

# Co-Cultivation of Sake Yeast and Kocuria Isolates From the Sake Brewing Process

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## Research Article

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# Abstract

*Kocuria* isolates collected from the sake brewing process have inhabited the Narimasa Sake Brewery in Toyama, Japan. To investigate the effect of these actinobacterial isolates on the growth and metabolism of sake yeast, co-cultivation of sake yeast and *Kocuria* isolates was performed in a medium containing tryptone, glucose, and yeast extract (TGY), and a solution containing *koji* (steamed rice covered with *Aspergillus oryzae*) and glucose. In the TGY medium, the ethanol concentration and the number of living cells of each microorganism were measured. In the *koji* solution, the concentrations of ethanol and organic acids (citric acid, lactic acid, and succinic acid) were measured. The results showed that in TGY media, the growth of each *Kocuria* isolate in the co-culture of the two *Kocuria* isolates was similar to that in each monoculture. However, the growth of both *Kocuria* isolates was inhibited in the co-cultures of sake yeast and *Kocuria* isolates. On the other hand, the growth and ethanol productivity of sake yeast did not differ between its monoculture and co-cultures with *Kocuria* isolates. In the *koji* solution, although the concentration of succinic acid did not differ between the monocultures of sake yeast and co-cultures with *Kocuria* isolates, the concentrations of citric acid and lactic acid were different, indicating that *Kocuria* isolates affected the microbial population and/or sake yeast metabolism, but the effects were not identical between the two isolates. This strongly suggests that bacteria inhabiting a sake brewery influence the flavor of sake products of the brewery.

## Introduction

During sake brewing, the *koji* mold *Aspergillus oryzae* converts rice starch into glucose, which is then converted into ethanol by the sake yeast *Saccharomyces cerevisiae*. Eventually, the ethanol concentration reaches approximately 20%. Most microorganisms present at the beginning of the sake production process die because of the high ethanol concentration, although some lactic acid bacteria can grow at high ethanol concentrations and cause spoiling<sup>1</sup>. Thus, sake undergoes pasteurization during storage, a process which was recorded 300 years before Pasteur reported his method.

Living bacteria cannot be detected in sake products, but bacterial DNA can be detected. Based on DNA sequence data, we obtained information on bacteria that have entered and briefly grown during sake brewing<sup>2-5</sup>. Living bacteria were previously isolated at the beginning of the sake production process<sup>6</sup>. Among these, bacteria belonging to the genus *Kocuria* were the most common isolates found in the Narimasa Sake Brewery (Brewery Toyama 1 in Terasaki and Nishida, 2020<sup>6</sup>). *Kocuria* belongs to the phylum Actinobacteria<sup>7</sup>. It has been reported that lactic acid bacteria belonging to the phylum Firmicutes play a role in the sake production process<sup>1-3,8</sup>. In addition, co-cultivation of sake yeast and lactic acid bacteria has also been performed<sup>9,10</sup>. However, to our knowledge, no bacteria belonging to Actinobacteria have been reported to be associated with sake brewing.

In this study, a medium containing tryptone, glucose, and yeast extract (TGY), a solution containing *koji* (steamed rice covered with *Aspergillus oryzae*), and glucose were used for cultivation (Fig. 1). Although the TGY medium was autoclaved and kept sterile, *koji* included *koji* mold and other microorganisms. *Koji*

is commercially available in Japan. Although sake yeasts produce ethanol in both the TGY medium and *koji* solution, environments in *koji* solution are more similar to those in sake brewing than those in the TGY medium.

*Kocuria* DNA was detected only in the products of Narimasa Sake Brewery, suggesting that the *Kocuria* isolates have inhabited the sake brewery. In addition, *Kocuria* DNA was detected not in clear sake, but in cloudy sake and *sake-kasu* (sake lee)<sup>6</sup>. This indicates that *Kocuria* cells do not lyse in sake even after they die because these cells are difficult to lyse using ethanol<sup>11</sup>. *Kocuria* cells are then squeezed via filtration and included in the *sake-kasu* fraction during the process of sake brewing.

The composition of amino acids and organic acids is associated with bacterial flora in the sake<sup>12,13</sup>. Some bacteria are alive and grow temporarily during the sake production process, but finally die in the final sake product. The *Kocuria* strains may have inhabited the Narimasa Sake Brewery, which may further play a key role in the interaction among microorganisms, and influence the flavor and taste of sake. If so, we could produce sakes with different flavors and tastes by exchanging the sake brewery-specific bacteria from different sake breweries for the sake production process. Taken together, the purpose of this study was to elucidate whether *Kocuria* isolate(s) can affect the growth and metabolism of sake yeast during co-cultivation.

