Journal of Bioscience and Bioengineering
VOL. 118 No. 6, 661–664, 2014

www.elsevier.com/locate/jbiosc

TECHNICAL NOTE

# Differentiation of industrial sake yeast strains by a loop-mediated isothermal amplification method that targets the *PHO3* gene

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Received 14 April 2014; accepted 28 May 2014
Available online 22 July 2014

**We developed a loop-mediated isothermal amplification method that targets the *PHO3* gene for discriminating sake yeast strains. Our data indicate that this assay is simple, rapid, and useful to use for differentiation of specific yeasts in sake mash.**



[**Key words:** Differentiation; Loop-mediated isothermal amplification; Sake yeast; *PHO3*; Acid phosphatase]

In recent years, the consumption of sake, a traditional Japanese alcoholic beverage, has increased worldwide. Sake is produced by fermenting steamed rice with *koji* (a culture of *koji* mold, *Aspergillus oryzae*, on steamed rice) and sake yeast (*Saccharomyces cerevisiae*) (1). Maintenance of the purity of the sake yeast strain during the fermentation process is essential for obtaining consistent product quality. Contamination with other yeasts/bacteria can adversely affect the product quality, for example, 4-vinylguaiacol production by contaminant bacteria/yeast can produce off-flavors in brews (2,3).

Commercial sake is commonly brewed using Kyokai sake yeast strains supplied by the Brewing Society of Japan. Kyokai no. 6, 7, 9, and 10 strains are frequently used in sake production (4), and several studies have characterized both the phenotypic and genomic characteristics of these strains (4–7). The yeast genes *PHO5* (encoding a repressible acid phosphatase) and *PHO3* (encoding a constitutive acid phosphatase), which are adjacently located on chromosome II, have been studied in detail (7,8). Previous studies have revealed that a large part of the *PHO3* locus is deleted in several Kyokai sake yeast strains (including Kyokai no. 6, 7, 9 and 10) because of *PHO5* and *PHO3* gene fusion. The fusion results in the loss of constitutive acid phosphatase (cACP) activity in the presence of high levels of inorganic phosphate.

Although the mechanisms underlying the differences in cACP activity between specific sake yeasts and wild type yeasts were previously unknown, cACP activity was routinely utilized as a phenotypic marker for monitoring yeast purity in sake mash. Mizoguchi and Fujita developed a colony staining method that used diazo-coupling for detecting cACP activity to distinguish Kyokai no. 6, 7, 9, and 10 strains from contaminant wild yeasts (9,10). In addition, another colony staining method that uses 5-bromo-4-chloro-3-indolyl phosphate disodium salt was also developed (11). Yeast identification methods that utilize phenotypic characteristics as discriminatory markers are frequently used to ensure yeast purity during brewery fermentation. However, yeast identification assays that are based on phenotypic characterization are time-consuming, and may not always yield accurate results.

The loop-mediated isothermal amplification (LAMP) method (12) is widely used for identifying contaminating microorganisms in food production (13). This assay is highly specific; it has a simple set-up and it can be used to amplify DNA rapidly under isothermal conditions. So far, no study has focused on detecting yeasts in sake mash by this method.

In this study, we developed a LAMP method that amplifies a region of the *PHO3* gene to discriminate between the widely used sake yeast strains and the other alcohol-producing yeast strains. We showed that the LAMP method enables rapid, simple, and cost-effective detection and is therefore more efficient than the identification methods that are based on phenotypic characterization.

The yeast strains that were used in this study are listed in Table 1. Strain S288C was purchased from Open Biosystems. The Kyokai no. 28 strain was obtained from the Brewing Society of Japan. The Niigata sake yeast strains G9 (11), G9NF (11) (a non-forming strain derived from G9), G9arg (14) (a non-urea-producing strain isolated from G9) were originally developed at the Niigata Prefectural Sake Research Institute. G9NFarg was screened for a non-urea-producing strain from G9NF strain by the positive selection method according to Kitamoto et al. (15). Other yeast strains were obtained from the National Research Institute of Brewing.

