Effects of AI-2 quorum sensing inhibitors on mitigating bacterial contamination in bioethanol production

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Abstract: In this paper, the activities of signal molecule AI-2 in the sole fermentation of *Lactobacillus plantarum* and co-fermentation of *Saccharomyces cerevisiae* and *L. plantarum* were investigated. Co-fermentation of *S. cerevisiae* and *L. plantarum* was found to reduce *L. plantarum* to secrete AI-2. Three AI-2 quorum sensing inhibitors (DMHF, D-Rib, and D-Gal) were added to the co-fermentation

system to interrupt the growth of *L. plantarum* further. It was found that 0.2 g/L DMHF and 18.0 g/L D-Gal could mainly decrease the activity of AI-2 by 38% and 36%, respectively, and significantly inhibit the biofilm formation of *L. plantarum*. Moreover, the ethanol concentrations of the groups added with 0.2 g/L DMHF and 18.0 g/L D-Gal were 14% and 103% higher than that of the control, respectively, within 30 hours of fermentation. This study provides basic data for the novel prevention of *L. plantarum* contamination in bioethanol production via quorum sensing control.

Keywords: Quorum sensing inhibitors; Signal molecule AI-2; *Saccharomyces cerevisiae*; *Lactobacillus plantarum*; Bioethanol

1. Introduction

As now well acknowledged, bioethanol, is a renewable green energy resource which is a potential substitute for fossil fuels. At present, microbial fermentation is the most promising method for the production of bioethanol. *S. cerevisiae* is widely used in industrial bioethanol production due to its high productivity and facile growing requirements (Widiastuti et al., 2011; Da et al., 2022). However, the microbial fermentation for bioethanol production is often contaminated by bacteria. As is known, lactic acid bacteria (LAB) are the main contaminating species because of their fast reproductivity, strong tolerance to ethanol, and lower pH. In the fermentation of *S. cerevisiae*, LAB is easy to grow, can consume nutrients, reduce

survival space, and secrete acidic substances to lower the pH of the fermentation system, leading to the decrease of bioethanol production (Roach et al., 2013; He et al., 2021). Therefore, effective inhibition of LAB contamination in fermentation has become a key challenge to developing the bioethanol industry.

Quorum sensing (QS) is a communication mechanism between microorganisms, conducted by secreting quorum sensing signal molecules (QSMs) to the environment during microbial growth. The expressions of particular genes would be regulated if the QSMs were secreted to a certain amount, and then favourable changes in the microbe's behavior could occurr (Whiteley et al., 2017; Mukherjee and Bassler, 2019). Regarding LAB, the QS system is divided into two categories according to the donor and receptor of QSMs. One is called intraspecies QS, using self-inducing peptides as QSMs, and the other is called interspecies communication QS using AI-2 (Autoinducer-2) as QSMs (Brexó and Sant'Ana, 2018; Kareb and Aïder, 2020). The signal molecule AI-2 is considered a 'bacterial Esperanto,' which can regulate the physiological activities of bacteria and communicate with fungi through the LuxS/AI-2 system (Rezzonico and Duffy, 2008). Studies have shown that the LuxS/AI-2 QS system could mediate the physiological activities of LAB, such as cell growth and biofilm formation (Gu et al., 2020; Gu et al., 2021). In bioethanol fermentation, LAB can form robust biofilms, which are helpful for the bacteria to grow in the fermentation facilities. The bacteria in the biofilm have higher resistance to antibiotics and preservatives, and the biofilm provides space for other microbes, contributing to more microbial contamination (Muthaiyan et al., 2011; Leathers et al.,

2014; Brexó and Sant'Ana, 2018). Therefore, it is necessary to inhibit the biofilm formation of LAB. So far, impeding biofilm formation by quenching the activity of signal molecule AI-2 is a relatively new method to prevent LAB contamination in the bioethanol fermentation system.

AI-2 quorum sensing inhibitors (QSIs) are used to quench the activity of signal molecule AI-2, that is, to block the synthesis, transmission, and reception of signal molecules in the LuxS/AI-2 QS system to prevent the intercellular communication of bacteria (Zhu and Li, 2012; Vipin, 2013). AI-2 analogs are common QSIs used to inhibit the formation of AI-2 intermediates. For example, furanone (DMHF), which has five-membered lactone rings similar to AI-2, can compete with AI-2, binding to the receptors and interfering with the regulation of the QS system (Ma et al., 2022). Besides, D-form sugars, like D-ribose (D-Rib) and D-galactose (D-Gal), have also been reported as QSIs to inhibit the AI-2 activity in the LuxS/AI-2 system (Niazy, 2020). So far, AI-2 QSIs are more widely used to inhibit the activity of AI-2 in pathogenic bacteria. Wu et al. (2019) found that DMHF could significantly inhibit the motility and AI-2 activity of *Listeria monocytogenes*. The biofilm inhibition rate of *Listeria monocytogenes* was 75.27%, and the production of the hemolytic ring completely ceased when the concentration of DMHF reached 200 μg/mL. Cho et al. (2016) showed that D-Rib and DMHF could destroy the biosynthesis of AI-2 in *Porphyromonas gingivalis*, inhibit the expression of *LuxS* gene, and finally prevent the occurrence of periodontal disease. However, the study of QSIs on the regulation of AI-2 secreted by LAB in the contaminated bioethanol production process has not

been reported.

