



Characterization of Mannitol-Producing Yeasts from Manalagi Apple Cider and Their Effects on Bacterial Nanocellulose Production by Acetic Acid Bacterium M3

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Abstract: This study aimed to isolate and identify mannitol-producing yeasts from apple cider and to evaluate their potential to support bacterial nanocellulose (BNC) production by acetic acid bacterium isolate M3. The experimental procedures included yeast isolation, mannitol quantification using the colorimetric method of Sánchez (1998), genus-level identification of the yeast isolates, compatibility testing, and consortium-based BNC production. Four yeast isolates, designated CA1, CA2, CA3, and CA4, were obtained from Manalagi apple cider. Colorimetric analysis showed that all isolates were capable of producing mannitol. Isolate CA1 produced the lowest mannitol concentration, at 0.06378 ± 0.00164 ppm, whereas isolate CA2 produced the highest concentration, at 0.11210 ± 0.00186 ppm. Based on morphological and biochemical characteristics, isolate CA1 was presumptively identified as belonging to the genus *Zygosaccharomyces*, whereas isolates CA2, CA3, and CA4 were presumptively assigned to the genus *Yarrowia*. Compatibility assays revealed no inhibition zones between any of the yeast isolates and acetic acid bacterium isolate M3. In the consortium-based BNC production assay, only the co-culture of yeast isolate CA1 and bacterial isolate M3 produced BNC; however, the yield was lower than that obtained from the M3 monoculture. These findings indicate that, under the conditions tested, the use of mannitol-producing yeasts in a microbial consortium did not enhance BNC production.

Keywords: Bacterial nanocellulose; microbial consortium; mannitol-producing yeast

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INTRODUCTION

Bio-based materials are increasingly being developed as alternatives to conventional materials because of their more environmentally friendly characteristics and their broad range of applications. Cellulose is one of the most abundant biopolymers in nature and is synthesized by various organisms, including plants, algae, oomycetes, and bacteria (Anderson et al., 2018; Noack & Persson, 2023). Cellulose produced by bacteria is commonly referred to as bacterial nanocellulose (BNC), with members of the genus *Komagataeibacter* recognized as efficient BNC producers (Barja, 2021).

BNC consists of a nanofibrillar network characterized by high purity and crystallinity, excellent water-holding capacity, and high mechanical strength (Lahiri et al., 2021). These properties support its use as a biomaterial in biomedical, food, and materials engineering applications, including tissue engineering, wound dressings, biodegradable packaging, and composite materials (Aditiawati et al., 2023). The expanding range of BNC applications has been accompanied by substantial market growth. The global BNC market was valued at approximately USD 412 million in 2023 and is projected to grow at a compound annual growth rate (CAGR) of 16–20% through

2030. More broadly, the nanocellulose industry, which includes BNC, was valued at USD 351.5 million in 2022 and is projected to exceed USD 1.5 billion by 2031 (Guevara et al., 2025). This increasing demand highlights the need for more efficient BNC production processes.

BNC production is influenced by the producing microorganism, medium composition, carbon and nitrogen sources, and fermentation conditions. Various acetic acid bacteria (AAB) can synthesize BNC from different carbon sources, although productivity varies among strains and culture conditions (Wang et al., 2018; Gorgieva & Trcek, 2019; Merli et al., 2021). Glucose is commonly used as a carbon source for bacterial cellulose production; however, a proportion of the glucose may be oxidized to gluconic acid. The accumulation of gluconic acid can reduce the culture-medium pH and inhibit cellulose biosynthesis (Peng et al., 2024). The use of alternative carbon sources may therefore help overcome this limitation. Gullo et al. (2019) reported that mannitol supplementation in *Komagataeibacter xylinus* cultures increased BNC production by 43% compared with glucose, while also reducing gluconic acid formation and maintaining a more stable medium pH. Similarly, La China et al. (2020) reported higher BC production by *K. xylinus* in mannitol-containing medium than in glucose-containing medium.

Mannitol can be produced by various microorganisms, including yeasts (Kang et al., 2023). Fructose is one of the substrates commonly used for mannitol production by yeasts, while some strains are also capable of producing mannitol from sucrose. Khan et al. (2009) reported that *Candida magnoliae* could produce mannitol using either fructose or sucrose as a carbon source. This metabolic capability could potentially be exploited to generate mannitol *in situ* in a consortium containing BNC-producing AAB, thereby reducing the need for direct mannitol supplementation to the culture medium. Mannitol-producing yeasts have been isolated from a variety of sources. For example, Meng et al. (2017) isolated *Candida parapsilosis* from sugarcane juice and demonstrated its ability to produce mannitol at high concentrations. Apple cider is a fermented product in which yeasts play an important role and exhibit considerable diversity throughout the fermentation process (Yu et al., 2022). This microbial diversity suggests that apple cider may serve as a potential source of mannitol-producing yeast isolates. However, the occurrence of mannitol-producing yeasts in Manalagi apple cider and their interactions with BNC-producing AAB have not yet been investigated.

