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Research article

Polyphasic identification of a *Zygosaccharomyces rouxii* isolated from grape juice concentrate and its control using thermal processing

Wu-Joo Lee[1], Barbara Nielsen[1], Frank L. Younce[2], Charles G. Edwards[2], and Gülhan Ünlü[1,2,3,*]

[1] Department of Animal, Veterinary and Food Sciences, University of Idaho, Moscow, USA
[2] School of Food Science, Washington State University, Pullman, USA
[3] Department of Chemical and Biological Engineering, University of Idaho, Moscow, USA

*** Correspondence:** E-mail: gulhan@uidaho.edu; Tel: +12088857771; Fax: +12088856420.

Abstract: A yeast, isolated from grape juice concentrate by a grape juice processing company in central Washington, was subjected to thermal processing in 52° Brix grape juice concentrate, commercial grape juice, and Yeast-Mold (YM) medium to determine *D*-values and *z*-values in the search for an effective means of control. At 50 ℃ in grape juice concentrate the *D*-value was 33.44 minutes, dropping rapidly as the temperature increased to 52.5 ℃, 55 ℃, 57.5 ℃, and 60 ℃. From this data, a *z*-value was determined to be 4.38 ℃. Grape juice and YM broth were similarly tested. Commercial grape juice at 45 ℃, 47 ℃, and 50 ℃ showed *D*-values of 9.80, 7.68, and 1.42, respectively, resulting in a *z*-value of 5.69 ℃. The isolate heat treated in YM broth at 40 ℃, 45 ℃, 46 ℃, 47.5 ℃, and 50 ℃ showed no effect at 40 ℃ but at higher temperatures, with a *D*-value of 2.28 at 45 ℃, 1.61 at 46 ℃, 1.05 at 47.5 ℃, and with a *z*-value of 7.46 ℃. This data suggests that an increase in the concentration of sugars in the environment has a protective effect on the yeast against elevated temperatures. Even so, pasteurization at 90 ℃ for thirty seconds appears to be an effective means of control for the yeast investigated. A yeast with the identical colony and cell morphology was isolated from 52° Brix grape juice concentrate from the same grape juice processing company. Both isolates were subjected to 28S DNA sequencing and FAME analysis for identification and determined to be *Zygosaccharomyces rouxii*, a known problem yeast in the grape juice and wine-making industry, to this point unknown in the central Washington region.

Keywords: *Zygosaccharomyces rouxii*, spoilage yeast, grape juice concentrate spoilage, osmotolerance, sour rot, thermal resistance, thermal processing, *D*-value, *z*-value, polyphasic identification

1. Introduction

Grape juice concentrate (GJC) is one of the major products of the grape juice industry. GJC is an essential component of many products in the food, beverage, and pharmaceutical industries due to its versatility and stability. Juice concentrates are considered more stable than other juice products due to high sugar concentrations, lower water activity (a_w), and low pH naturally preserving these products. This commonly allows for room temperature storage, and coupled with greatly reduced volumes also minimizes transportation and storage costs [1–3].

Zygosaccharomyces rouxii has been identified as the main spoilage yeast in grape juice concentrates (GJC) [1]. While little has been reported on the ecology of *Z. rouxii* in the environment or in the production facilities [1], a study examining yeast existence in spoiled grape juice concentrates in Argentina identified *Z. rouxii* as the only yeast isolated [2]. While samples without visible spoilage had a more diverse yeast population, *Z. rouxii* was isolated at a higher frequency [3]. *Zygosaccharomyces* contamination appears to be limited to grapes damaged by sour rot [5,6].

Z. rouxii is a serious problem in the fruit juice industry due to its extreme osmotolerance and its ability to grow at low pH levels, as low as 1.5 under some conditions, and low temperatures, as low as 5 ℃ [2,4]. Isolates of *Z. rouxii* are usually under the detection level (<1 CFU/50 mL) in freshly prepared grape juice (preconcentrate) but tend to predominate during subsequent processing steps [1]. *Z. rouxii* spoilage in grape juice is fermentative, characterized by the presence of alcoholic, esoteric, or other unwanted odors, and/or gas production leading to bubbling and foaming, with possible expansion and deformation of packaging [1,7].

