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Identification of Lactic Acid Bacteria Inhibiting the Growth of *Saccharomyces cerevisiae* in Molasses Fermentation

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Abstract. Ethanol production by *Saccharomyces cerevisiae* is essential for the biofuel industry, but microbial contamination by Lactic Acid Bacteria (LAB) can significantly reduce yield and fermentation efficiency. This research aimed to identify Lactic Acid Bacteria (LAB) causing significant inhibition of *Saccharomyces cerevisiae* growth. LAB isolates were screened and identification was carried out by carbohydrate fermentation using the 50 CHL Analytical Profile Index (API) Kit, and the LAB isolates (MC_2K_1, MP_2K_1, and MP_2K_2) were identified as *Lactobacillus rhamnosus* and isolate MC_2K_2 as *L. pentosus*. The results demonstrated that bacteriocins did not significantly inhibit *S. cerevisiae* following heat treatment, indicating that metabolic byproducts, particularly lactic acid, were primarily responsible for yeast inhibition. Co-culture of LAB and *S. cerevisiae* supernatants was used to determine inhibition by metabolites produced by LAB. A higher inhibitory effect occurred in the supernatant from 48 hours of incubation with lower CFU results. The highest inhibition rate reached 42% in isolate MC_2K_1 and the incubation time of 6 hours with all isolates significantly inhibited the growth of *S. cerevisiae*. These findings challenge the prevailing assumption that bacteriocins are the primary inhibitors of yeast and highlight the importance of metabolic byproducts as dominant inhibitory factors. This insight contributes to developing targeted contamination control strategies, ultimately enhancing ethanol production efficiency.

Keywords: Ethanol; Inhibitory Power; Lactic Acid Bacteria; Molasses; *Saccharomyces cerevisiae*

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INTRODUCTION

Ethanol production is a vital process in the biofuel industry, which relies on *Saccharomyces cerevisiae* primarily due to its high fermentative capacity and ethanol tolerance. Ethanol production efficiency is essential to supplying global biofuel demands and optimizing yields and productivity required well-designed fermentation methods. For reliable and effective ethanol production, yeast viability and performance must be maintained during fermentation to achieve consistent and efficient ethanol output (Gozal et al., 2024).

The decrease in the quality of ethanol is influenced by the ability of contaminants that can inhibit the growth of *S. cerevisiae*. One of the contaminants that can reduce the population of *S. cerevisiae* is Lactic Acid Bacteria (LAB) such as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus* (Brexó & Sant'Ana, 2017; Liu *et al*., 2022; Worley-Morse *et al*., 2015). Subsequently, LAB can release compounds such as lactic acid and bacteriocins that inhibit the growth of *S. cerevisiae*, reducing yeast growth by about 90% (Kostinek et al., 2007; Lindgren & Dobrogosz, 1990; Liu et al., 2022).

The decrease in ethanol production can reach approximately 30% due to decreased viability of *S. cerevisiae* cells(Maicas, 2020; Malode et al., 2021; Nandy & Srivastava, 2018). Additionally, quality issues may arise, which include, the formation of impure ethanol, toxins, secondary compounds, yeast flocculation, and foaming (Beckner et al., 2011; Worley-Morse et al., 2015). LAB contaminants can inhibit the fermentation process by decreasing ethanol production by 15-

30 g/L and increasing glucose residues by 30-50 g/L. An increase in the concentration of lactic and acetic acids is also found in the fermentation process (Leathers et al., 2014; Valerio et al., 2004).

LAB are bacteria capable of releasing increasing concentrations of acid into their environment as their population grows. This elevated acid concentration can negatively impact yeast cell viability and hinder ethanol production by causing cytoplasmic acidification, disrupting cell homeostasis, reducing growth rates, and prolonging the yeast's lag phase. Notably, acetic acid, a metabolic byproduct of LAB, is particularly toxic, amplifying toxicity by approximately 30 times compared to ethanol (Basso et al., 2014; Elshaghabee et al., 2016; Zuehlke et al., 2013).

