in a real-time manner (Fig. 1) (Kitagaki and Shimoi 2007). As a result, it turned out that in the early stage of sake brewing, yeast mitochondria are filamentous, forming long tubules. As brewing proceeds, the tubules start to fragment and are converted into dotted structures in the later phase of sake brewing.

9. Mitochondrial morphology and organic acid production profile

Mitochondrial morphology of yeast is regulated by several mitochondrial proteins. Among these proteins, Dnm1, a dynamin-related GTPase, was first identified as an essential regulator of mitochondrial fission in yeast (Bleazard et al. 1999). Deletion in DNM1 results in a networked structure of mitochondria. The same mitochondrial morphology is caused by deletion of FIS1 (McNiven et al. 2000), a 17-kDa integral protein localized at the outer mitochondrial membrane. Fis1-contains a single transmembrane domain with a protein-protein interaction domain facing the cytosol, which is responsible for mitochondrial fragmentation during vegetative growth. Kitagaki and his colleagues (Kitagaki et al. 2008) investigated whether mitochondrial morphology affects metabolism during sake brewing by disrupting the FIS1 gene (K7 haploid fis1::natMX4) of sake yeast. Furthermore, a sake yeast mutant deleted in FIS1 gene with visualized mitochondria was also generated. Sake was brewed using the mutant strain, and its mitochondrial morphology was investigated. Mitochondria remained networked throughout brewing, indicating that Fis1 regulates mitochondrial morphology during sake brewing (Figs. 2A, B). Moreover, the content of malic acid increased in fis1 relative to the wild-type strain (Fig. 2C). These results indicate that structural change of brewery yeast mitochondria plays a role in the metabolism of organic acids during alcoholic fermentation and that inhibition of mitochondrial fragmentation during alcoholic brewing leads to higher production of malate. Malate is found in apples and is the compound responsible for the “tartness” of sour apple flavoring. Thus, increasing the malate content during alcohol fermentation is favorable and this strategy provides a novel approach for breeding brewery yeast (Kitagaki et al. 2008; Kitagaki and Kitamoto 2013).

As described below, Kitagaki and his colleagues have also found that a decrease in mitochondrial activity increases malate productivity in sake yeast (Motomura et al. 2012). Therefore, it can be hypothesized that fis1 mutation causes a decrease in mitochondrial activity, and subsequently increases malate concentration.

10. Breeding of a low pyruvate-producing sake yeast strain by targeting mitochondrial transport

Sake contains the highest concentration of ethanol among the brewed alcohol beverages in the world. Recently, the consumption of sake in Japan has decreased, probably because of the too high concentration of ethanol contained in sake. Due to the recent consumer’s demand of low-ethanol beverages in Japan, lowering the content of ethanol in sake is desired. However, when low-ethanol sake is manufactured by filtering the mash at a low concentration of ethanol, an off-flavor, diacetyl is formed.
Diacetyl is a butter-like critical off-flavor (Inoue et al. 1968), which has a very low detection threshold (0.15 mg/l), contained in beverages. Diacetyl is formed from \( \alpha \)-acetolactate by non-enzymatic oxidative decarboxylation during storage of alcoholic beverages. \( \alpha \)-Acetolactate is synthesized from pyruvate by the pathway as follows; C2 carbon of pyruvate is attacked by thiamine pyrophosphate ylid, carboxylic carbon is decarboxylated to form hydroxyethyl-thiamine pyrophosphate carboanion, and hydroxyethyl-thiamine pyrophosphate carboanion attacking the C2 carbon of pyruvate to form \( \alpha \)-acetolactate (Fig. 3). \( \alpha \)-Acetolactate is used for the biosynthesis of leucine and valine (Lewis and Weinhouse 1958). During sake brewing, the concentration of \( \alpha \)-acetolactate correlates well with that of pyruvate (Sato et al. 1981). Pyruvate is converted to acetaldehyde (Dohi et al. 1974) and acetate (Akamatsu et al. 2000; Goto-Yamamoto and Dang 2006), which are known to impart unpleasant flavors, during manufacturing of sake. Keeping low pyruvate levels has been thought to be critical for reduction of these off-flavors during sake brewing and, therefore, the concentration of pyruvate during sake brewing has generally been utilized as an index to determine the timing of the process of filtering the mash in the sake brewing industry. Therefore, researchers have tried to address this issue by breeding sake yeast mutants with low pyruvate productivity. For example, a mutant resistant to 2-deoxyglucose was isolated as a pyruvate-underproducing sake yeast. A sake yeast mutant which overexpresses the gene encoding Jen1 (Tsuboi et al. 2003), which imports pyruvate across the plasma membrane (Akita et al. 2000), was constructed. A sake yeast mutant resistant to a pyruvate analog (Fukuda et al. 1998) was isolated as a pyruvate-underproducing sake yeast. However, there was no practical sake yeast which underproduces pyruvate because of its deterioration in fermentation ability.

