Isolation of lactic-acid tolerant *Saccharomyces cerevisiae* from Cameroonian alcoholic beverage

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ABSTRACT

We investigated yeast strains used in Cameroonian microbreweries, and identified a *Saccharomyces cerevisiae* strain (OCY3) with an excellent capacity for alcoholic fermentation. OCY3 showed higher tolerance to lactic acid and better fermentation performance under acidic conditions than a representative Japanese *sake* yeast, Kyokai No. 7, and a wine yeast, EC1118.

[Key words: lactic acid stress, Cameroon, distilled spirits, vacuolar fragmentation, *Saccharomyces cerevisiae*]

In sub-Saharan African nations including Cameroon, indigenous alcoholic beverages are still dominant, and they accounted for more than 80% of the total alcoholic beverage consumption in 1999 (1). They play important roles in sub-Saharan African culture not only as a refreshment and social lubricant, but also as an energy source, religious beverage, tribute for authority, source of cash income, and reward for work (2).

In the middle and Southern areas of Cameroon, people make a distilled spirit from cassava called “*mebwalam*” (3). Although making *mebwalam* is quite popular in Cameroon, brewing technology has generally not been sophisticated and natural fermentation is the most popular way of brewing. Furthermore, there is no available information regarding the fermentation ability of yeast strains used in Cameroon.

In order to promote a greater understanding of yeast strains used in Cameroon, we

collected yeast from microbreweries in Andom village, Lom-et-Djerem division, East region, Cameroon. Typical ethanol concentration of *mebwalam* was 20.7-26.6% (3). Fermentation samples were collected from *mebwalam*, aseptically diluted, and streaked on yeast selection (YS) medium (2% glucose, 1% yeast extract, 2% peptone, 5% ethanol, 1% lactic acid, 0.1% sodium propionate, and 2% agar). After incubation for 2 days at 28°C, colonies on the plates were purified by repeated streaking on YPD medium (2% glucose, 1% yeast extract, 2% peptone, and 2% agar), and the shapes of the cells were observed by light microscopy. As a consequence of microscopic analysis, we isolated budding yeast strains and named them OCY1-59. Through analysis of the sequences of internal transcribe spacers (ITS) of rRNA (4, 5), it was clarified that OCY strains comprise *Saccharomyces cerevisiae*, *Hanseniaspora guilliermondii*, and *Issatnchenkia orientalis*.

Next we carried out an alcoholic fermentation test to identify yeast strains effective for alcohol production. Yeast cells were pre-cultured in 3 mL of YPD medium and these starter cultures were added to 250 mL of YPHD medium (25% glucose, 1% yeast extract, and 2% peptone) at pH 5.5. Alcoholic fermentation was carried out without shaking at 28°C for 8 days. Ethanol concentrations were measured using gas chromatography (AL-2; Riken Keiki Co., Tokyo, Japan). Brix was measured by using a refractometer (Master-T, Atago Co. Ltd., Tokyo). We focused on the strain OCY3 (*S. cerevisiae*) since it showed the highest efficiency for ethanol production (data not shown). To further characterize OCY3, we compared it with the representative Japanese *sake* yeast Kyokai No. 7 (K7) and European wine yeast EC1118.

We examined the assimilation of various sugars as carbon sources. Yeast cells were

used to inoculate 50 mL of synthetic medium (1% sugar and 0.68% yeast nitrogen base (YNB) w/o amino acids) and cultured at 28°C for 7 days. All sugars were of special grade and purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). YNB was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). D-(+)-glucose, D-(-)-fructose, D-(+)-mannose, raffinose, and maltose can be efficiently assimilated by OCY3 cells, as well as K7 and EC1118 cells. OCY3 cells showed better growth than K7 and EC1118 cells in the media containing D-(+)-galactose, methyl- α -D-glucopyranoside, or D-(+)-trehalose. On the other hand, OCY3 hardly grew in the medium with glycerol. D-(+)-xylose, L-(+)-arabinose, L-(+)-rhamnose, L-(-)-fucose, and lactose cannot be assimilated by OCY3 cells.

Since yeast cells are often subjected to various stresses during the process of brewing and bioethanol production, it is generally considered that a higher stress tolerance of yeast cells can contribute to improvement of their fermentation efficiency (6, 7). Indeed, several yeast strains with high stress tolerance have been reported and applied to efficient bioethanol production (8, 9). Therefore, we examined the tolerance of OCY3 cells to various kinds of stress, employing the methods previously reported (10-13). There was no significant difference in the tolerance to ethanol stress (10-15%), hyperosmotic stress (1 M NaCl and 18-60% glucose), heat shock (37-42°C), freeze-thaw stress, **hydrochloric acid**, **sulfuric acid**, and oxidative stress (0.4-10 mM H₂O₂) among OCY3, K7, and EC1118 (data not shown). **We also compared the growth at high temperature (38-42°C) but no significant difference was observed among OCY3, K7, and EC1118 (data not shown).**

