J. Gen. Appl. Microbiol.

doi 10.2323/jgam.2021.03.002

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Full Paper

**Quantitative stability of the folates highly accumulated in a non-Kyokai
sake yeast**

(Received December 1, 2020; Accepted March 4, 2021; J-STAGE Advance publication date: August 7, 2021)

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Running head: Folate accumulation in a sake yeast

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Summary

Pressed sake cake, a by-product of sake brewing, is a rich dietary source of folates, which are important vitamins for humans. However, considerable losses of folates occur during storage and cooking. We have previously reported that Km67, the house sake yeast strain of Kiku-masamune sake brewery, can accumulate high folate levels. In this study, we found that the folate content of pressed sake cakes produced with Km67 remained at approximately their maximum level after the fermentation activity stopped. To elucidate the mechanisms of high folate accumulation in Km67, we analyzed the expression of 23 folate-metabolizing genes. The expression of *ABZ1* and *FOL3* was almost always higher in Km67 than in Kyokai no. 701 yeast (K701), which suggested that enhanced expression of the genes involved in folate biosynthesis was a mechanism of high folate accumulation in Km67. We found that the folates of Km67 pressed sake cakes were quantitatively stable at 4°C under refrigerated storage conditions. In addition, the homocysteine content of Km67 pressed sake cakes was almost always higher than that of K701 pressed sake cakes. This result suggests that a reason for high folate accumulation in Km67 yeast is the need to reduce the intracellular concentration of homocysteine. Our results provide biologically meaningful information on folate metabolism in yeast.

Key words: folate; homocysteine; pressed sake cake; *Saccharomyces cerevisiae*; sake yeast

1 **Introduction**

2 Pressed sake cake is a secondary product of the traditional sake (Japanese rice
3 wine) manufacturing process. It is thought to be beneficial to human health as it
4 contains high proportions of protein, carbohydrates, and vitamins such as folates
5 (Izu et al., 2006; Kawamoto et al., 2011; Saito et al., 1994). The B-vitamin folate
6 is required for central cellular functions, such as the biosynthesis of various
7 amino acids and purines, cell replications, and cell growth, and plays major roles
8 in preventing several diseases, e.g., cardiovascular disease, certain cancers,
9 and neural tube defects. This discovery led to worldwide recommendations for
10 women planning a pregnancy to take folic acid supplements from before
11 conception until the 12th gestational week to ensure optimal maternal status
12 during the period of neural tube closure in early pregnancy (De-Regil et al., 2015;
13 Lucock, 2000; Selhub and Rosenberg, 1996; Witthoft et al., 1999). Rich folate
14 sources include some vegetables and nuts in addition to pressed sake cakes,
15 however, folates are labile and prone to oxidation. Therefore, considerable
16 losses of folates have been reported under the storage and cooking conditions
17 typically used by retailers and consumers (Dang et al., 2000; Hawkes and Villota,
18 1989; McKillop et al., 2002).

19 We previously reported that pressed sake cakes obtained by brewing with Km67
20 yeast, which has played a central role in industrial sake brewing at
21 Kiku-masamune Sake Brewing Co. Ltd. (Hyogo, Japan), contained large
22 amounts of folates, and that the Km67 type-*HMT1* gene was related to the
23 mechanisms of high folate accumulation in Km67 yeast. It has been suggested
24 that the Hmt1p of Km67 exhibits higher activity than the Hmt1p of Kyokai no. 7.

1 However, *HMT1* may not be the only factor that modulates high folate
2 accumulation in Km67 (Shibata et al., 2020). Thus, we performed additional
3 studies to clarify the mechanisms underlying high folate accumulation in Km67
4 compared with Kyokai yeasts, which have been widely distributed by the
5 Brewing Society of Japan. We found that Km67 yeast could accumulate large
6 amounts of folates and that the folates were quantitatively stable during
7 long-term storage. This advantage of Km67 may be closely related to the fact
8 that pressed sake cakes produced by Km67 yeast had the highest folate content
9 among those collected from various sake manufacturers (Shibata et al., 2020).
10 However, the mechanisms of high folate accumulation and stability in Km67
11 yeast remain unclear.