This study aims to establish a novel way to prevent LAB contamination using QSIs. The AI-2 activities in the sole fermentation of *L. plantarum* and the co-fermentation of *S. cerevisiae* and *L. plantarum* were detected, respectively. Furthermore, QSIs (DMHF, D-Rib, and D-Gal) were added to the sole fermentation of *S. cerevisiae* to explore the effects of QSIs on the yield of bioethanol. Also, QSIs (DMHF, D-Rib, and D-Gal) were added to the sole fermentation of *L. plantarum* to investigate AI-2 activity, bacterial metabolism, and biofilm formation. On this basis, the effects of adding QSIs on the cell growth, metabolism, AI-2 activity, and bioethanol yield in the co-fermentation of *S. cerevisiae* and *L. plantarum* were investigated, which was set up to simulate the contamination of *L. plantarum* in the regular bioethanol fermentation process.

2. Materials and methods

2.1 Strains and QSIs

In this work, *Saccharomyces cerevisiae GIM2.188*, *Lactobacillus plantarum ATCC8014,* and *Vibrio harveyi BB170* used in this study were all purchased from the Guangdong Microbial Conservation Culture Collection Center. DMHF (≥ 98%), D-Rib (biotech grade), and D-Gal (biotech grade) were purchased from Shanghai Macklin Biochemical Co. Ltd., 2216E liquid medium for *V.harveyi BB170* activation, were purchased from Qingdao Hibo Biotechnology Co., Ltd.

2.2 Media and culture conditions

Yeast-Peptone-Dextrose (YPD) medium (2% peptone, 2% glucose, 1% yeast extract, based on w/v) was used to cultivate *Saccharomyces cerevisiae GIM2.188* at 28℃, 180 rpm (revolutions per min) in a shaking incubator. de Man-Rogosa-Sharpe (MRS) medium (1% peptone, 1% beef extract, 0.5% yeast extract, 0.2% diammonium hydrogen citrate, 2% glucose, 0.5% sodium acetate, 0.2% potassium phosphate, 0.058% magnesium sulfate, 0.025% manganese sulfate, based on w/v, 0.1% (v/v) Tween 80) was used to cultivated *Lactobacillus plantarum ATCC8014* at 37℃. *V.harveyi BB170* was cultured in an autoinducer bioassay (AB) medium at 30 ℃, 100 rpm. The AB medium (1L) was prepared by dissolving sodium chloride (17.5 g/L), magnesium sulfate heptahydrate (12.3 g/L), and acid hydrolyzed casein (excluding vitamins, 2 g/L) in 960 mL deionized water, adjusting the pH value to 7.5 using 3 mol/L potassium hydroxide, sterilizing at 121 ℃ for 15 min, and then adding 10 mL 1M sterile potassium phosphate buffer (pH 7.0) and 10 mL 0.1M L-arginine (filtered through 0.22 μm) and 20 mL 50 % (w/v) sterile glycerol.

2.3 Experimental methods

2.3.1 Preparation of cell-free fermentation supernatant (CFS)

Sole fermentation of *S. cerevisiae*: The *S. cerevisiae* was activated till the density of 4×10^5 cells/mL was achieved by counting with a blood cell plate counter, and then it was inoculated to the 180 mL YPD medium. The fermentation broth was sampled at 0, 3, 6, 9, 12, 24, and 30 h during the cultivation. The fermentation broth was centrifuged at 8000 rpm for 10 min and filtered with a 0.22 μm filter to obtain CFS

for detecting ethanol concentrations and reducing sugar. CFS was stored at -25 ℃ before determination.

Sole fermentation of *L. plantarum*: The activated *L. plantarum* was inoculated to a 180 mL YPD medium with a density of 1×10^8 cells/mL counting by the dilution coating method. The fermentation broth was sampled at 0, 4, 8, 12, 24, 36, and 48 h during the cultivation. The fermentation broth was centrifuged at 8000 rpm for 10 min and filtered with a 0.22 μm filter to obtain CFS for detecting lactic acid concentrations and reducing sugar. The activity of signal molecule AI-2 in the sole fermentation of *L. plantarum* was detected. The supernatant used for measuring AI-2 activity was collected every 3 h. The pH of the supernatant was adjusted to 7.0 with 1 mol/L KOH solution. Then, the supernatant was filtered with a 0.22 μm filter and stored at -25 ℃ before determination.

Co-fermentation of *S. cerevisiae* and *L. plantarum*: *L. plantarum* was inoculated into the fermenter culturing with sole *S. cerevisiae* at the concentration of 1×10^8 cells/mL to simulate the contamination situation of *L. plantarum* in the industrial bioethanol fermentation process. The fermentation broth was sampled according to the method in the sole fermentation of *S. cerevisiae*. After centrifugation at 8000 rpm for 10 min, the supernatant was filtered with a 0.22 μm filter for the subsequent detection of the concentrations of bioethanol, lactic acid, and reducing sugar. To detect the activity of AI-2 in co-fermentation, 2% glucose in the YPD medium was replaced with 12% skim milk. The CFS was prepared in the same way as the sole fermentation system and was stored at -25 ℃ before determination.

V.harveyi BB170 was activated and inoculated in AB medium for 12 h. Then, the fermentation broth was sampled and centrifuged at 8000 rpm for 10 min, and the supernatant was filtered with a 0.22 μm filter to obtain CFS working as a positive control. MRS medium and AB medium were filtered using 0.22 μm filters as a medium and negative control, respectively. CFS was stored at -25 ℃ before determination.

2.3.2 Addition of QSIs in sole/co-fermentation

DMHF (0.2, 0.1, 0.05 g/L), D-Rib (15.0, 7.0, 1.5 g/L) and D-Gal (18.0, 9.0, 1.8 g/L) were added in the sole fermentation of *S. cerevisiae* (SC+QSIs), respectively. To ensure that no reduction of the ethanol yield occurred, the highest concentrations of QSIs were added in the sole fermentation of *L. plantarum* (LP+QSIs), and the same amount of deionized water was added as the control group (LP). The QSIs that could inhibit the AI-2 activity of *L. plantarum* were selected and added in the co-fermentation of *S. cerevisiae* and *L. plantarum* (SC+LP+QSIs), and the same amount of deionized water was added as the control group (SC+LP).

