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ORIGINAL RESEARCH PAPER

Improved 2-methyl-1-propanol production in an engineered *Bacillus subtilis* by constructing inducible pathways

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Abstract High-level constitutive gene expression can result in cellular metabolic imbalance and limit production. To circumvent these problems, a P_{alsSD}-controlled auto-inducible 2-ketoisovalerate biosynthetic pathway and a P_{spac}-controlled IPTG-inducible Ehrlich pathway were constructed in *Bacillus subtilis* to modulate gene expression. Based on the precise gene expression characteristics of the two inducible pathways, the optimal IPTG induction time point and dose for 2-methyl-1-propanol biosynthesis were determined as 9.5 h and 300 μM, respectively. Under the optimized conditions, strain BSUΔL-03 with inducible pathways produced up to 3.83 ± 0.46 g 2-methyl-1-propanol/l, which was about 60 % higher than BSUL04 with constitutive pathways.

Keywords *Bacillus subtilis* · Inducible expression · Metabolic balance · 2-Methyl-1-propanol · Pathway engineering

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Introduction

2-Methyl-1-propanol, also called *iso*-butanol, is a promising substitute of the current fossil fuels. It can be biosynthesized by utilizing 2-ketoisovalerate (KIV) biosynthetic pathway and the heterologous Ehrlich pathway in several engineered microorganisms (Atsumi et al. 2008; Li et al. 2012) (Fig. 1). However, the production is relatively low and needs to be improved in these strains.

To improve the production of 2-methyl-1-propanol, previous studies mainly focused on pathway modifications but have neglected the adaptation of the reconstructed pathways to the host. Consequently, the continuous metabolic requirements for 2-methyl-1-propanol biosynthesis are in conflict with the changing demands for cellular survival and growth during high-level enzyme expression, which leads to unbalanced metabolism and low conversion yield (Keasling 2012; Medema et al. 2011; Zhang et al. 2012). The native genes (and the associated enzymes) follow the temporal expression principle, which shows clear evolutionary benefits (Zaslaver et al. 2004). Pathway regulation is, however, of importance to improve the

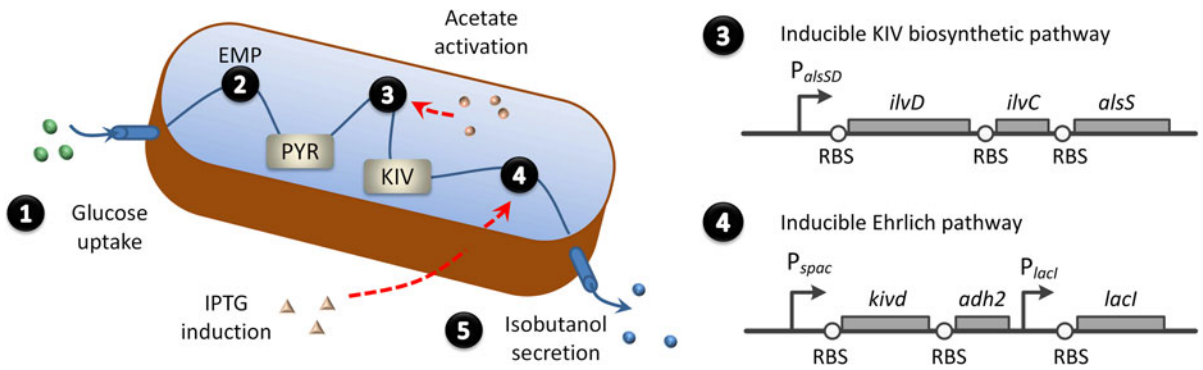

Fig. 1 Schematic diagram of 2-methyl-1-propanol biosynthesis and the inducible pathway design. Genes *ilvD*, *ilvC*, *alsS*, *kivd* and *adh2* encode dihydroxy-acid dehydratase, ketol-acid reductoisomerase, acetolactate synthase, keto-acid decarboxylase, and alcohol dehydrogenase, respectively. *EMP* glycolysis, *PYR* pyruvate, *KIV* 2-ketoisovalerate, *RBS* ribosome binding site

adaptation of the reconstructed pathways to the host and balance the cellular metabolism (Keasling 2012; Medema et al. 2011; Zhang et al. 2012). As inducible gene expression system (IGES) can start gene expression when necessary, it is considered as an effective tool for pathway modulation in metabolic engineering (Biswas et al. 2012; Yadav et al. 2012). Nevertheless, studies about the 'just-in-time' gene expression by IGES are rather limited in 2-methyl-1-propanol producers.