Aggregation also occurs when *Lactobacillus* interacts with *S. cerevisiae*. During the logarithmic phase, flocculation in *S. cerevisiae* yeast can reduce contact with the substrate, leading to decreased ethanol production. Contaminants such as *L. fermentum* can influence gene expression in *S. cerevisiae*, affecting the yeast's ability to hydrolyze sugars and contributing to flocculation (Carvalho-Netto et al., 2015; Tiukova et al., 2014). According to (Carvalho-Netto et al., 2015), it is known that self-aggregation does not occur in yeast when contamination takes place, hence the genes that cause flocculation by yeast do not experience increased function. Binding interactions occur in *L. fermentum* and *S. cerevisiae* when mannose-binding proteins bind to yeast cells due to the high mannose content in the cell walls of *S. cerevisiae* (Bassi et al., 2018; Tiukova et al., 2014).

The inhibition of *S. cerevisiae* is also influenced by the lactic acid produced as a metabolite by LAB. The lactic acid produced decreased the media's pH to 3.88 within 24 hours, compared to the culture media, which was 5.64. However, the acidic environment is not the main key to inhibiting the growth of *S. cerevisiae* (Liu *et al*., 2022). Inhibition occurs with the co-culture of LAB and *S. cerevisiae* as LAB undergoes logarithmic phase growth until it reaches a certain number of cells which will later begin to inhibit the growth of *S. cerevisiae* (De Oliva-Neto et al., 2013; He et al., 2021; Liu et al., 2022; Lopes et al., 2016).

Metabolites produced by LAB can inhibit the growth of *S. cerevisiae*, and when obtained in the form of supernatants, these metabolites can cause inhibition ranging from 35% to 75% in the growth of *S. cerevisiae*. However, co-culture of LAB and yeast will cause stronger inhibition of *S. cerevisiae* than LAB monoculture alone. This difference in inhibition ability can be caused by different gene expressions for metabolism and transport pathways. According to (Liu *et al*., 2022), the interaction between *L. plantarum* and *S. cerevisiae* can cause changes in the response of the cells. This research found that there is an increase in the regulation of genes in the lamBDCA system in adult LAB (24 hours) causing *S. cerevisiae* to increase the regulation of stress response genes to overcome oxidative stress, competition for nutrients, and stimulants such as cell contact and metabolites. Meanwhile, activities such as DNA replication and growth, including the cell cycle decrease have the potential to cause cell death and low growth rates (Liu et al., 2022; Worley-Morse et al., 2015)

The decreasing quality of ethanol is caused by one of the decreased viability of *S. cerevisiae*. The growth of *S. cerevisiae* can be inhibited by compounds produced by LAB which can be extracted in the form of Cell-Free Supernatant (CFS) and Neutralized Cell-Free Supernatant (NCFS). In line with these findings, not all contaminants in ethanol fermentation significantly inhibit the growth of *S. cerevisiae*.

Therefore, the primary purpose of this research is to investigate the inhibitory mechanisms of LAB isolates against *S. cerevisiae* during ethanol fermentation, with particular emphasis on differentiating between the effects of bacteriocins and metabolic byproducts. The most inhibitory LAB strains will also be identified, and their time-dependent effects on yeast inhibition. The final objective is to provide a comprehensive understanding of strain-specific inhibition, which can inform the development of targeted contamination control strategies and optimize ethanol production processes.

The findings from this research are anticipated to advance the scientific understanding of LAB-induced inhibition in ethanol fermentation, particularly in differentiating between strain-specific and metabolite-driven inhibition. This knowledge can guide the development of targeted contamination control strategies, ultimately improving the sustainability and efficiency of bioethanol production.

METHODS

Sample Preparation

The laru seed cultures were obtained from

ethanol fermentation using molasses from the alcohol production center in Bekonang Village, Mojolaban District, Sukoharjo Regency, Central Java. The laru seed was given molasses every 2 days to ensure the growth of *S. cerevisiae* and LAB.

Isolation and Selection Stage of Yeast and Lactic Acid Bacteria Isolates

Isolation of LAB and *S. cerevisiae* was carried out by inoculating 0.1 mL of samples diluted with 0.9 mL of NaCl solution to reach a dilution of 10^{-2} and 10^{-3}. Using the spread plate method, in each dilution series, 0.1 ml of solution was taken to be grown on Man Rogosa Sharpe Agar (MRSA-$CaCO_3$ 1%) medium for LAB isolation and on Potato Dextrose Agar (PDA) for *S. cerevisiae* isolation. Incubation of LAB isolates was carried out at 37˚C for 48-72 hours, while incubation of *S. cerevisiae* isolates was carried out at 30˚C for 48 hours. Re-inoculation was carried out on LAB colonies perfectly round, milky white, and transparent, and produced a clear zone around the colony. The isolates obtained were LAB candidates that contaminated the fermentation process and could inhibit the growth of *S. cerevisiae* (Amarantini et al., 2020). Inoculation of *S. cerevisiae* was carried out using the streak plate method on milky white colonies.