2.4 Analysis methods

2.4.1 AI-2 activity

The optimized bioluminescence assay based on the report of Taga and Xavier (2011) was applied in this work. In brief, 1% (v/v) activated *V.harveyi BB170* was cultured in AB medium at 30 ℃, 100 rpm until $OD_{600\ nm}$ of the cell culture reached

0.9~1.2. The culture fluid was diluted with fresh AB medium at 1:5000 (v/v). The positive, negative, and medium control test samples were mixed with the diluted *V. harveyi BB170* culture at 1:10 (v/v), respectively, cultivating at 30 ℃, 100 r/min. Within 0~5 h, 200 μL fluid was sampled and placed into a black 96-well plate every 30 min. The fluorescence intensity was detected in the chemiluminescence mode of the multi-function microplate reader (BioTek Cytation 5, US), and the curve of fluorescence intensity changes over time was drawn. The fluorescence intensity of each sample was measured when the fluorescence intensity of the negative control reached the lowest value, and the relative fluorescence intensity was calculated to represent the activity of the signal molecule AI-2. The calculation formula is as follows:

$$\text{Relative fluorescence intensity} = \frac{\text{Fluorescence intensity value of sample}}{\text{Fluorescence intensity value of medium control}}$$

The inhibition rate of QSIs on AI-2 activity was calculated as follows:

$$\text{Inhibitory rates (\%)} = \frac{\text{Relative fluorescence intensity of control} - \text{Relative fluorescence intensity of sample}}{\text{Relative fluorescence intensity of control}}$$

2.4.2 Growth curves and concentrations of ethanol, lactic acid, and reducing sugar

As reported in our previous work (Tian et al., 2023), the $OD_{600\ nm}$ value was measured using a spectrophotometer once the fermentation broth was diluted 5-fold. The growth curve was plotted analyzed against the X-axis of time and the Y-axis of $OD_{600\ nm}$. The concentrations of reducing sugar, ethanol, and lactic acid in CFS were determined by using the 3,5-dinitrosalicylic acid method (DNS), potassium

dichromate method and p-hydroxybiphenyl method, respectively (G., 2002; Peng et al., 2019).

2.4.3 Effect of QSIs on biofilm formation of *L. plantarum*

The activated *L. plantarum* (1×10^8 cells/mL) was inoculated in an MRS medium supplemented with different types of QSIs. 200 μL of bacterial suspension was added to 96-well plates and placed in an incubator at 37 ℃ for 24 h. After cultivation, the biofilm formation was determined by the crystal violet staining method (O'Toole, 2011).

2.4.4 Effects of QSIs on biofilm morphology of *L. plantarum*

The observation of biofilm morphology was carried out by using an optical microscope (x400). The activated *L. plantarum* (1×10^8 cells/mL) was inoculated in MRS medium. Then 1 mL fluid was sampled and placed in 24-well plates, added with 100 μL QSIs, and cultured at 37 ℃ for 24 h. Meanwhile, sterile water was used to replace QSIs as a control group. The floating cells in the well were removed, and the slides were washed with sterile water. After staining with 500 μL crystal violet (0.4 %) for 20 min, sterile water was used again to rinse the slides 5 times slowly and then the slides were dried under sterile conditions. The biofilm structure was observed by using an optical microscope at 40×10.

The biofilm morphology was also observed by scanning electron microscopy (SEM). A sterile cell climbing sheet (Φ14 mm) was placed in 24-well plates. The culture method was the same as above. The floating cells in the well were removed,

and sterile water was used to rinse the cell climbing sheet 3 times slowly. After drying the cell climbing sheet under sterile conditions, it was fixed in 2.5% glutaraldehyde solution at 4 ℃ for 12 h. Then sterile water was used again to rinse the cell climbing sheet 3 times to remove the residual glutaraldehyde. Finally, the cell climbing sheet was dehydrated in 40%, 70%, 90%, and 100% ethanol for 15 min, followed by vacuum drying and gold spray. The biofilm structure was observed using SEM at 5000 times magnification.

2.5 Statistical analysis

All statistical analyses were performed using SPSS 22.0. One-way or two-way analysis of variance (ANOVA) and two-tailed t-tests were used to determine the significant differences between groups. $P<0.05$ was considered to be significantly different.

3. Results and discussion

3.1 Activity of AI-2 in sole fermentation of *L. plantarum*

The activity of the signal molecule AI-2 of *L. plantarum* was quantitatively detected by using *V.harveyi BB170,* which only senses the signal molecule AI-2 due to the lack of *LuxN* gene that is used for sensing the signal molecule AI-1 (Bassler et al., 1994). As shown in Fig.1, the fluorescence intensity of each sample showed a decreasing trend and then an increasing tendency. The initial fluorescence intensity came from the AI-2-induced luminescence produced by *V. Harveyi BB170*. However,

after dilution of *V.harveyi BB170* fluid, the cell density decreased, causing the fluorescence to gradually quench, and the concentration of AI-2 could not reach the threshold to induce luminescence. The fluorescence intensity of the negative control decreased to the lowest level within 3 h of cultivation. The cell density gradually increased after 3 h and more AI-2 was produced to reach the threshold to induce luminescence, obtaining a gradual increase in fluorescence intensity. The changes in fluorescence intensity in the medium control were consistent with the negative control, indicating that the fresh MRS medium did not contain the signal molecule AI-2. AI-2 was produced in the positive control, and the fluorescence intensity rose at 2.5 h. Hence, compared to the fluorescence intensity of *V. harveyi BB170* cultivated at 3 h, the relative fluorescence intensity of the supernatant of *L. plantarum* could be obtained, presenting the activity of the signal molecule AI-2.