Bacillus subtilis is considered as an appropriate 2-methyl-1-propanol biosynthetic host because of its valuable features, such as high solvent tolerance and extensive substrate utilization capacity (Li et al. 2011). The purpose of this work was to improve 2-methyl-1-propanol production by constructing the inducible KIV and Ehrlich pathways. The optimal induction conditions were determined by monitoring gene transcription profiles. Cell growth and production profiles were also evaluated. The maximal titer of the inducible 2-methyl-1-propanol producing strain was much higher than that of the constitutive one.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Supplementary Table 1. *Escherichia coli* JM109 was used to propagate all plasmids and cultivated in Luria–Bertani (LB) medium in test tubes at 250 rpm. *Bacillus subtilis* strains were cultivated in 400 ml LBGSM-I medium (Li et al. 2011) in a fed-batch Pro fermentation system (DASGIP). Inoculation was 1 % (v/v). Dissolved O_2 was maintained at 5 ± 1 % of air saturation by varied agitation from 50 to 200 rpm with an aeration of 0.5 vvm. The pH was kept on 7 ± 0.1 with 2 M NaOH and 2 M HCl. All strains were cultivated at 37 °C. Antibiotics were supplemented when necessary as follows: 100 μg ampicillin/ml, 1 μg erythromycin/ml, 10 μg kanamycin/ml, and 20 μg spectinomycin/ml.

DNA manipulations

All primers used in this work are listed in Supplementary Table 2. The inducible heterologous pathways were designed as shown in Fig. 1. Promoter P_{spac}, gene *lacI* along with its promoter and ribosome binding site was amplified from plasmid pMUTIN4. The P_{spac} and *lacI* PCR products were digested with *Sac*I–*Kpn*I and *Xma*I–*Pvu*I, respectively, and sequentially cloned into pHPL04 at the corresponding sites, generating pHPL11. Fragment PKAL was amplified from pHPL11, digested with *Sph*I–*Sal*I and then cloned into pDGM to yield the integration plasmid pDGMPKAL. Promoter P_{alsSD} and gene *alsS* were amplified from *B. subtilis* genomic DNA and digested with *Hin*dIII–*Kpn*I and *Afe*I–*Xho*I, respectively. The P_{alsSD} and *alsS* PCR products were then cloned into pHPL08 in sequence, creating pHPL12 and pHPL13.

Strain BSUΔL-01 was obtained by introducing plasmid pHPL11 into BSUΔL. Strain BSUΔL-02 was created by integrating plasmid pDGMPKAL into the chromosome of BSUΔL. Strain BSUΔL-03 was generated by introducing plasmid pHPL13 into BSUΔL-02.

Strain transformation, selection and verification were carried out as described by Li et al. (2011).

Enzyme activity assay

The crude cell extracts were obtained by sonication (10 s pulse with 5 s intervals, total 10 min) of cell suspension and followed by centrifugation (17,900×*g* for 10 min at 4 °C) to remove cell debris. Enzyme activity of acetolactate synthase, keto-acid decarboxylase and alcohol dehydrogenase were assayed as described (Smith et al. 2010). Enzyme activity of ketol-acid reductoisomerase and dihydroxy-acid dehydratase was analyzed as described by Leyval et al. (2003).