Martorell et al. showed that some strains of *Z. rouxii* can grow in microbiological media containing 5M glucose (90% w/v; 900 g/L or 90° Brix) [8]. Rojo et al. demonstrated that storage for 1–2 weeks at 5 ± 3 ℃ allowed an increase of the *Z. rouxii* concentration from less than 1 cell to greater than 4 x 10^4 cells in 50 g GJC [1]. Dakal et al. report that *Z. rouxii* survives at a water activity (a_w) of 0.80 in the presence of ionic solutes (salts) and all the way down to a_w of 0.65 in the presence of non-ionic solutes (sugars) [9]. Growth also occurred in holding tanks during steps in the pre-concentration and concentration processes at 5 ℃ [1]. Moreover, the concentration of sugars appears to have a protective effect on *Z. rouxii*, protecting the yeast from extremes in pH and temperature, as demonstrated in Shearer et al. [5] and Corry [6].

Rojo et al. found that pH was the environmental factor that had the highest impact on delaying the spoilage of a product by *Z. rouxii*. At a high Brix of 64–68°, the pH would have to be less than 1.7 to eliminate the risk of spoilage by *Z. rouxii* [2]. A pH of 2 would provide delay in spoilage for a limited time at isothermal conditions, perhaps long enough to satisfy the consumer. Due to *Z. rouxii*'s tolerance of low pH, weak acids commonly used as preservatives in sugar-containing low pH foods (such as fruit juices, beverages, wine, dressings, and sauces) are ineffective [8].

Z. rouxii has been shown to be sensitive to elevated heat. Jermini and Schmidt-Lorenz concluded that no osmotolerant yeast cells or spores should be encountered after mild heat treatments of 1.5 h at 60 ℃, 40 minutes at 61 ℃, 20 minutes at 62 ℃, or 5 minutes at 63 ℃, at a_w of 0.85 and pH 4.5 [7]. Rojo et al. found that thermal pasteurization could be lethal for their assessed population of *Z. rouxii* in 68° Brix concentrated grape juice (pH 3.2), with a 7-log reduction in 90 seconds at 75 ℃ or 5 seconds at 85 ℃. 75 ℃-pasteurization of concentrated grape juice for 160 seconds (10 seconds at 85 ℃) led to a 12-log reduction of *Z. rouxii*, achieving commercial sterilization. Temperatures below 75 ℃ were not shown to be lethal in the study [8].

Understanding possible contaminants encountered in grape juice processing is necessary for their control. *A problem yeast* was recently found in a 52° Brix grape juice concentrate sample from central Washington. If left unchecked, the spoilage could potentially affect several thousands of gallons of concentrate, with its accompanying economic impact. The objectives of this study were to identify, using the polyphasic approach, the causative agent and determine its thermal resistance (*D*-values and *z*-values) to aid in the control of this undesirable contaminant.

2. Materials and methods

2.1. Microorganisms, microbiological growth media, and growth conditions

All yeasts were cultured using yeast-mold (YM) broth (Becton-Dickinson, Sparks, MD, USA) or YM broth supplemented with 1.5% agar (Fisher Bioreagents, Fair Lawn, NJ, USA) prior to aerobic incubation at 30 ℃. *Zygosaccharomyces rouxii* 10687 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Frozen stocks were prepared by growing yeast in YM broth at 30 ℃ to mid-late logarithmic growth stage before adding sterile 50% (v/v) glycerol and storage at -80 ℃. Additional agar media obtained from Becton-Dickinson were potato dextrose agar (PDA), Dichloran Rose Bengal chloramphenicol (DRBC), and yeast extract-peptone-dextrose (YPD). Bacteria were cultured using PDA (due to them being initially isolated off of PDA) and tryptic soy agar (TSA, Criterion, Hardy Diagnostics, Santa Maria, CA, USA) and grown 3 days at 30 ℃.

2.2. Isolation and characterization of microorganisms in GJC

Three containers representing samples of 52° Brix GJC were obtained from a central Washington grape juice processor. After mixing each by shaking, samples were removed from all three containers and transferred to YM, PDA, DRBC, and YPD agars for growth at 30 ℃ under aerobic and anaerobic conditions, the latter using BBL GasPak jars with BD GasPak anaerobic sachets with indicator #260001 (Both Benton-Dickinson, Sparks, MD, USA).

2.3. Genotypic and phenotypic identification of yeast and bacterial isolates

To confirm the identity of the industrial isolate studied, one colony provided by the grape juice processing company was prepared according to the protocol on the MIDI Labs (Newark, DE, USA) web page for polyphasic testing. The two yeast isolates from the GJC, designated JC1 and JC2, detailed below, were also sent to MIDI Labs for identification. Polyphasic testing involves both 28S rRNA gene sequencing (genotypic method) and Fatty Acid Methyl Ester (FAME) analysis (phenotypic method) independently run to increase the accuracy of the identification.