## Results And Discussion

### Monoculture and co-culture of *Kocuria* isolates in TGY medium

In this study, the *Kocuria* isolates TGY1120\_3 and TGY1127\_2 were used, which were identified based on different colony colors (Fig. 2). Comparison of monocultures of the isolates TGY1120\_3 and TGY1127\_2 at different temperatures showed that these two *Kocuria* isolates grew better at 15 °C than at 5 °C or 30 °C (Fig. 3A). Comparison of the 16S rDNA sequence showed that the isolates TGY1120\_3 (accession number: NZ\_AP022830) and TGY1127\_2 (NZ\_AP022834) belonged to *K. koreensis* and *K. uropygioeca*, respectively. The optimal growth temperature of *K. koreensis* was between 30 °C and 37 °C<sup>14</sup>, while that of *K. uropygioeca* was 35 °C<sup>15</sup>. Considering that sake brewing was performed at 15 °C, the isolates TGY1120\_3 and TGY1127\_2 probably adapted to grow at 15 °C. If this is true, these *Kocuria* isolates have inhabited and evolved in the Narimasa Sake Brewery for a long time.

The growth of either isolate TGY1120\_3 or TGY1127\_2 in the co-culture of these two isolates was similar to that in each monoculture (Fig. 3A). The maximum growth rate of isolate TGY1127\_2 was higher than that of the isolate TGY1120\_3 (Fig. 3A). To elucidate this, a cluster analysis<sup>6</sup> of the eight growth patterns at 15 °C and 30 °C was performed using the software R (<http://www.R-project.org/>). The results indicated that the isolates TGY1120\_3 and TGY1127\_2 were clustered separately, except for TGY1127\_2, during co-culture at 30 °C (Fig. 3B).

### Co-culture between sake yeast and *Kocuria* isolates in TGYm2 medium

Sake yeast grew in co-cultures with *Kocuria* isolate(s) as well as in monoculture (Fig. 4). In addition, the change in ethanol concentration was similar among monocultures and in three different co-cultures (Fig. 4). In both co-cultures of isolate TGY1120\_3 and sake yeast and that of isolate TGY1127\_2 and sake yeast, the *Kocuria* isolates did not grow but they survived (Fig. 4). The isolates TGY1120\_3 and TGY1127\_2 survived up to 19 and 25 days, respectively (Fig. 4).

On the other hand, in the co-culture containing sake yeast and two *Kocuria* isolates, the *Kocuria* isolates grew from 0 to 5 days (Fig. 4) and the number of living cells was limited to  $10^5$ - $10^6$  cells/mL after five days (Fig. 4). During the same period, the number of living cells decreased (Fig. 4). In the co-culture containing sake yeast and both *Kocuria* isolates, the isolates TGY1120\_3 and TGY1127\_2 survived up to 14 and 25 days, respectively (Fig. 4). Interestingly, although the ethanol concentration was above 5% after eight days in the monoculture and co-cultures of sake yeast and either *Kocuria* isolate, it was below 5% after eight days in the co-cultures containing sake yeast and both *Kocuria* isolates. Considering that the growth of sake yeast did not differ among the four different culture conditions (Fig. 4), the ethanol productivity of sake yeast may decrease after eight days in the co-cultures of sake yeast and the two *Kocuria* isolates. The low ethanol productivity of sake yeast may be associated with the growth of *Kocuria* isolates from 0 to 5 days of co-cultivation.

*Kocuria* DNA was not detected in clear sakes, but was detected in cloudy sakes and *sake-kasu*<sup>6</sup>, because *Kocuria* cells are difficult to lyse using ethanol<sup>11</sup>. In this study, TGYm2 medium used after 34 days of incubation in a co-culture of sake yeast and both *Kocuria* isolates was centrifuged to separate it into a precipitate and supernatant. Quantitative PCR (qPCR) was performed on each of them using primers specific to genomic DNA of the isolates TGY1120\_3 and TGY1127\_2. Living cells were not detected after 34 days of incubation. The qPCR results showed that *Kocuria* DNA was detected in the precipitate but not in the supernatant, supporting the idea that *Kocuria* cells are difficult to lyse using ethanol. In addition, the Cq value of TGY1120\_3-specific qPCR was higher than that of the TGY1127\_2-specific qPCR, with a difference of approximately 3 (Table 1), indicating that the number of dead or non-culturable<sup>16</sup> cells in cultures containing the isolate TGY1127\_2 was eight times greater than that in the cultures containing the isolate TGY1120\_3. This indicates that the *Kocuria* cells of isolates TGY1120\_3 and TGY1127\_2 are difficult to lyse after co-cultivation.

### **Co-culture of sake yeast and *Kocuria* isolates in *koji* solution**

The change in ethanol concentration during co-cultivation of sake yeast and the *Kocuria* isolate TGY1120\_3 was similar to that during monocultivation of sake yeast (Fig. 5). On the other hand, ethanol productivity in the co-culture of sake yeast and isolate TGY1127\_2 was slightly higher than that in the monoculture of sake yeast (Fig. 5). During co-culture with sake yeast, ethanol concentration in the co-culture with both *Kocuria* isolates was higher than those in the other cultures (Fig. 5). The cause of this ethanol productivity in the co-culture of sake yeast and both *Kocuria* isolates is uncertain. It is possible that the existence of *Kocuria* isolates became a stress for sake yeast, and hence the yeast produced more ethanol.