Based on our findings of the mitochondrial structure of sake yeast during sake brewing as described above, it is possible to conduct the breeding strategy to decrease pyruvate concentration by absorbing pyruvate into the mitochondria, or enhancing the turnover of pyruvate in mitochondria during alcoholic fermentation. Although genes encoding pyruvate transporters have recently been identified (Bricker et al. 2012), utilization of genetically modified organisms for beverages is still open for discussion. Therefore, we attempted to obtain a mutant which has an increased mitochondrial pyruvate transport by natural occurrence or mutagenesis. It has been hypothesized that mutants resistant to an inhibitor of mitochondrial pyruvate transport should have a fortified pyruvate transport into mitochondria or alter pyruvate metabolism (Fig. 4).

![Breeding strategy of low pyruvate-producing sake yeast](image-url)

Kitagaki and his colleagues isolated mutants resistant to ethyl \( \alpha \)-transcyanocinnamate, an inhibitor of mitochondrial pyruvate transporter, as expected to fortify pyruvate transport to decrease these off-flavor substances.
Fig. 5. Pyruvate levels of sake brewed with strains resistant to ethyl α-transcyanocinnamate on laboratory, pilot and factory scales. (A) Laboratory scale test. Sake brewing was performed as described in Fig. 1. The results are mean values, and bars represent standard errors for six independent brewing experiments from respective precultures. Open circles represent pyruvate levels for Kyokai No. 7, and closed squares represent those for TCR7 (n = 6; **p < 0.005, unpaired one-tailed Student’s t-test). Reprinted with permission from Biosci. Biotech. Biochem. 74(4), Horie et al., Breeding of a low pyruvate-producing sake yeast by isolation of a mutant resistant to ethyl alpha-transcyanocinnamate, an inhibitor of mitochondrial pyruvate transport, 843–847, Fig. 2a, © 2010, Japan Society for Bioscience, Biotechnology, and Agrochemistry. (B) Pilot scale test. Sake was brewed on a pilot scale (total 12 kg rice). Diamonds represent pyruvate levels of Kyokai No. 7 and rectangles represent those of TCR7. (C) Factory scale test. Sake was brewed on a factory scale (total 1 ton rice). F-4 is a sake yeast appropriate for brewing of ginjo-type sake. F-4 TCR is a mutant of F-4 resistant to ethyl α-transcyanocinnamate. Diamonds represent pyruvate levels of F-4 and rectangles represent those of F-4 TCR. Reprinted with permission from Journal of Brewing Society of Japan, 106(5), Hirata et al., Brewing characteristics of sake yeast resistant to an inhibitor of mitochondrial pyruvate transport on a pilot scale, 323–331, Fig. 2, © 2011, Brewing Society of Japan.

tochondrial pyruvate transport (Paradies and Ruggiero 1988), and quantified pyruvate concentrations in sake brewed with the mutants (Horie et al. 2010). Some of the sake mash brewed with the resistant mutants contained lower concentration of pyruvate relative to that of the parent strain Kyokai No. 7 (data not shown). To elucidate the mechanism more precisely, sake brewing profiles of a mutant, designated TCR7, were investigated.

The mutant, TCR7, had a fermentation ability similar to the wild type sake yeast (statistically not significant). Sake brewed with TCR7 contained a significantly lower concentration of pyruvate (30% lower than the parent strain). However, the other organic acids were not significantly altered, indicating that sake brewing with TCR7 decreased pyruvate levels without significantly altering the taste of sake relative to the parent strain. TCR7 also produced lower levels of pyruvate during sake brewing on a pilot-scale (total 12 kg rice) (Fig. 5B) (Hirata et al. 2011). Furthermore, a sake yeast mutant resistant to ethyl α-transcyanocinnamate was obtained from a sake yeast F-4, which is a progeny of
Kyokai No. 9, a strain appropriate for brewing of ginjo-type sake, and was designated F-4 TCR. F-4 TCR produced low levels of pyruvate on a factory-scale (total 1 ton rice) without affecting the fermentation ability (Sasaki et al., 2011) relative to its parent strain (F-4). Indeed, α-acetolactate, the direct precursor of diacetyl, produced by F-4 TCR was decreased to less than 20% relative to the parent strain on a factory scale without affecting final ethanol concentration (Table 1) (Sasaki et al., 2011). Therefore, it was concluded that this approach is a practical method for breeding brewery yeast strains.