12 Hence, in the present study, we have investigated these mechanisms.

14 **Materials and methods**

15 **Strains and media**

16 The nonfoaming sake yeast *Saccharomyces cerevisiae* Km67 was a house
17 stock derived from the ATCC32694 strain, which was isolated from
18 Kiku-masamune sake mash between 1966 and 1973. Kyokai no. 701
19 (nonfoaming variants of Kyokai no. 7) was provided by the Brewing Society of
20 Japan (Tokyo, Japan). Km67 does not belong to the Kyokai yeasts, which have
21 been widely distributed by the Brewing Society of Japan (Takao et al., 2018).
22 YPD medium containing 1% yeast extract (Difco Laboratories, Detroit, MI, USA),
23 2% bacto-peptone (Difco Laboratories), and 2% glucose (Fujifilm Wako Pure

Chemical Corporation, Osaka, Japan) was used as a rich medium for yeast growth.

Fermentation test

Laboratory-scale sake brewing was carried out according to the method reported by Namba et al. (1978) using 1 kg of α -rice and rice-koji with a polishing ratio of 70%. The sake mash temperature was maintained at 15°C throughout the 25-day fermentation period. The fermentation was monitored by measuring the alcohol concentration and sake meter value using an alcohol densitometer (Alcomate AL-3, Riken Keiki, Tokyo, Japan) and a density/specific gravity meter (DA-510, KEM Co, Ltd., Japan), respectively. Seven samples were taken at 4, 8, 11, 14, 18, 21, and 25 days after the start of fermentation. At each sampling, approximately 40 g of sake mash was centrifuged, and a portion of the obtained precipitate (pressed sake cake) was then freeze-dried. Yeast cells were recovered from a second portion of the precipitate and stored at -80°C prior to RNA preparation.

Quantitative real-time polymerase chain reaction (PCR) targeting folate-metabolizing genes

Total RNA was extracted from yeast cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA quality was evaluated by measuring the ratio of optical densities at 260 and 280 nm. cDNA was synthesized from 1 μ g total RNA in a final volume of 20 μ L using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Folate-metabolizing gene-specific primers for quantitative real-time

(qRT)-PCR analysis were designed using Primer3plus (<http://primer3plus.com/>) and are shown in Table S1 (Averesch et al., 2016; Gelling et al., 2004; Subramanian et al., 2005). SYBR Premix EX Taq II (Takara Bio, Kusatsu, Japan) was combined with 2- μ L cDNA and 10 μ M primers in a 25- μ L reaction volume. Amplification was performed on a Thermal Cycler Dice Real Time System II (Takara Bio) under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The $\Delta\Delta$ Ct method was used for relative quantification of gene expression (Livak and Schmittgen, 2001); levels were normalized to that of *TFC1*, the *transcription factor class C1* gene, and are expressed as a percentage of control levels.

Low-temperature preservation test of pressed sake cakes

A second laboratory-scale sake brewing was carried out as described above and the fermentation was monitored. The sake mash was centrifuged at Day 14 after the start of fermentation, at which time fermentation activity began to cease. The obtained precipitate (pressed sake cake) was stored at 4°C. Each week, a portion of the precipitate stored at 4°C was freeze-dried and was subjected to the analyses described below.

Analysis of folate, S-adenosyl-methionine, and S-adenosyl-L-homocysteine

In this study, the sum of the main folate forms in yeast, tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-CH₃-THF) was analyzed following the methodology described by Patring et al. (2005). For analysis of S-adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH), 0.3-g samples of pressed sake cake were weighed into plastic centrifuge tubes with 5

1 mL of Milli-Q water and vortexed. Extractions were performed by freezing and thawing the tubes, followed by centrifugation. The SAM and SAH concentrations of the supernatants were analyzed using an Agilent Technologies 1200 series high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA). The separation of SAM and SAH was performed on a TSKgel ODS-80T_M column, 250 x 4.6 mm, 5 μ m (Tosoh, Tokyo, Japan) at 35°C. The flow rate was 1.0 mL/min; the injection volume 10 μ L; and the thermostated autosampler temperature was 4°C. A diode array detector (DAD) was used for the detection and quantification of SAM and SAH, with the DAD channel set at 254 nm. The mobile phase used was 18% methanol, pH 3.0, with 40 mM NH₄H₂PO₄ and 8 mM CH₃(CH₂)₆SO₃Na (all Fujifilm Wako Pure Chemical Corporation) under isocratic conditions.

Analysis of methionine and homocysteine

Samples for the analysis of methionine and homocysteine were prepared by disrupting pressed sake cakes with glass beads using a multi-bead shocker (Yasui Kikai, Osaka, Japan) in Milli-Q water at 4°C. The free methionine and homocysteine concentrations in the supernatants recovered by centrifugation were analyzed using an amino acid analyzer (L-2000 series, Hitachi, Tokyo, Japan). The total methionine concentration was analyzed using the same method after 24 h hydrolysis of samples in 6N HCl in vacua at 110°C. The peptide-form methionine concentration was calculated by subtracting the free methionine concentration from the total methionine concentration.

