

Article

Identification and Characterization of Yeast Species Isolated from *Cornus kousa* Fruits in Japan

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Abstract: The *Cornus kousa* tree, which is of Asian origin, is often cultivated for ornamental purposes and used in traditional medicine. The tree produces sugar-rich fruits, which are potential habitats for natural yeasts. The identification of new yeast strains has many advantages for the industry and research. This study aimed to isolate and identify yeast species from *C. kousa* fruits and to understand their microbial ecology. Ripe and rotten fruits, which had fallen on the ground naturally, were collected and soaked in culture media, followed by plate spreading for colony growth. The morphological examination revealed three distinct colony types, including two from the ripe fruits and one from the rotten fruits. The analysis of the internal transcribed spacer 1 region indicated three yeast strains corresponding to the three colony types: *Torulaspora delbrueckii* and *Pichia kluyveri* from the ripe fruits and *Saccharomyces cerevisiae* from the rotten fruits. The metabolic characterizations demonstrated that all three yeasts efficiently consumed glucose and produced alcohol. *S. cerevisiae* exhibited the strongest fermentation ability and the highest growth rate. These findings showed that *Cornus kousa* fruit is a source of diverse yeast species, with distinct species associated with different states of fruit decomposition.

Keywords: *Cornus kousa*; Japanese dogwood; Yamaboushi; wild yeast species; *Torulaspora delbrueckii*; *Pichia kluyveri*; *Saccharomyces cerevisiae*



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1. Introduction

Cornus kousa, commonly referred to as kousa dogwood, Japanese dogwood, or Yamaboushi, belongs to the genus *Cornus* within the family of the same name [1,2]. The tree originated in Asia and has a broad distribution across Japan, from Honshu to the Ryukyu Islands [3]. *C. kousa* was introduced to Europe and America over 156 years ago, during the Edo period, and is now cultivated for ornamental purposes in those regions [3]. During summer in Japan, the tree produces white blossoms, followed by the production of fruits from late summer to autumn. The mature fruit is characterized by its palatability and significant sweetness, coupled with nutritional value [3]. Despite its fruit's edibility [1,4], the *C. kousa* is typically utilized for ornamental landscaping; it also grows in the wild within Japan but is not harvested for food consumption. To a certain extent, the fruit of *C. kousa* is

known for its biochemical profile and biological activity, and has been used in traditional Asian medicine for various purposes [4].

An analysis of the primary metabolites revealed a high sugar content in *C. kousa* fruit. The sugar concentration increases as the fruit matures [1] and comprises mainly fructose and glucose; fructose predominates at all ripeness levels. Comparative analysis has also shown that the *C. kousa* fruit contains higher total sugar levels than other *Cornus* species [1]. The isolation of natural yeast species often focuses on substrates with abundant sugar, such as fruits, blossoms, or tree saps. Previous research has successfully isolated yeasts from various glucose-rich sources, including raisins [5], cherry blossoms [6], udachi petals [7], and the tree sap from *Castanopsis sieboldii* [8], emphasizing the importance of sugar-rich environments for the isolation of wild yeasts. Therefore, this fruit may contain several yeast species and could be a useful target for discovering new yeast species. However, no previous studies have attempted to isolate yeast from this fruit. Hence, the aim of this study was to verify whether it is possible to isolate natural yeast species from this fruit and to understand their microbiological characteristics.

2. Materials and Methods

2.1. Sample Collection and Pre-Culture

In this study, we collected fruits from a tree of *C. kousa* that grows naturally on the campus of the University of Tsukuba in Ibaraki, Japan. The tree stands at 3.9 m in height and produces a large amount of fruit during the Japanese mid-autumn season (Figure 1A). The ripe or rotten *C. kousa* fruits that had fallen naturally on the ground beneath the tree (Figure 1B) were collected. The collection was conducted on 10 November 2023 at 4:00 PM; it was a clear day with a temperature of 15.6 °C. The weight of the individual fruits was as follows: Ripe fruits 1–4 weighed 5.66, 3.15, 8.47, and 3.82 g, respectively; rotten fruits 5–8 weighed 2.64, 1.31, 1.91, and 2.29 g, respectively. The ripe fruits harvested in this study were defined as having an intact shape and as being free of invasion by microorganisms or insects. On the other hand, the rotten fruits were defined as having been invaded by microorganisms or insects.