Yeast genomic DNA was isolated using the Dr. GenTLE kit for Yeast (Takara Bio, Shiga, Japan). Approximately 5 ng of the yeast genomic DNA was used for each LAMP assay reaction. Yeast DNA was extracted from sake mash by using the boiling DNA extraction method (16) with some modifications. Briefly, 10 μL of sake mash

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1389-1723/$ – see front matter 
http://dx.doi.org/10.1016/j.jbiosc.2014.05.019

**TABLE 1.** Specificity of various yeast strains analyzed by using the loop-mediated isothermal amplification (LAMP) assay and diazo-coupling stain.

| Yeast no. | Yeast strain | LAMP method[a] | Diazo-coupling stain[b] |
|---|---|---|---|
| | Sake yeast | | |
| 1 | Kyokai no. 1 | + | + |
| 2 | Kyokai no. 2 | − | − |
| 3 | Kyokai no. 3 | + | + |
| 4 | Kyokai no. 4 | + | + |
| 5 | Kyokai no. 5 | + | + |
| 6 | Kyokai no. 6 | − | − |
| 7 | Kyokai no. 7 | − | − |
| 8 | Kyokai no. 8 | + | + |
| 9 | Kyokai no. 9 | − | − |
| 10 | Kyokai no. 10 | − | − |
| 11 | Kyokai no. 11 | − | − |
| 12 | Kyokai no. 12 | − | − |
| 13 | Kyokai no. 13 | − | − |
| 14 | Kyokai no. 14 | − | − |
| 15 | Kyokai no. 15 | − | − |
| 16 | Kyokai no. 28 | + | + |
| | Niigata sake yeast | | |
| 17 | G9 | − | − |
| 18 | G9NF[c] | − | − |
| 19 | G9arg[d] | − | − |
| 20 | G9NFarg[d] | − | − |
| | Shochu yeast | | |
| 21 | S-2 | + | + |
| 22 | SH-4 | + | + |
| | Wine yeast | | |
| 23 | KW-1 | + | + |
| 24 | W-3 | + | + |
| 25 | OC-2 | + | + |
| | Laboratory yeast | | |
| 26 | S288C | + | + |

[a] +, positive reaction; −, negative reaction.
[b] +, positive reaction; −, negative reaction.
[c] G9NF is a non-foaming strain isolated from G9.
[d] G9arg and G9NFarg are non-urea-producing strains from G9 and G9NF, respectively.

was suspended in 10 μL of 0.05 M NaOH, and then incubated at 98°C for 10 min. The mixture was cooled at room temperature, and then 20 μL of 0.1 M Tris−HCl buffer (pH 7.5) was added. The suspension was vortexed for 5−10 s and the cell debris was separated by centrifugation (13,000 ×*g* for 1 min). The supernatant contained yeast genomic DNA and was directly used for LAMP assay.

A set of four LAMP primers were designed using the standard laboratory yeast strain S288C detectable target *PHO3* locus (Fig. 1A, B). This DNA sequence was obtained from the Saccharomyces Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces/). Next, the specific primers for the LAMP reaction were designed using the online PrimerExplorer V4 software (Eiken Chemical, http://primerexplorer.jp/elamp4.0.0/index.html). The structure and sequences of the primers are shown in Fig. 1A and B. The LAMP reaction was performed using a Loopamp DNA amplification kit according to the manufacturer's instructions (Eiken Chemical, Tokyo, Japan). The LAMP mixtures contained 40 pmol of primer FIP, 40 pmol of primer BIP, 5 pmol of primer F3, 5 pmol of primer B3, 12.5 μL of 2× reaction mixture, 1 μL of *Bst* DNA polymerase, 1 μL of a fluorescent detection reagent (Eiken Chemical), and 2 μL of the DNA sample, and distilled water was added to make the final volume up to 25 μL. The amplification for the LAMP assay was performed in a PCR thermal cycler MP (Takara Bio); the reaction mixtures were incubated at 63°C for 60 min, and then heated at 95°C for 2 min to terminate the reaction. After the reaction was completed, the reaction product could be detected by the naked eye (17). Thus, LAMP assay analysis can be performed without using expensive sophisticated equipment. The green colorof the positive LAMP reaction and the orange color of negative LAMP reaction were used to distinguish wild type yeast from the specific sake yeast. Evaluations under ultraviolet light were also performed using the ImageMaster VDS (Pharmacia, Uppsala, Sweden). In addition, LAMP products were resolved by performing electrophoresis with 2% agarose gels by applying a voltage of 100 V for 40 min. The gel was then stained with Gel Red (Biotium, Hayward, CA, USA). The final amplification products appeared with a ladder-like pattern in agarose gel electrophoresis due to the presence of stem-loop DNA structures in the LAMP product.