Yeast–AAB consortia have been applied in BNC production; however, the coexistence of these microorganisms does not necessarily enhance BNC yield. Devanthi et al. (2021) reported that co-culturing *Dekkera bruxellensis* with *Komagataeibacter intermedius* increased bacterial growth but reduced BNC yield by 24%. This finding indicates that enhanced microbial coexistence or growth does not necessarily translate into improved BNC production. Therefore, the interactions between mannitol-producing yeasts and AAB require further investigation. To date, the potential of mannitol-producing yeasts isolated from Manalagi apple cider and their interaction with acetic acid bacterium isolate M3, maintained in the Microbiology Laboratory, Department of Biology, Universitas Negeri Malang, have not been evaluated.

Accordingly, this study aimed to isolate and identify mannitol-producing yeasts from Manalagi apple cider and to evaluate their ability to coexist with acetic acid bacterium isolate M3 in a consortium system. Mannitol concentration was used to assess the ability of the yeast isolates to produce mannitol, whereas BNC dry weight, yield, and productivity were used to evaluate the effect of the consortium on BNC

production. We hypothesized that mannitol-producing yeasts would be compatible with isolate M3 and increase mannitol availability, thereby enhancing BNC production. This study is expected to provide a scientific basis for the development of yeast–AAB consortia as a potentially more efficient and sustainable strategy for BNC production.

METHOD

Research Design

This study employed both exploratory and experimental approaches. The exploratory phase was conducted to isolate and identify yeasts from Manalagi apple cider and to evaluate the compatibility of the yeast isolates with the BNC-producing bacterial isolate M3. The experimental phase aimed to determine which yeast isolate was most effective in supporting BNC production.

A non-factorial Completely Randomized Design (CRD) was used, with the treatment factor consisting of mannitol-producing yeast isolates that were able to coexist with acetic acid bacterium (AAB) isolate M3. Experimental units were assigned by simple randomization, with three replicates for each treatment. The resulting data were analyzed using one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$.

Equipment and Materials

The equipment used in this study included a laminar airflow (LAF) cabinet, analytical balance, micropipettes, micropipette tips, vernier caliper, autoclave, oven, microcentrifuge tubes, microscope, microscope slides, coverslips, shaker, and centrifuge. The materials included glucose, urea, yeast extract (HiMedia®), peptone (HiMedia®), sucrose, fructose, galactose, maltose, lactose, ammonium sulfate, glacial acetic acid, absolute ethanol, sodium periodate, pH 3 buffer, acetylacetone, ammonium acetate, sodium thiosulfate, chloramphenicol, agar, 0.9% NaCl, NaOH, methylene blue, 0.5% safranin, 0.5% malachite green, crystal violet, 1% CuSO₄, and a 0.5 McFarland standard solution.

Sample Collection and Yeast Isolation

Sampling was conducted at the U.D. Dani Perkasa Manalagi apple cider production facility in Batu City, Indonesia. A 500-mL sample of Manalagi apple cider was collected at the final stage of fermentation after approximately two months. The collected cider sample was incubated at 25–30 °C for 24 h (Mathew et al., 2019).

The sample was then inoculated into Yeast Peptone Dextrose (YPD) medium for microbial enrichment. The enrichment culture was incubated on a rotary shaker at 120 rpm for 3 days at 25–30 °C (Goudar et al., 2022). Following incubation, yeast isolation was performed using serial dilution and spread-plating techniques. A 1-mL aliquot of the culture was mixed with 9 mL of sterile saline solution (0.9% NaCl), followed by serial dilution to 10⁻⁶ (Galli et al., 2022).

Aliquots from each dilution were spread onto Yeast Glucose Chloramphenicol Agar (YGCA), which was used as a selective medium to distinguish yeast colonies from other microorganisms (Moeini et al., 2004). Inoculation was performed in triplicate for each dilution.

Screening of Mannitol-Producing Yeasts

The isolated yeasts were cultured in liquid YPD medium modified with respect to the carbon source and containing 1% yeast extract, 2% peptone, and 10% fructose (Gonçalves et al., 2019). A 1.5-mL aliquot of each culture was transferred into a 1.5-mL microcentrifuge tube and centrifuged twice at 12,000 rpm for 15 min to separate the supernatant.