The change in citric acid concentration was different between the monoculture of sake yeast and the co-cultures containing yeast and *Kocuria* isolates (Fig. 5). However, the concentration of citric acid was similar for the first five days of culture between the monoculture of sake yeast and co-cultures of yeast and *Kocuria* isolate(s) (Fig. 5), and the citric acid concentration in the co-culture of sake yeast and isolate TGY1120\_3 was higher than that in the monoculture of sake yeast at 17 and 23 days. On the other hand, citric acid concentrations in the co-cultures containing sake yeast and isolate TGY1127\_2 and in the co-cultures containing sake yeast and both *Kocuria* isolates were higher at 17 days but lower at 23 days than in the monoculture of sake yeast (Fig. 5).

The lactic acid concentration increased in the monoculture of sake yeast (Fig. 5). Due to the growth of lactic acid bacteria, lactic acid concentration increases during the production of sake fermentation starters *kimoto*<sup>2</sup> and *yamahai-moto*<sup>3</sup>. In the *koji* solution, lactic acid concentration peaked after 10 days and then decreased after 17 days in the co-cultures of yeast and *Kocuria* isolates (Fig. 5). This may indicate that the *koji* solution, containing lactic acid bacteria and *Kocuria* isolates, may affect the growth and/or metabolism of lactic acid bacteria.

The change in succinic acid concentration was similar among the four culture conditions. It decreased after 5 days of culture, then increased up to 23 days (Fig. 5). This indicates that the TCA cycle changed from oxidative metabolism to reductive metabolism in the sake yeast, starting from five days in culture<sup>17</sup>.

The amino acid compositions of the final cultures of *koji* solution were similar among the four culture conditions (Fig. 6). It indicates that *Kocuria* isolates do not affect the amino acid metabolism in sake yeast.

## Conclusions

*Kocuria* isolates did not affect the growth of sake yeast during co-cultivation. However, the composition and concentration of organic acids were influenced by the presence of *Kocuria* isolate(s) in the *koji* solution. These results strongly suggest that the addition or temporary growth of *Kocuria* isolate(s) during sake brewing could alter the flavor and taste of sake. Thus, bacteria that have entered and briefly grown during sake brewing may have had effects on sake flavor and taste when they were grown with sake yeast.

## Methods

### Strains used in this study

Sake yeast (*Kyokai* yeast strain 1401)<sup>18</sup> and the *Kocuria* isolates<sup>6</sup> TGY1120\_3 and TGY1127\_2 were used in this study.

### Media used in this study

For the cultivation of *Kocuria* isolates, we used the following media: TGY (tryptone 5 g/L, glucose 1 g/L, yeast extract 3 g/L), TGYm1 (tryptone 20 g/L, glucose 20 g/L, yeast extract 10 g/L), TGYm2 (tryptone 20 g/L, glucose 300 g/L, yeast extract 10 g/L), TGY agar (yeast extract 3 g/L, tryptone 5 g/L, glucose 1 g/L, agar 15 g/L, cycloheximide 500 ng/mL), and TGYm1c agar (yeast extract 10 g/L, tryptone 20 g/L, glucose 20 g/L, agar 15 g/L, cycloheximide 500 ng/mL).

For the cultivation of sake yeast (*Saccharomyces cerevisiae* strain 1401), we used the following media: TGYm1 and TGYm1k agar (yeast extract 10 g/L, tryptone 20 g/L, glucose 20 g/L, agar 15 g/L, kanamycin 500 ng/mL).

To cultivate a mixture of *Kocuria* isolate(s) and sake yeast, we used the medium TGYm2.

### **Monoculture of *Kocuria* isolates**

The *Kocuria* isolate TGY1120\_3 or TGY1127\_2 was statically cultured in TGY medium at 5 °C, 15 °C, or 30 °C. Sampling was performed at 0, 5, 8, 14, 19, 25, and 33 days of incubation. Each sample was diluted 1:10, and 5 µL of each sample was spread on TGY agar to count the number of colonies.

### **Co-culture of *Kocuria* isolates**

The *Kocuria* isolates TGY1120\_3 and TGY1127\_2 were statically cultured in TGY medium at 15 °C or 30 °C. Sampling was performed at 0, 5, 8, 14, 19, 25, and 33 days of incubation. Each sample was diluted 10 times, and 5 µL of each sample was spread on TGY agar to count the number of colonies. The colony colors of the isolates TGY1120\_3 and TGY1127\_2 were cream and orange, respectively (Fig. 2). Thus, the number of colonies could be counted according to the presence of different colors.

### **Co-culture of sake yeast and either *Kocuria* isolates**

*Kocuria* isolate TGY1120\_3 and sake yeast strain 1401, or isolate TGY1127\_2 and yeast strain 1401 were statically cultured together in TGYm2 medium at 15 °C. Sampling was performed at 0, 5, 8, 14, 19, 25, and 33 days of incubation. Each sample was diluted 10 times, and 5 µL of each sample was spread on TGYm1c agar to count the number of *Kocuria* isolate colonies, and on TGYm1k agar to count the number of sake yeast colonies. In addition, the ethanol concentration of each broth at each sampling time was determined using HPLC.