Screening and Characterization of *S. cerevisiae*

Screening of *S. cerevisiae* was carried out by observing cell morphology through methylene blue staining and by slide culture. The morphology of *S. cerevisiae* was observed by marking dead cells with a blue color. The slide culture technique was carried out to observe the morphology of budding cells microscopically. The morphology that appeared was in the form of prolate spheroids with visible budding cells indicating growth in *S. cerevisiae* (Zakhartsev & Reuss, 2018).

Screening and Characterization of Lactic Acid Bacteria

LAB screening was carried out with several tests such as gram staining, morphological observation, catalase test, and low pH tolerance test. The catalase test was carried out by the administration of 2% H_2O_2 solution to the isolate with a negative reaction obtained marked by the absence of foam on the isolate. The screening was carried out to ensure that the isolate obtained was in accordance with the characteristics of LAB, namely gram-positive bacteria with a purple color and a bacillus shape under microscope observation (Amarantini et al., 2020).

Further screening is a low pH tolerance test on LAB isolates to ensure the growth ability of LAB at low pH. Subsequently, the low pH tolerance test begins with the preparation of MRSB media in a test tube with a modification, namely the administration of 0.1N HCl until the media pH is 2, 3, and 4. Inoculation is carried out on MRSB media and incubated for 8 hours and continued by carrying out the streak plate method in 3 different quadrants according to pH. Incubation is required for 72 hours to observe the growth of the isolate in a low-pH environment. Isolate growth was calculated by scoring growth (+) and no growth (-). In addition, qualitative screening was observed from the color changes produced by the media after incubation for 72 hours (Amarantini et al., 2019).

Identification of LAB using API 50 CHL Kit

Identification of LAB was carried out based on the phenotype test of the 50CHL Analytical Profile Index (API) kit (BioMérieux, USA) by examining the ability of LAB biochemical metabolism to react with various sources of carbohydrates contained in each cupule (Pyar & Kok, 2019).

Testing the Inhibitory Power of CFS and NCFS Solutions against *S. cerevisiae*

The inhibition stage begins by extracting CFS, and this is carried out by taking cell-free supernatant through a centrifugation process at a speed of 10,000 rpm for 10 minutes. The harvesting results obtained CFS and then neutralized at pH 6.5 using NaOH to produce NCFS for bacteriocin testing. The extract results can be stored in the refrigerator until needed for the next stage. The inhibition test was carried out using the good diffusion method by adding NCFS to the *S. cerevisiae* population grown using cotton swap on PDA agar (Amarantini et al., 2021).

Co-Culture Test of BAL and S. cerevisiae

The inhibitory activity between BAL and yeast isolates was determined by co-culture testing of BAL and *S. cerevisiae* supernatants following existing procedures (Liu *et al*., 2022). with slight modifications. *S. cerevisiae* culture was grown on YPDB media and *Lactobacillus sp.* culture on MRSB media overnight. The initial number of cells used was 10^7 CFU/mL for *S. cerevisiae* and 10^8 for BAL isolates (Liu *et al*., 2022).

BAL supernatant was prepared by inoculating 10^8 CFU/mL culture on MRSB media, incubated at 37˚C, and shaken at 200 rpm for 48 hours. Furthermore, the culture was centrifuged for 10 minutes at 6000 rpm, and the supernatant was separated from the cells using a pipette (Liu *et al*., 2022).

In the co-culture test, *S. cerevisiae* culture and supernatant from *Lactobacillus sp*. were combined with a concentration ratio of 1:1. In the control treatment, H_2O and MRSB were used as substitutes for the supernatant of the LAB isolate. The inhibitory power against the growth of *S. cerevisiae* was observed based on the results of the calculation of the growing colonies (Colony Forming Unit/CFU) using YPDA medium added with 200 ppm chloramphenicol every 0, 6, 12, 24, and 48 hours respectively (Liu *et al*., 2022).