The resulting supernatant was subjected to colorimetric analysis using the method described by Sánchez (1998). Mannitol concentrations were determined based on a standard curve prepared using mannitol solutions ranging from 5 to 150 nM in 0.1 mL of liquid fructose-modified YPD medium.

Yeast Identification

1. Macroscopic and microscopic morphological observation

Macroscopic morphology was evaluated based on colony size, shape, elevation, margin, texture, and color. Microscopic observations included cell shape and size, the presence or absence of hyphae or pseudohyphae, and budding characteristics.

2. Growth test in liquid medium

Growth in liquid medium was evaluated by inoculating 48–72-h-old yeast isolates into 10 mL of liquid YPD medium. The cultures were examined for sediment formation and the presence of pellicles on the medium surface (Mamun-Or-Rashid et al., 2022).

3. Ascospore staining

Ascospore staining was performed using a modified Schaeffer–Fulton method. One loopful of an isolate grown on CMA medium was transferred onto a microscope slide, suspended, and stained with 2–3 drops of 0.5% malachite green. The preparation was then steamed for approximately 5 min.

The slide was rinsed with distilled water for 30 s, counterstained with 2–3 drops of 0.5% safranin, and allowed to stand for 30 s. It was then rinsed again with distilled water and examined under a microscope at 400× magnification. Ascospores appeared green, whereas vegetative cells appeared red (Kurniatuhadi et al., 2023).

4. Capsule test

Capsule staining was performed using a smear method on microscope slides. Yeast isolates were placed on a slide and stained with crystal violet for approximately 5 min, followed by rinsing with 1% CuSO₄ solution. The preparations were then examined microscopically.

A positive result was indicated by the presence of a clear halo surrounding purple-stained cells, representing the capsule. A negative result was characterized by purple-stained cells without a surrounding clear zone (Pratama et al., 2024).

5. Sugar fermentation test

Sugar fermentation tests were performed by inoculating yeast isolates into 5 mL of fermentation medium in test tubes. Liquid YPD medium supplemented separately with glucose, sucrose, maltose, galactose, or lactose as the carbon source was used.

The isolates were incubated at room temperature for 72 h and monitored for changes in medium color and gas formation. A positive result was indicated by a change in medium color from green to yellow, reflecting organic acid formation resulting from yeast metabolism, together with gas accumulation in the Durham tube. A negative result was indicated by the absence of both a color change and gas formation in the Durham tube (Musa et al., 2023).

6. Urease test

The urease test was performed by inoculating yeast isolates onto slanted Urea Agar Base medium (Christensen's Urea Agar) in test tubes, followed by incubation for 7 days. The cultures were examined periodically for urease activity. A positive result was indicated by a color change from yellow to orange, reflecting urease activity by the yeast isolate (Pratama et al., 2024).

Compatibility Test and Consortium Formation Between Mannitol-Producing Yeasts and Isolate M3

Compatibility between the yeast isolates and isolate M3 was evaluated using the cross-streak method on YPDA medium. The test microorganism (isolate M3) and indicator microorganisms (yeast isolates) were streaked against each other on the agar surface. The assay was conducted in triplicate, and the medium was initially incubated for 48 h to allow yeast growth (Aouizerat et al., 2020).

Following incubation, the indicator microorganism was streaked in separate straight lines intersecting the initial streak at a 90° angle. The agar plates were then reincubated for an additional 3 days (Hossain, 2024).

The mannitol-producing yeast isolates were subsequently co-cultured with bacterial isolate M3 at a 1:1 ratio in liquid Hestrin and Schramm (HS) medium modified by using sucrose as the carbon source (Mohammadkazemi et al., 2015). Cell density was standardized using a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 cells/mL (Waluyo et al., 2024). The inoculated media were covered with aluminum foil and incubated statically at room temperature for 14 days (Aditiawati et al., 2023).

BNC Harvesting

After 14 days of fermentation, the resulting BNC pellicles were harvested and washed with distilled water. The BNC was subsequently treated with 0.5% NaOH at 90 °C for 30 min to remove bacterial cells. The pellicles were then immersed in distilled water for 24 h to neutralize the pH (Saleh et al., 2022).