Furthermore, *koji* solution (*koji* 100 g/L, glucose 200 g/L) was used in the co-culture of sake yeast and *Kocuria* isolates. *Koji* from Shin-mura Koujimisō in Toyama, Japan, was used in this study, and the resulting mixture of *koji*, glucose, and water was called the *koji* solution. Co-cultures containing sake yeast and TGY1120\_3, and sake yeast and TGY1127\_2 were statically grown in *koji* solution at 15 °C. Sampling was performed at 0, 5, 10, 17, and 23 days of incubation. The concentrations of ethanol and organic acids in each sample were detected using HPLC.

### **Co-culture of sake yeast and both *Kocuria* isolates**

The *Kocuria* isolates TGY1120\_3 and TGY1127\_2 and the sake yeast strain 1401 were statically cultured together in TGYm2 medium at 15 °C. Sampling was performed at 0, 5, 8, 14, 19, 25, and 33 days of incubation. Each sample was diluted 10 times, and 5 µL of each sample was spread on TGYm1c agar to count the number of colonies of *Kocuria* isolates, and on TGYm1k agar to count the number of sake yeast colonies. In addition, the ethanol concentration of each broth at each sampling time was determined using HPLC.

In addition, co-cultures containing sake yeast and both *Kocuria* isolates (TGY1120\_3 and TGY1127\_2) were statically performed in *koji* solution at 15 °C. Sampling was performed at 0, 5, 10, 17, and 23 days of incubation. The concentrations of ethanol and organic acids in each sample were detected using HPLC.

### **Quantitative PCR (qPCR) for DNA-specific detection of *Kocuria* isolates**

One milliliter of TGYm2 medium, at 34 days of incubation, containing a co-culture of sake yeast and both *Kocuria* isolates was centrifuged to separate it into a precipitate and supernatant. The precipitate was then resuspended in 100 µL of water. The TGY1120\_3 DNA-specific primers used in this study were 5'-CTGGAGATCGGTGGGTTTAC-3' and 5'-TGAGAATCGCGAACCCCTTC-3'. The TGY1127\_2 DNA-specific primers used in this study were 5'-CGACAAGACTCACCTGTTCG-3' and 5'-CTCGACGTCGTAGCAGATCA-3'. Approximately 2.5 µL of each sample was used for qPCR, which was performed using a LightCycler Nano (Roche, Basel). The cycling conditions were as follows: 95 °C for 10 min, followed by 45 cycles of denaturation (95 °C for 10 s), annealing and extension (70 °C for 30 s). After the final extension, a melting curve cycle was performed from 60 °C to 95 °C, at increments of 0.1 °C/s, to confirm the absence of non-specific PCR products. The quantification cycle (C<sub>q</sub>) values were obtained using the LightCycler Nano Software (Roche, Basel, Switzerland).

### **Quantification of ethanol and organic acids using HPLC**

High-performance liquid chromatography (HPLC) analysis of the TGY medium and *koji* solution during cultivation was carried out using a Shimadzu LC-10 HPLC system equipped with an Aminex HPX-87H column (7.8 mm × 300 mm; Rio-Rad, Tokyo). Five microliters of each sample solution was injected and isocratically eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL/min. The column temperature was maintained at 35 °C, and the compounds were monitored with a UV detector at 210 nm and a Shodex RI-71 refractive index detector (Showa Denko, Tokyo). The retention time of each compound was as follows: 9.6 min for citric acid, 12.4 min for succinic acid, 13.4 min for lactic acid, and 22.0 min for ethanol.

### **Quantification of amino acids using HPLC**

The amino acid compositions of the final cultures of *koji* solution were assessed via HPLC by IDEA Consultants, Inc. (<https://ideacon.jp/>). One mL of each sample and 3 mL of 80% methanol were mixed with vigorous stirring. After that, the solution was sonicated for 5 min and then centrifuged at 3,000 rpm for 10 min. The supernatant was filtered using 0.2 µm pore size filter. Labeling was performed by auto-injector program (IDEA Consultants).

# Declarations

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## Author contributions

M.T., A.I., S.Y., M.Y., and H.T. carried out the experiments. H.N. designed the experiments. M.T., H.T., and H.N. wrote the paper.

## Competing interests

The authors declare no competing interests.

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## Tables

Table 1. Results of quantitative PCR.

Sample	Primers	Cq value
Supernatant	TGY1127_2	Not detected
Supernatant	TGY1127_2	Not detected
Supernatant	TGY1127_2	Not detected
Precipitate	TGY1127_2	19.8
Precipitate	TGY1127_2	20.8
Precipitate	TGY1127_2	21.4
Supernatant	TGY1120_3	Not detected
Supernatant	TGY1120_3	Not detected
Supernatant	TGY1120_3	Not detected
Precipitate	TGY1120_3	17.7
Precipitate	TGY1120_3	17.7
Precipitate	TGY1120_3	17.6

