Promoting Cell Growth And Poly-Y-Glutamic Acid Production By Boosting The Synthesis of Alanine And D-Alanyl-D-Alanine In Bacillus Licheniformis

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Promoting cell growth and poly-γ-glutamic acid production by boosting the synthesis of alanine and D-alanyl-D-alanine in *Bacillus licheniformis*

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Abstract

Objective The production of some bio-chemicals affected by the cell growth. This study aimed at promoting the cell growth by overexpressing the synthesis of peptidoglycans tetrapeptide tail components to improve poly-γ-glutamic acid (γ-PGA) production.

Results L-alanine, D-alanine and D-alanyl-D-alanine are primary precursors for the synthesis of peptidoglycans tetrapeptide tail. The addition of L-alanine and D-alanine significantly increased both the cell growth and production of γ-PGA. Then, several genes encoding key enzymes for L/D-alanine and D-alanyl-D-alanine biosynthesis were overexpressed respectively, including *ald* (encoding alanine dehydrogenase), *dal* (encoding alanine racemase) and *ddl* (encoding D-alanine ligase). The results showed that the overexpression of genes *ald*, *dal* and *ddl* increased the production of γ-PGA by 19.72%, 15.91% and 60.90%, and increased the microbial biomass by 15.58%, 18.34% and 49.85%, respectively. Moreover, we demonstrated that the overexpression of genes *ald*, *dal* and *ddl* increased γ-PGA production mainly by enhancing cell growth rather than providing more precursors.

Conclusions This work illustrated the importance of the L/D-alanine and D-alanyl-D-alanine synthesis to the cell growth and the high yield of γ-PGA, and provided an effective strategy for producing γ-PGA.

Keywords

Bacillus licheniformis; Alanine and D-alanyl-D-alanine synthesis; Cell growth; Poly-γ-glutamic acid; Metabolic engineering

Introduction

Bacterial cell walls are characterized by the presence of peptidoglycans, macromolecules built from sugars and peptides, which help to maintain cell shape and integrity and balance the intracellular osmotic pressure (Brown et al. 2020). Bacterial cell wall synthesis is closely related to cell growth, and disruption of proper bacterial cell wall formation makes the cell highly sensitive to common environmental pressure, such as high salinity or antibiotics (Das et al. 2011). Thus, the methods of engineering cell wall component were generally developed to improve cell integrity and production of biochemicals. For instance, Son et al. suppressed cell lysis and increased squalene production by approximately 12% through activating the cell wall integrity pathway (Son et al. 2020). In addition, elevation of membrane cardiolipin biosynthesis and repression of the cell division initiator protein FtsZ also increased the OD_{600} by 86% and increased the HA titer by 204% (Westbrook et al. 2018). Therefore, engineering cell wall component or shape might be feasible strategies to increase metabolites production.

L-alanine (L-Ala), D-alanine (D-Ala) and D-alanyl-D-alanine are important components of the tetrapeptide tail in peptidoglycans of bacterial cell wall, which play an indispensable role in the normal growth of bacteria (**Fig. 1**) (Das et al. 2011). In recent studies, D-alanine was found to be essential for cell growth, biofilm formation and interspecific competition (Qiu et al. 2016), and it was often used as a screening marker for bacterial auxotroph to construct food-grade expression systems (Xia et al. 2007).

Poly-γ-glutamic acid (γ-PGA), a multifunctional biopolymer made up of D-glutamic acid and / or L-glutamic acid monomer by γ-amide bond, was selected as a research objective (Luo et al. 2016). Generally, numerous methods were developed to improve the γ-PGA production, such as increasing the supply of precursors, blocking the synthesis of by-products, boosting energy supplement, et al (Cai et al. 2018; Feng et al. 2015). While, the high viscosity of γ-PGA decreased the concentrations of dissolved

oxygen and limited the absorption or utilization of nutrients during the fermentation, which property hindered the cell growth and the synthesis of γ-PGA (Hsueh et al. 2017). Previous study in our lab found that the decrease of negative charge on cell wall surface could significantly improve cell growth and γ-PGA production in *B. licheniformis* (He et al. 2019). So, cell wall properties are closely related to cell growth and γ-PGA synthesis. In this study, alanine dehydrogenase (encode by *ald*), alanine racemase (encode by *dal*), D-alanyl-D-alanine ligase (encode by *ddl*) were respectively overexpressed to explore the effects of L/D-alanine and D-alanyl-D-alanine which associated with peptidoglycans tetrapeptide tail synthesis on cell growth and γ-PGA production.

