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Effect of $MgSO_4$ on Lactic Acid Production in *Rhizopus oryzae* Fermentation

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We investigated the effects of $MgSO_4$ on L-lactic acid production by the *Rhizopus oryzae* strain RLC41-6 in order to improve the production rate and quality of L-lactic acid. We also studied the effects of $MgSO_4$ on intracellular lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) activity. Different concentrations of $MgSO_4$ were added to the fermentation medium before culture of *R. oryzae* at 36°C for 36 h. L-lactic acid concentration was determined by reverse phase high performance liquid chromatography, and the activity and intracellular expression of LDH and ADH were examined via polyacrylamide gel electrophoresis and kinetic methods. Although increasing $MgSO_4$ concentrations led to increased lactic acid production by *R. oryzae*, $MgSO_4$ had no effect on LDH expression or activity. Furthermore, $MgSO_4$ promoted mycelium growth and inhibited ADH activity. With an initial $MgSO_4$ concentration of 0.075%, maximum mycelium production of 36.8 g/L was achieved and L-lactic acid was the only fermentation product, at ~138 g/L with a 92% conversion rate. An appropriate concentration of $MgSO_4$ can inhibit the conversion of pyruvic acid into malic acid and fumaric acid as well as both ADH activity and alcohol synthesis, thereby enhancing the conversion of glucose into lactic acid in *R. oryzae*.

Key words: L-lactic acid; *Rhizopus oryzae*; Lactate dehydrogenase; Alcohol dehydrogenase; $MgSO_4$.

Lactic acid is widely applied in the food, medicinal, agricultural, and chemical industries. Polylactic acid can be used to produce completely biodegradable plastics and is widely applied in pharmaceutical and medical industries[1].

Rhizopus oryzae is characterized as a filamentous fungus that belongs to the Ascomycetes class. It has been adopted in lactic acid fermentation industries worldwide, because it can be used to produce L-lactic acid of high purity. *Rhizopus oryzae* can convert glucose into pyruvic acid via the Embden–Meyerhof–Parnas (EMP) pathway. Then the pyruvic acid is utilized in one of four pathways: (1) ethanol production via pyruvate decarboxylase and alcohol dehydrogenase (ADH); (2) oxaloacetate formation from pyruvate carboxylase, which further generates malic acid and fumaric acid; (3) direct generation of lactic acid from L-lactate dehydrogenase (LDH); and (4) the tricarboxylic acid cycle[2]. Thitiprasert *et al*.[3] added an ADH inhibitor into the fermentation of *R. oryzae* and found that less pyruvic acid was converted to ethanol and more lactic acid obtained. Therefore, the products of *Rhizopus* fermentation are dependent on the fermentation conditions, in addition to being strongly dependent on the characteristics of the specific *Rhizopus* strains. Based on the results of metabolic flux analysis, Bai

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et al.[4] concluded that the theoretical conversion rate for *R. oryzae* conversion of glucose into lactic acid is 98.2%. By adopting a homogeneous design, Yao *et al.*[5] studied the relationships between lactic acid production and five factors including the concentrations of glucose, cow dung crude protein, $ZnSO_4$, $MgSO_4$, and KH_2PO_4. They concluded that the $ZnSO_4$ concentration had the greatest effect on lactic acid production, followed by the concentrations of cow dung crude protein and KH_2PO_4, and that the effect of $MgSO_4$ on lactic acid metabolism was minimal. Pan *et al.*[6] studied the effects of zinc and magnesium ions in *R. oryzae* metabolism and reported that magnesium has a mild inhibitory effect on ADH activity but enhances LDH activity.

To improve the production of L-lactic acid, the amounts of pyruvic acid used in the pathways for ethanol and oxaloacetate production must be reduced. We hypothesized that an appropriate concentration of inorganic salts can increase the production of lactic acid and inhibit the production of fumaric acid and other pyruvic acid metabolites during *R. oryzae* fermentation. In order to increase the production of L-lactic acid by *R. oryzae*, we previously studied the effects of many inorganic salts on lactic acid metabolism[7]. In the present study, we extended our research by investigating the relationships between $MgSO_4$ concentration and lactic acid production by *R. oryzae* as well as LDH expression.