## Figures

## Cultures using TGYm2 medium

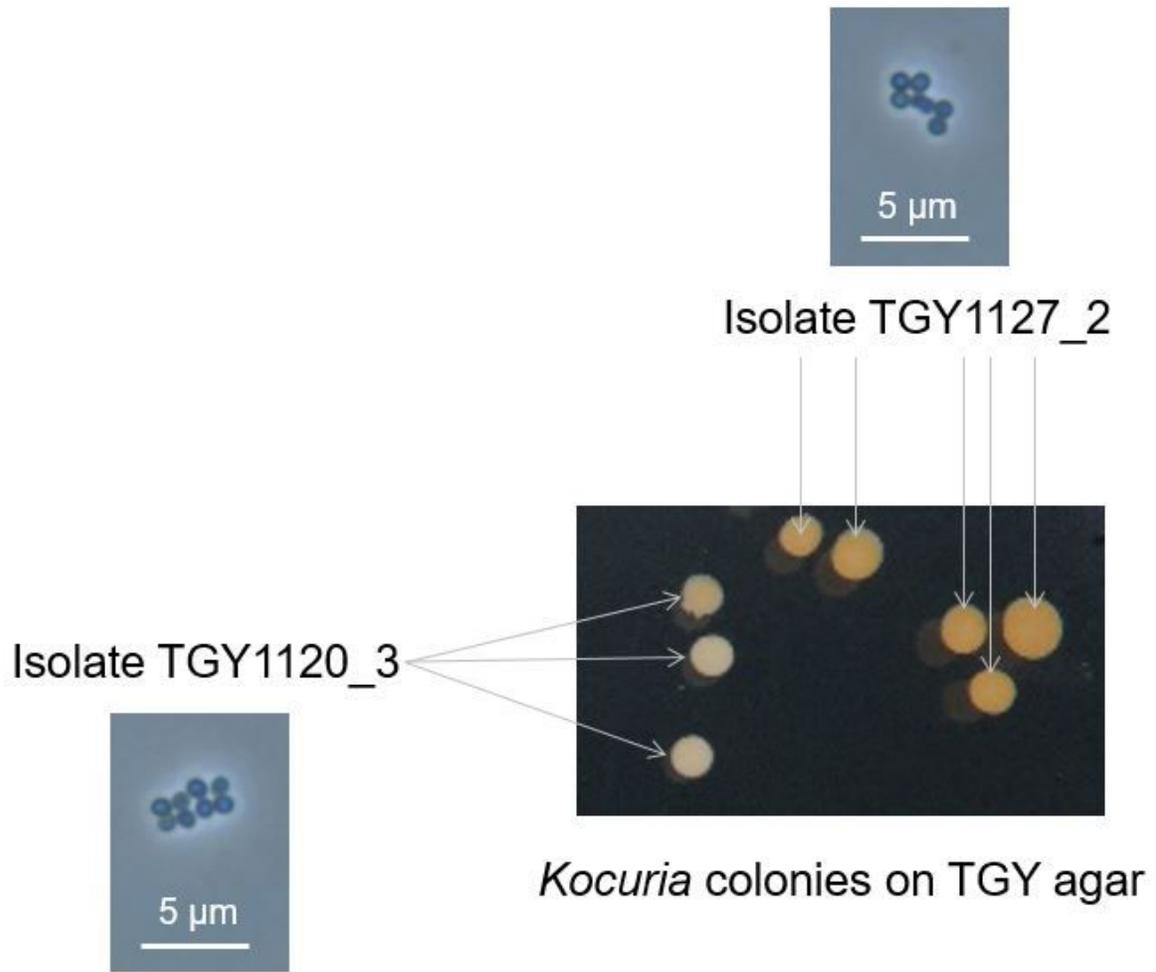


## Cultures using *koji* solution



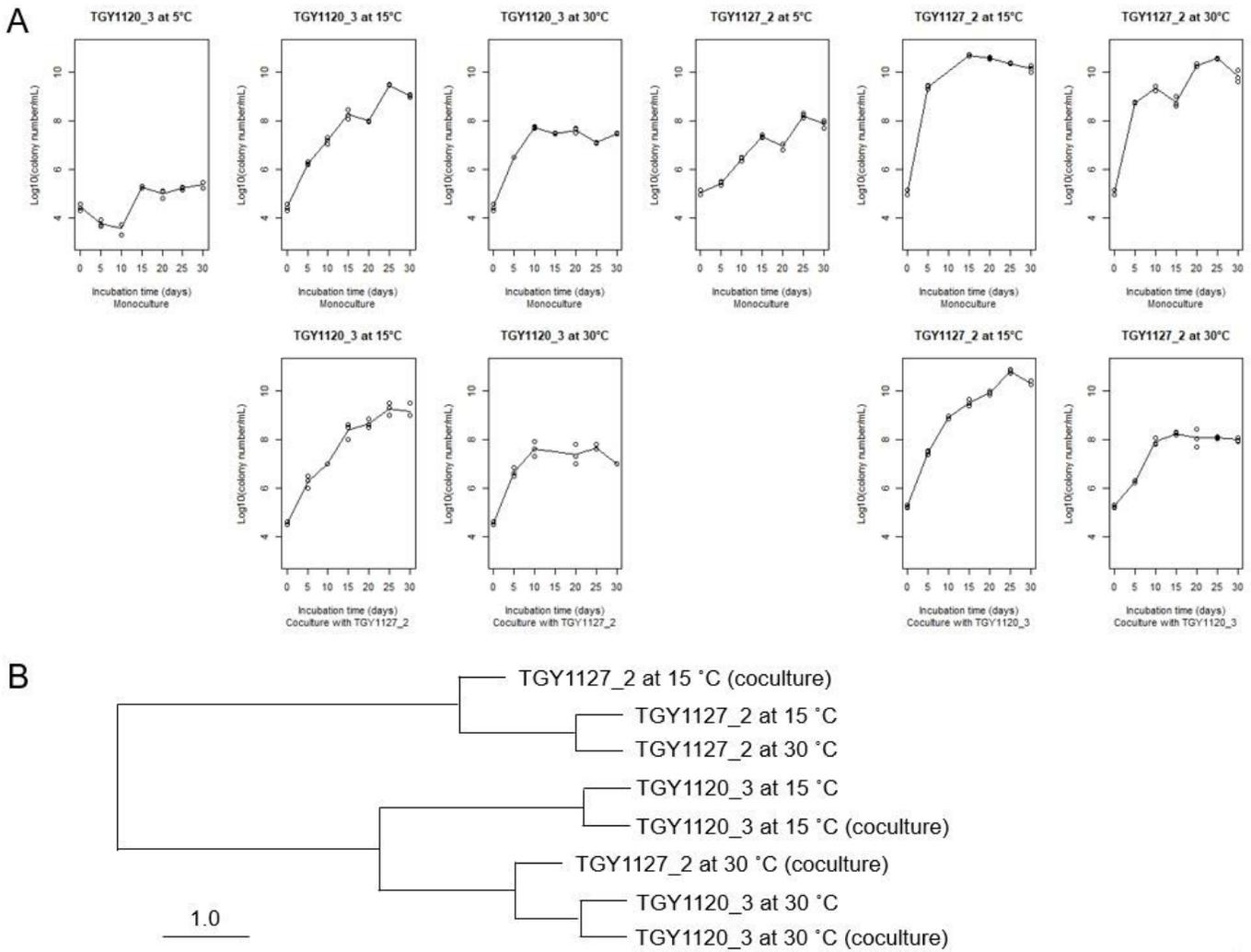
**Figure 1**

Cultivation of sake yeast and *Kocuria* isolate(s) in TGYm2 medium and koji solution. TGYm2 medium contained tryptone 20 g/L, glucose 300 g/L, and yeast extract 10 g/L. Koji solution contained koji 100 g/L and glucose 200 g/L.