Bacterial isolates designated JC3, JC4, and JC5 from GJC were also identified at MIDI Labs using MALDI-TOF (matrix-assisted laser desorption ionization-time of flight mass spectrometry) analysis. Two of the bacterial isolates needed further identification so 16S rRNA gene sequencing was performed at MIDI Labs.

2.4. Generation of survival and thermal death time curves

An overnight *Z. rouxii* culture was grown at 30 ℃ under agitation (220 rpm), then cooled on ice. Cell density was determined by spectrophotometry (at 600 nm), then cells were diluted to a concentration of 10^5 CFU/mL in (1) 52° Brix GJC, (2) 16–18° Brix commercially available grape juice (GJ, Welch's 100% grape juice), or (3) YM broth (less than 1.2° Brix, with glucose and malt extract accounting for the sugars). 1 mL aliquots were prepared for heat treatment. A Fisher dry block incubator fitted with an aluminum heat block sized for 1.5 mL microcentrifuge tubes (Dry Block Incubator Fisher Scientific #11-718-2; Microfuge tubes Fisher Scientific, # 05-408-129) was utilized for temperature maintenance. 400 µL deionized H_2O was added to each cell of the block to assure consistent thermal contact with the tubes to aid in rapid heat transfer and temperature stability of samples. The temperature of the sample was monitored and recorded at 6-second intervals using a type T thermocouple connected to a data acquisition module (model USB-Temp, Measurement Computing, Norton, MA, USA). Temperatures employed included 40, 42.5, 45, 47.5, 50, 52.5, 55, 57.5, 60, and 70 ℃. Samples were removed from the heat block and placed on ice to stop the heat treatment, serially diluted in 0.1% sterile peptone water (Difco, Becton-Dickinson, Sparks, MD, USA), and plated in duplicate by spreading onto YM agar. The plates were incubated at 30 ℃ for 72 hours. The resulting yeast colonies on YM agar plates were enumerated and the $\log_{10}$ number of viable yeast cells was graphed against the time of heating to generate the survival curves and their accompanying *D*-values (the time required at heating temperature to reduce the number of viable yeast cells 10-fold (1 log or 90%). The logarithms of *D* values plotted against the heating temperatures led to the thermal death time curves illustrating the *z* values, the number of degrees of temperature change necessary to change the *D*-value by a factor of 10.

2.5. Heat resistance/sensitivity of bacterial isolates

All three bacterial isolates were inoculated in duplicate in 5 mL tryptic soy broth (TSB) and incubated overnight at 30 ℃ and 220 rpm. 100 µL of overnight culture was added to 900 µL of TSB, GJ, and GJC in 1.5 mL microcentrifuge tubes, mixed well, and heat treated at 65 ℃ (6 minutes), 75 ℃ (4 minutes), 85 ℃ (1 minute), and 90 ℃ (0. 5 minutes). After treatment, 100 µL samples were removed from the treatment tube and spread on the surface of YM agar plates and incubated at 30 ℃ for 24 hours. 100 µL samples were also added to 2 mL YM broth and incubated at 30 ℃ for 20 hours at 220 rpm in a shaking incubator. YM medium was selected as it was the medium used when initially screening for yeast in GJC and the bacteria was isolated initially on it.

2.6. Testing for germination of ascospores after heat treatment

Using the temperature data collected above, a test was devised to check for post-heat treatment ascospore germination with *Z. rouxii* industrial isolate. GJC was challenged with the isolate at a concentration of 10^5 CFU/mL. Heat treatments were performed in a Fisher dry block incubator described above as follows: 65 ℃ (6 minutes), 75 ℃ (4 minutes), 85 ℃ (1 minute), and 90 ℃ (0.5 minutes). *Z. rouxii*-challenged, and heat-treated GJC samples (1 mL) were incubated at 30 ℃ for up to 5 weeks to encourage the germination of any ascospores that may be present in the heat-treated GJC. Weekly, starting at 0 weeks, 10 YM plates were each spread with 100 µL of sample and incubated for 3 days at 30 ℃. In addition, 5 mL YM broth was inoculated with 250 µL sample in duplicate and

incubated at 30 ℃ under 220 rpm agitation. 1 mL of this yeast culture was then pelleted at 10,000 rpm for 10 minutes using a bench-top microcentrifuge (Eppendorf, Model 5415, New York, USA) to visually check the appearance of any yeast by means of phase-contrast and light microscopy using a Zeiss AXIO Imager A2 microscope (Jena, Germany).