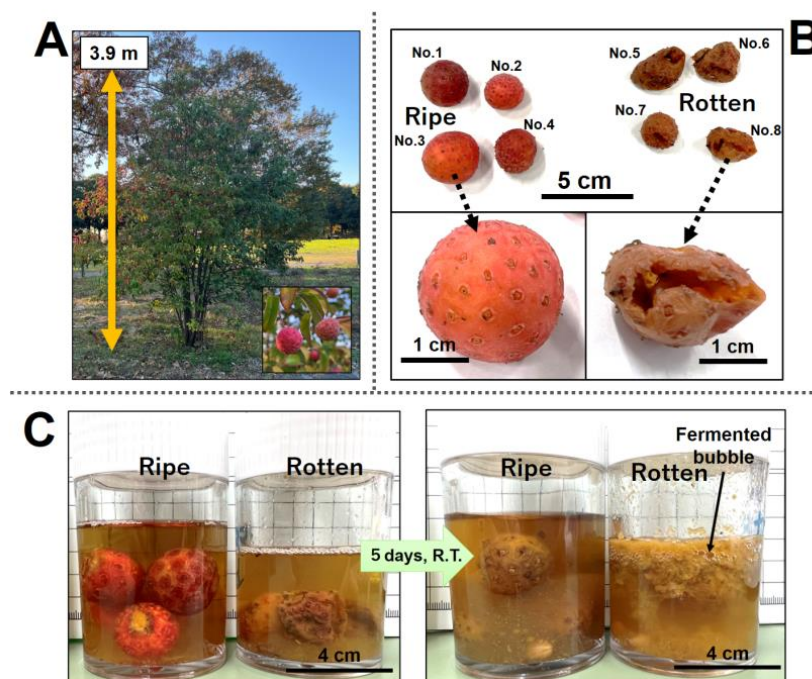


Figure 1. Harvested fruits and pre-culture in this experiment. (A) *C. kousa* tree and its fruits; (B) harvested ripe and rotten fruits; (C) pre-culture of the ripe and rotten fruits. R.T.: room temperature.

In the pre-culture experiment, pooled specimens of four ripe or rotten fruits were immersed in 50 mL of Yeast Peptone Dextrose (YPD) medium supplemented with 2% glucose and chloramphenicol at a final concentration of 10 µg/mL within a plastic container (Figure 1C). These pre-cultures were incubated at a room temperature of approximately 25 °C for five days to increase the number of yeast cells. This method comprises an enrichment culture and is useful when the number of fungi and bacteria on fruits and flowers is expected to be low. An enrichment culture with antibiotics can amplify the target microorganisms, thus increasing the efficiency of colony formation after plating. In this experiment, since the number of starting yeast species was determined to be low, this culture method was used as a pre-culture.

Throughout this incubation period, the containers were opened to permit air exchange once a day and then inverted 10 times to facilitate oxygenation.

2.2. Plating of the Pre-Culture Solutions

From the 5-day pre-cultures of *C. kousa* ripe and rotten fruits, we removed the pieces of the immersed fruits by filtering through a cell strainer (100 µL mesh holes, Cat# 3-6649-03, AS ONE, Nishi-ku, Osaka, Japan) to obtain a clear culture filtrate. The filtrates were then subjected to a 10-fold serial dilution from 1 to 1/10,000 using Milli-Q (MQ) water (Merck Millipore, Burlington, MA, USA). Finally, 100 µL of each dilution were spread on a YPD agar plate that included chloramphenicol (with the same concentration as above), followed by incubation at 30 °C in an incubator for three days to allow the growth of colonies.