Fig. 1

Materials and methods

Strains and plasmids. The strains and plasmids used in this study were listed in **Table 1**. *B. licheniformis* WX-02 (CCTCC M208065) was served as the original strain for the construction of recombinant strains. The *ald*, *dal* and *ddl* overexpressed vector pHY-*ald*, pHY-*dal* and pHY-*ddl* were constructed based on shuttle vector pHY300PLK.

Table 1

Medium and cultivation conditions. LB medium (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2) was served as the basic medium for the cultivation of *B. licheniformis* and *E. coli*, and 20 μg/mL kanamycin, 50 μg/mL ampicillin or 20 μg/mL tetracycline were added into the medium when necessary. The seed culture of *B. licheniformis* was prepared in 250 mL flasks with 50 mL LB medium, and incubated in the rotatory shaker with 180 rpm at 37ºC for 10-12 h until OD_{600} reached 4.0~4.5. Then, the seeds were transferred into the γ-PGA production medium, consisting of (per liter) 80 g glucose, 10 g sodium citrate, 8 g NH_4Cl, 1 g $CaCl_2$, 1 g $K_2HPO_4 \cdot 3H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $ZnSO_4 \cdot 7H_2O$ and 0.15

g $MnSO_4 \cdot 7H_2O$ at pH 7.2. The fermentation was performed in the rotatory shaker with 220 rpm at 37ºC for 36 h.

Construction of the *ald*, *dal and ddl* overexpressed strain. The method for constructing the *ald* overexpression strain was used as an example and which referred to the previous method (Cai et al. 2018). Briefly, P43 promoter from *B. subtilis* 168, *ald* gene and *amyL* terminator from *B. licheniformis* WX-02 were amplified by the corresponding primers (**Table 2**), fused by SOE-PCR, and the fused fragment was inserted into pHY300 at the restriction sites *Xba* I and *Eco*R I. PCR verification and DNA sequence confirmed that the *ald* expression vector was constructed successfully, named pHY-*ald*. Then, pHY-*ald* was electro-transferred into *B. licheniformis* WX-02 to construct the *ald* overexpression strain, named WX-02/pHY-*ald*. The strain harboring the empty pHY300PLK was used as the control. Engineered strains WX-02/pHY-*ald*, WX-02/pHY-*dal* and WX-02/pHY-*ddl* were constructed with the same method.

Table 2

Analytical methods

Cell growth curve was determined by measuring the optical density at 600 nm (OD_{600}) in LB medium. γ-PGA titer and biomass were detected by the method described in the previous research (He et al. 2019). Concentrations of acetoin, 2,3-butanediol and acetic acid were quantified using Thermo Scientific GC-MS (Thermo, USA) equipped with TG-WAXMS column (30 m × 0.32 mm ID, 0.25 μm film) by an optimized method from previous research (Qi et al. 2014). The method for determination of alanine and glutamic acid concentrations were accorded to the previous reported method (Yuan et al. 2019) by Thermo Scientific GC-MS (Thermo, USA) equipped with TG-5MS column (30 m × 0.32 mm ID, 0.25 μm film). The values shown represent the means of three independent experiments and the error bars represent standard deviations of three values.

Results and discussions

The addition of L-alanine or D-alanine can promote the cell growth and γ-PGA production. In order to investigate the effects of L-alanine or D-alanine on cell growth, growth curves of *B. licheniformis* WX-02 were measured upon addition of different concentrations of exogenous L-alanine or D-alanine to the LB media (**Fig. 2**). Firstly, the OD_{600} of WX-02 in LB medium with L-alanine addition were detected (**Fig. 2A**), which showed a significant increasement in cell growth when 0.1 g/L L-alanine was added to the medium compared with the control group (no L-alanine addition). It was worthy for us to note that the cell growth does not always improve with the concentration of added L-alanine increases. The OD_{600} of cells were even decreased when 0.2, 0.3 and 0.4 g/L of L-alanine were added to the medium compared with that of 0.1 g/L. These results suggested that the addition of L-alanine can boost bacterial cell growth, but the concentration of added L-alanine was need to be considered.