MATERIALS AND METHODS

Strains

The *R. oryzae* RLC41-6 strain (using PW352 as the original strain) was obtained via ionizing-radiation breeding for screening[8]. Medium: Spore-producing medium consisted with 600 g potato, 20 g glucose, 20 g agar, and 1 g $CaCO_3$ per liter of improved potato dextrose agar (PDA) medium; the seed medium consisted of 10 wt% glucose, inorganic salts at the indicated concentrations, 2 wt% $CaCO_3$ (individually sterilized); and the fermentation medium contained 15 wt% glucose, inorganic salts, and 5 wt% $CaCO_3$ (individually sterilized).

Slant culture conditions

36°C, 4–7 days. Liquid seed culture conditions: Specific amounts of spore suspension were added to 50 mL seed medium in a 250-mL conical flask to achieve an initial cultured spore concentration of 1.0×10^6 spores/mL. The liquid seed was cultured at 36°C with oscillation at 200 rpm for 12 h. Fermentation culture conditions: The cultured seed liquid was added to a concentration of 4 vol% in 50 mL fermentation medium in a 250-mL conical flask and cultured at 36°C with oscillation at 200 rpm for 36 h.

Qualitative determination of L-lactic acid in the fermentation broth

Reversed phase high performance liquid chromatography (RP-HPLC)[9] was performed using a Luna C18 3.9 nm × 300 nm column, a mobile phase of methanol:water:phosphoric acid (10-90-0.3), a flow velocity of 0.8 mL/min, a column temperature of 25°C, and a detection wavelength of 210 nm. Quantitative determination of L-lactic acid in the fermentation broth: The EDTA titrimetric method and SBA40C enzyme electrode method were used as previously described[10,11].

Determination of LDH expression and activity

Polyacrylamide gel electrophoresis methods and kinetic methods were applied as previously described[12]. Determination of ADH activity: Kinetic methods were applied as previously described[13].

RESULTS AND DISCUSSION

Effect of $MgSO_4$ concentration on fermented L-lactic acid purity

$MgSO_4$ provides magnesium and sulfur for cell growth. Magnesium is an activator of many enzymes and plays an important role in the stability of ribosomes, cell membranes, and nucleic acids. Sulfur is a component of amino acids such as cysteine and methionine as well as coenzyme and glutathione. In this study, different concentrations of $MgSO_4$ were added to *R. oryzae* cultures in order to observe the effects of $MgSO_4$ concentration on L-lactic acid production. Fig. 1 shows representative HPLC chromatograms of the fermentation products of RLC41-6 after culture in medium with different initial concentrations of $MgSO_4$ for 36 h. Without the addition of $MgSO_4$, complex metabolism products of strains were observed and more products such as fumaric acid, malic acid, and citric acid were formed. With the addition of $MgSO_4$, the amounts of heteroacids