2.3. Morphological Observation and Isolation of Wild Yeast Species

After yeast colony formation, the plates were visualized using a stereomicroscope to observe the colony morphology and counted the number of grown colonies. Next, we selected three colonies (three replicates; $n = 3/\text{group}$) with distinct morphologies and separately resuspended each of them in 100 µL of MQ water. The colony suspension was added in a counting chamber to observe the morphological characteristics of the isolated cells.

2.4. Sequencing

Internal transcribed spacer 1 (ITS1) was chosen to determine the identity of the isolated yeasts. To prepare genomic DNA templates, 10 µL of yeast-like cell-containing colony suspensions were subjected to DNA extraction using Cica genus DNA extraction reagent ST (Cat#08210-96; KANTO KAGAKU, Chuo-ku, Tokyo, Japan) according to the manufacturer's instructions. Then, the DNA templates were utilized to amplify the ITS1 region by performing PCR using a KOD plus kit (Cat#KOD-201; TOYOBO, Kitaku, Osaka, Osaka, Japan) and a 300 nm primer pair (forward 5'-GTAACAAGGTTTCCGT-3'; reverse 5'-CGTTCTTCATCGATC-3' [9]) in a 20 µL reaction. The reactions were conducted in a thermal cycler with the following settings: 98 °C for 2 min; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 60 s; and 4 °C continuously [9]. A sample of each of the PCR products was loaded onto a 2% agarose gel for electrophoresis followed by ethidium bromide staining. The gel was visualized under UV to detect amplification and the size of the PCR products. The rest of the PCR products were purified using NucleoSpin Gel and a PCR clean-up kit (Cat#U0609B; Takara Bio, Kusatsu, Shiga, Japan) before being subjected to Sanger sequencing using the forward primer; the latter was performed by AZENTA (Shinagawa-ku, Tokyo, Japan). The obtained nucleotide sequences were uploaded to BLAST at <https://blast.ncbi.nlm.nih.gov/Blast.cgi> (accessed on 13 December 2023) to identify the yeast species.

2.5. Metabolic Activity Assays

To investigate the metabolic characteristics of the isolated yeast species, we conducted assays to measure their cell proliferation, glucose utilization, and ethanol production. Initially, a suspension of 10^5 cells was inoculated into 6 mL of YPD medium, which

contained a glucose concentration of 10%, and shaken at 200 rpm on an orbital shaker, using a plastic tube with a half-open lid. The measurements for each metabolic parameter were taken at 7 time points, at 8 h intervals, over a 48 h period. For each time point, 150 μ L of cell suspension were sampled and used to assess the respective parameters.

The cell proliferation rate was determined by measuring the optical density at 600 nm (OD600) using a 96-well microplate, with 100 μ L of cell suspension per well. A standard curve correlating the cell numbers with OD600 values facilitated the quantification of the cell growth.

For glucose concentration analysis, we employed a LabAssay glucose kit (Catalogue #638-50971; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Following the manufacturer's instructions, the total reaction volume was adjusted to 102 μ L, with a 2 μ L template volume for each well in a 96-well microplate.

Ethanol levels were quantified using a Serotec ALC-L kit (Catalogue #A735-00; Serotec, Sapporo, Japan); the manufacturer's guidelines were followed to adjust the total reaction volume to 106 μ L, incorporating a 2 μ L template for each assay in a 96-well microplate.

2.6. Statistics

Statistical analyses were conducted using GraphPad Prism (version 10.2.0). Graphical representations of the data were generated with Microsoft Excel 2019. In the experiments ($n = 3$ /group) described in Section 2.5, the normal distributions of the data from each group at the 24 h mark were confirmed using Shapiro–Wilk tests. Subsequent analysis of variance was carried out with a two-way ANOVA. Post hoc comparisons between the means were conducted using the Tukey test, with the threshold for statistical significance set at $p < 0.05$.

3. Results

3.1. Fermentation Was Notably Observed in the Pre-Culture of Rotten *C. kousa* Fruits

The wild yeasts in *C. kousa* fruits were initially pre-cultured by immersing whole fruits in selective media to increase the number of yeast cells. After a five-day period of the pre-culture, bubbles appeared in the culture of the rotten fruits, indicating fermentation (Figure 1C). In contrast, bubbling was scarcely seen in the pre-culture of the ripe fruits. This observation suggests that there was a higher abundance of yeast cells in the decomposing fruits compared to the ripe fruits. It is plausible that the yeast population is larger in rotten fruits given that the sugar content is highest at the overripe stage [1].