The relationship between the cell density and AI-2 produced by *L. plantarum* is shown in Fig.2. Before 12 h, the relative fluorescence intensity gradually increased with the growth of cells, reaching a maximum of 9.2. At 12 h of cultivation, the growth of *L. plantarum* reached the late logarithmic phase and began to become stable, and the highest activity of AI-2 was noted. Within 12 h of cultivation, a positive correlation between the cell density and AI-2 of *L. plantarum* was found. After 12 h, a decline in the growth of *L. plantarum* occurred, but the cell density almost remained stable, while the fluorescence intensity significantly reduced. Hence, the CFS of *L. plantarum* was collected at 12 h to detect the activity of AI-2 by measuring the fluorescence intensity in this study.

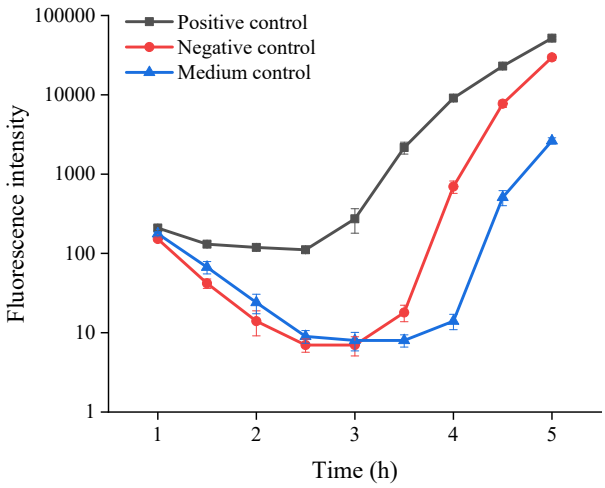


Fig. 1. Fluorescence intensity of *Vibrio harveyi bb170*

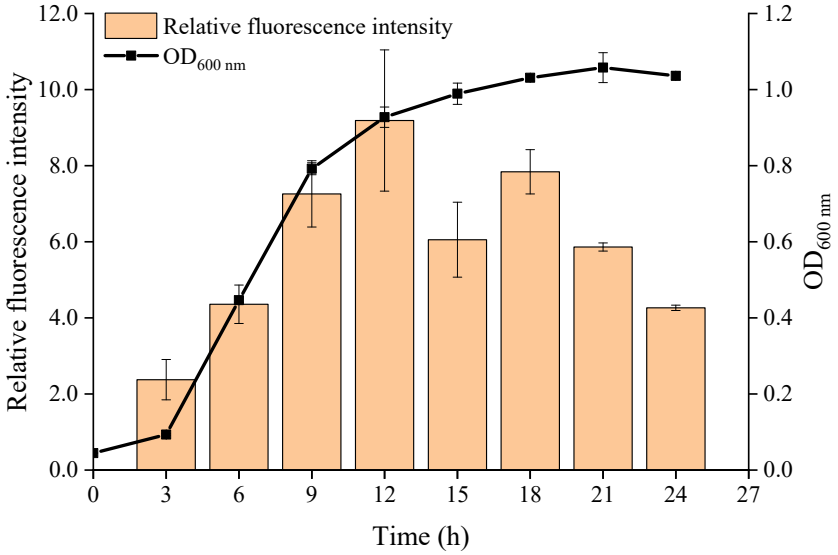


Fig. 2. Relationships between AI-2 and cell density of *L. plantarum*

3.2 Activity of AI-2 in co-fermentation of *S. cerevisiae* and *L. plantarum*

S. cerevisiae and *L. plantarum* were co-cultured in the YPD medium. The activity of AI-2 was not detected in the sterile fermentation supernatant. When the pH of the supernatant was adjusted to 7.0, the activity of AI-2 still could not be detected. The reason was that the concentration of AI-2 in the co-fermentation reactor was too

low, and the high concentrations of glucose in the YPD medium interfered with the luminescence of *V. harveyi BB170* (Yevgeniy and Michael, 2006; Zhao et al., 2018). Therefore, skim milk was used to replace the glucose in the YPD medium. The activities of AI-2 in co-fermentation are shown in Fig.3. The relative fluorescence intensity in the sole fermentation of *S. cerevisiae* (SC) was lower than that in the medium control, which indicated that *S.cerevisiae* could not produce signal molecule AI-2. Being different from the trend of AI-2 activities of *L. plantarum* growing in the MRS medium (Fig.2), it took longer to produce AI-2 in the sole fermentation of *L. plantarum* (LP) when the skim milk replaced the glucose in the medium, and no obvious AI-2 was detected within 12 h fermentation. This result was due to the various utilizations of glucose and skim milk by *L. plantarum*. Skim milk is composed of lactose, which is more challenging than glucose to metabolize by *L. plantarum*. Therefore, *L. plantarum* grew slowly in skim milk medium, and its ability to produce AI-2 was relatively weakened.

It can be seen in Fig. 3 that the AI-2 activity of co-fermentation of *S. cerevisiae* and *S. cerevisiae* (SC+LP) was lower than that of sole fermentation of *L. plantarum* (LP), which was consistent with the results of Gu et al. (2021). The reason was that with the increase of culture time, *S. cerevisiae* grew rapidly and began to compete with *L. plantarum* for nutrients, which reduced the ability of *L. plantarum* to secrete the signal molecule AI-2. It indicated that *S. cerevisiae GIM2.188* could inhibit *L. plantarum ATCC8014* from secreting AI-2 in the co-fermentation reactor. However, the symbiotic relationship between yeast and lactic acid bacteria is complex. Studies

have shown that the co-fermentation of yeast and LAB can promote or inhibit the production of signal molecule AI-2 (Guo, 2015).