2.7. Assessment of the viability of yeast cells

The *Z. rouxii* industrial isolate, along with a known strain of *Z. rouxii* (ATCC 10687), were grown in YM broth at 22–24 ℃, 220 rpm agitation, and standardized to a concentration of 10^5 CFU/mL for a cell viability study. Cells were heat-challenged at 65 ℃ and 85 ℃ using a Fisher dry block incubator, with samples periodically removed and placed on ice to stop the heat reaction. Unheated samples of the industrial isolate and the ATCC culture were used as controls. Samples were centrifuged (1 minute, 13,200 rpm) to pellet in an Eppendorf microcentrifuge (Eppendorf, Model 5415, New York, USA) and most of the liquid was removed. Yeast cells were resuspended in a small volume of YM broth, then stained with 0.1% methylene blue (Alfa Aesar, Haverhill, MA, USA, 10µL/100µL sample) on a microscope slide. After a 5-minute incubation, wet mounts were observed at 400X on a light microscope (Olympus BH-2, Shinjuku, Tokyo, Japan) for cell viability. Dead cells (blue- unable to eliminate methylene blue) were differentiated from viable cells (colorless, methylene blue metabolized) [14,15].

3. Results

3.1. Isolation and characterization of microorganisms in GJC

Two yeast colony morphologies were isolated from the 52° Brix GJC. The first isolate (designated JC1) produced approximately 3.477 log CFU/mL small (2–3 mm in diameter), round, drop-like, smooth, and milky-white colonies on YM agar, similar to the isolate provided by a grape juice processing company in central Washington. The second isolate (designated JC2), approximately 2.057 log CFU/mL, formed larger (4–5 mm in diameter) colonies on YM agar, similar in appearance to JC1 and exhibiting a yeasty aroma. Three distinct types of bacterial colonies designated JC3, JC4, and JC5, were also isolated on YM agar in very small concentrations, less than 1 CFU/mL. The bacteria were isolated on YM medium, usually used for culturing yeasts, fungi, and molds. We continued to work on YM for consistency. All isolates were examined under light microscopy. Isolates JC1 and JC2 were confirmed as actively budding yeast.

3.2. Genotypic and phenotypic identification of isolates

All yeast isolates were sent to MIDI Labs for polyphasic testing. JC1 and the industrial isolate obtained from the grape juice processing company were identified as *Zygosaccharomyces rouxii*.

The second yeast isolate, JC2, showed more ambiguity, with the closest match being *Saccharomyces bayanus/pastorianus*. These are common yeasts used in winemaking and lager beer brewing and are readily available in the environment [9].

Isolate JC3 (3.477 log CFU/mL) showed 8-13 mm in diameter, off-white, concentric, flat to slightly raised, wavy, wrinkled colonies, dry in appearance on the top, but viscous and slimy underneath, exhibiting a possible zone of inhibition of 4–7 mm diameter on spread plates—likely

against the predominant yeasts. Staining showed Gram (+) thin rods of various lengths and many spores. Three representative isolates of isolate JC3 were sent to MIDI Labs for MALDI-TOF analysis where all were identified as *Bacillus amyloliquefaciens*. Interestingly, this bacterial species is known to produce a couple of known antibiotics, barnase, and plantazolicin [10], possibly explaining the exhibited zone of inhibition.

Isolate JC4 (less than 1 CFU/mL) was flat to slightly raised, beige, 4 mm in diameter, smooth and round. It exhibited no growth on DRBC but grew similar colonies when streaked on YM, PDA, and YPD (colonies slightly larger on YM). Staining showed Gram (+) uniform medium rods in clusters with spores. Isolate JC4 was identified as *Bacillus pumilus*, a common soil bacterium, at MIDI Labs using MALDI-TOF and 16S rRNA gene sequencing.

Isolate JC5 (less than 1 CFU/mL) was very small, only 1–2 mm in diameter, round, smooth, and slightly raised. No growth occurred on DBRC. Growth on PDA and YPD resembled that on YM. This isolate was sometimes difficult to produce, not always growing in broth medium from a picked colony. Staining revealed Gram-variable medium to long rods with subterminal spores in singles and pairs. Staining was indeterminate, sometimes appearing mixed. Some had a segmented appearance. MALDI-TOF identification at MIDI Labs found the closest match to be *Paenibacillus rhizosphaerae*, with a confidence level of "species, closely related." 16S rRNA gene sequencing, ordered to help clarify, found the closest match to be *Paenibacillus cineris*, again with a confidence level of "species, closely related." The Gram-staining results being varied appears to be typical for this organism. Several instances in the literature reviewed refer to it as being "varied" [18,19]. *Paenibacillus* species have been isolated in a wide variety of climate zones from the Arctic to the tropics and from marshes to deserts. Most are soil-borne, associated with plant roots where they have been shown to promote plant growth [11].