3.2. Dispersion of the Pre-Culture Supernatants on Agar Media Resulted in Heterogeneous Growth of Colonies

To investigate the diversity and to isolate the yeast species present in *C. kousa* fruits, we spread dilutions of the pre-culture on selective agar media to facilitate the formation of colonies. Given the same number of immersed fruits and the same volume of pre-culture media, the total number of colonies that developed from the rotten *C. kousa* fruits was 31-fold higher than that from the ripe fruits (Figure 2A). This correlated with the robust fermentation observed in the rotten fruit pre-culture.

Next, we examined the morphological characteristics of the colonies. Under the stereomicroscope, the colonies collected from the *C. kousa* fruits were heterogeneous, with three distinct morphological types. From the ripe fruits, two types of colonies were found: one had a round shape; the other exhibited a large and irregular shape. The third colony type from the rotten fruits was round but larger than the round-shaped colonies from the ripe fruits (Figure 2B). Subsequently, the colonies were harvested as three replicates of each type (for a total of nine colonies), and they were resuspended in MQ water to observe the morphology of the cells under the microscope.

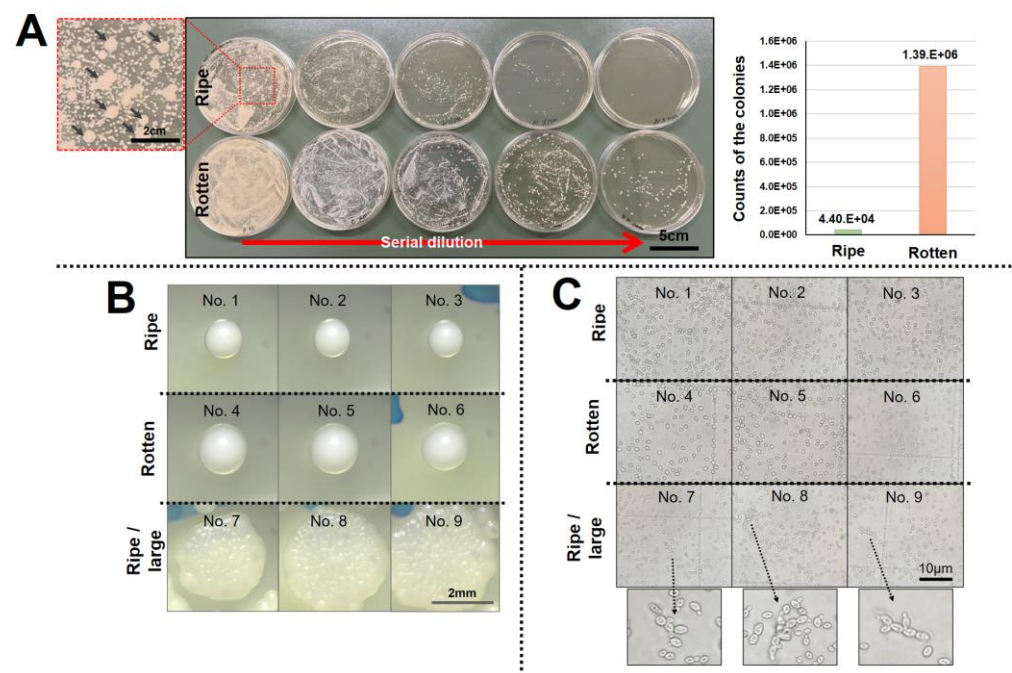


Figure 2. Colony formation and morphological examination. (A) Colony formation at 3 days after plating serial dilutions of the pre-culture solutions (left side) and colony counting (right side). The observed large and irregularly shaped colonies were from the ripe fruits, as shown in the enlarged photo (right side, arrow); (B) enlarged photos of a total of 9 colonies of each shape; (C) micrograph after each colony was suspended in MQ water.