Determination of BNC Yield

The thickness of the harvested BNC pellicles was measured using a vernier caliper. The pellicles were subsequently dried at 70 °C for 24 h to determine their dry weight. BNC production yield and productivity were calculated according to Jafari et al. (2024) using the following equations:

$$\text{BNC production yield (g/L)} = \frac{\text{BNC dry weight (g)}}{\text{Culture medium volume (L)}}$$

$$\text{BNC productivity (g/L/day)} = \frac{\text{BNC dry weight (g)}}{\text{Culture medium volume (L)} \times \text{cultivation time (day)}}$$

RESULTS AND DISCUSSION

Isolation and Morphological Characteristics of Mannitol-Producing Yeasts

Isolation on selective Yeast Glucose Chloramphenicol Agar (YGCA) yielded four yeast isolates from Manalagi apple cider, designated CA1, CA2, CA3, and CA4. The ability of each isolate to synthesize mannitol was evaluated using colorimetric analysis according to Sánchez (1998). All four yeast isolates were able to produce mannitol, as indicated by the color change observed during the colorimetric assay. This color development results from the Hantzsch reaction involving ammonium acetate, acetylacetone, and aldehydes generated through the oxidation of mannitol by sodium periodate (Chades et al., 2018). The principal product of the Hantzsch reaction is a 1,4-dihydropyridine compound, which is responsible for color formation in the assay. The conjugated double-bond system of 1,4-dihydropyridine enables absorption of light within the visible spectrum, resulting in the characteristic yellow coloration (Christiaens et al., 2014).

Mannitol concentrations produced by the yeast isolates were determined using a standard curve prepared with mannitol concentrations ranging from 5 to 150 nM and

measured at an absorbance wavelength of 412 nm. The standard curve showed a positive linear relationship between mannitol concentration and absorbance. The resulting linear regression equation was $y = 0.0016x + 0.4923$, with a coefficient of determination (R^2) of 0.74. According to Hair et al. (2011), an R^2 value of 0.74 can be categorized as strong. This coefficient indicates that approximately 74% of the variation in the data could be explained by the linear regression model.

The colorimetric analysis confirmed that all four yeast isolates were capable of producing mannitol. One-way ANOVA ($p < 0.05$) indicated significant differences in mean mannitol concentrations among isolates CA1, CA2, CA3, and CA4. Mannitol concentrations obtained from three replicates are presented as mean $\pm$ SD in Figure 1. Isolate CA1 produced the lowest mannitol concentration, at 0.06378 ± 0.00164 ppm, whereas isolates CA2, CA3, and CA4 produced higher concentrations than CA1. Differences in mannitol-producing capacity among yeast isolates may be associated with genus-specific mannitol metabolic pathways (Suprahman et al., 2022). Mannitol biosynthesis in yeasts involves several enzymes, including mannitol dehydrogenase (MDH), mannitol-1-phosphate dehydrogenase (M1PDH), and mannitol-1-phosphate phosphatase (MPP). Yeasts may also produce mannitol from sucrose after its hydrolysis into glucose and fructose (Gonçalves et al., 2019). Nevertheless, the mannitol-producing capacity of the isolates examined in this study was considerably lower than that reported for the lactic acid bacterium *Leuconostoc pseudomesenteroides*, which can produce approximately 0.65 g/L, or 650 ppm, mannitol (Lejaniya et al., 2019).

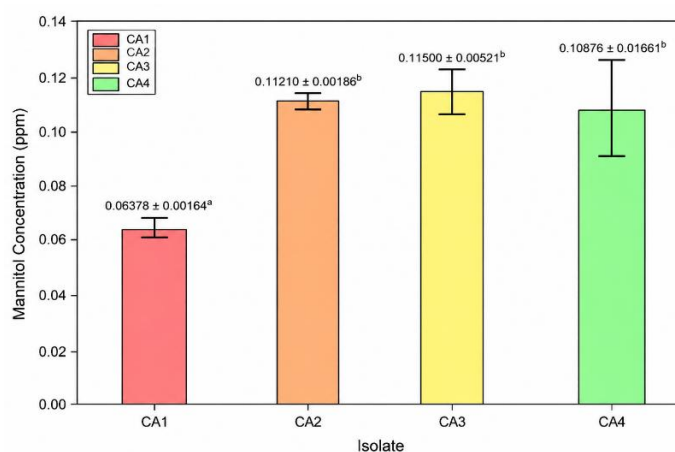


Figure 1. Mannitol concentration produced by each isolate

Yeast characterization was subsequently performed through macroscopic and microscopic morphological observations. Biochemical characterization included growth in liquid medium, ascospore staining, capsule testing, sugar fermentation, and urease testing. The four isolates exhibited distinct macroscopic colony characteristics, including differences in colony morphology and visual appearance (Figure 2). These phenotypic variations provided preliminary indications of possible taxonomic differences among the isolates. The macroscopic characteristics of each isolate are summarized in Table 1.