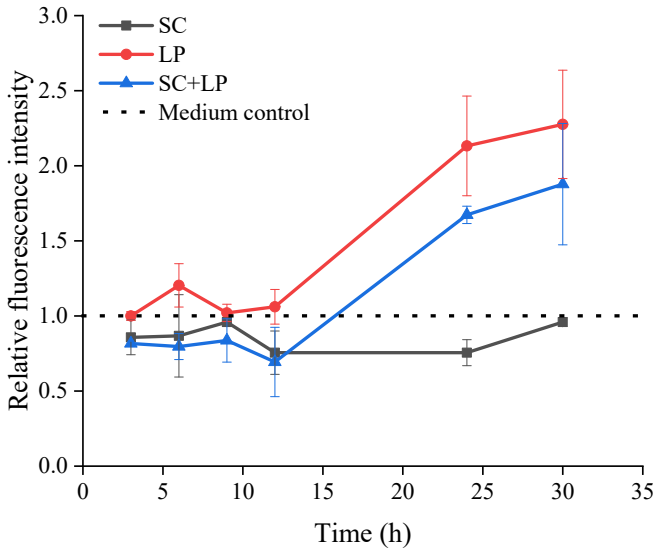


Fig. 3. Activity of AI-2 in co-fermentation of *S. cerevisiae* and *L. plantarum*. (Note: SC means *S. cerevisiae* and LP means *L. plantarum*)

3.3 Effects of AI-2 quorum sensing inhibitor on sole fermentation of *S. cerevisiae*

Compared to the control group, DMHF had no significant effects on the growth of *S. cerevisiae* and ethanol yields (P>0.05) (Fig.4 (a) and Fig.4 (A)). D-Rib had no significant effects on the growth of *S. cerevisiae,* too (Fig.4 (b)). Adding 15.0 g/L and 7.5 g/L D-Rib enhanced the ethanol yields at the beginning of fermentation. Still, the final ethanol yields were 18% and 19% lower than that of the control group (P<0.05) at the end of fermentation, respectively (Fig.4 (B)). In addition, the content of reducing sugar in each group increased with the adding dosage of D-Rib at 30 h, indicating that D-Rib was not consumed by *S.cerevisiae GIM2.188* as a fermentation

substrate. On the other hand, D-Gal is a reducing hexanal sugar, which can be converted by *S. cerevisiae* into glucose-6-phosphate through the Leloir pathway, and then glucose-6-phosphate can be further converted into pyruvate through the EMP pathway based on the metabolism of *S. cerevisiae* (Bro et al., 2005; Lee et al., 2011). As shown in Fig.4 (c), D-Gal promoted the growth of *S. cerevisiae*. Higher cell intensities were observed with the increase of D-Gal concentrations. Also, the final ethanol yields significantly increased by adding 18.0 g/L and 9.0 g/L D-Gal, reaching 81% and 36% higher than that of the control group ($P<0.05$), respectively (Fig.4 (C)). Although *S. cerevisiae* can use D-Gal as a carbon source to produce ethanol, the ethanol yield is lower than that using glucose as a substrate. Moreover, D-Gal is more expensive. Therefore, D-Gal applied in this work was considered a QSI affecting the activity of AI-2 of *L. plantarum* rather than a carbon source for the growth of *S. cerevisiae* to produce ethanol. Because these three QSIs (DMHF, D-Rib, and D-Gal) all had no inhibition on the growth of *S.cerevisiae* to produce ethanol, the highest concentrations of 0.2 g/L DMHF, 1.5 g/L D-Rib and 18.0 g/L D-Gal were selected to apply in the sole fermentation of *L. plantarum.*

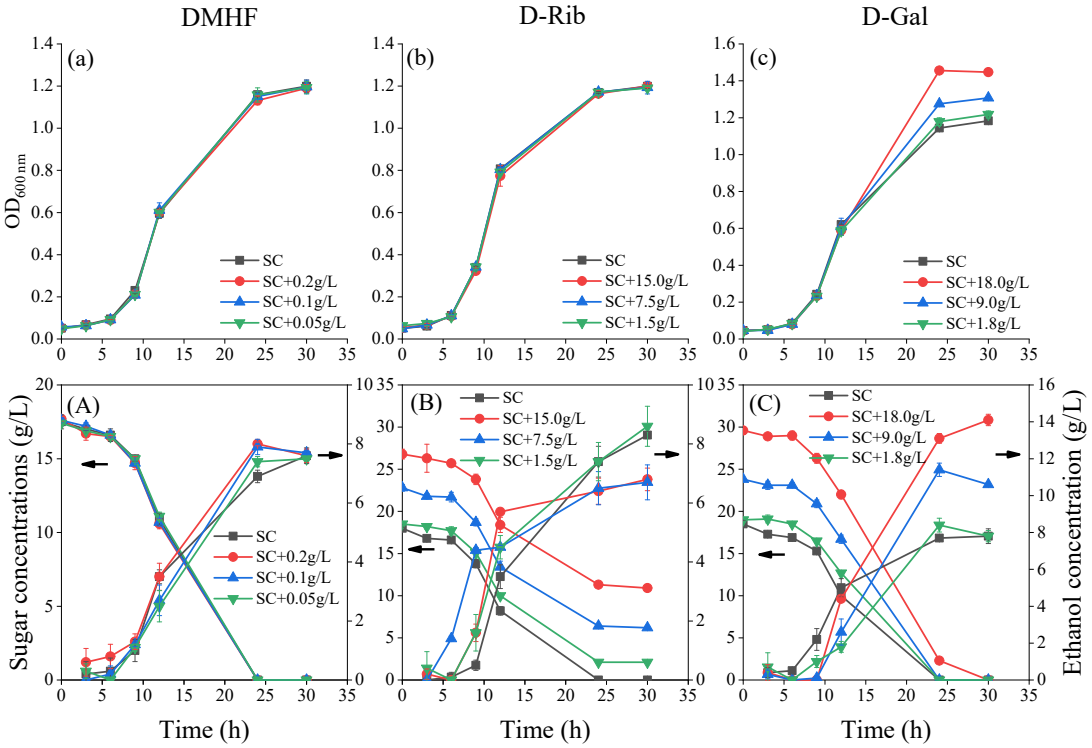


Fig. 4. Effects of QSIs on the cell density, sugar concentration, and ethanol production in sole fermentation of *S. cerevisiae*. (Note: SC means *S. cerevisiae*)