The microscopic examination revealed that all of the colonies had budding yeast-like cells. In addition, the irregularly shaped colonies from the ripe fruits displayed cells that were joined together and aggregated (Figure 2C). The size of the yeast cells that originated from the rotten fruits was larger than that from the ripe fruits (Figure 2C). The heterogeneity in the morphology of the cells and colonies suggests that there is a diversity of natural yeast species in *C. kousa* fruits.

3.3. Genetic Analysis Revealed the Identity of Yeast Species Inhabiting *C. kousa* Fruits

To further confirm that the budding yeast-like cells were indeed yeasts, we examined the ITS1 regions from the genomes of cells from the three colony types using PCR. As illustrated in Figure 3A, the PCR target products were shown to have distinct sizes of about 400, 450, and 100 bp, indicating different yeast species in those colonies. Furthermore, to determine the identity of these yeast species, we subjected the PCR products of the ITS1 regions to Sanger sequencing. The chromatogram of the sequencing is shown in Figure 3B. The BLAST search of the sequences of the ITS1 regions identified three yeast species from the three isolated colony types, as predicted. The yeast species from the small, round colonies from the ripe fruit (No. 1 to 3; Figure 3B) were *Torulaspora delbrueckii* (T.d.). The colonies of a large and irregular shape from the ripe fruit (No. 7 to 9; Figure 3B) were *Pichia kluyveri* (P.k.). Lastly, the relatively large colonies from the rotten fruit (No. 4 to 6; Figure 3B) contained *Saccharomyces cerevisiae* (S.c.) (Figure 3C). These yeast species were subjected to the following metabolic analyses.

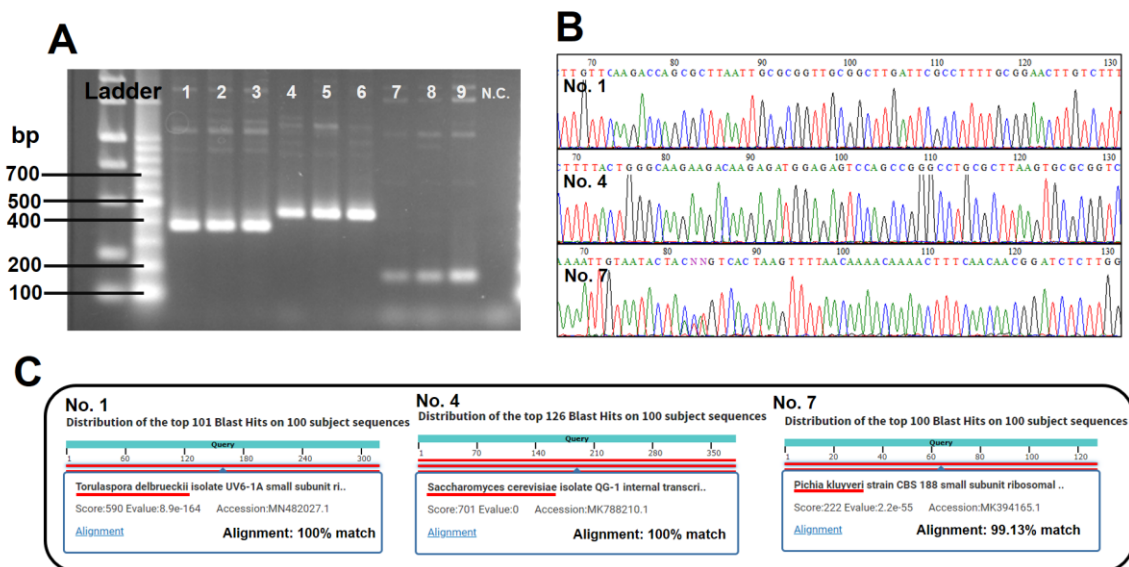


Figure 3. Genetic testing for the ITS1 region. (A) Electrophoresis photo showing that the lane and colony numbers correspond (No. 1 to 9); N.C. means negative control. (B) Chromatograms of the Sanger sequences of representative samples. (C) Results of the BLAST search with alignment rates (%) and their accession numbers, which identified each yeast species.