Then, the effect of D-alanine on cell growth was explored in WX-02 (**Fig. 2B**). The addition of D-alanine in medium promoted the cell growth of WX-02, and the OD_{600} increased most with 0.1 g/L D-alanine addition. From the above results, it was found that an appropriate concentration of L/D-alanine could promote the cell growth, and the high concentration of L/D-alanine could hinder cell growth.

Fig. 2

The effects of L-alanine and D-alanine on γ-PGA synthesis were also investigated by adding different concentrations of L-alanine and D-alanine into γ-PGA fermentation medium. The results showed that the supplement of L-alanine and D-alanine could effectively improve γ-PGA production (**Fig. 3**). The titer of γ-PGA was increased by 14.92% compared with the control (without L-alanine addition) when 0.2 g/L L-alanine was added into the medium (**Fig. 3A**). In addition, the γ-PGA titer was also respectively increased by 5.89%, 6.19% and 6.04% when adding 0.1, 0.2 and 0.3 g/L of D-alanine

(**Fig. 3B**). Therefore, the results indicated that the addition of L-alanine and D-alanine with appropriate concentrations was beneficial to the cell growth and further facilitated γ-PGA production.

Fig. 3

Overexpression of *ald*, *dal* and *ddl* promotes γ-PGA production. L-alanine and D-alanine are primary precursors for D-alanyl-D-alanine synthesis, and D-alanyl-D-alanine is subsequently involved in the synthesis of tetrapeptide tails during peptidoglycan assembly (**Fig. 1**). In order to explore effects of endogenous generation of L-alanine, D-alanine and D-alanyl-D-alanine on the synthesis of γ-PGA, alanine dehydrogenase (Ald), alanine racemase (Dal) and D-alanine ligase (Ddl) were individually overexpressed in *B. licheniformis* WX-02 to construct engineered strains WX-02/pHY-*ald*, WX-02/pHY-*dal* and WX-02/pHY-*ddl*, and the strain WX-02/pHY300 which harboring the empty pHY300PLK was used as the control. The results showed that the overexpression of the genes *ald, dal* and *ddl* increased the γ-PGA titer by 19.72%, 15.91% and 60.90%, respectively (**Fig. 4A**). The biomass of WX-02/pHY-*ald*, WX-02/pHY-*dal* and WX-02/pHY-*ddl* were also increased by 13.96%, 15.58% and 49.85%, respectively (**Fig. 4A**). It was worth noting that the γ-PGA yield of WX-02/pHY-*ald*, WX-02/pHY-*dal* and WX-02/pHY-*ddl* had no significant difference compared with that of the control strain (**Fig. 4A**), which suggested that the overexpression of *ald, dal* and *ddl* enhanced γ-PGA production mainly by promoting cell growth.

The concentration of intracellular alanine was detected to verify the availability of *ald, dal* and *ddl* overexpression. Compared with the control, the intracellular alanine concentration of WX-02/pHY-*ald* and WX-02/pHY-*dal* increased by 272.24% and 235.95%, respectively, and the concentration of intracellular alanine decreased by 55.12% in WX-02/pHY-*ddl* (**Fig. 4B**). Those results illustrated that the overexpression of *ald, dal* and *ddl* in WX-02 performed their functions correctly. In addition, glutamic

141 acid is the direct precursor for γ-PGA synthesis and the accumulation of glutamic acid affected the γ-
142 PGA production (Tian et al. 2017). The accumulation of glutamic acid in *ald*, *dal* and *ddl* overexpressed
143 strains had no significant difference compared with the control strain (**Fig. 4B**), which further indicated
144 that γ-PGA synthesis was promoted by enhancing cell growth rather than affecting its precursors
145 supplement when *ald*, *dal* and *ddl* were overexpressed.