3.4 Effects of AI-2 quorum sensing inhibitors on sole fermentation of *L. plantarum*

AI-2 can promote LAB's growth and increase EPS production, which is the main component of LAB biofilm, and it also enhances the environmental tolerance of LAB (Gu et al., 2021; Zhang et al., 2022). In this study, AI-2 QSIs were added to inhibit the activity of AI-2 secreted by *L. plantarum*. Simultaneously, the effects of AI-2 QSIs on cell growth, biofilm formation, cell morphology, and lactic acid yield were investigated. As shown in Fig. S1, compared to the control group (LP), the relative fluorescence intensity of *L. plantarum* was significantly reduced in the groups in which 0.2 g/L DMHF and (or?) 18.0 g/L D-Gal was added, which proved that AI-2

QSIs had a significant inhibit on AI-2 activity. Fig.5 (a) showed that the addition of 0.2 g/L DMHF had the highest inhibition rate of 38% on the activity of AI-2, followed by the addition of 18.0 g/L D-Gal with an inhibition rate of 36%, confirming the results in Fig. S1. The addition of 1.5 g/L D-Rib did not inhibit the activity of AI-2. As an AI-2 analog, D-Rib can only compete with AI-2 at an appropriate concentration. The inhibition effect also varies with the AI-2 receptor. According to Zheng et al. (2022), 19.65% and 28.57% of the inhibition rates were obtained when 200 μmol/L D-Rib was added in the sole fermentation of *Lactobacillus fermentum 2-1* or *Lactobacillus fermentum 221*, respectively. It can be seen from Fig.S2 (a) that the addition of three QSIs had no significant effect on the growth of *L. plantarum* (P>0.05), which was consistent with the results of Zheng et al. (2022). The addition of three QSIs also had no significant effects on the yield of lactic acid (P>0.05) (Fig.S2 (b)). Compared to the control group, most reducing sugar remained in the reactors with D-Gal at 48 h, indicating that *L. plantarum ATCC8014* cannot use D-Gal as a carbon source for metabolism.

The effects of three QSIs on the biofilm formation of *L. plantarum* were determined. As shown in Fig.5 (b), the addition of 0.2 g/L DMHF and 18.0 g/L D-Gal both had significant inhibitory effects on the biofilm formation of *L. plantarum* (P>0.05). The results of Ma et al. (2022) showed that DMHF blocked the LuxS/AI-2 QS system of *Lactobacillus fermentum L1*, successfully inhibited the expression of QS-related gene *LuxS* and enolase gene *eno* in the glycolysis pathway, and decreased the contents of AI-2, conjugated linoleic acid and cell density of *Lactobacillus*

359 *fermentum L1*. Similarly, Wu et al. (2019) found that DMHF significantly inhibited
360 the AI-2 activity *Listeria monocytogenes* and inhibited the biofilm formation of
361 *Listeria monocytogenes*. The biofilm inhibition rate of *Listeria monocytogenes*
362 reached 75.27% when 200 mg/L DMHF was applied. The results of Ryu et al. (2016)
363 showed that D-Gal significantly inhibited the biofilm formation of periodontal
364 pathogens without affecting the growth of bacteria, which was consistent with the
365 results of this study. Moreover, Jing et al. (2021) found that the addition of 0.2 mol/L
366 D-Gal could reduce the biofilm formation via down-regulating the expression of *LuxS*
367 gene of *Lactobacillus plantarum LIP-1* and the key gene *cysE* for biofilm formation.
368 The effects of three QSIs on the morphology and biofilm of *L. plantarum* were
369 explained using an optical microscope and SEM. It can be seen from Fig.6 (A) and (a)
370 that the *L. plantarum* cells in the control group were interconnected to form a
371 relatively complete biofilm structure, indicating that the cell adhesion was strong.
372 Fig.6 (C) and ??(c) ??showed that there was a certain degree of ?time? gap in the
373 biofilm after adding 1.5 g/L D-Rib, and some free cells appeared, which was
374 consistent with the results of Zheng et al. (2022). In Fig.6 (B, b) and (D, d), it can be
375 seen that after adding 0.2 g/L DMHF and 18.0 g/L D-Gal, less biofilm was formed
376 and the bacteria were dispersed. It can also be seen from the SEM images that
377 compared to the control group, the addition of 0.2 g/L DMHF and 18.0 g/L D-Gal
378 resulted in sparse biofilm and large voids. The addition of D-Gal also
379 generatedwrinkles on the surface of *L. plantarum*. It indicated that the addition of 0.2
380 g/L DMHF and 18.0 g/L D-Gal reduced the adhesion of *L. plantarum* and decreased

the bacterial aggregation, resulting in the inhibition of biofilm formation, which was consistent with the results of Wang (2019) and Jing et al. (2021). According to the above results, 0.2 g/L DMHF and 18.0 g/L D-Gal were applied as QSIs for the subsequent co-fermentation of *L. plantarum and S. cerevisiae*.