3.4. The Identified Yeast Species Exhibited Variable Rates of Proliferation, Alcohol Production, and Glucose Utilization

Each assay characterized the metabolic state of each yeast species. The cell proliferation assays showed that *P.k.* had the fastest cell proliferation rate and that *T.d.* had the slowest rate at 24 h; on the other hand, there was a slight difference between *S.c.* and commercial dry yeast (*D.y.*). The growth of all of the yeast species was saturated at 24 to 32 h (Figure 4A).

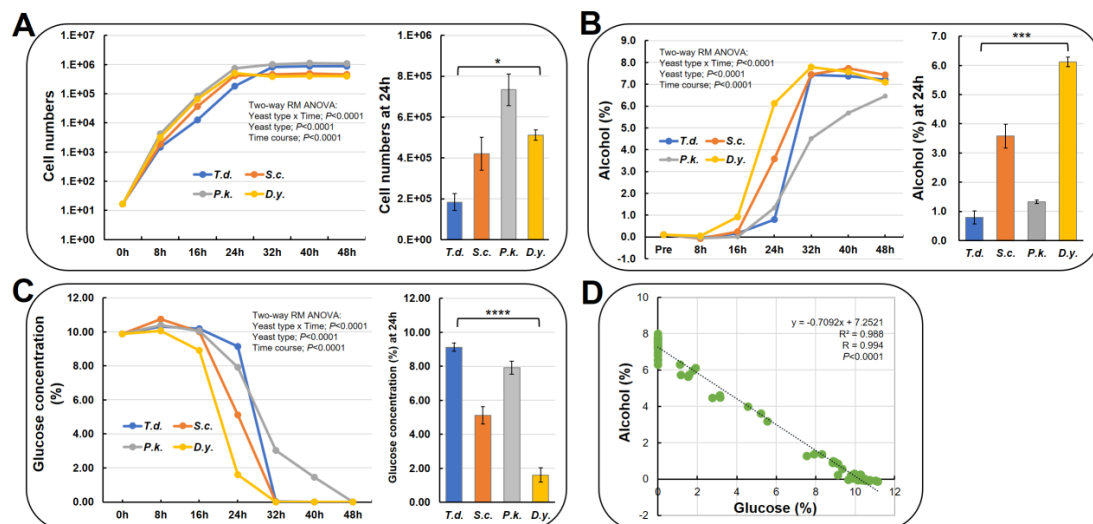


Figure 4. Characterization of the metabolic activity. (A) Cell growth curve (left) for 48 h and average cell numbers of each yeast species ($n = 3/\text{group}$) at 24 h; (B) alcohol-producing curve for 24 h and average alcohol (%) of each yeast species ($n = 3/\text{group}$) at 24 h; (C) glucose consumption (glucose concentration) for 48 h and average glucose (%) of each yeast species ($n = 3/\text{group}$) at 24 h; (D) correlation analysis between alcohol (%) and glucose (%), and a total of 72 samples were plotted. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ on all group pair comparisons. *T.d.*: *Torulaspora delbrueckii*; *P.k.*: *Pichia kluyveri*; *S.c.*: *Saccharomyces cerevisiae*; and *D.y.*: dry yeast (commercial yeast).

The alcohol-producing capacity at 24 h was highest in *D.y.*, followed by *S.c.* All of the yeast species isolated in this study had an alcohol-producing capacity, with concentrations ranging from 6.3 to 7.5% at 48 h (Figure 4B).

The glucose consumption ability corresponded to the capacity for alcohol production. In contrast to the alcohol production results, at 24 h the glucose in the medium was lowest in *D.y.*, followed by *S.c.* All of the yeast species exhibited glucose consumption, and all glucose was almost completely consumed by *T.d.*, *S.c.*, and *D.y.* but not by *P.k.* at 32 h (Figure 4C). The alcohol concentration showed a significant and strong correlation with the glucose consumption (Figure 4D).

These results indicate that all of the yeast species proliferate rapidly and have alcohol production and glucose consumption abilities.