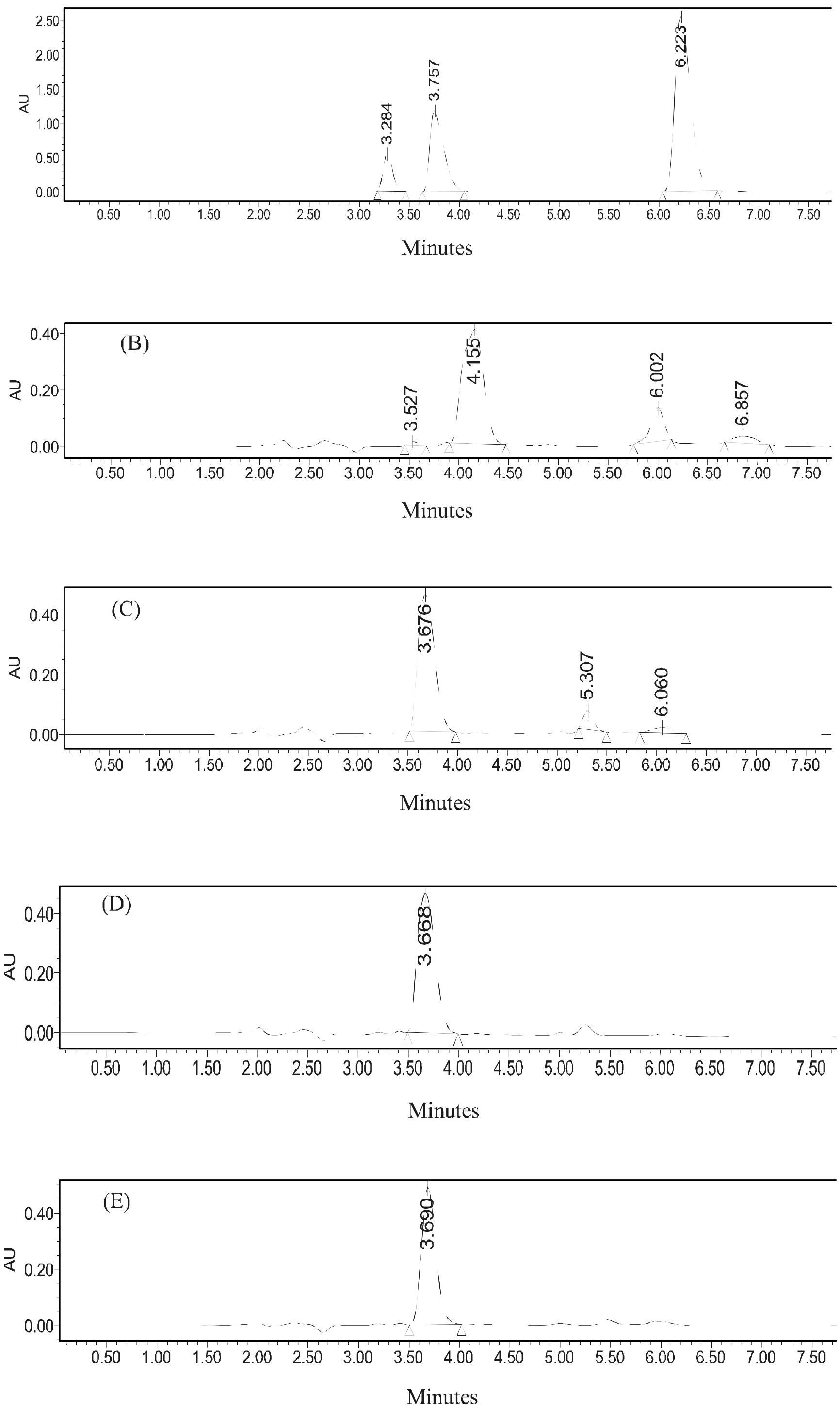

Fig.1. HPLC analysis of RLC41-6 fermentation products. A: Standard HPLC chart, showing peaks from left to right for malic acid, L-lactic acid, and fumaric acid; B: with a [$MgSO_4$] of 0.000%, peaks from left to right represent malic acid, L-lactic acid, unknown acid 1, and fumaric acid; C: with a [$MgSO_4$] of 0.025% peaks from left to right represent L-lactic acid, unknown acid 2, and fumaric acid; D: with a [$MgSO_4$] of 0.050%, a peak for L-lactic acid; and E: with a [$MgSO_4$] of 0.075%, a peak for L-lactic acid.