146 **Fig. 4**

147 **Effects of genes *ald*, *dal* and *ddl* overexpression on by-products synthesis.** Acetoin, 2,3-
148 butanediol, acetic acid and lactic acid are main by-products generated during γ-PGA synthesis, which
149 consumed partial carbon fluxes. Many previous studies have shown that the production of the target bio-
150 chemicals can be increased by reducing the synthesis of by-products (Ma et al. 2018). Meanwhile, the
151 synthesis of by-products requires pyruvate as the precursor, and pyruvate is also the direct precursor for
152 alanine synthesis. Thus, the accumulations of acetoin, 2,3-butanediol, acetic acid and lactic acid were
153 detected in *ald*, *dal* and *ddl* overexpressed strains. The results showed that the by-products were
154 respectively reduced by 14.10%, 8.77% and 36.62% when overexpressing gene *ald*, *dal* and *ddl*
155 compared with the control strain (**Table 3**). It showed us that the overexpression of genes *ald*, *dal* and
156 *ddl* leaded a significant decrease in the synthesis of by-products, which was more conducive for cell
157 growth and γ-PGA synthesis.

158 **Table 3**

159 **Conclusion**

160 γ-PGA is a kind of multifunctional biopolymer with many applications, and its high viscosity
161 hinders the oxygen transformation and the nutrients absorption and utilization, which further affects the
162 cell growth and γ-PGA synthesis. In this study, we confirmed that the synthesis of L/D-alanine and D-

alanyl-D-alanine can promote the cell growth, and the enhancement of alanine and D-alanyl-D-alanine was an effective approach to improve the γ-PGA production.

Author contributions SW Chen designed experiments, contributed reagents and materials. Z Zhang, PH He, SY Hu, YQ Yu and XT Wang performed the experiments. Z Zhang and PH He drafted the manuscript.

Acknowledgments

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Declarations

Conflict of interest

The authors declare no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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Figure captions

Fig. 1 Graphical representation of alanine synthesis (A) and peptidoglycan assembly (B). PYR: pyruvate; OAA: oxaloacetic acid; CIT: citric acid; ICIT: isocitric acid; AKG: α-ketoglutaric acid; SucCoA: succinyl-coenzyme A; SUC: succinic acid; FUM: fumaric acid; MAL: malic acid; AceCoA: acetyl coenzyme A; L-Glu: L-glutamic acid; D-Glu: D- glutamic acid; L-Ala: L-alanine; D-Ala: D-alanine; L-Lys: L-lysine; 2,3-BDO: 2,3- butanediol.

Fig. 2 Effects of L/D-alanine addition with different concentrations on the growth curves of *B. licheniformis* WX-02. (A): Adding L-alanine; (B): Adding D-alanine.

Fig. 3 Effects of L/D-alanine addition with different concentrations on cell growth and γ-PGA production. (A): Adding L-alanine; (B): Adding D-alanine.

Fig. 4 Effects of genes *ald*, *dal* and *ddl* overexpression on the production of γ-PGA (A) and the accumulation of intracellular alanine and glutamic acid (B). The concentration of intracellular alanine and glutamic acid were detected in mid-log phase.

240 **Table 1** The strains and plasmids used in this research

Strains and plasmids	Relevant properties	Source
Strains		
Escherichia coli DH5α	*sup*E44 Δ*lac*U169 (f 80 *lacZ*ΔM15) *hsd* R17 *recA*1 *gyr*A96 *thi*1 *rel*A1	Stored in this lab
Bacillus licheniformis WX-02	Wide-type CCTCC M208065	Stored in this lab
WX-02/pHY-*ald*	WX-02 harboring pHY-*ald*	This study
WX-02/pHY-*dal*	WX-02 harboring pHY-*dal*	This study
WX-02/pHY-*ddl*	WX-02 harboring pHY-*ddl*	This study
Plasmids		
pHY300PLK	*E. coli* and *B. s* shuttle vector; Amp^r, Tet^r	This study
pHY-*ald*	*ald* expression vector	This study
pHY-*dal*	*dal* expression vector	This study
pHY-*ddl*	*ddl* expression vector	This study