4. Discussion

This research describes the successful first attempt to isolate a yeast species from *C. kousa* fruits in Japan. We established these yeast strains and gave them three names, as shown below. *T.d.* and *P.k.* were isolated from fresh fruit and named TX-Y-1 and TX-Y-2; *S.c.* from rotten fruit was named TX-Y-3. Moreover, we sought further analysis and social implementation, and this yeast can be provided to researchers around the world for use in collaborative research. In addition, we encourage interested researchers to contact us.

Among the three identified yeast species, two species, *T.d.* and *P.k.*, coinhabited in the ripe fruit, while *S.c.* was observed mainly in the rotten fruit. This suggests that *S.c.* potentially outcompetes other yeast species. However, whether *S.c.* has a faster growth efficiency or possesses mechanisms to inhibit the proliferation of other yeast species still needs to be clarified. Elucidating these factors in subsequent research could provide valuable insights for the development of improved yeast collection methods. In this study, we were able to isolate three yeast species from *C. kousa* fruits. Furthermore, it was demonstrated that the yeast species that could be isolated depending on the rotting state of the fruit. Therefore, our findings would be of great help to other researchers in their attempts to isolate yeast species from other fruits, not only from *C. kousa*, in order to increase their success rate. In particular, targeting rotten fruit to try to isolate yeast species is a reasonable strategy.

We obtained important findings by observing the colonies using a stereomicroscope. In this study, as shown in Figure 2, two round colonies were observed, each with a different size. Irregularly shaped colonies were also observed. Genetic testing showed that the three distinctly shaped colonies were different yeast species. Moreover, glucose consumption and alcohol production ability also differed among the yeast species. Hence, it is highly likely that the size and shape of colonies indirectly reflect the functions and characteristics of the yeast species. Therefore, it is very important to carefully observe the size and shape of colonies when attempting to isolate wild yeast. This is because the types of yeast that is isolated differs depending on the shape and size. As in the case of this experiment, multiple yeast species may be present in a collected sample, and careful observation of colonies using a stereomicroscope may lead to the successful isolation of more yeast species. In particular, since *P.k.* was recessive, with relatively few colonies formed, careful observation, as in this experiment, is reasonable when isolating recessive yeast species.

The relevant literature includes investigations into the interaction between *S.c.* and *Aspergillus carbonarius*, a pathogenic fungus. For instance, one study [10] demonstrated that *S.c.* could hinder the growth of the fungus and decrease its production of Ochratoxin A (OTA), a mycotoxin. These findings suggest that *S.c.* may impede the growth of other yeast species as well. Furthermore, it raises the possibility that the *S.c.* strains identified in the current study could be employed to prevent infections caused by pathogenic fungi. Additionally, the present study showed a clear difference in the yeast community between the ripe and rotten fruits. Therefore, this suggests the possibility of a new approach that targets the different maturity stages of fruits to improve the success rate of yeast isolation and examines the diversity of yeast species inhabiting the fruit.

The *T.d.* isolated in this study could be used in the production of wine. *T.d.* is probably the most widely used non-*Saccharomyces* yeast in wine production today. Moreover, it has been claimed that it has multiple advantages over traditional *S. cerevisiae* strains [11,12]. *T.d.* has been shown to be relatively smaller in size and more like budding yeast than *S.c.* [11], which is consistent with the results of this study. Accordingly, there is a possibility that this *T.d.* can also be utilized in wine production. Another possibility is to use both *T.d.* and *S.c.* to make wine, which seems to be an excellent approach. A previous study has shown that a multi-culture of *T.d.* with different *S.c.* strains possessing distinct aromatic characteristics, could generate the desired and diversified aromatic profiles of the final wine [12]. Therefore, we are considering the use of both yeast species in our future studies, with the aim of producing original wines. We are then going to use mass spectrometry to analyze in detail the taste and odor molecules that characterize the wines produced by these yeast species.