Inhibitory Power Analysis Through ANOVA

The inhibitory activity of LAB supernatants against *S. cerevisiae* was assessed by counting the number of growing colonies (Colony Forming Unit/CFU) on YPDA medium supplemented with 200 ppm chloramphenicol. The data obtained from the inhibitory power tests were statistically analyzed using Analysis of Variance (ANOVA). Additionally, the inhibitory effects at different time intervals (0, 6, 12, 24, and 48 hours) were examined to see if there were any significant differences. The results demonstrated statistically significant differences in inhibitory activity among the LAB isolates ($p < 0.01$), indicating that certain isolates exhibited superior inhibitory potency compared to others, and the results are presented as mean ± standard deviation. These results imply that the inhibitory results were highly impacted by the type of isolate and the length of incubation. In order to determine if exposure length had a significant impact on the inhibitory power of LAB isolates against *S. cerevisiae*, ANOVA was used in this research.

RESULTS AND DISCUSSION

Lactic Acid Bacteria (LAB) that Inhibits the Growth of *S. cerevisiae* in Molasses-Based Ethanol Fermentation

The colonies grown on MRSA media formed two types of isolates that were able to produce a clear zone based on the shape of the growing colonies. This clear zone is an early marker to determine the occurrence of lactic acid and $CaCO_3$ reactions in the growing colonies. Subsequently, colonies grown are milky white with differences in size, namely large and small, found in all samples with an even number of colonies in each sample (Amarantini et al., 2020).

Five colonies are originating from MRSA media and four colonies originating from PDA media that were re-inoculated to purify the isolate using the streak plate method. Figure 1 shows all streak results and all isolates on MRSA media form a clear zone. Isolate MC_2K_1 has a perfectly round colony with a shiny milky white color and isolate MP_2K_1 has an imperfectly round colony with a transparent white color. *Saccharomyces cerevisiae* colonies grown on PDA media are milky white with budding cells.

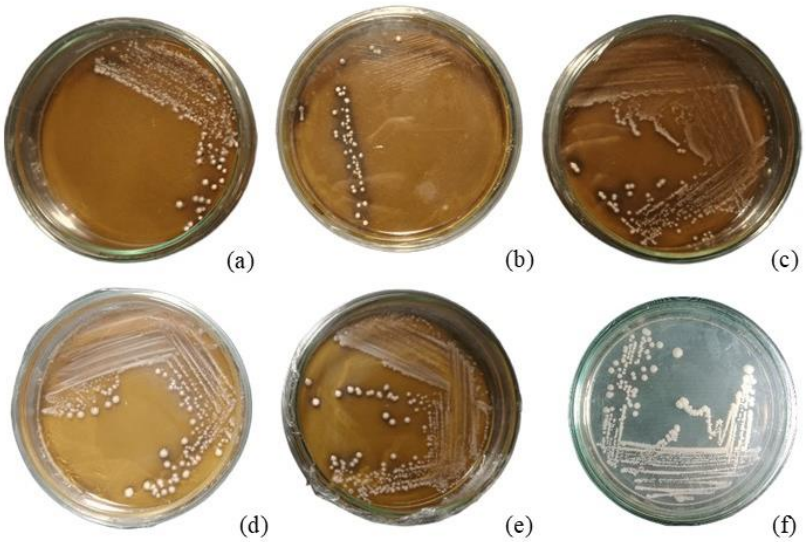

Figure 1. Streak Plate Results of Isolates (a) MC_2K_1, (b) MC_2K_2, (c) MC_3K_1, (d) MP_2K_1, (e) MP_2K_2 on MRSA media and (f) *S. cerevisiae* on PDA media

Table 1. BAL Isolates with Characteristics of Morphology and Physiology of Screening Results

Code Sample	Isolate Code	Gram/Shape	Catalase	Low pH Tolerance Test		
				pH 2	pH 3	pH 4
MC_2	MC_2K_1	+ / Short Thick Stem	-	+	-	+
	MC_2K_2	+ / Short Thin Stem	-	+	-	+
MC_3	MC_3K_1	+ / Short Thick Stem	-	-	+	+
MP_2	MP_2K_1	+ / Long Thin Stem	-	+	+	+
	MP_2K_2	+ / Long Thin Stem	-	-	+	+

Note : Low pH Tolerance Test (pH 2, pH 3, and pH 4 represent pH values used to evaluate bacterial tolerance). The numbers 2, 3, and 4 in the Low pH Tolerance Test column indicate the pH values at which the bacterial isolates were tested for their ability to survive in acidic conditions. The symbols (+) and (-) denote whether the isolates demonstrated tolerance or not at the respective pH level.