1 **Results and discussion**

2 **Transitions in the folate content during fermentation**

3 To investigate the relationship between folate accumulation in yeasts and the
4 fermentation process, we performed a sake brewing comparison between Km67
5 yeast and Kyokai no. 701, which is a representative strain among Kyokai yeasts.
6 The folate content was higher in pressed sake cakes produced with Km67 than
7 in those produced with K701 throughout the fermentation period, except for the
8 fourth day. The alcohol concentration and sake meter value suggested that
9 fermentation activities of Km67 and K701 stopped on the 18th day (Fig. S1). The
10 folate, especially THF, contents of pressed sake cakes produced with K701
11 gradually decreased from the 18th day onwards; however, those of pressed sake
12 cakes produced with Km67 remained at approximately the maximum level (Fig.
13 1A). These results indicated that Km67 could not only accumulate large amounts
14 of folates, but also that the folates accumulated in Km67 were quantitatively
15 stable. This advantage of Km67 may be closely related to the fact that pressed
16 sake cakes produced with Km67 had the highest folate content among those
17 collected from various sake manufacturers (Shibata et al., 2020). However, it is
18 unclear why the folates accumulated in Km67 are quantitatively stable.
19 Additional studies are required to investigate the various conceivable reasons.
20 Further, the ratio of THF to the total folate content was higher than that of
21 5-CH₃-THF to the total folate content (Fig. 1A–C).

22

23 **Expression of genes involved in folate metabolism**

1 To elucidate the mechanism of high folate accumulation in Km67 yeast, we
2 measured the expression of 23 genes involved in folate metabolism by
3 qRT-PCR of yeast cells recovered from the sake mash.

4 The results of *ABZ1*, *FOL3*, *DFR1*, and *MET6* genes are shown in Fig. 2. The
5 expression of the genes differed between Km67 and K701 throughout the
6 fermentation period. Therefore, we focused on these four genes.

7 The expression of *ABZ1* and *FOL3* was almost always higher in Km67 than in
8 K701 throughout the fermentation period (Fig. 2A, B). The genes *ABZ1* and
9 *FOL3* encode para-aminobenzoate synthase and dihydrofolate synthetase,
10 respectively, leading to dihydrofolate (DHF) and THF biosynthesis (Table S1, Fig.
11 3) (Averesch et al., 2016; Gelling et al., 2004). It was suggested that high
12 expression of the genes involved in folate biosynthesis was a possible
13 mechanism of high folate accumulation in Km67. However, the expression of
14 *DFR1*, which has a role in folate biosynthesis, was almost always lower in Km67
15 than in K701 throughout the fermentation period (Fig. 2C). It is assumed that
16 high expression of *DFR1* did not result in enhanced THF biosynthesis because
17 of the low productivity of the substrate DHF in K701 (Fig. 3).

18 *MET6* expression was higher in Km67 than in K701 except for the fourth day,
19 which was the same pattern observed in the folates of pressed sake cakes (Figs.
20 1A, 2D). *MET6* encodes cobalamin-independent methionine synthase
21 (Subramanian et al., 2005). Folate content in *S. cerevisiae* strains was
22 reportedly increased by addition of methionine in the medium (Kanai et al., 2013).
23 Therefore, high expression of *MET6* may also be a factor involved in high folate
24 accumulation in Km67.

1 In general, the gene expression does not always correlate with the enzymatic
2 activity. Translation and post-translational protein control are also important.
3 Therefore, additional studies are needed to test the above hypotheses.
4 The expression of other folate-metabolizing genes are shown in Fig. S2. The
5 expression of the other 19 genes did not seem to differ much between Km67 and
6 K701 throughout the fermentation period.

7 8 **Low-temperature preservation test of pressed sake cakes**

9 Considering the refrigerated storage conditions typically used by retailers and
10 consumers, we performed a low-temperature (4°C) preservation test of pressed
11 sake cakes. The folate content of pressed sake cakes produced with K701
12 gradually decreased from the start of the low-temperature preservation test, and
13 little remained after 8–12 weeks of storage (Fig. 4A). However, the folate content
14 of pressed sake cakes produced with Km67 remained at approximately the
15 maximum level after 6 weeks of storage and was greater than 300 µg per 100 g
16 dried pressed sake cake after 12 weeks of storage (Fig. 4A). This result
17 indicated that the folates accumulated in Km67 were quantitatively stable under
18 4°C storage conditions. The ratio of THF to the total folate content was higher
19 than that of 5-CH₃-THF to the total folate content after 0–6 weeks of storage, but
20 was lower after 7–12 weeks (Fig. 4A–C). It has been reported that 5-CH₃-THF is
21 moderately stable, but THF is very labile, especially in an oxygen atmosphere
22 and when heated (Jones and Nixon, 2002). Therefore, the THF content may
23 have gradually decreased during 4°C storage.