Real-time quantitative PCR (RT-qPCR)

Gene transcription profile was determined by RT-qPCR (Bio-Rad) using the *TransScript* II Green Two-Step qRT-PCR SuperMix (Trans). Total RNA was extracted by Trizol reagent (Qiagen) and then treated with DNase I (Fermentas). Transcriptional level was normalized with the *gapA* gene, which was used as internal control. Data analysis was performed according to the comparative C_T method. Primers used are prefixed with RT and listed in Supplementary Table 2.

Analytical methods

Cell growth was monitored from the OD_{600} values and converted to the cell dry weight (CDW); an OD_{600} of 1 = 0.325 g CDW/l. Glucose was measured using a glucose biosensor analyzer. Extracellular 2-methyl-1-propanol and acids were quantified by GC-FID and HPLC, respectively. Detailed procedures for metabolite analysis were performed as described previously (Li et al. 2011).

Results

Construction of inducible pathways for 2-methyl-1-propanol biosynthesis

An effective Ehrlich pathway is essential for 2-methyl-1-propanol biosynthesis. To alleviate metabolic burden resulting from the high-level constitutive gene expression, an inducible Ehrlich pathway was constructed by employing the IPTG-controlled hybrid promoter P_{spac} (Fig. 1). This pathway was ineffective without IPTG induction (Fig. 2a, b), meaning it was tightly regulated by P_{spac}. Moreover, it improved 2-methyl-1-propanol biosynthetic capacity by about 30 % than the constitutive one at 24 h.

The inducible KIV biosynthetic pathway was further designed to direct more carbon flux toward 2-methyl-1-propanol. Strains harboring the inducible KIV biosynthetic pathway showed a 47 % titer increase than the parents at 24 h. Besides, the conversion capacity of this inducible pathway was significantly promoted by 0.2 % acetate. Further details are shown in Fig. 2c, d. Since acetate is one of the byproducts of 2-methyl-1-propanol (Li et al. 2011), this inducible KIV biosynthetic pathway can be modulated without external acetate induction.

Investigation of the optimal conditions for 'just-in-time' gene expression

The consecutive metabolites conversion is of great significance to improve 2-methyl-1-propanol production. Therefore, it is essential to coordinate the expression of KIV biosynthetic pathway and Ehrlich pathway. As shown in Fig. 3a, gene transcriptional level of KIV biosynthetic pathway was significantly increased at 10 h and almost kept constant thereafter. Figure 3b shows that 30 min induction results in the highest transcriptional level of Ehrlich pathway at 10 h. Therefore, 9.5 h is considered as the optimal time point to induce the expression of Ehrlich pathway. In addition, 300 μM IPTG was determined as the optimal induction dose according to the P_{spac} transcriptional strength analysis (Fig. 3c).

Effect of inducible pathways on cell growth and 2-methyl-1-propanol production

Cell growth and 2-methyl-1-propanol biosynthesis profiles of the engineered strain BSUΔL-03 and BSUL04 were compared to evaluate the capacity of inducible pathway. The maximal specific growth rate and CDW of BSUΔL-03 was 0.25 ± 0.02 h^{-1} and 3.53 ± 0.21 g/l, respectively, which were 38 and 29 % higher than that of BSUL04. As shown in Fig. 4, BSUΔL-03 produced more 2-methyl-1-propanol than BSUL04 after 5 h induction. Besides, it showed a continuous upward trend till 60 h, which was 10 h longer than BSUL04. During 2-methyl-1-propanol accumulation period,