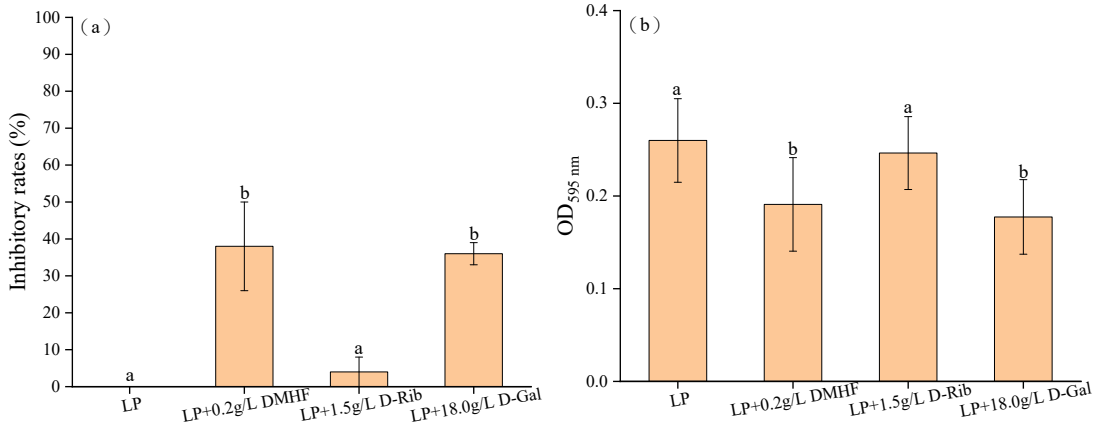


Fig. 5. Inhibition effects of QSIs on AI-2 activity (a) and biofilm formation (b) of *L. plantarum*. (Note: LP means *L. plantarum*)

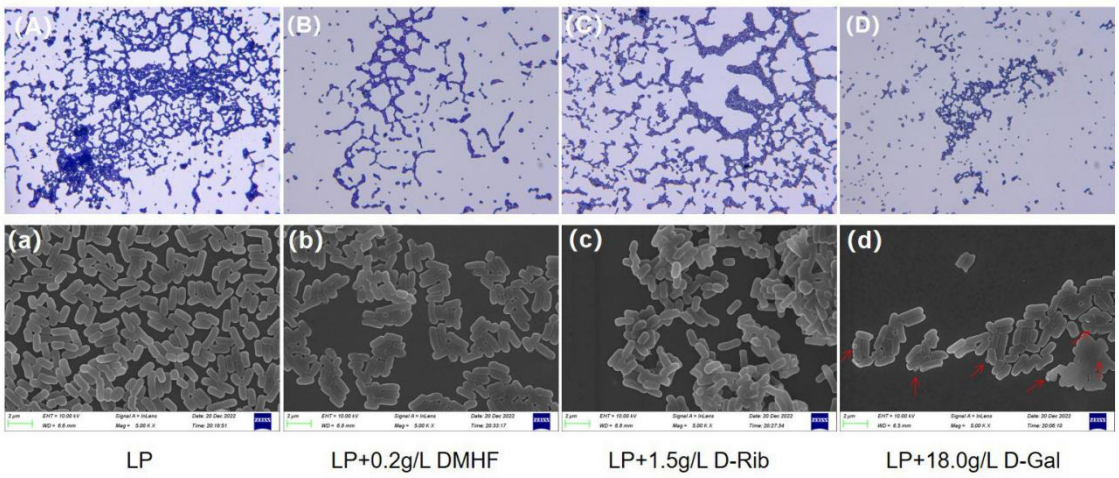


Fig. 6. Effect of QSIs on biofilm morphology of *L. plantarum*. (Note: LP means *L. plantarum*, Figures (A)~(D) and (a)~(d) are images observed by optical microscope at 400 times magnification and scanning electron microscope at 5000 times magnification, respectively)

3.5 Effects of AI-2 quorum sensing inhibitors on co-fermentation of *S. cerevisiae*

and *L. plantarum*

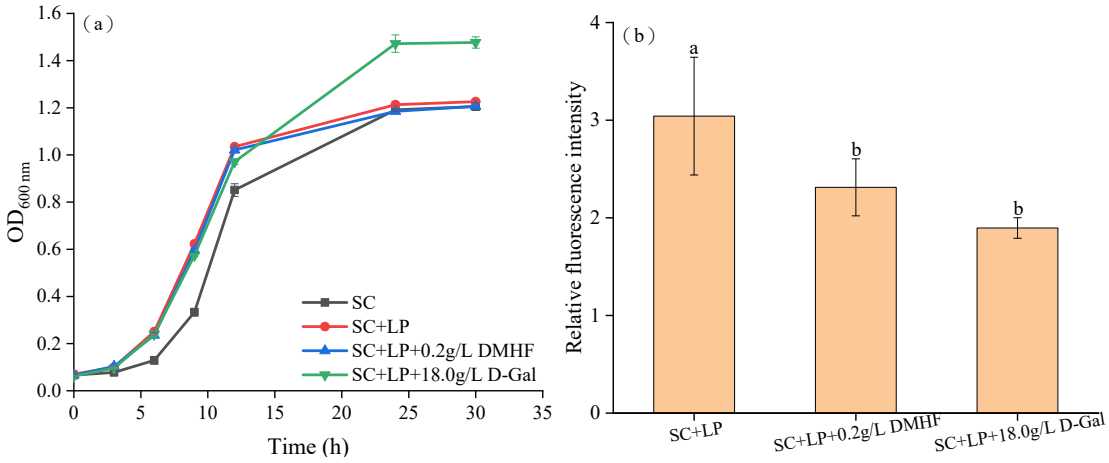
The addition of 0.2 g/L DMHF had no significant effect on cell growth in the co-fermentation system (P>0.05) (Fig.7(a)). The addition of 18.0 g/L D-Gal inhibited the cell growth at 9~12 h of fermentation. The reason may be that D-Gal inhibited the biofilm formation of *L. plantarum* at this stage, resulting in severe environmental stress on *L. plantarum* and inhibited growth. It was consistent with the result that the addition of 0.2 g/L DMHF and 18.0 g/L D-Gal both significantly reduced activities of AI-2 in the co-fermentation system (P<0.05) (Fig.7 (b)). However, the addition of 18.0 g/L D-Gal significantly promoted the cell density at 24~30 h of co-fermentation (P<0.05). The possible reason was that D-Gal could be used as a carbon source to promote the metabolism of *S. cerevisiae* (Hartl et al., 2007).