241

242

243 **Table 2** PCR primers used in this study

Primers	Sequence
pHY-F	GTTTATTATCCATACCCTTAC
pHY-R	CAGATTTCGTGATGCTTGTC
P43-F1	GCGAATTCTGATAGGTGGTATGTTTTCG
P43-R1	CTTTCGGTACGCCGATAATCATGTGTACATTCCTCTCTTACCTA
ald-F	TAGGTAAGAGAGGAATGTACACATGATTATCGGCGTACCGAAAG
ald-R	GAAATCCGTCCTCTCTGCTCTTCTATGCGCCGGCGGCTGACGAC
TamyL-F1	GTCGTCAGCCGCCGGCGCATAGAAGAGCAGAGAGGACGGATTTC
TamyL-R1	GGTCTAGACGCAATAATGCCGTCGCACT
P43-F2	GCGAATTCTGATAGGTGGTATGTTTTCG
P43-R2	TATAGAATGGTTTTAAGCTCATGTGTACATTCCTCTCTTACCTA
dal-F	TAGGTAAGAGAGGAATGTACACATGAGCTTAAAACCATTCTATA
dal-R	GAAATCCGTCCTCTCTGCTCTTTCAGGATTGATCAGGCAAGATC
TamyL-F2	GATCTTGCCTGATCAATCCTGAAAGAGCAGAGAGGACGGATTTC
TamyL-R2	GGTCTAGACGCAATAATGCCGTCGCACT
P43-F3	GCGAATTCTGATAGGTGGTATGTTTTCG
P43-R3	CCAATCCTAATCTTGTCTTCAAGTGTACATTCCTCTCTTACCTA
ddl-F	TAGGTAAGAGAGGAATGTACACTTGAAGACAAGATTAGGATTGG
ddl-R	GAAATCCGTCCTCTCTGCTCTTTTAAAATGTATGTTTAATCTG
TamyL-F3	CAGATTAAACATACATTTTAAAAGAGCAGAGAGGACGGATTTC
TamyL-R3	GGTCTAGACGCAATAATGCCGTCGCACT

244

245

246 **Table 3** Acetoin, 2,3-butanediol, lactate and acetic acid synthesis by engineered strains

Strains	Acetoin (g/L)	2,3-Butanediol (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	By-products (g/L)
WX-02/pHY300	6.31±0.17	14.88±0.72	6.69±0.14	0.85±0.03	28.73
WX-02/pHY-*ald*	6.82±0.06	11.65±0.43	5.74±0.20	0.48±0.03	24.68
WX-02/pHY-*dal*	9.89±0.10	10.62±0.17	5.03±0.21	0.66±0.02	26.21
WX-02/pHY-*ddl*	5.65±0.13	7.45±0.68	4.69±0.19	0.43±0.02	18.21