1 To elucidate the factors contributing to quantitative stability of folates in Km67,
2 we analyzed methionine cycle-related substances (methionine, SAM, SAH, and
3 homocysteine) of pressed sake cakes produced with Km67 and K701 during 4°C
4 storage.

5 The free methionine contents of pressed sake cakes produced with Km67 and
6 K701 were small at the start of the low-temperature preservation test, but
7 gradually increased and, after 5 weeks of storage, those of Km67 were higher
8 than those of K701 (Fig. 5A). The result of peptide-form methionine analysis is
9 shown in Fig. S3. Unlike the free methionine, the peptide-form methionine
10 contents of Km67 and K701 were almost equal, and then gradually decreased,
11 and little peptide-form methionine remained in pressed sake cakes of both Km67
12 and K701 after 9–12 weeks of storage. These results suggested that the free
13 methionine gradually increased because of release from the peptide-form
14 methionine by yeast peptidases which leaked out from the cells during storage
15 (Hara et al., 1977). However, the peptide-form methionine contents of Km67 and
16 K701 were almost equal through the preservation test period. Therefore, as
17 described below, we suggest that the conversion of homocysteine into
18 methionine contributes to higher free methionine contents in pressed sake cakes
19 of Km67 than in those of K701.

20 The SAM content was clearly lower in pressed sake cakes produced with Km67
21 than in those produced with K701 (Fig. 5B). It has been suggested that
22 Km67-Hmt1p exhibits higher activity for converting SAM into SAH than
23 K7-Hmt1p does (Shibata et al., 2020). The result of the SAM comparison in this
24 study is consistent with this hypothesis.

1 The SAH contents of pressed sake cakes produced with Km67 and K701 were
2 undetectable (data not shown). SAH reportedly exerts a strong feedback
3 inhibitory activity on the transmethylation reaction, and is rapidly hydrolyzed into
4 homocysteine (Yi et al., 2000). Thus, we assume that SAH exists in pressed
5 sake cakes at an undetectable level.

6 We observed a difference in the homocysteine content in pressed sake cakes
7 produced with Km67 and K701. The homocysteine content in those produced
8 with K701 was very low throughout the preservation test period (Fig. 5C). In
9 contrast, those produced with Km67 gradually increased during the first 4 weeks
10 of the preservation test, then gradually decreased, and were undetectable after
11 11–12 weeks of storage (Fig. 5C). Homocysteine inhibits the growth of yeast and
12 this inhibitory effect is due to endoplasmic reticulum (ER) stress (Kumar et al.,
13 2006). Thus, we suggest that one reason for high folate accumulation in Km67
14 yeast is the need to reduce the intracellular concentration of homocysteine by
15 converting homocysteine into methionine. In this reaction, the methyl group is
16 supplied by 5-CH₃-THF (Fig. 3). Therefore, the need to reduce homocysteine in
17 yeast may be associated with the transition of the ratio of THF and 5-CH₃-THF to
18 the total folate content. Kacprzak et al. (2003) demonstrated that increased
19 intracellular homocysteine concentration resulted in upregulated expression of
20 *MET6*, which catalyzes the remethylation of homocysteine to methionine. This
21 result is consistent with our finding of high *MET6* expression in Km67. On the
22 other hand, during 5-12 weeks of storage, formation of THF was not observed
23 despite methylation of homocysteine and formation of methionine (Figs. 4A-C,
24 5A-C). There are some pathways of THF formation other than demethylation of

1 5-CH₃-THF (Fig. 3). Therefore, we assume that the results of homocysteine and
2 methionine are not always correlated with the result of THF.

3 A summary of the comparison of methionine cycle-related substances in
4 pressed sake cakes produced with Km67 and K701 is shown in Fig. 6. It is
5 assumed that the different Hmt1p activities in Km67 and K701 lead to SAM
6 accumulation in K701 and homocysteine accumulation in Km67. Km67 yeast
7 may accumulate large amounts of folates to reduce intracellular homocysteine,
8 which is toxic for yeast. This may result in the observed difference in free
9 methionine contents between Km67 and K701 after 5 weeks of storage.

10 In this study, we suggest a reason for the accumulation of large amounts of
11 folate by Km67 yeast, and provide biologically meaningful information on folate
12 metabolism in yeast. However, the mechanisms of high folate accumulation in
13 Km67 yeast remain unclear. Additional studies, including analyses of
14 metabolome and enzymatic activity, are needed.