However, OCY3 showed higher tolerance to lactic acid stress than K7 and EC1118

(Fig. 1). Tolerance to acetic acid and propionic acid of OCY3 cells was higher than that of K7 cells but slightly lower than or almost comparable to that of EC1118 cells (Fig. 1). The method to assess the susceptibility to organic acid was described previously (14). *L*-lactic acid and other acids were purchased from Nacalai tesque (Kyoto, Japan). In the fermentation industry, lactic acid is one of the most important weak acids. Lactic acid functions as a flavor enhancer in traditional sourdough breadmaking (15), and lactic acid production has been achieved using metabolically engineered *S. cerevisiae* (16). On the other hand, lactic acid has adverse effects on yeast cells (17), and increased lactic acid levels caused by lactic acid bacteria contaminating the fermentation broth during industrial bioethanol production often markedly reduces the fermentation ability of yeast cells (18). Therefore, lactic acid-tolerant yeast strains offer a significant advantage for efficient bioethanol production, and so are desired by the bioenergy industry (18, 19).

We carried out a fermentation test under acidic conditions in 250 mL of YPHD medium at 28°C (Fig. 2). The pH of the medium was adjusted to 2.6, 2.8, 3.0, and 5.5 with lactic acid. In these acidic media, OCY3 cells showed a smooth increase in the concentration of ethanol rather than K7 and EC1118 cells. Although EC1118 cells showed the highest final concentration of ethanol at pH 3.0, OCY3 cells finally produced a higher ethanol concentration than the others at pH 2.8. OCY3 cells could carry out alcoholic fermentation even at pH 2.6. These results clearly indicate that OCY3 cells can perform satisfactory alcoholic fermentation under acidic conditions caused by lactic acid.

It has been reported that yeast cells induce vacuolar fragmentation in response to lactic acid stress and that many vacuolar proteins play important roles in adaptation to lactic acid

(19). In order to obtain clues to understand the high tolerance for lactic acid of OCY3 cells, we examined the vacuolar morphology. Vacuolar membranes were visualized with FM4-64 using the method of Suzuki *et al* (19). Vacuolar types were categorized into 3 groups depending on the number of vacuoles per cell: Type I, more than 5 small and highly fragmented vacuoles per cell; Type II, 3 or 4 mildly fragmented vacuoles per cell; Type III, 1 or 2 large and swollen vacuoles per cell. Under non-stressed conditions, OCY3 cells showed a slightly higher proportion of Type III vacuoles than K7 and EC1118 cells (Fig. 3). Lactic acid stress caused an increased number of highly fragmented vacuoles and a decreased number of swollen vacuoles in all strains. However, the degree of variation in the vacuolar morphology of OCY3 was the most distinctive among the 3 strains. The marked response of vacuoles may partly contribute to the tolerance to lactic acid of OCY3 cells. Although further detailed study is necessary, we suggest that vacuolar functions play roles in the high tolerance to lactic acid of OCY3.

In this study, we identified a lactic acid-tolerant yeast strain that was highly effective for alcoholic fermentation in Cameroon. For microbrewers in sub-Saharan Africa, it is difficult to produce alcoholic beverages under aseptic conditions, and contamination by bacteria, including lactic acid bacteria, in cassava mash can easily occur. Besides, in most cases, brewing is commonly carried out at air temperature in the tropics. Therefore, to prevent the spoilage of alcoholic beverages, it is important to maintain acidic conditions by lactic acid produced with lactic acid bacteria in sub-Saharan Africa (3, 20). In this situation, it is reasonable that a lactic acid-tolerant yeast, OCY3, was isolated from a Cameroonian microbrewery. The utilization of OCY3 as a yeast starter may facilitate safe and efficient

138 brewing in Cameroon.

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FIGURE LEGENDS

FIG 1.

OCY3 cells showed higher tolerance to lactic acid than K7 and EC1118 cells. Yeast cells in an exponential phase of growth were resuspended in fresh SD medium to obtain an initial OD₆₀₀ value of 0.1, and treated with lactic acid (1.0 - 2.5%), propionic acid, and acetic acid at 28°C for 60 min. They were then spotted (10 µL) on to plates of SD medium in 10-fold serial dilutions and incubated at 28°C for 48 h.

FIG 2.

Ethanol production under acidic conditions. Cells were used to inoculate 250 mL of YPHD medium with turbidity adjustment of 0.5 at 600 nm. The pH of the medium was adjusted with lactic acid. The fermentation was carried out without shaking at 28°C for 7-10 days. Data are shown as means ± S.D. of three independent experiments. Symbol legends: *sake* yeast K7 (circle), wine yeast EC1118 (square), and OCY3 (triangle). Ethanol concentrations and brix in the medium are indicated as closed and open symbols, respectively.

FIG 3.

Rapid vacuolar fragmentation caused by lactic acid stress in OCY3 cells. Vacuolar membranes were stained with FM-4-64. Yeast cells in an exponential phase of growth were treated with or without 1.5% lactic acid for 15 min in SD medium. (A) Vacuolar

235 fragmentation was classified into three types based on the number of vacuoles per cell, and
236 shown as the proportion of vacuolar types among 200 cells. Numbers of vacuoles per cell
237 were as follows: *empty bar*, one or two; *gray bar*, three or four; *filled bar*, more than five.
238 (B) Representative pictures of FM4-64-stained cells. The white bar indicates 5 μm .
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