P.k. has also frequently been used in winemaking. It has become increasingly popular in winemaking and wine quality improvement, and the strains are sold commercially [13]. The metabolism of this yeast allows it to produce various volatile molecules such as esters and varietal thiols, which increase the quality of specific varietal wines or neutral ones. However, it is considered to be a low- or non-fermentative yeast, and subsequent inoculation with a more fermentative yeast such as *Saccharomyces cerevisiae* is indispensable to the achievement of a properly fermented alcohol [13]. Therefore, by combining the *P.k.* and *S.c.* that we isolated in this study, fruity and aromatic wine could be produced.

P.k. was among the yeast species isolated in this study. As shown in Figure 2A, it has a distorted shape, and only about 30 colony traces were observed. It is therefore a recessive yeast. However, it had the highest growth rate on the YPD medium and the lowest alcohol production and glucose consumption. Therefore, this yeast species may have a metabolic profile in which cell proliferation is a priority. Health foods that incorporate various yeast species have become a hot topic in recent years due to increasing health literacy. Consuming dried yeast is thought to have some health benefits. If researchers want to create a supplement that requires the ingestion of whole yeast, it is recommended that they choose a species that multiplies quickly, such as *P.k.* If it were to be put on a production line, its rapid proliferation would create the possibility of significantly reducing costs. We are aiming for the industrial application of these yeast species and believe that *P.k.*, which proliferates rapidly, is a suitable ingredient for health supplements. We are developing the next strategy.

Several previous reports investigated improvement in the intestinal environment by yeast species. In an experiment in which rats were fed beer yeast alone or in combination with yogurt, bowel movements and symptoms of constipation were improved [14,15]. In addition, data from other studies in humans have been reported. In a randomized, double-blind, placebo-controlled trial, 500 mg per day of EpiCor® fermentate (yeast ferment; Cargill, Incorporated, MN, USA) taken orally by humans improved bowel discomfort and stool consistency and frequency, and relieved constipation symptoms [16]. These animal and human results suggest that yeast species definitely improve the human intestinal environment. *S.c.* was used in these studies. Therefore, the strain of *S.c.* found in this study could also be utilized in the development of supplements that help to improve the intestinal environment. However, it is unclear whether the *T.d.* and *P.k.* found in this study might also be effective in relieving constipation.

In general, yeast species tend to have many dietary fibers (β -glucan) in their cell walls; β -glucan can improve constipation, and it is also used in various commercially available health supplements. Therefore, *T.d.* and *P.k.* may be useful supplements for improving the intestinal environment and relieving constipation. Further animal studies, including safety studies, are needed to confirm this.

Additional studies are required to confirm the potential application of yeast strains isolated from *C. kousa* fruit in the wine and food industry. Therefore, we offer the yeast

strains isolated in this study to the global research community for collaborative projects. Researchers with an interest in this area are welcome to contact us for potential collaboration.

5. Conclusions

In this study, the fruit of *Cornus kousa* were shown to be a novel source of diverse natural yeasts. In particular, *Torulaspora delbrueckii* and *Pichia kluyveri* were isolated from ripe fruit, while *Saccharomyces cerevisiae* was isolated from rotten fruit. These yeasts could have valuable applications in various industries, such as food and beverage production or biotechnology. Finally, this study offered a valuable method of targeting fruits at various stage of ripeness from specific trees for discovering wild yeast strains and exploring the diversity and dynamics of yeasts residing on fruits.

Author Contributions: Conceptualization, T.S. and N.O.; methodology, T.S., N.O., H.-S.Y. and Y.M.; validation, T.S., N.O. and K.D.M.N.; formal analysis, T.S., N.O., K.D.M.N. and A.H.; investigation, T.S., N.O. and K.D.M.N.; writing—original draft preparation, T.S., N.O., A.H. and K.D.M.N.; writing—review and editing, T.S., N.O., K.D.M.N., A.H., Y.T., Y.K., H.-S.Y., W.G. and Y.M.; visualization, T.S., N.O., A.H. and K.D.M.N.; supervision, T.S. and Y.T.; project administration, T.S. and N.O.; funding acquisition, N.O., Y.T., Y.K. and T.S. All authors have read and agreed to the published version of the manuscript.

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