Fig.7 (c) showed that the ethanol concentration in the co-fermentation group (SC+LP) was significantly lower than that of the control group (SC), and the ethanol production decreased by 28% within 30 h of fermentation. The addition of 0.2 g/L DMHF and 18.0 g/L D-Gal increased the ethanol concentration to 6.6 g/L and 11.7 g/L, respectively, which were 14% and 103% higher than that of the co-fermentation group (SC+LP) (P<0.05), indicating that the addition of DMHF and D-Gal reduced the negative effect of *L. plantarum* on the ethanol production. Moreover, the ethanol concentration in the co-fermentation group with 18.0 g/L D-Gal was 46% higher than that of the control group (SC) at 30 h, even though the ethanol production was inhibited at the early stage of fermentation due to the too-high concentration of reducing sugar for the growth of *S. cerevisiae* leading to the lag of ethanol

418 metabolism (Chen, 2017). D-Gal could promote ethanol production as a carbon
419 source in the co-fermentation group and furthermore, it could also reduce the negative
420 effect of *L. plantarum* as a QSI in the co-fermentation process.

421 Compared to the group of SC+LP, the concentration of lactic acid was increased
422 significantly when 18.0 g/L D-Gal was added (P<0.05) (Fig.7 (d)). The reason was
423 that the addition of galactose may protect *L. plantarum* cells from the hindrance of
424 intracellular lactic acid concentration, thus resulting in lactic acid accumulation
425 (Tendekayi et al., 2001). However, the accumulation of lactic acid would reduce the
426 pH in the system and inhibit the growth of *S.cerevisiae* because the solubility of
427 undissociated lactic acid in the plasma membrane and the insolubility of dissociated
428 lactic acid lead to cytoplasmic acidification and protons drive failure (Majdiah et al.,
429 2017). Therefore, the addition of D-Gal in the co-fermentation system led to the
430 increase of lactic acid, which would affect the fermentation of *S. cerevisiae* to
431 produce ethanol.

432



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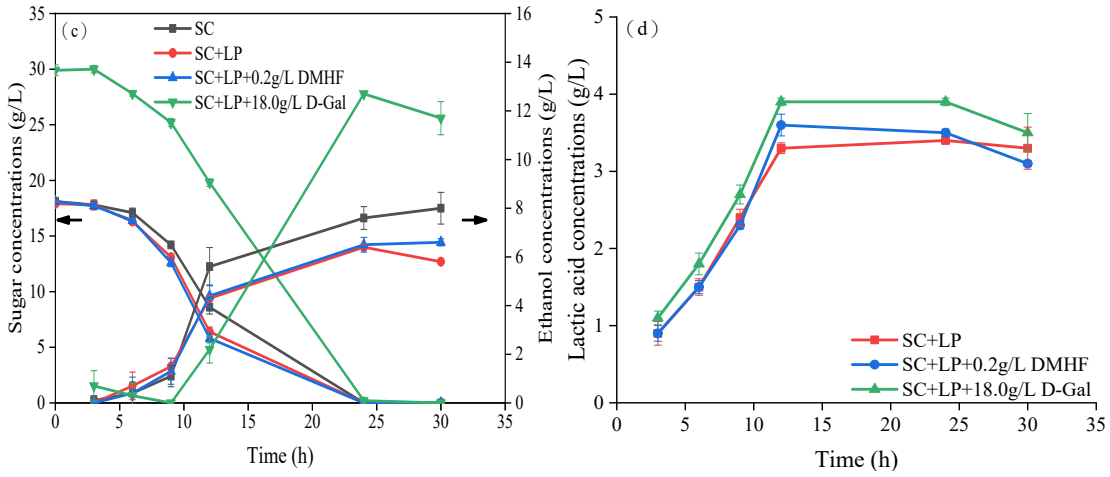

Fig. 7. Effects of QSIs on the cell density (a), AI-2 activity (b), sugar concentration and ethanol production (c), lactic acid concentration (d) in co-fermentation of *S. cerevisiae* and *L. plantarum*. (Note: SC means *S. cerevisiae* and LP means *L. plantarum*)

4. Conclusions

The cell density of *L. plantarum* was positively correlated with the activity of signal molecule AI-2. The highest activity of AI-2 was observed at 12 h of sole fermentation of *L. plantarum*. *S. cerevisiae* inhibited *L. plantarum* from secreting AI-2 in their co-fermentation reactor. The addition of 0.2 g/L DMHF could reduce 38% of AI-2 activity in the sole fermentation of *L. plantarum* and significantly inhibit biofilm formation but had no significant adverse effect on the ethanol yield in the sole fermentation of *S.cerevisiae*. In the co-fermentation system of *S.cerevisiae* and *L.plantarum*, the ethanol concentrations in the groups which were containing 0.2 g/L DMHF and 18.0 g/L D-Gal were 14% and 103% higher than that in the group without OSIs, and the activities of AI-2 were also significantly inhibited with QSIs addition.

CRediT authorship contribution statement

Jun Tian: Investigation, Validation, Data curation, Writing - Original draft

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Liheng Zhang: Investigation, Formal analysis, Data curation

Xianzhi Meng: Validation, Writing, Editing

Arthur J. Ragauskas: Supervision, Review & Editing

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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