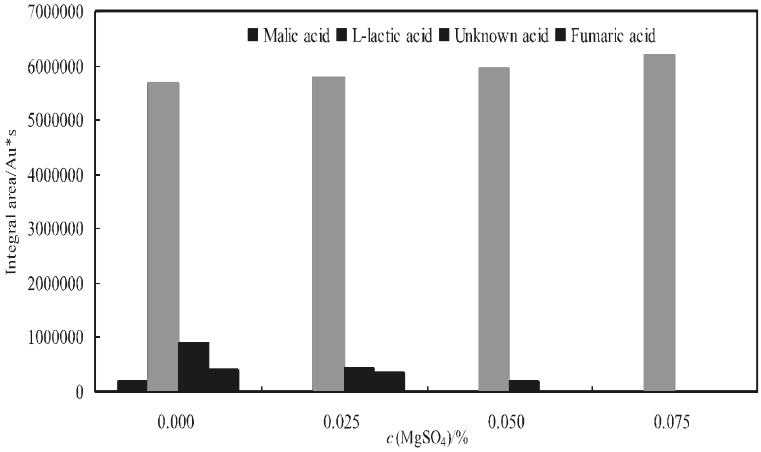

Fig. 2. Effects of $MgSO_4$ concentration on the levels of different RLC41 fermentation products

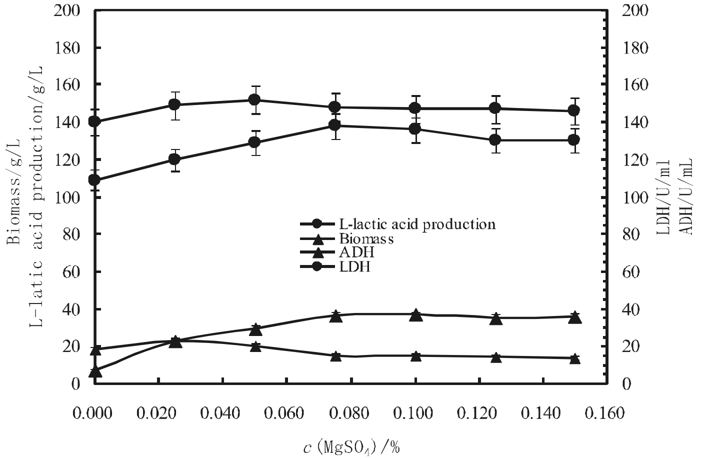

Fig. 3. Effects of $MgSO_4$ concentration on L-lactic acid and biomass production as well as LDH and ADH activity

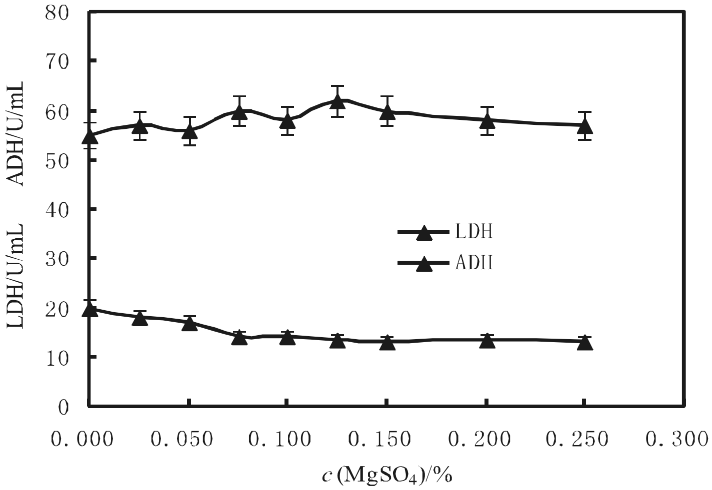

Fig. 4. Effects of $MgSO_4$ concentration on the in vitro activities of LDH and ADH

such as malic acid, citric acid, and fumaric acid were reduced sharply. With a $MgSO_4$ concentration of 0.025 wt%, fumaric acid and an unknown heteroacid were present in the fermentation products, but with a higher $MgSO_4$ concentration of more than 0.05 wt%, essentially no heteroacid was produced.

The areas (integrals) of the elution peaks for each organic acid on the HPLC chromatograms were calculated. A positive relationship was identified between the peak areas and concentrations of each organic acid, and the peak areas corresponding to each product were plotted against the initial $MgSO_4$ concentrations (Fig. 2). With a $MgSO_4$ concentration of less than 0.025%, the concentrations of heteroacids such as fumaric acid, malic acid, and citric acid in the fermentation products were higher. Further, with a $MgSO_4$ concentration of more than 0.050%, the quality of L-lactic acid produced by these strains was higher, and heteroacids basically were not produced. With increasing $MgSO_4$ concentrations of up to 0.150%, heteroacid production basically ceased in *R. oryzae* fermentation. In addition, the production of L-lactic acid was also slightly reduced with higher $MgSO_4$ concentrations. The presence and concentration of $MgSO_4$ can affect the acetylformic acid metabolic pathway and lead to the production of different metabolites.