3.3. Generation of Survival and Thermal Death Time Curves

The industrial yeast isolates identified as *Z. rouxii* was heat treated in YM broth at 40 ℃, 45 ℃, 46 ℃, 47.5 ℃, and 50 ℃. The heat treatment showed no effect at 40 ℃, a *D*-value of 2.28 at 45 ℃, 1.61 at 46 ℃, 1.05 at 47.5 ℃ (Figure 1A), and total death at 50 ℃. The *z*-value was calculated to be 7.46 ℃ (Figure 2).

The industrial yeast isolates heat-treated in commercial grape juice at 45 ℃, 47 ℃, and 50 ℃ showed *D*-values of 9.80, 7.68, and 1.42, respectively. (Fig. 1B) These readings result in a *z*-value of 5.69 ℃ (Figure 2).

The industrial isolate was unaffected by heat treatment when tested in GJC until the temperature approached 50 ℃. At 50 ℃ the *D*-value was calculated to be 33.44 minutes. The *D*-value rapidly dropped as the temperature increased, 6.84 minutes at 52.5 ℃, 1.04 minutes at 55 ℃, and 0.714 minutes at 57.5 ℃. (Figure 1C) All cells were killed before the temperature reached 65 and 70 ℃. From this data, a *z*-value was calculated and determined to be 4.38 °C (Fig. 2). The thermal death curve generated with heat treatment in YM broth appeared to be much steeper than the curve generated with heat treatment in GJC (Figure 2).

3.4. Testing for germination of ascospores after heat treatment

After the plating and incubation of the yeast cells heat treated at 65 ℃, 75 ℃, 85 ℃ and 90 ℃, there were no colonies on any plate for weeks 0–5. This indicates that there were no surviving yeast cells or their ascospores after heat treatment at the previously described times. Microscopy performed

on stained and wet mounts of the pelleted heat-treated and incubated cells revealed intact yeast cells in all samples, but as nothing grew when these heat-treated cells were inoculated in YM broth, a medium known to support the growth of this yeast, it appears that the yeast cells were totally inactivated.