Table 2. Identification of LAB Isolates Based on Carbohydrate Fermentation Using API 50 CHL

LAB Isolates	API Identification	ID (%)
MC_2K_1	*Lactobacillus rhamnosus*	99.5
MC_2K_2	*Lactobacillus pentosus*	99.7
MP_2K_1	*Lactobacillus rhamnosus*	99.9
MP_2K_2	*Lactobacillus rhamnosus*	95.0

The BAL screening stage was carried out using four tests, namely gram staining, catalase testing, and low pH testing. Based on microscopic observations, all BAL isolates showed gram-negative characteristics with a bacillus shape and negative catalase (Table 1). The pH test proved that all BAL isolates were able to grow in an acidic environment (Brexó & Sant'Ana, 2017). Differences were observed between the MC_2K_1 and MP_2K_1 isolates in the shape of rod cells after gram staining. The MC_2K_1 isolate had a short bacillus shape when compared to the MP_2K_1 isolate which tended to have a long bacillus cell shape. The bacillus shape of the MP_2K_1 isolate looked like an elongated chain compared to the MC_2K_1 isolate which tended to cluster and did not form a chain. Based on the screening test, four isolates were then selected MC_2K_1, MC_2K_2, MP_2K_1, and MP_2K_2 for phenotype testing.

The identification results using the database from API-web™ API 50 CHL V5.1 Software (Pyar & Kok, 2019) are presented in Table 2. Isolates MC_2K_1, MP_2K_1, and MP_2K_2 belong to the same species, namely *L. rhamnosus*, marked by an ID exceeding ≥95%, and isolate MC_2K_2 belongs to the species *L. pentosus* with an ID value of 99.7%.

The Impact of Bacteriocins in Inhibiting the Growth of *S. cerevisiae*

Bacteriocin activity can be determined by the agar well diffusion method (Amarantini et al., 2019). Bacteriocin secretion is influenced by various factors, including incubation time, with longer incubation periods leading to an increased production of bacteriocins in LAB. This research used bacteriocin results with an incubation time of 48 hours. The inhibition test used CFCS that had been neutralized at pH 6.5 using 1N NaOH. Neutralization was carried out to determine the ability of bacteriocins to inhibit the growth of *S. cerevisiae*. Furthermore, the NFCS solution was heated at 100˚C for 10 minutes to determine the inhibitory activity.

Figure 2. Bacteriocin inhibition test in NCFS solution against *S. cerevisiae* using the Agar Well Diffusion method.

The results in Figure 2 show the inability of bacteriocin to inhibit the growth of *S. cerevisiae*. The lack of inhibition may be due to bacteriocin degradation during heat treatment or an increase in the bacteriocin's pH. In addition, media pH, temperature, and environmental conditions can greatly affect the formation of bacteriocin where the optimum bacteriocin secretion is at pH 4 and 5 (Reuben et al., 2019). Therefore, further testing is needed, namely co-culture, to determine the inhibitory ability of metabolites originating from LAB isolates such as lactic acid in inhibiting the growth of *S. cerevisiae*.

Co-culture of LAB Supernatant in Inhibiting the Growth of *S. cerevisiae*

Further testing to determine LAB inhibition against *S. cerevisiae* was carried out using the co-culture technique, namely mixing yeast culture with LAB supernatant in the form of CFS. Inhibition will be observed in the number of colonies that decrease or are static based on the results of the calculation of growing colonies (Colony Forming Unit/CFU) (Liu *et al*., 2022).

Table 3 shows that significant inhibition (<1%) occurred in all types of isolates with the highest ability observed in isolate MC_2K_1 which has an average value of 12.44 (log CFU). The difference in inhibition ability can be caused by the number of metabolites contained in the supernatant. The number of metabolites is largely determined by the number of cells and incubation time as observed in Figure 1. Isolate MP_2K_2 has the lowest inhibition ability compared to other isolates. Subsequently, isolate MC_2K_1 was significantly able to provide the highest inhibition against *S. cerevisiae* which was observed at an incubation time of 6 hours with a level reaching 42% when compared to the H_2O. This level of inhibition is in line with previous investigations, which reported an inhibition rate of 45.30% (He et al., 2021; Liu et al., 2022).

The 48-hour BAL supernatant is known to have a lower pH reaching 3.01 compared to 24 hours. During the stationary phase (over 24 hours), LAB generates significant amounts of metabolites that inhibit the growth of *S. cerevisiae* (Liu *et al*., 2022). Longer incubation time will affect the increase in the number of cells to about 83% of the initial number (Figure 3).