15 Dimethyl trisulfide (DMTS) is one of the main components responsible for hineka,
16 the aroma associated with deteriorated Japanese sake during storage. It has
17 been suggested that the yeast methionine metabolism is involved in the
18 production of a major precursor compound of DMTS (Isogai et al., 2006;
19 Wakabayashi et al., 2013). Therefore, elucidation of all the mechanisms
20 underlying high folate accumulation and understanding of the methionine
21 metabolism in yeast will also facilitate improvement of the quality of Japanese
22 sake.

23 24 **Acknowledgments**

Yusuke Shibata, Toshinari Takahashi, and Tasuku Yamada are employees of Kiku-masamune Sake Brewing Co., Ltd. The authors declare no other conflicts of interest.

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22

Figure legends

Fig. 1. Comparison of folate, tetrahydrofolate (THF), and 5-methyltetrahydrofolate (5-CH₃-THF) contents in pressed sake cakes produced with Km67 and K701 throughout the 25-day fermentation period.

Folate refers to the sum of THF and 5-CH₃-THF in this study.

A: folate, B: tetrahydrofolate (THF), C: 5-methyltetrahydrofolate (5-CH₃-THF).

Km67: unfilled circle, K701: filled triangle.

Fig. 2. Comparison of the expression levels of *ABZ1*, *FOL3*, *DFR1*, and *MET6* in Km67 and K701 throughout the fermentation period. Expression levels are shown as fold-changes relative to those of Km67 at Day 4 of fermentation. A: *ABZ1*, B: *FOL3*, C: *DFR1*, D: *MET6*.

Fig. 3. Overview of folate metabolism in *Saccharomyces cerevisiae* from previous reports (Averesch et al., 2016; Gelling et al., 2004; Subramanian et al., 2005). The enzymatic reactions are indicated by the genes encoding the enzymes in italics. The enzymes corresponding to the genes are listed in Table S1. DHF: dihydrofolate, THF: tetrahydrofolate, 5-CH₃-THF: 5-methyltetrahydrofolate, 5,10-CH₂-THF: 5-10-methylenetetrahydrofolate, 5,10-CH⁺-THF: 5,10-methenyltetrahydrofolate, 10-CHO-THF: 10-formyltetrahydrofolate, SAM: S-adenosyl-methionine, SAH: S-adenosyl-L-homocysteine, PABA: para-aminobenzoic acid, -CH₃: methyl group.

Fig. 4. Comparison of folate, tetrahydrofolate (THF), and 5-methyltetrahydrofolate (5-CH₃-THF) contents in pressed sake cakes produced with Km67 and K701 during 4°C storage. Folate refer to the sum of

1 THF and 5-CH₃-THF in this study. A: folate, B: tetrahydrofolate (THF), C:
2 5-methyltetrahydrofolate (5-CH₃-THF). Km67: unfilled circle, K701: filled
3 triangle.

4 Fig. 5. Comparison of the methionine, *S*-adenosyl-methionine (SAM), and
5 homocysteine contents in pressed sake cakes produced with Km67 and K701
6 during 4°C storage. A: methionine, B: *S*-adenosyl-methionine (SAM), C:
7 homocysteine. Km67: unfilled circle, K701: filled triangle.

8 Fig. 6. Summary of the results from our experiment on methionine cycle-related
9 substances in the pressed sake cakes produced with Km67 and K701. -CH₃:
10 methyl group. Folate refer to the sum of tetrahydrofolate (THF) and
11 5-methyltetrahydrofolate (5-CH₃-THF) in this study.

12

13 Fig. S1. Comparison of alcohol concentration and sake meter value in Km67 and
14 K701 throughout the fermentation period. A: alcohol concentration, B: sake
15 meter value. Km67: unfilled circle, K701: filled triangle.

16 Fig. S2. Comparison of the expression level of 19 folate-metabolizing genes in
17 Km67 and K701 throughout the fermentation period. Expression levels are
18 shown as fold-changes relative to that of Km67 at Day 4 of fermentation.

19 Fig. S3. Comparison of the peptide-form methionine contents in pressed sake
20 cakes produced with Km67 and K701 during 4°C storage. Km67: unfilled
21 circle, K701: filled triangle.

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(Fig. 1. Shibata et al.)

(Fig. 2. Shibata et al.)

(Fig. 3. Shibata et al.)

(Fig. 4. Shibata et al.)

(Fig. 5. Shibata et al.)

(Fig. 6. Shibata et al.)