11. Control of yeast mitochondrial activity affects malic and succinic acid production

Sake yeasts are exposed to various conditions affecting the mitochondrial state in the sake industry. For example, the size of the fermentation tank affects the surface area of the mash, leading to different oxygen availability. Further, temperature of the raw materials, such as water and rice, affects oxygen solubility for sake yeast. Furthermore, availability of oxygen is high for yeast that localizes to the surface of the mash, while it is low for yeast cells near the bottom of the tank. However, the involvement of the mitochondrial states of sake yeast in the production profile of organic acids has not been investigated in detail.

Therefore, we investigated the effect of the mitochondrial activity of sake yeast on organic acid production profile during sake brewing. Two propagation conditions were designed: one is “respirative condition” where yeast cells were cultured in a non-fermentable carbon source (3% w/v glycerol) with aerobic shaking of the flask, and the other is “fermentative condition” where yeast cells were cultured in a glucose-containing medium (10% w/v glucose) in a static culture.

First, mitochondrial morphology of the sake yeast in these conditions were investigated. Exposure to the fermentative condition caused the mitochondria to exhibit long tubule structures, with little branches elongating along the surface of the cell (Fig. 6A). In contrast, exposure to the respirative conditions caused the mitochondria to be distributed all over the cell with short filamentous and branched structures or clumpy structures (Fig. 6B). To examine the influence of the mitochondrial activity on organic acid production, the organic acid profiles of these yeasts were investigated after alcoholic fermentation for 48 h. Respirative sake yeast produced significantly increased citric acid.
(123%), decreased pyruvic acid (63.0%), decreased malic acid (74.6%), increased succinic acid (180%) and increased acetic acid (129%) during the alcoholic fermentation phase, relative to that of fermentative yeast (Fig. 7A).

The role of mitochondrial state of yeast cells on these changes were investigated with the compound carboxylcyanide p-trifluoromethoxyphenylhydrazone, a specific uncoupler of mitochondrial membrane potential. When carboxylcyanide p-trifluoromethoxyphenylhydrazone was added to the culture, the production of malic acid significantly increased (374%) and succinic acid decreased (87.9%, although not statistically significant ($p < 0.05$)) (Fig. 7B). Notably, these changes were almost similar to those observed upon the transfer from the respirative to fermentative condition. These results suggest that the mitochondrial state during the propagation stage is responsible for the difference in the organic acid production between the respirative and fermentative yeast.

To develop a technology to alter the organic acid profile by regulating mitochondrial activity, respirative yeast cells were exposed to a medium containing 10% (w/v) of glucose, incubated as a static culture, and then...
used for alcoholic fermentation. When yeast cells were exposed to these conditions, where mitochondrial activity decreased, for 12 h and 24 h, production of malic acid increased significantly (Fig. 7C), while the production of succinic acid decreased significantly compared to the untreated cells (24 h cultivation). These results indicated that the physiological state of sake yeast can be converted from the respirative condition to the fermentative condition by incubating yeast cells under static conditions for 12 h or 24 h in a medium containing 10% glucose. Taken together, these results allow us to propose a new scheme of organic acid production in brewery yeast (Fig. 8). In fermentative sake yeast cells, a major pool of pyruvate in the cytosol is not transported into the mitochondria, but remains in the cytosol and reduced to lactate or carboxylated to oxaloacetate. Oxaloacetate is then reduced to malate, which is driven by accumulated NADH. In respirative sake yeast cells, a major pool of pyruvate in the cytosol is transported to mitochondria, where it is converted aerobically to acetyl-CoA, or succinic acid by aldol-condensation with oxaloacetate. These results indicate, for the first time, that production profiles of organic acids in brewery yeast can be manipulated by changing the mitochondrial state of yeast (Motomura et al. 2012).

12. Concluding remarks

Through these studies, the structures and roles of yeast mitochondria during sake brewing was first elucidated. These findings led to the establishment of
novel and practical fermentation technologies such as high malate production, low pyruvate production and manipulation of malate and succinate. These results indicate that metabolisms and events that occur in mitochondria will be promising targets for breeding of sake yeasts.

References


McNiven MA, Cao H, Pitts KR, Yoon Y. The dynamin fam-