Table 1. Macroscopic characteristics of yeast isolates

Isolate	Shape	Elevation	Margin	Texture	Color
CA1	Circular	Raised	Entire	Mucoid	White
CA2	Irregular	Raised	Undulate	Dry	Yellowish white

Isolate	Shape	Elevation	Margin	Texture	Color
CA3	Filamentous	Crateriform	Lobate	Dry	Yellowish white
CA4	Irregular	Crateriform	Undulate	Dry	Yellowish white

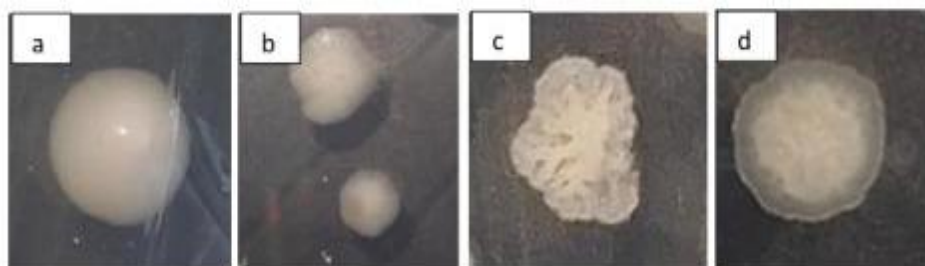


Figure 2. Colonies of yeast isolates (A : CA1 yeast isolate colony, B : CA2 yeast isolate colony, C : CA3 yeast isolate colony, D : CA4 yeast isolate colony)

Microscopic observations (Table 2) showed that all isolates reproduced asexually through budding. Budding is a major reproductive mechanism that enables yeasts to rapidly increase their populations under nutrient-rich conditions (Sun & Gresham, 2021). Isolate CA2 differed from the other isolates by exhibiting cylindrical cells. Isolate CA1 also displayed distinct characteristics, particularly in cell size and the absence of hyphae or pseudohyphae.

The formation of pseudohyphae or hyphae in yeasts can occur in response to environmental stress, including nutrient limitation and changes in temperature or pH (Chow et al., 2021). Rupert & Rusche (2022) reported that pseudohyphal formation can play an important role in yeast virulence. Pseudohyphal structures can facilitate adaptation to host environments, tissue penetration, and resistance to host immune defenses.

Table 2. Microscopic characteristics of yeast isolates

Isolate	Cell Shape	Hyphae/Pseudohyphae	Budding	Size (µm)	Figure
CA1	Oval	Absent	Present (multilateral)	5 × 2.5	
CA2	Cylindrical	Present (pseudohyphae)	Present (multilateral)	7.5 × 2.5	
CA3	Oval	Present (pseudohyphae)	Present (multilateral)	7.5 × 2.5	
CA4	Oval	Present (pseudohyphae)	Present (multilateral)	7.5 × 2.5	

Table 3. Sugar fermentation test

Isolate	Glucose		Sucrose		Maltose		Galactose		Lactose	
	W	G	W	G	W	G	W	G	W	G
CA1	+	+	+	+	–	–	+	+	–	–
CA2	–	–	–	–	–	–	–	–	–	–
CA3	–	–	–	–	–	–	–	–	–	–
CA4	–	–	–	–	–	–	–	–	–	–

Note: W = change in medium color; G = gas formation; + = positive; – = negative.

The sugar fermentation assay evaluated five carbon sources: glucose, sucrose, maltose, galactose, and lactose. Bromothymol blue was added to the test medium as a pH indicator; the medium changed to yellow when the pH decreased as a result of organic acid production (Maikan et al., 2022). Isolates CA2, CA3, and CA4 did not produce positive fermentation reactions with any of the tested carbon sources. In contrast, isolate CA1 produced positive reactions with glucose, sucrose, and galactose (Table 3). Differences in carbon-source utilization may reflect metabolic diversity among the yeast isolates and provide additional phenotypic information for taxonomic differentiation (Da Silva Fernandes et al., 2022).

Carbon sources are essential substrates in yeast metabolism because they provide energy and precursors for cellular biosynthesis. Yeasts can metabolize carbon substrates into simpler compounds through different metabolic pathways. Heterofermentative metabolism may generate products such as ethanol, which is relatively neutral in pH and may therefore produce little or no response in pH-sensitive indicators in the culture medium (Cheng et al., 2025).

Growth tests in liquid medium showed that all yeast isolates formed sediment but did not form a ring at the liquid surface. Pellicle formation was observed in isolates CA2, CA3, and CA4, whereas isolate CA1 did not form a pellicle (Table 4). Pellicle formation in yeasts represents an adaptive strategy that may improve access to oxygen and thereby confer an advantage for survival under particular environmental conditions (Fleischmann & Sripuntanagoon, 2011). Some yeast genera also possess polysaccharide-based capsules that function as adaptive structures in response to environmental factors such as osmolarity, pH, CO₂ availability, nutrient concentration, and temperature changes (Dos Santos et al., 2021).