Table 3. Number of colonies (log CFU) of *S. cerevisiae* resulting from co-culture of BAL supernatant in various.

LAB Isolates	Incubation Time					Average/SD
	0 hour	6 hours	12 hours	24 hours	48 hours	
MC_2K_1	10.34	10.00	13.04	14.34	14.49	$12.44^{a}\pm2.15^{*}$
MC_2K_2	9.84	10.60	13.30	14.60	14.67	$12.60^{a}\pm2.25^{*}$
MP_2K_1	9.90	10.69	13.18	14.54	14.61	$12.58^{a}\pm2.18^{*}$
MP_2K_2	9.95	10.69	13.32	14.60	14.62	$12.64^{a}\pm2.19^{*}$
MRSB	9.90	11.17	14.19	15.05	15.11	$13.08^{ab}\pm2.39$
H_2O	10.17	14.39	14.60	15.20	15.28	$13.93^{b}\pm2.13$
Average/SD	$10.03^{a}\pm0.18^{*}$	$11.12^{b}\pm1,48^{*}$	$13.55^{c}\pm0.59^{*}$	$14.71^{d}\pm0.30$	$14.78^{d}\pm0.29$	

Note: *Significant (<1%). Statistical analysis using ANOVA demonstrated a significant difference in inhibition rates among LAB strains ($p < 0.05$).

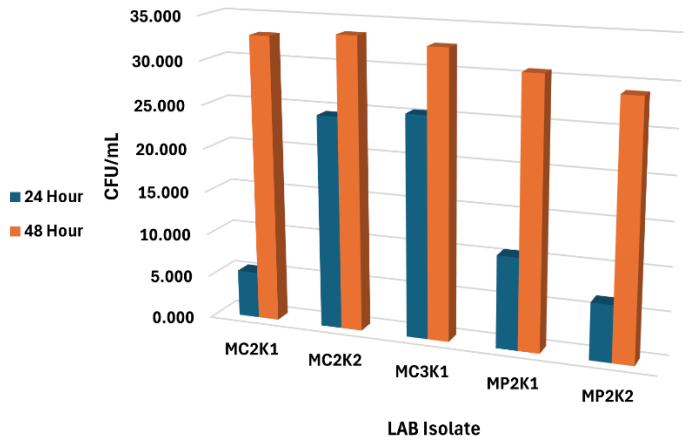

Figure 3. Comparison of the growth of LAB isolates incubated for 24 and 48 hours.

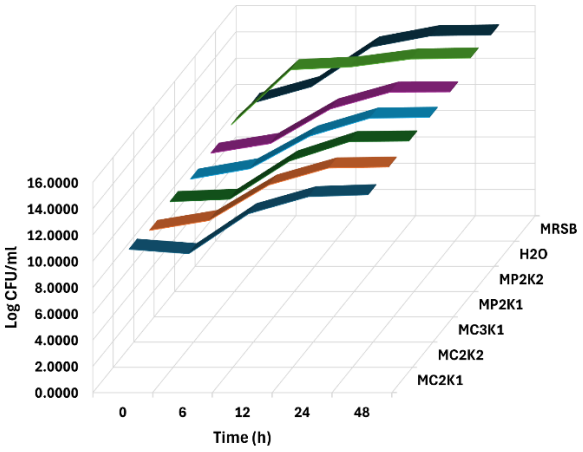

Figure 4. Effect of LAB Supernatant Co-Culture on *S. cerevisiae* Growth

The inhibitory ability of metabolites produced by LAB such as lactic acid was observed by examining the growth curve of *S. cerevisiae* (Figure 4). Based on the graph an increase occurred in all treatments. These data prove that there was no decrease or lethal effect on the growth of *S. cerevisiae*. The inhibition that occurred in *S. cerevisiae* was static, characterized by stagnant or low growth in the LAB supernatant treatment compared to the control treatment. Based on the inhibition test using the co-culture technique, it can be proven that all LAB isolates were significantly able to inhibit the growth of *S. cerevisiae* statically.