The cACP activity of all yeast strains used in this study was assessed by performing diazo-coupling staining according to a protocol described by Mizoguchi and Fujita (10). Each strain was grown in YPD broth (2% glucose, 2% peptone, and 1% yeast extract) until the culture reached stationary phase, and then 2 μL of the medium was spotted onto a high-phosphate solid medium containing 1% glucose, 0.2% peptone, 0.15% yeast extract, 0.1% $KH_2PO_4$, 0.04% $MgSO_4$, 0.027% citric acid, and 3% agar (TTC-basal media, purchased from the Brewing Society of Japan). After incubating the spotted high-phosphate medium plates at 30°C for 48 h, the plates with yeast colonies were covered with staining agar (50°C) that contained 1.5% agar, 0.01 M potassium acetate buffer (pH 4.0), 0.5% Fast Blue B Salt (Tokyo Kasei Kogyo, Tokyo, Japan), and 0.05% monosodium 1-naphthyl phosphate salt monohydrate (Tokyo Kasei Kogyo). The colonies that secreted cACPs were visualized as dark-red colonies. For detecting yeast strains in sake mash, the mash was diluted (1:10,000) in water, and then 0.1 mL of diluted mash was plated on the high-phosphate agar. The colonies were stained as described above.

To test whether the LAMP assay can accurately discriminate for the various yeast strains, we performed the assay by using purified yeast DNA. Representative results of the LAMP analyses are shown in Fig. 1C, and the data are summarized in Table 1. The cACP-producing yeasts (S288C, yeast no. 26 in Table 1) were distinguished from cACP-negative yeasts (Kyokai no. 7, yeast no. 7 in Table 1) based on color development following LAMP amplification. These results were corroborated by gel electrophoresis analysis of the LAMP products. The electrophoresis data revealed that the yeasts that were identified as cACP-positive by color visualization of LAMP reaction products also produced the characteristic ladder-like pattern due to the stem-loop structure of the LAMP products (Fig. 1C). Furthermore, to confirm that the LAMP products corresponded with the known yeast genomic structures, the amplicons were digested using a restriction enzyme BslI (10 U/μL; New England Biolabs, Ipswich, MA, USA) for 3 h at 55°C. The digestion products were resolved on a Spreadex gel EL600 (Elchrom Scientific, Cham, Switzerland) by using the ORIGINS electrophoresis system (Elchrom Scientific), and were stained with Gel Red. The BslI enzyme digests the sequence between F1c and B1c region of the LAMP amplicon to produce approximately 105 and 100 bp fragments, which are the predicted theoretical DNA fragment sizes (105−107 bp and 100−102 bp) that are obtained from the known DNA sequences (Fig. 1D). Thus, all assays, including the LAMP assay, indicate that S288C strain has a genomic DNA sequence that corresponds to the *PHO3* locus.

Among the 26 yeast strains examined, the LAMP assay could identify Kyokai no. 2, 6, 7, 9, 10, 11, 12, 13, 14, 15, and all Niigata sake yeast strains. It has been previously reported that these discriminable Kyokai sake yeast strains do not have the *PHO3* gene (18). On the other hand, we confirmed that the parent strain of Niigata sake yeasts (strain G9, yeast no. 17 in Table 1) also has the *PHO5-3* locus fusion structure by sequence analysis (data not shown). These finding indicate that this LAMP method can be adopted only for the sake yeasts that have been verified to be the *PHO3*-deleted strains, and that the assay design does not allow precise distinction among strains (i.e., Kyokai no. 7 and Kyokai no. 9, or Kyokai no. 8 and S288C); however, the data obtained using the newly developed technique corresponded to that of the previously established diazo-coupling method. Thus, this LAMP assay could be used to distinguish specific sake yeast strains from other *S. cerevisiae* strains.