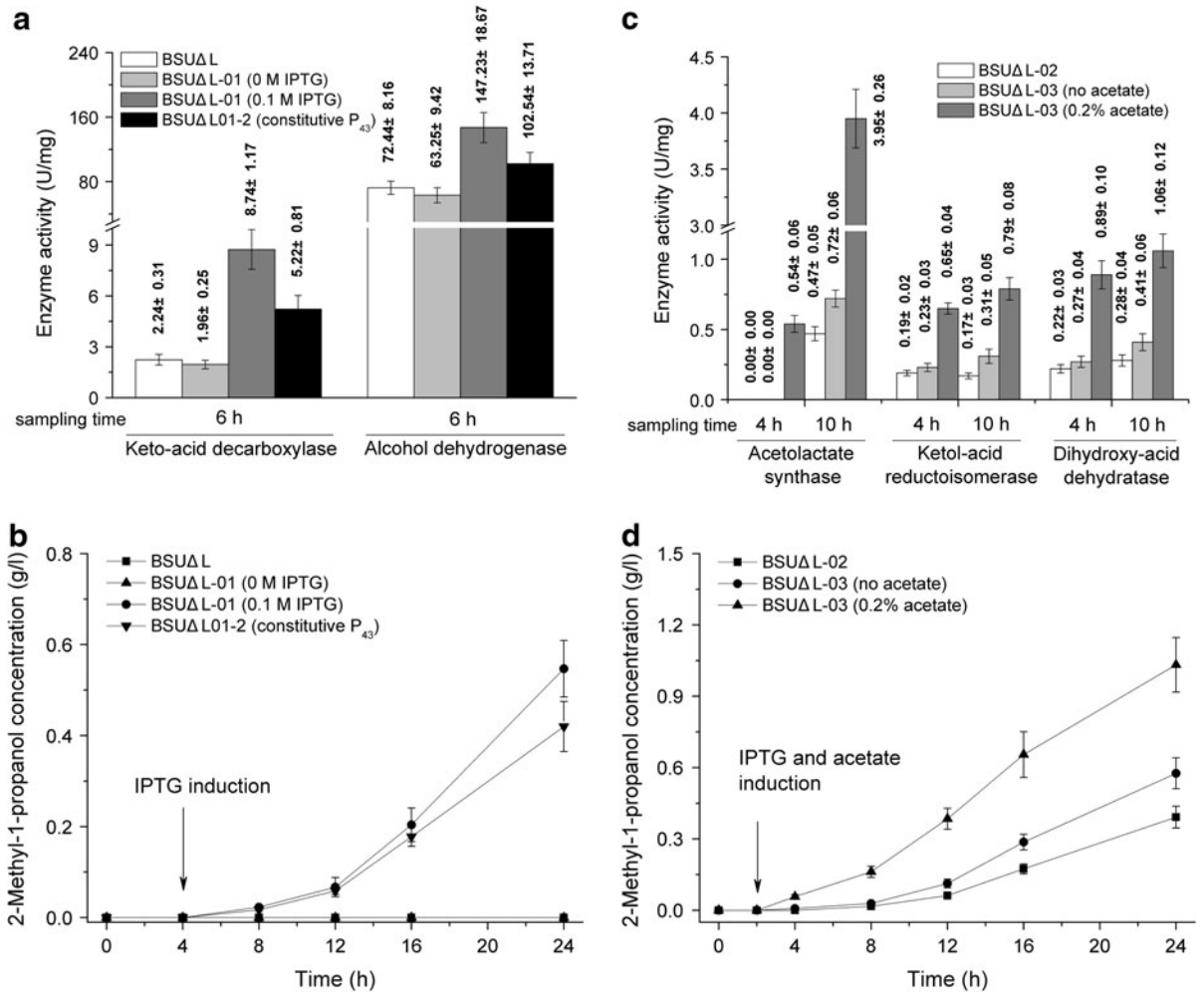

Fig. 2 Characterization of the inducible pathways in engineered *B. subtilis*. **a**, **c** Activity assay of enzymes involved in Ehrlich pathway and KIV biosynthetic pathway, respectively; **b, d** influence of inducible Ehrlich pathway and KIV biosynthetic pathway on 2-methyl-1-propanol biosynthesis, respectively. Strains were cultivated for 24 h. Data shown are the means of three independent experiments and *error bars* represent standard deviations

BSUΔL-03 showed a 20 % higher average production rate (up to 0.06 ± 0.01 g/l h) than BSUL04, as well as an approximately 60 % increase of 2-methyl-1-propanol production (up to 3.83 ± 0.46 g/l). In conclusion, these data demonstrate the advantages of the inducible pathways in production improvement.