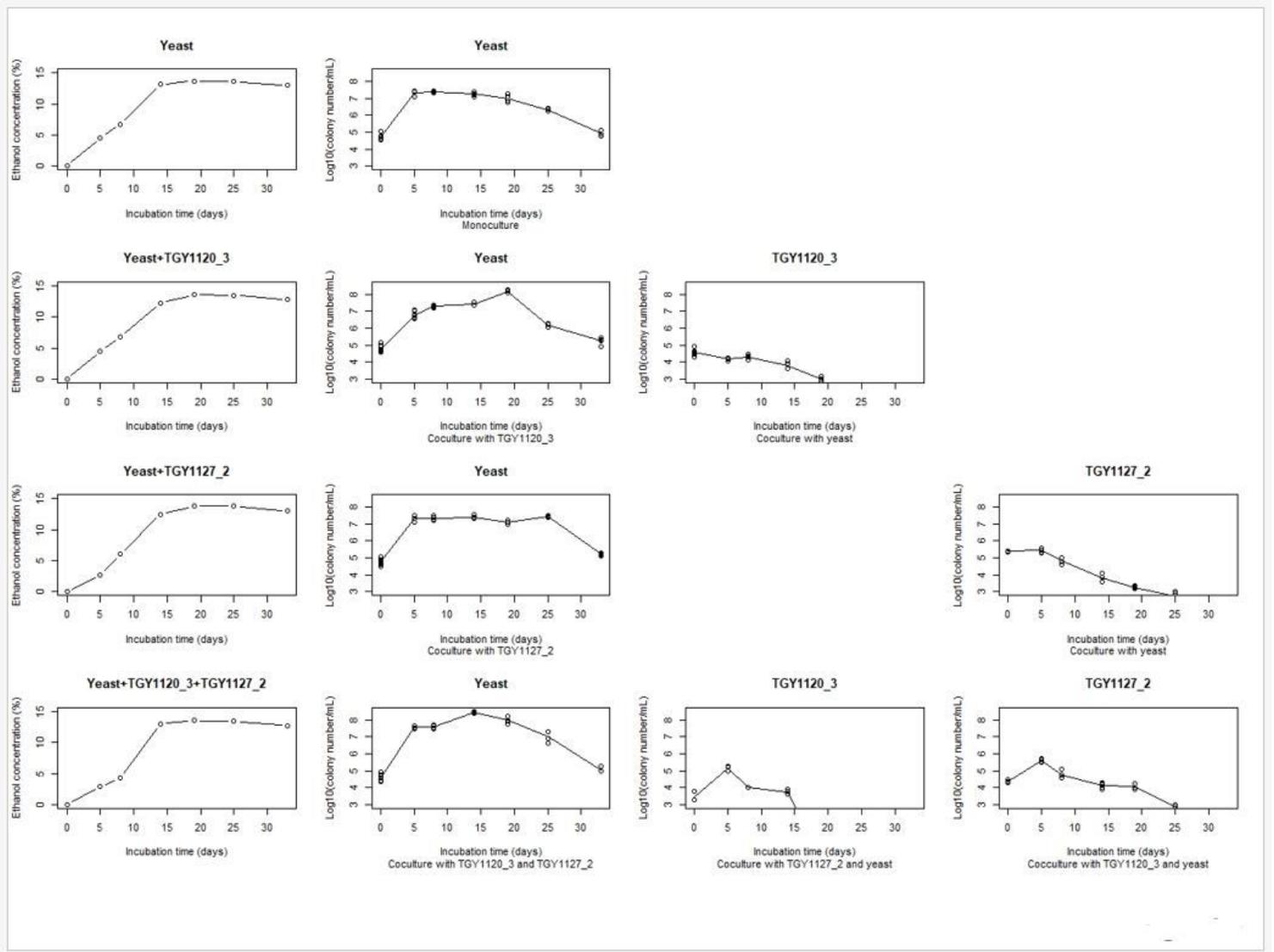
**Figure 2**

Colonies of *Kocuria* isolates grown on TGY agar. The isolates TGY1120\_3 and TGY1127\_2 appeared as cream and orange colonies, respectively. Phase contrast microscopy images were captured using an Olympus BX51 microscope.