Ascospore staining further revealed differences between CA1 and isolates CA2, CA3, and CA4 (Table 4). Ascospore characteristics are important phenotypic criteria for identifying yeast genera, particularly within the Ascomycota. Relevant characteristics include ascospore shape and number as well as the properties of the ascus. Ascospores are involved in sexual reproduction and are produced through meiotic division within specialized structures known as asci (K. Zhang et al., 2017). Isolate CA1 exhibited globose ascospores within persistent asci, whereas isolates CA2, CA3, and CA4 exhibited round to hat-shaped ascospores associated with deliquescent asci.

Table 4. Biochemical characteristics of yeast isolates

Isolate	Sediment	Ring	Pellicle	Capsule Test	Urease Test	Ascospore Characteristics
CA1	Present	Absent	Absent	Present	Negative	Persistent ascus with round/globose ascospores
CA2	Present	Absent	Present	Absent	Positive	Deliquescent ascus with round to hat-shaped ascospores

Isolate	Sediment	Ring	Pellicle	Capsule Test	Urease Test	Ascospore Characteristics
CA3	Present	Absent	Present	Absent	Positive	Deliquescent ascus with round to hat-shaped ascospores
CA4	Present	Absent	Present	Present	Positive	Deliquescent ascus with round to hat-shaped ascospores

The urease test is an important phenotypic assay for yeast identification (Khotimah et al., 2023). Christensen Urea Agar (CUA) detects the ability of yeasts to produce urease through changes in medium color. Urease catalyzes the hydrolysis of urea by cleaving its carbon–nitrogen (C–N) bonds, producing carbon dioxide and ammonia (Rashid et al., 2020). The resulting accumulation of ammonia increases the pH of the medium, which is detected by the phenol red indicator (Khotimah et al., 2023).

Isolate CA1 showed a negative urease reaction, as indicated by the absence of a color change to orange. In contrast, isolates CA2, CA3, and CA4 produced an orange color, indicating positive urease activity (Figure 3).

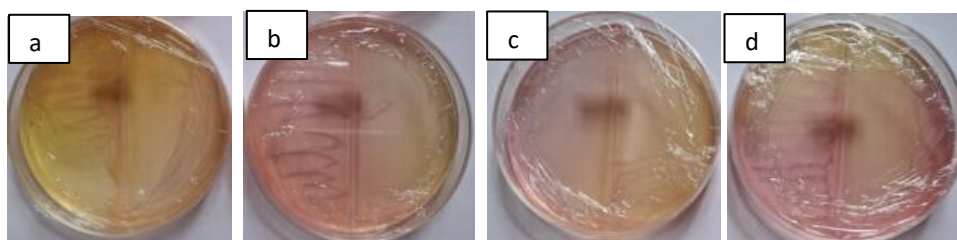


Figure 3. Urease test (A : Yeast isolate CA1, B : Yeast isolate CA2, C : Yeast isolate CA3, D : Yeast isolate CA4)

The yeast isolates were identified based on their macroscopic and microscopic morphology, biochemical characteristics, and sexual reproductive features. Comparison with the taxonomic characteristics described in *The Yeasts: A Taxonomic Study* by Kurtzman et al. (2011) indicated that the isolates exhibited phenotypic similarities to the genera *Zygosaccharomyces* and *Yarrowia*.

Genus *Zygosaccharomyces*

Members of the genus *Zygosaccharomyces* generally have spherical, elliptical, or elongated cells. Pseudohyphae may be formed, although true hyphae are absent. Asexual reproduction occurs by multilateral budding. The genus can produce spherical to elliptical ascospores, typically with one to four ascospores per ascus. The asci are persistent and may be conjugated or unconjugated. *Zygosaccharomyces* can ferment glucose, while some species are also capable of fermenting other sugars. Members of this genus may form pellicles in liquid medium and generally test negative for urease activity (Kurtzman et al., 2011).

Several of these characteristics were observed in isolate CA1, including oval cells, multilateral budding, a negative urease reaction, and the ability to ferment glucose. CA1 also possessed a capsule. The absence of pseudohyphae in CA1 may be related to sufficient nitrogen availability because pseudohyphal formation can be induced under nitrogen-limited conditions (de Paula et al., 2023). Ascospore staining revealed persistent asci containing one to four round or globose ascospores formed through conjugation between cells and buds. Collectively, these phenotypic characteristics support the tentative assignment of isolate CA1 to the genus *Zygosaccharomyces*.

Genus *Yarrowia*

Members of the genus *Yarrowia* typically possess spherical, elliptical, or elongated cells. Both pseudohyphae and true hyphae may be formed, whereas asexual reproduction occurs by multilateral budding. Ascospores may be spherical, hemispherical, hat-shaped, or slightly angular, with one to four ascospores per unconjugated ascus. *Yarrowia* generally does not ferment glucose, can form a pellicle in liquid medium, and exhibits strong urease activity (Kurtzman et al., 2011).