MRSB and H_2O treatments were also carried out to determine the differences in controls presenting minimum nutrients (H_2O) and controls with maximum nutrients (MRSB). It can be seen in Figure 4 that there is no significant difference in the nutrients administered. This result indicates that low nutrients are not a significant factor in reducing the growth of *S. cerevisiae*. Figure 4 indicates that *S. cerevisiae* has similar log CFU values at 24 and 48 hours of incubation. Significant inhibition was not observed during these periods, likely due to the robust growth of *S. cerevisiae* after the logarithmic phase, which may have rendered LAB metabolites ineffective at inhibiting *S. cerevisiae* once a certain concentration was reached (Liu *et al*., 2022). The inability of LAB to inhibit *S. cerevisiae* could also be due to the possibility that LAB and yeast co-exist during the fermentation process. Some research states that BAL such as *Lactobacillus* can significantly inhibit the growth of *S. cerevisiae*, but other cases can occur when *Lactobacillus* in certain amounts can increase the ability of *S. cerevisiae* to survive environmental stress. Metabolites produced by *Lactobacillus* will increase reactions such as maintenance and structural restoration of *S. cerevisiae* (He et al., 2021).

The results of this research provide new insights into the inhibitory interaction between LAB and *S. cerevisiae* in molasses-based ethanol fermentation. While previous research has indicated that bacteriocins can contribute to yeast inhibition, this research demonstrates that their effect alone is not significant following heat treatment (Mokoena, 2017). Instead, LAB metabolites, particularly lactic acid, play a predominant role in inhibition, suggesting that metabolic byproducts rather than direct antimicrobial activity are responsible for the observed effects.

Additionally, this research identifies *L. rhamnosus* MC2K1 as the most inhibitory strain, exhibiting the highest inhibition (42%) at 6 hours of incubation. This finding is particularly significant as prior research on LAB contamination has predominantly focused on species-level interactions without quantifying strain-specific inhibitory effects (Liu et al., 2022; Sieuwerts et al., 2018). Moreover, the time-dependent nature of LAB inhibition observed in this research introduces a novel perspective on yeast-LAB interactions, demonstrating that the competitive dynamics between these microorganisms fluctuate throughout fermentation rather than remain constant.

By demonstrating that LAB inhibition is primarily metabolite-driven, strain-specific, and time-dependent, this research challenges existing perspectives on microbial contamination in ethanol fermentation. These findings underscore the necessity of further research into strain-

specific inhibitory mechanisms and the metabolic pathways responsible for yeast suppression. A deeper understanding of these factors could facilitate the development of targeted strategies for controlling LAB contamination in industrial ethanol production, shifting the focus from reliance on antimicrobial compounds like bacteriocins to the regulation of metabolic byproducts that influence yeast viability(Sandesh & Ujwal, 2021; Takagi et al., 2016).

From an industrial perspective, these insights are particularly pertinent for optimizing ethanol production. LAB contamination is a persistent challenge in bioethanol fermentation, often leading to reduced yeast viability, inefficient fermentation, and lower ethanol yields. This research provides a foundation for developing more accurate contamination control strategies by determining the predominant function of LAB metabolic byproducts in yeast inhibition. Also, to minimize LAB-induced stress on *S. cerevisiae*, industrial fermentation processes could be optimized by modulating environmental factors such as pH, nutrient availability, and organic acid production. By emphasizing strain-specific interactions and metabolic influences, this research contributes to the design of more efficient, targeted microbial management strategies, ultimately improving both the productivity and sustainability of industrial fermentation systems (Labuschagne & Divol, 2021).

CONCLUSION

This research identified four LAB isolates (MC2K1, MP2K1, MP2K2) as *Lactobacillus rhamnosus* and one isolate (MC2K2) as *Lactobacillus pentosus*. The inhibitory potential of LAB-derived bacteriocins, tested through NCFS solutions, demonstrated no significant inhibition against *S. cerevisiae* after heat treatment, indicating that bacteriocins alone may not be responsible for yeast inhibition. However, LAB metabolites, particularly lactic acid, played a crucial role in inhibiting *S. cerevisiae* growth. Among the isolates, MC2K1 exhibited the highest inhibition rate (42%) at 6 hours of incubation, suggesting strain-specific inhibitory effects. These findings challenge the conventional perspective that bacteriocins are the principal inhibitors and highlight the significance of metabolic byproducts as the dominant factor in yeast inhibition. Future research should focus on identifying the specific inhibitory metabolites and evaluating their effects under industrial fermentation conditions. Furthermore, investigating the temporal dynamics of LAB inhibition throughout prolonged fermentation periods would be beneficial to develop targeted contamination control strategies.

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