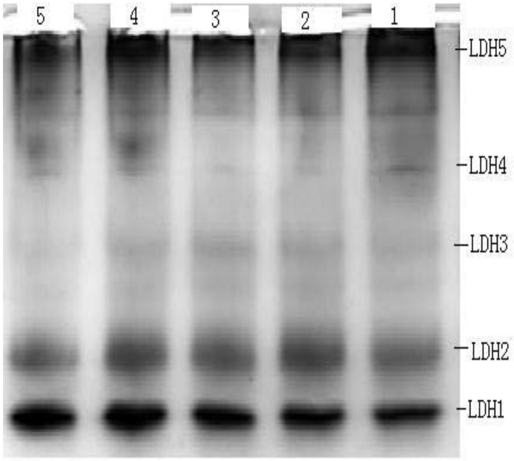

Fig. 5. Effect of $MgSO_4$ concentration on LDH isoenzyme levels. Lane 1: [$MgSO_4$] 0.025%, Lane 2: [$MgSO_4$] 0.050%, Lane 3: [$MgSO_4$] 0.075%, Lane 4: [$MgSO_4$] 0.100%, and Lane 5: [$MgSO_4$] 0.125%.

Relationships between $MgSO_4$ concentration and LDH activity, ADH activity, biomass production, and L-lactic acid production

The RLC41-6 strain of *R. oryzae* was cultured in fermentation medium containing different concentrations of $MgSO_4$. The effects of $MgSO_4$ on mycelium biomass and lactic acid production during fermentation as well as LDH and ADH metabolism were observed (Fig. 3). With no $MgSO_4$, concentrations of 7.2 g/L mycelium biomass and 110 g/L L-lactic acid were produced. With a $MgSO_4$ concentration of 0.025%, 22.8 g/L mycelium biomass and 120 g/L L-lactic acid were produced. With a $MgSO_4$ concentration of 0.075%, 36.8 g/L mycelium biomass and 138 g/L L-lactic acid were produced, and the conversion rate to lactic acid from glucose was 92%. In the fermentation system, with the initial increase in $MgSO_4$ concentration from 0.025 wt% to 0.075 wt%, the LDH activity remained constant at 150–155 U/mL, and ADH activity decreased by 22% from 18 to 14 U/mL. Increasing magnesium ion concentration had no obvious effect on LDH activity but tended to decrease ADH activity.

The effects of $MgSO_4$ on LDH and ADH activity were further studied in vitro (Fig. 4). Different concentrations of $MgSO_4$ were added to each enzyme reaction system, and the LDH and ADH activities were determined. As shown in Fig. 4, LDH activity was constant independent of the presence or concentration of $MgSO_4$ in the reaction system. However, the addition of $MgSO_4$ had a mild inhibitory effect on ADH activity. $MgSO_4$ can promote cell growth in the fermentation system, and it affects the efficiency of the conversion of acetylformic acid into lactate by pathways other than LDH, for example, by inhibiting ADH activity to reduce conversion of acetylformic acid into ethanol.

Effect of $MgSO_4$ concentration on the composition and expression of LDH produced during RLC41-6 fermentation

In the studied medium, increasing $MgSO_4$ concentration had essentially no effect on the composition and expression of LDH isoenzymes (Fig. 5). By contrast, in our previous research examining the effects of $ZnSO_4$ on lactic acid production by *R. oryzae*, changes in $ZnSO_4$ concentration did affect of the levels of LDH isoenzymes (except for LDH_1, but especially for LDH_5)[7].