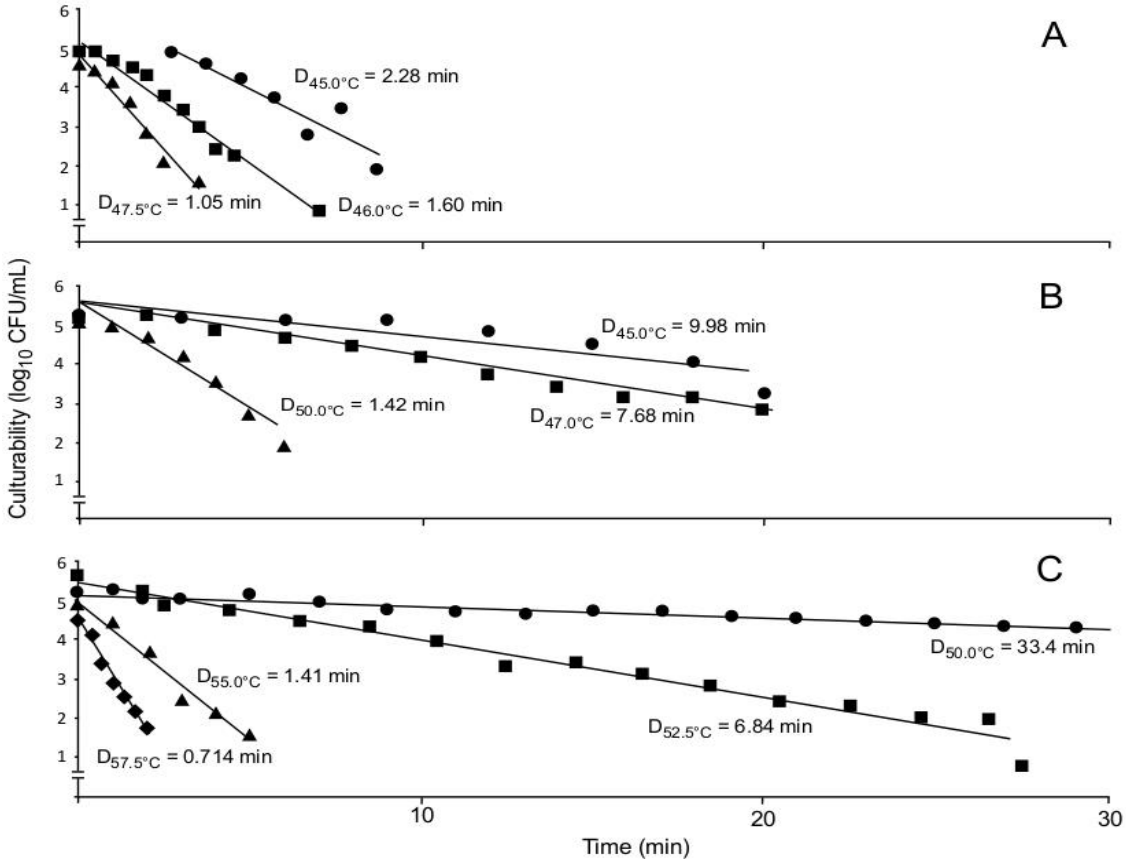

Figure 1. *Z. rouxii* was heat treated in YM media (1A), commercial grape juice (1B), and 52º Brix grape juice concentrate (1C). Survival curves were generated, and D-values were calculated as shown.

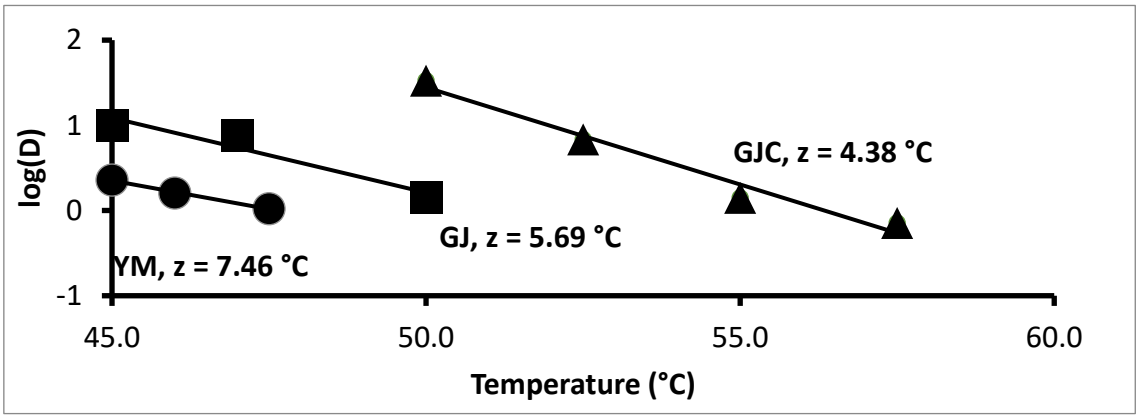

Figure 2. z-values, as calculated from log(D) values vs. temperature obtained from heat treatments of *Z. rouxii* in YM media, commercial grape juice (GJ), and 52°º Brix grape juice concentrate (GJC).

3.5. Assessment of the viability of yeast cells

At 5 minutes of incubation, with very few exceptions (<1/100), all methylene blue had cleared from the control yeast cells (not subjected to any heat treatments) in both the industrial isolate and the ATCC culture of *Z. rouxii*. Methylene blue is differential in that it will initially stain both living and dead cells, but cellular metabolism will clear out the stain in living cells, which appear colorless, while dead cells will still be stained blue. This method has been in common use for decades, is rapid, and delivers results almost as accurate as culturing methods [14,15]. This clearing of the untreated cells indicates that the cells were living before being subjected to heat treatment. Timepoint 0 was taken immediately as the test samples reached the desired temperatures of 65 ℃ and 85 ℃. At time 0, all yeast cells remained deep blue after the 5-minute incubation, indicating that the cells were dead. This result was true at all time points. Interestingly, the shape of the dead yeast cells appeared normal with budding evident. This matches the results we observed in previous heat treatments, where the cells appeared normal under the microscope (Figure 3) but there was no growth of any yeast when the samples were plated on YM agar or introduced into YM broth. The cells, while appearing normal under microscopy, were no longer viable, or at least were no longer metabolizing or culturable.