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248

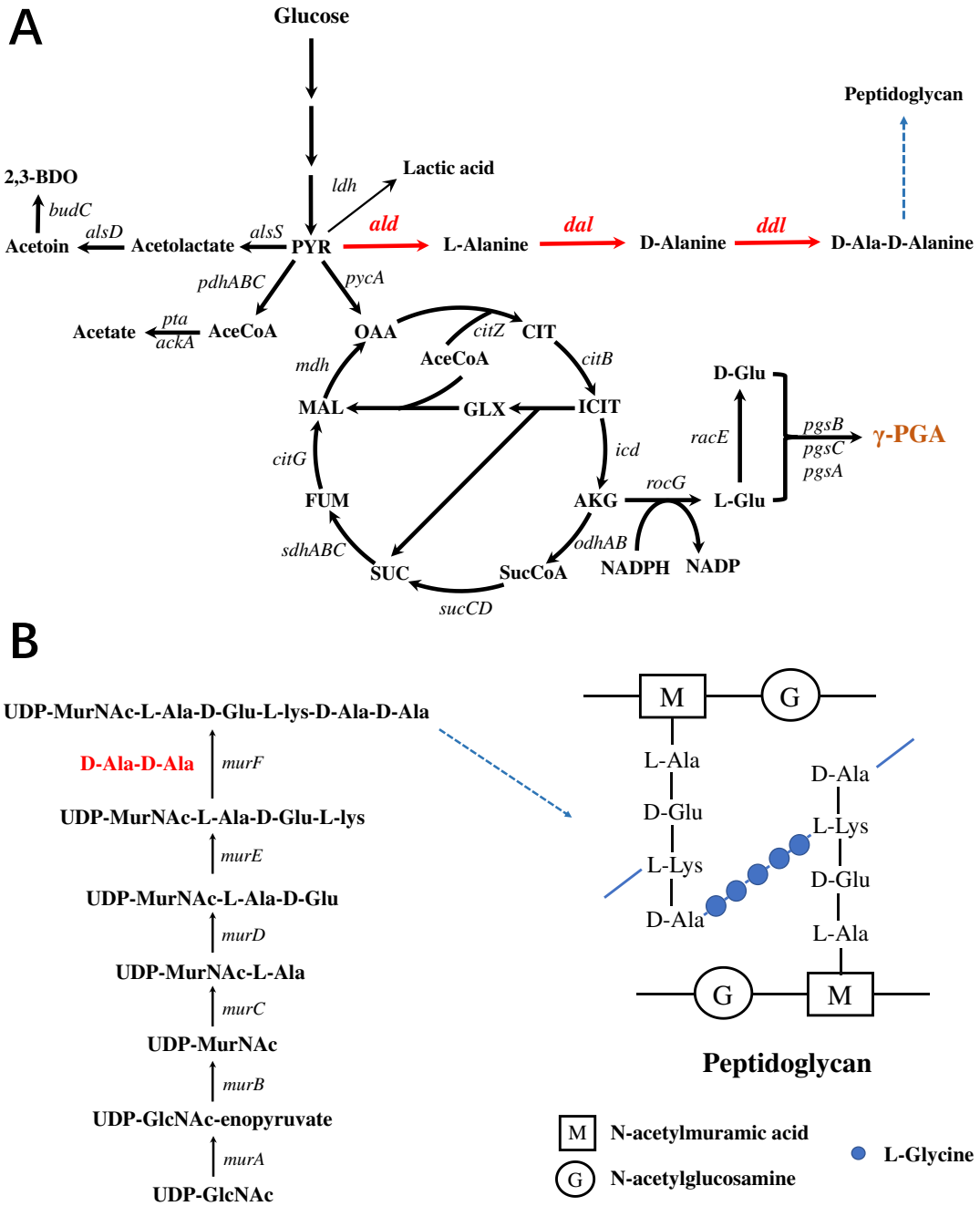
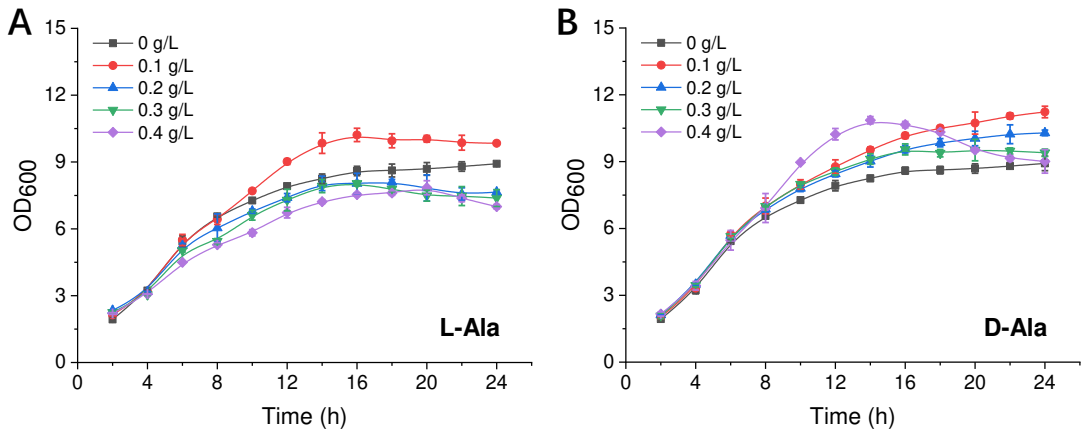


Fig. 1 Graphical representation of alanine synthesis (A) and peptidoglycan assembly (B). PYR: pyruvate; OAA: oxaloacetic acid; CIT: citric acid; ICIT: isocitric acid; AKG: α-ketoglutaric acid; SucCoA: succinyl-coenzyme A; SUC: succinic acid; FUM: fumaric acid; MAL: malic acid; AceCoA: acetyl coenzyme A; L-Glu: L-glutamic acid; D-Glu: D- glutamic acid; L-Ala: L-alanine; D-Ala: D-alanine; L-Lys: L-lysine; 2,3-BDO: 2,3- butanediol.