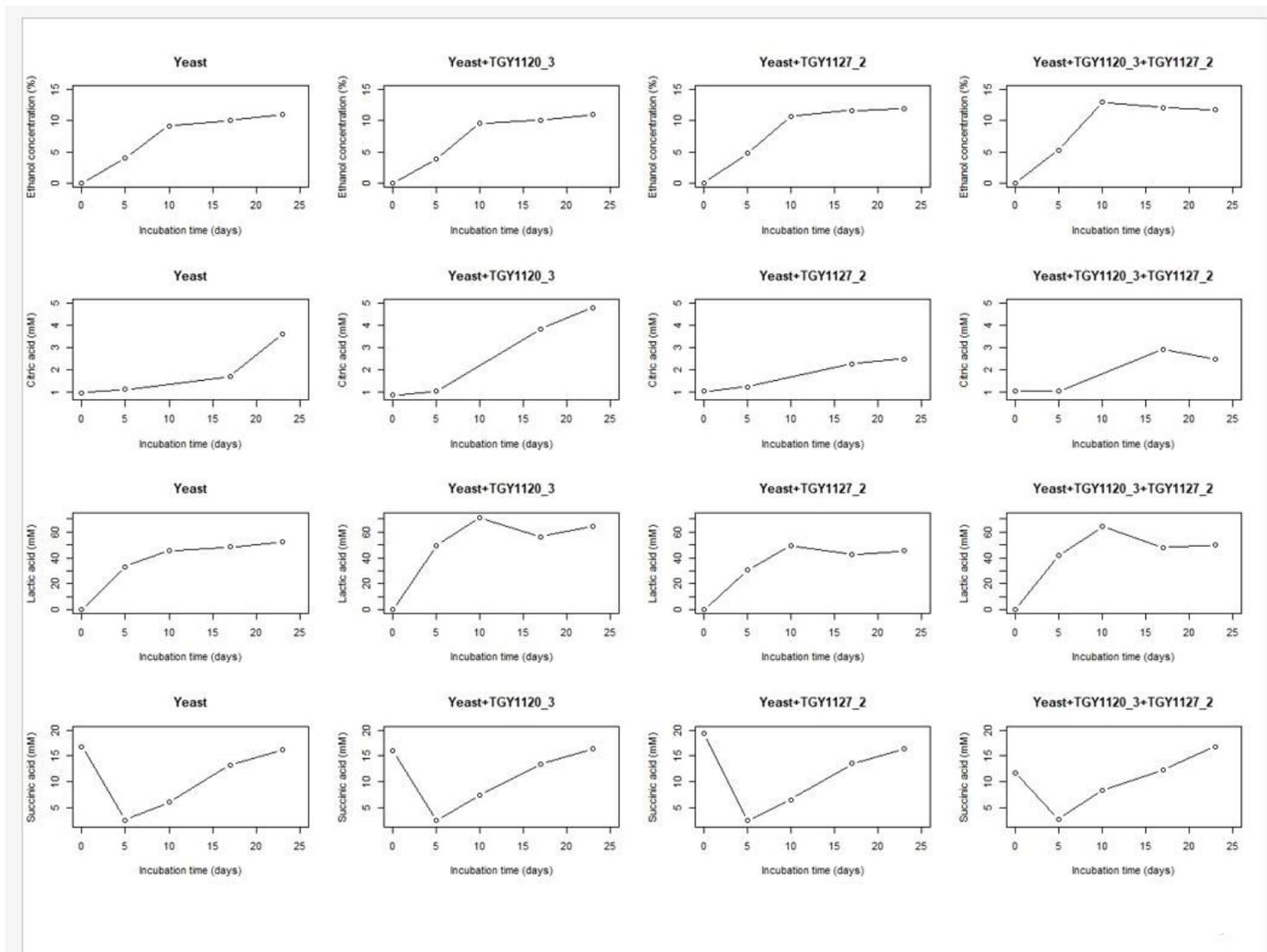
**Figure 3**

A) Growth of *Kocuria* isolates in monoculture and co-cultures in TGY medium. The incubation temperatures were 5 °C, 15 °C, and 30 °C. B) Phylogenetic relationships based on the growth patterns of *Kocuria* isolates under different culture conditions.



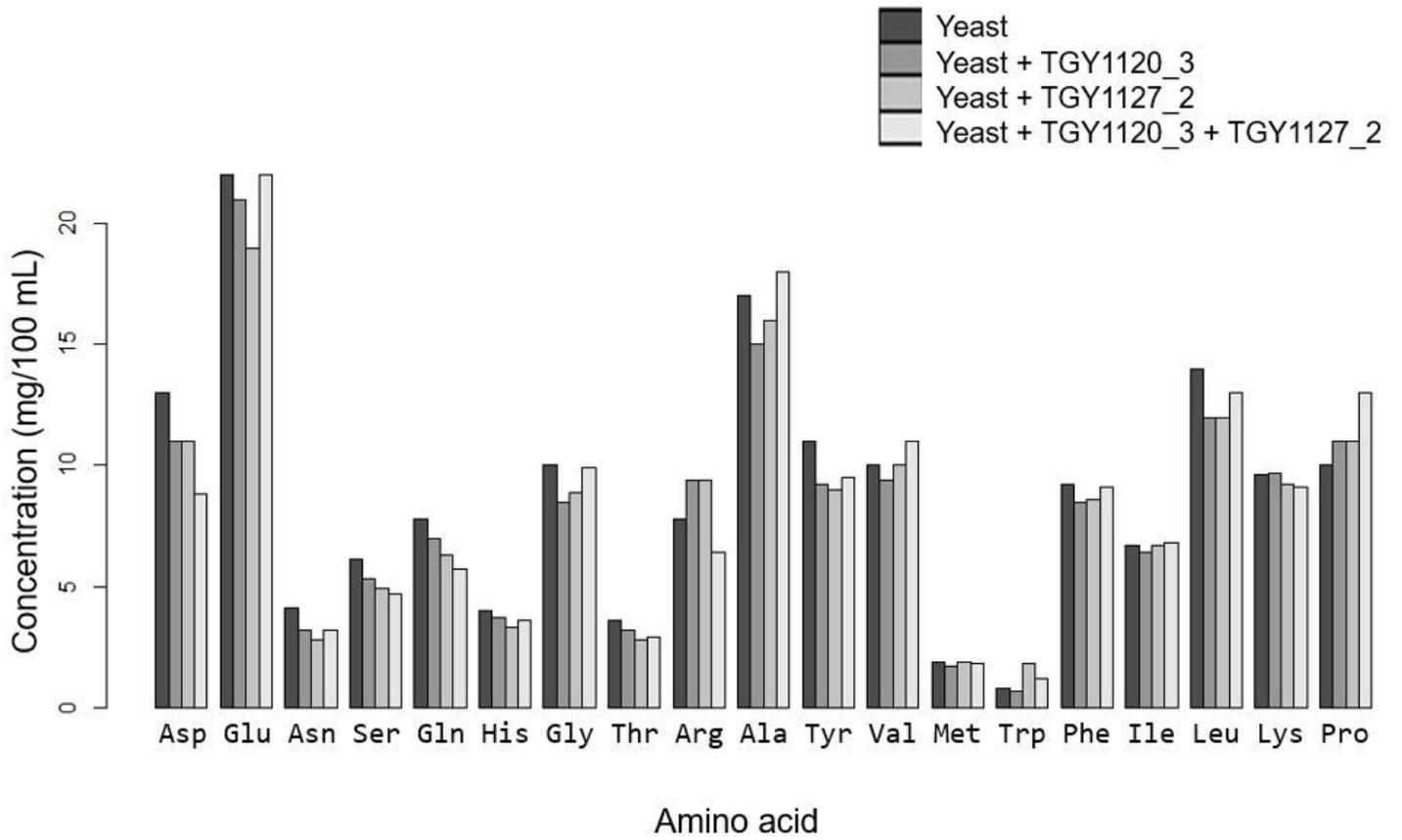
**Figure 4**

Ethanol concentration and growth of sake yeast, *Kocuria* isolate TGY1120\_3, and TGY1127\_2 in monocultures and co-cultures in TGYm2 medium. The incubation temperature was 15 °C.



**Figure 5**

Changes in ethanol, citric acid, lactic acid, and succinic acid concentrations in monocultures and co-cultures in koji solution. The incubation temperature was 15 °C.



**Figure 6**

Amino acid compositions of the final cultures of koji solution.