These characteristics were generally consistent with those observed for isolates CA2, CA3, and CA4. These isolates exhibited cylindrical or oval cells, multilateral budding, negative glucose fermentation, pellicle formation, and positive urease activity. Ascospore observations showed round ascospores, with one to four spores associated with unconjugated asci. Taken together, these phenotypic characteristics support the tentative classification of isolates CA2, CA3, and CA4 as members of the genus *Yarrowia*.

Compatibility of Mannitol-Producing Yeasts with Isolate M3

Yeasts can produce mannitol through a branch of glycolytic metabolism involving fructose-6-phosphate and the enzymes mannitol-1-phosphate dehydrogenase (M1PDH) and mannitol-1-phosphate phosphatase (MPP) (Gonçalves et al., 2019). Mannitol performs several physiological functions in yeasts, including osmotic protection, free-radical scavenging, carbohydrate storage, and, in certain species, involvement in reproductive development. Mannitol produced by yeasts may also serve as an alternative carbon source for other microorganisms, including acetic acid bacterium isolate M3, through metabolic interactions within a microbial consortium (S. S. Wang et al., 2018).

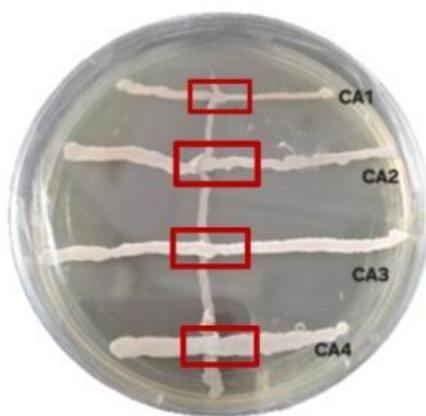


Figure 4. Compatibility test between yeast isolates and acetic acid bacterium isolate M3

Compatibility testing of the four yeast isolates (CA1, CA2, CA3, and CA4) with isolate M3 using the cross-streak method revealed no inhibition zones at the intersections of microbial growth (Figure 4). These results indicate that none of the yeast isolates inhibited the growth of isolate M3 under the conditions tested. Therefore, all four isolates were considered potentially suitable for co-culture with isolate M3.

Cell densities were standardized using a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 cells/mL. However, the actual viable cell populations expressed as CFU/mL may differ between microorganisms because bacterial and yeast cells differ considerably in size. Bacterial cells are generally smaller than yeast cells. Lozano et al. (2018) reported that a 0.5 McFarland standard for bacteria corresponds to approximately 10^7 – 10^8 CFU/mL, whereas Souza et al. (2020) reported

that, for yeasts, a 0.5 McFarland standard corresponds to approximately 10^8 CFU/mL. Therefore, although isolate M3 and the yeast isolates were standardized to the same turbidity, their actual cell numbers may not have been equivalent.

Co-culture experiments between isolate M3 and each of the four yeast isolates demonstrated that not all yeast isolates supported BNC formation. Isolates CA2, CA3, and CA4 produced relatively high mannitol concentrations but failed to support detectable BNC formation when co-cultured with isolate M3. In contrast, the consortium containing isolate CA1 produced BNC, although the amount was lower than that produced by the M3 monoculture (Figure 5 and Table 5).