CONCLUSIONS

The addition of different concentrations of $MgSO_4$ into the fermentation medium could influence the metabolism of *R. oryzae*, resulting in changes to the fermentation products. Our results showed that $MgSO_4$ could promote mycelium growth, but did not have obvious effects on LDH expression or activity. In the production of lactic acid by *R. oryzae*, an appropriate concentration of $MgSO_4$ could inhibit the conversion of pyruvic acid into malic acid and fumaric acid and mildly inhibit ADH activity as well as the conversion of pyruvic acid into alcohol, thus enhancing the metabolism of glucose to lactic acid by *R. oryzae*. With an initial $MgSO_4$ concentration of 0.075%, the maximal mycelium biomass concentration produced by *R. oryzae* was 36.8 g/L, and HPLC analysis showed that with this concentration of $MgSO_4$, L-lactic acid was the only fermentation product, with a concentration of ~138 g/L and a conversion rate of 92%.

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REFERENCES

1. Chen D.Y., Wang J.P., Bai Y. Research progress on the synthesis of lactic acid esters and the their derivatives. *Chemical Industry and Engineering Progress*. 2002; **21**(4): 243-246.
2. Meussen B, Graaff L, Sanders J.M., Weusthuis R.A. Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals. *Applied Microbiology and Biotechnology*, 2012; **94**(4): 875-886.
3. Thitiprasert S, Sooksai S, Thongchul N. In vivo regulation of alcohol dehydrogenase and lactate dehydrogenase in *Rhizopus oryzae* to improve L-lactic acid fermentation. *Applied Biochemistry and Biotechnology*. 2011; **164**(8):1305-1322.
4. Bai D.M., Fu W.M., Zhao X.M., Dai H.X., Li X.G., Xu S.M. The optimization of L-lactic acid production by *Rhizopus oryzae* R1021 fermentation via the metabolic flux analysis. *Journal of WuXi University of Light industry*. 2002; **21**(6): 554-558.
5. Yao W, Zhu J, Sun B, Miller C. Development and optimization of a culture medium for L-lactic acid production by *Rhizopus oryzae* using crude protein from dairy manure as nitrogen source . *Journal of Environmental Science and Health"Part A: Toxic Hazardous Substances and Environmental Engineering*. 2009; **44**(12): 1306-1313.
6. Pan L.J., Fu P, Zheng Z, Luo S.Z., Jiang S.T. Studies on the screening of *Rhizopus oryzae* ADH mutant strains and regulation of its zinc and magnesium ions. *Acta Microbiologica Sinica*. 2006; **46**(4): 586-590.
7. Ge C.M., Pan R.R., Zhang J. The effect of ZnSO4 on the lactic acid produced by the fermentation of *Rhizopus oryzae*. *Acta Microbiologica Sinica*. 2013; **53**(5): 510-515.
8. Zhou X, Ge C, Yao J, Pan R, Yu Z. Optimization of L-lactic acid production of *Rhizopus oryzae* mutant RLC41-6 by ion beam implantation at low-energy. *Plasma Science and Technology*. 2005; **7**(5): 3078-3080.
9. Bai D.M., Zhao X.M., Hu Z.D. Application of reversed-phase high performance liquid chromatography in the determination of organic acid in the lactic acid fermentation broth of *Rhizopus oryzae*. *Chinese Journal of Chromatography*. 2001; **31**(1): 8-11.
10. Fan Y.H., Wang L, Liu D, Li W, Yang Y, Zhen Z.M., Yu Z.L. Studies on analytical methods of lactic acid content in fermentation broth. *Biotechnology*. 2007; **17**(1): 54-55.
11. Sun S.Q., Yang J.H., Zhang L.Q., Yang Y, Shi J.G., Liu C.S. Determination of L-lactic acid in malolactic fermentation solution with enzyme electrode. *Shandong Science*. 2009; **22**(3): 7-10.
12. Li J.W., Xiao N.G, Yu R.Y. Biochemical experimental principles and methods. *Beijing: Peking University Press*. 1994; 354-358.
13. Xu Z.N. The effect of culture conditions of *Rhizopus oryzae* on dehydrogenase activity and composition of its products. *Journal of Zhejiang University*. 2003; **37**(6): 743-747