255

256 **Fig. 2 Effects of L/D-alanine addition with different concentrations on the growth curves of *B.***

257 ***licheniformis* WX-02.** (A): Adding L-alanine; (B): Adding D-alanine.

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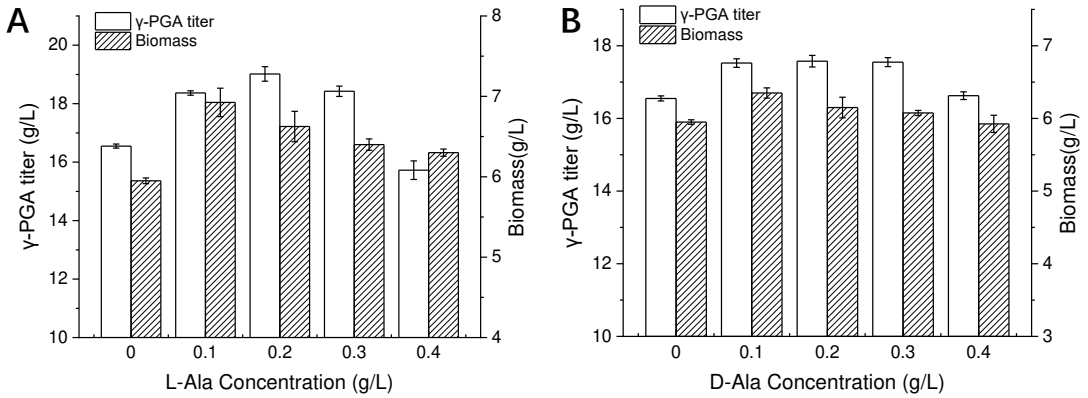

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260 **Fig. 3 Effects of L/D-alanine addition with different concentrations on cell growth and γ-PGA**

261 **production.** (A): Adding L-alanine; (B): Adding D-alanine.

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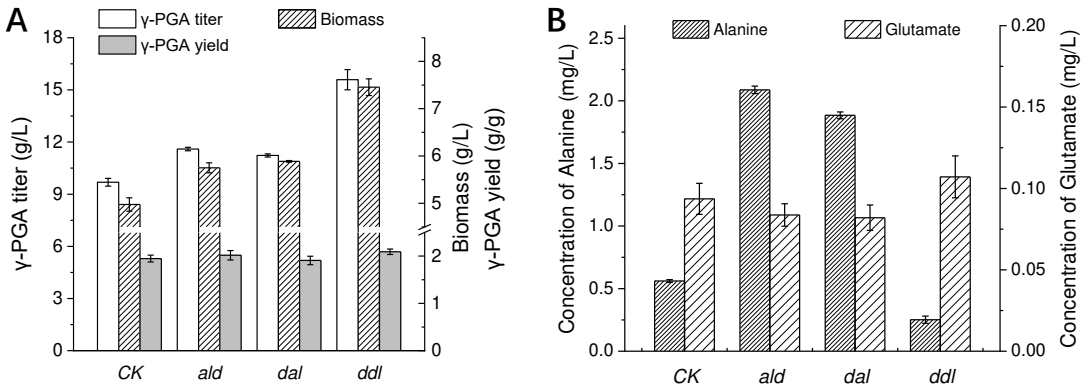

263

264 **Fig. 4 Effects of genes *ald*, *dal* and *ddl* overexpression on the production of γ-PGA (A) and the**

265 **accumulation of intracellular alanine and glutamic acid (B).** The concentration of intracellular alanine

266 and glutamic acid were detected in mid-log phase.

267