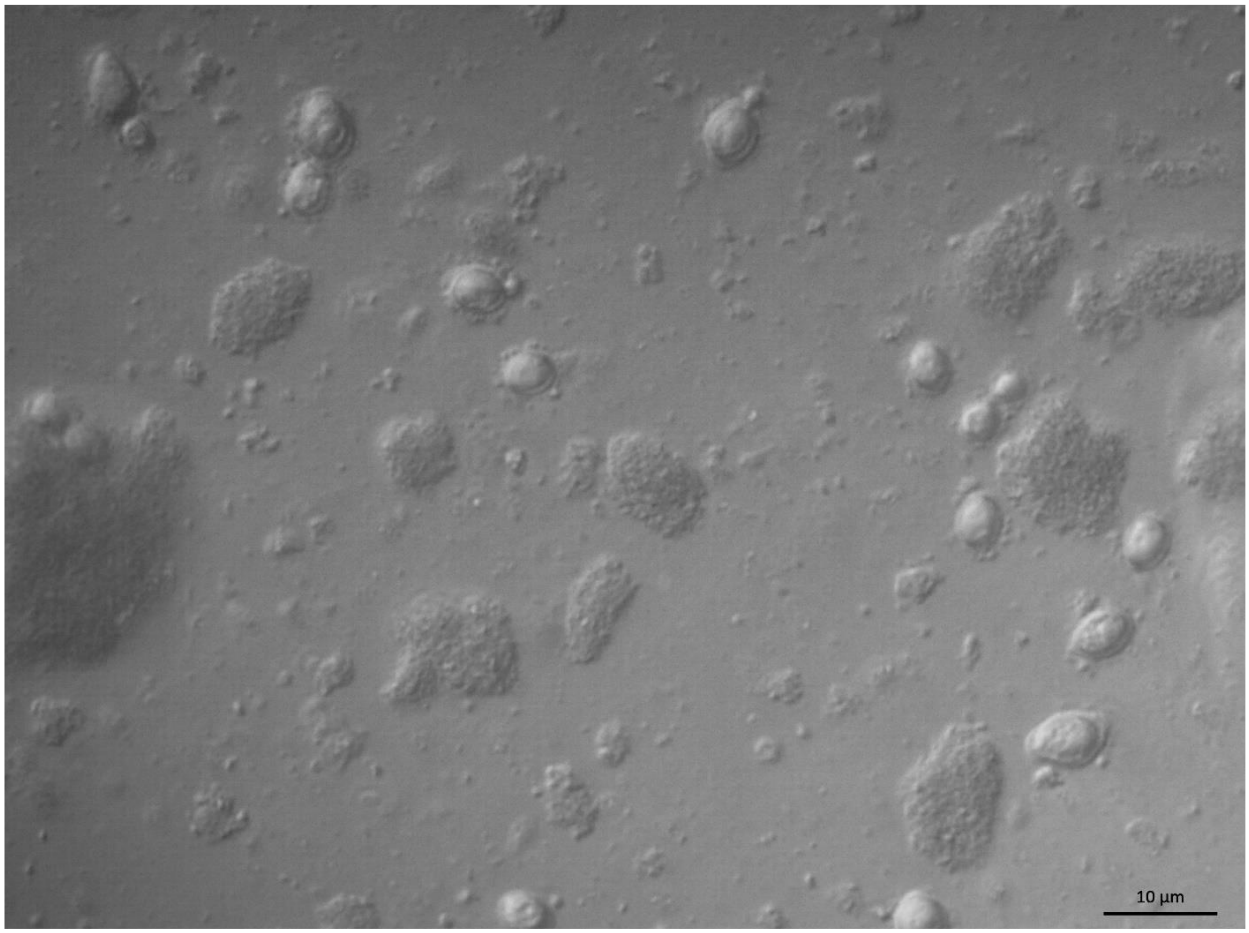

Figure 3. 85 ℃ treated *Z. rouxii* in GJC after 3-week incubation at 30 ℃. Yeast cells still exhibit normal morphology with evident budding but are not viable.

4. Discussion

With the identification of JC1 as *Z. rouxii*, this is the first known isolation of this problem yeast from a central Washington state grape juice concentrate producer. Signs of spoilage by *Z. rouxii* are consistent with the excessive CO_2 production observation made by the grape juice processing company which first isolated the organism and provided samples of it for this study. *Zygosaccharomyces* spp. have been shown to be tolerant of extreme conditions. Zuehlke et al. report a *Zygosaccharomyces* species, *Z. bailii*, showing exceptional tolerances to ethanol and glucose, and exhibiting growth over a pH range of 2.5 to 7 at 22 ℃ and 3.5 to 4.5 at 8 ℃ [12].