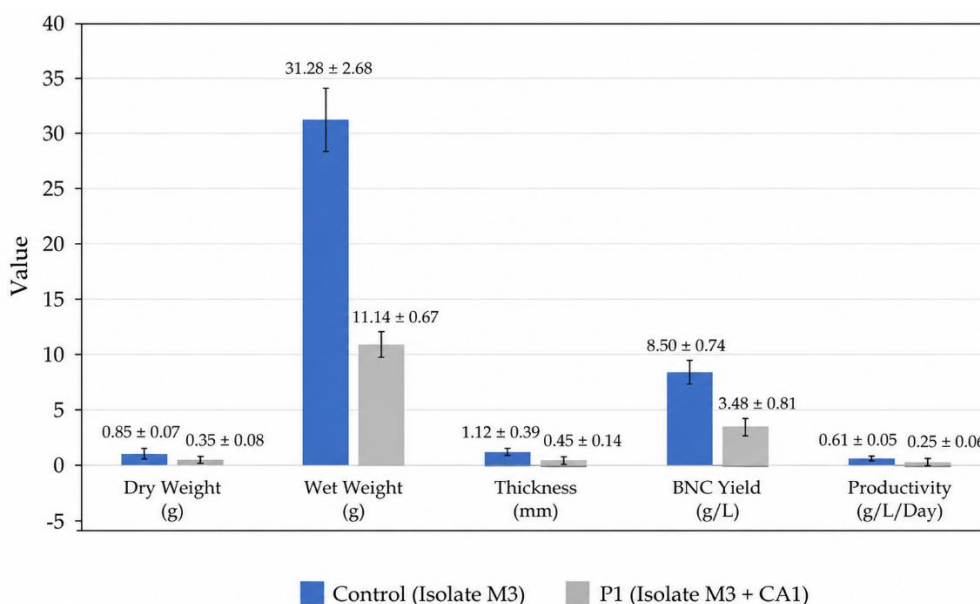


Figure 5. Comparison of mean bacterial nanocellulose (BNC) production

Table 5. Comparison of bacterial nanocellulose (BNC) production

Treatment	Dry Weight (g)	Wet Weight (g)	Thickness (mm)	BNC Yield (g/L)	Productivity (g/L/day)
Control (isolate M3)	0.85 ± 0.32	31.28 ± 17.67	1.12 ± 0.39	8.50 ± 3.19	0.61 ± 0.23
P1 (isolate M3 + CA1)	0.35 ± 0.18	11.14 ± 0.39	0.45 ± 0.14	3.48 ± 1.81	0.25 ± 0.13

BNC production by isolate M3 in monoculture tended to be slow during the first four days of cultivation, followed by an increase after the initial lag period. Overall, the experimental results showed that co-cultivation with the mannitol-producing yeast isolates did not enhance BNC production. One possible explanation is competition for nutrients because the yeast isolates and bacterial isolate M3 may have overlapping nutritional requirements.

Large-scale BNC production commonly uses sucrose as a carbon source. However, sucrose must first undergo hydrolysis and further metabolism to generate glucose and fructose that can be utilized during BNC biosynthesis (Beluhan et al., 2022). In aqueous HS medium, sucrose can undergo gradual hydrolysis, producing the monosaccharides glucose and fructose (Gao et al., 2014). These invert sugars can subsequently be utilized by isolate M3 to support cellular metabolism, including the production of BNC and organic acids.

The co-culture treatments differed markedly in their ability to support BNC formation. Detectable BNC was produced only by the consortium consisting of isolate M3 and isolate CA1, which exhibited phenotypic characteristics resembling those of

the genus *Zygosaccharomyces*. However, BNC production in this consortium was lower than that observed in the M3 monoculture (Figure 6 and Table 5), suggesting that the presence of isolate CA1 did not enhance BNC synthesis under the culture conditions used.

In contrast, no BNC was produced by the consortia containing isolate M3 together with CA2, CA3, or CA4. Phenotypic characterization suggested that these three isolates were similar to members of the genus *Yarrowia*. Thus, under the conditions evaluated in this study, the three putative *Yarrowia* isolates did not support BNC formation by isolate M3.

Overall, the use of consortia consisting of acetic acid bacterium isolate M3 and the tested yeast isolates did not improve BNC production. These findings demonstrate that establishing a microbial consortium does not necessarily result in a beneficial effect on BNC biosynthesis. Instead, the success of a consortium appears to depend on the specific combination of microorganisms and the culture conditions employed. A limitation of the present study is that the physiological and metabolic mechanisms underlying the reduced BNC production were not directly investigated. Consequently, the mechanisms responsible for the observed reduction in BNC formation cannot yet be fully explained.

CONCLUSION

This study yielded four yeast isolates from Manalagi apple cider, all of which were capable of producing mannitol. Compatibility testing showed that all four isolates could coexist with acetic acid bacterium isolate M3. The yeast isolates produced mannitol at concentrations ranging from approximately 0.06 to 0.11 ppm. Based on morphological and biochemical characterization, isolate CA1 exhibited characteristics consistent with the genus *Zygosaccharomyces*, whereas isolates CA2, CA3, and CA4 showed characteristics resembling those of the genus *Yarrowia*.

Evaluation of BNC production in the yeast–bacterium consortia showed that only the consortium comprising yeast isolate CA1 and bacterial isolate M3 produced bacterial nanocellulose (BNC). However, the amount of BNC produced was lower than that obtained from the positive control consisting of M3 monoculture. These findings indicate that, under the conditions tested in this study, the use of mannitol-producing yeast isolates in consortium with isolate M3 did not enhance BNC production.

RECOMMENDATION

Future studies should focus on optimizing yeast–bacterium co-culture interactions through more comprehensive evaluation of physiological parameters, metabolic profiles, and culture conditions. Such analyses are needed to better understand intermicrobial interactions and to identify conditions that may improve BNC synthesis.

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