All of the *D*-values resulting from the yeast experiments above appear to be in the range of those seen in *Z. rouxii* by Jermini and Schmidt-Lorenz, who performed their experiments using a variety of broth and agar media of differing water activities (malt extract, mycophile, yeast extract, supplemented with glucose to vary the a_w from 0.963–0.858) [7]. As can be seen in our results, the increase in the concentrations of sugars (YM broth at less than 1.2° Brix < commercial grape juice at 14–16° Brix < GJC at 52° Brix) appears to have a protective effect on the survival of the yeasts, as the yeast heat treated in GJC survived much longer at 50 ℃ than it did in grape juice (Figure 1). A comparison of 1A, 1B, and 1C clearly shows the protective effect of the increased concentration of sugars in the juice and juice concentrate. No cells survived at 50 ℃ in YM broth. Yeast was able to survive at significantly higher temperatures, over 65 ℃ in GJC, than the yeast heat treated in commercial grape juice and YM broth. This is also in agreement with published literature where yeast samples were placed in 1) carbohydrate solutions of varying concentrations or in a variety of fruit juices and heat-treated-grape juice concentrate pasteurized 90 seconds at 75 ℃ and 80 ℃ and 5 seconds at 85 ℃ [8]; 2) YPD media in water baths of temperatures from 40–60 ℃ in 2.5 ℃ increments or YPD + various concentrations of glucose at temperatures ranging from 40–60 ℃ in 5 ℃ increments for 25 minutes [8]; 3) malt extract, mycophile, and yeast extract with 2 levels of a_w due to added glucose concentrations and heat treated between 45 ℃ and 64 ℃ [11,12]; 4) various sugars in phosphate buffer or PEG heated to 65 ℃ [10]: and 5) citrate buffer at 3 pH levels and in various juice products heat treated at 5 temperatures [21]. The Food and Agriculture Organization (FAO) of the United Nations Agricultural Services Bulletin 146, entitled "Principles and practices of small– and medium– scale fruit juice processing" presents a generalized flowchart for grape juice production, showing a pasteurization step of 90 ℃ for 30 seconds before concentration [13]. The sugar found in grapes is mostly glucose when the grapes are unripe. As the grape ripens, fructose increases in concentration until when fully ripened, the grape contains roughly a 50/50 glucose-fructose ratio. Fructose is dominant in overripe grapes [22]. Corry shows that glucose and fructose show statistically similar thermal resistance for osmophilic yeasts [6]. Our results and the results of others show this pasteurization step should be sufficient to kill all yeast contaminants. Unfortunately, microbiological recontamination can occur post-pasteurization due to deficiencies in sanitary procedures in packaging and storing [8].

There is a chance that *Z. rouxii* can enter a Viable but non-Culturable (VBNC) state as a defense mechanism against moderate heat damage. Many non-spore-forming bacteria and a few yeasts have been shown to enter a VBNC status when placed under chemical or physical stress [23]. VBNC status has been shown in at least two yeast varieties, *Saccharomyces cerevisiae* and *Lachancea thermotolerans*, during alcoholic fermentation using heat-treated cells as their negative (dead) control [24]. While viability is restored to VBNC cells when conditions return to optimum, we observed no such growth in heat-treated *Z. rouxii* after incubating in grape juice concentrate at favorable conditions up

to 5 weeks. We have not been able to generate *Z. rouxii* ascospores for heat treatment during our experiments. This is in line with the findings of Jermini and Schmidt-Lorenz, who found that it was not possible to investigate the heat resistance of spores only due to the observation that mature ascospores of *Zygosaccharomyces* spp. tend to remain enclosed in their ascus [7].

Isolate JC5, a bacterium closely related to *P. cineris* or *P. rhizospaerae*, seems to be extremely heat tolerant. This seems to be a characteristic of other species in the genus [25] and may be worth further study. Some members of this genus have been known to be able to ferment glucose, yielding ethanol and CO_2 [26] which may also be worth studying, as we balance their usefulness in agriculture to promote plant growth versus their product contamination potential.

5. Conclusions

Z. rouxii is a known problem yeast in winemaking, though it has not until this point been reported to be a problem in Eastern Washington vineyards or in grape juice concentrate-producing facilities in the region. Although high sugar concentrations have been shown to have a protective effect on *Z. rouxii*, pasteurization at 90 °C appears to be highly effective against these yeast cells. Avoiding fruit exhibiting symptoms of sour rot and excluding damaged fruit is essential in avoiding contamination. As difficult-to-clean surfaces could be a reservoir of this yeast during processing, sanitation is important.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors report no affiliations, funding, or financial holdings that may be perceived as affecting objectivity.

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