Discussion

We have engineered IGES-mediated inducible biosynthetic pathways to modulate gene expression and improve 2-methyl-1-propanol production based on three considerations: (1) Regulation of genes for 'just-in-time' expression is important for metabolic balance. (2) Regulation in prokaryotes primarily happens at transcriptional level (van Hijum et al. 2009). (3) Inducible gene expression system is an effective tool to modulate gene transcription.

The most widely used IGES in *B. subtilis* is mediated by the IPTG-inducible hybrid promoter P_{spac}. Therefore, P_{spac} was used to construct the inducible Ehrlich pathway for 2-methyl-1-propanol production. Genes *alsS*, *ilvC* and *ilvD* are responsible for KIV biosynthesis. They are located at different loci of *B. subtilis* chromosome. The native transcriptions of these genes are subject to complex regulation by transcription factor CcpA and/or global regulators

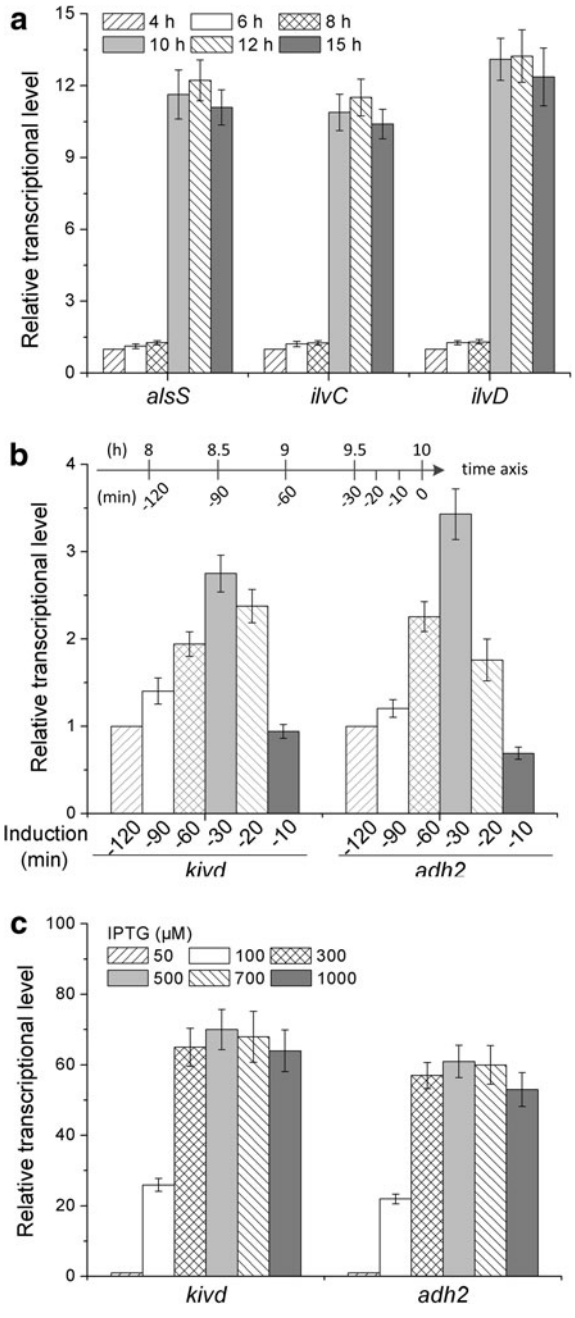

Fig. 3 Analysis of gene transcription profile strain BSUΔL-03. **a** Quantitative analysis of transcription profiles of KIV pathway without external acetate induction. **b** Influence of different IPTG induction time on transcriptional level of Ehrlich pathway at 10 h. The relative value of each gene expressed at 4 h (*a*) and 8 h/−120 min (*b*) were set as calibrator. **c** Influence of IPTG induction dose on the maximal transcriptional level of Ehrlich pathway. The relative values of each gene expressed with 50 μM IPTG was set as calibrator. Data shown are the means of three independent experiments and *error bars* represent standard deviations

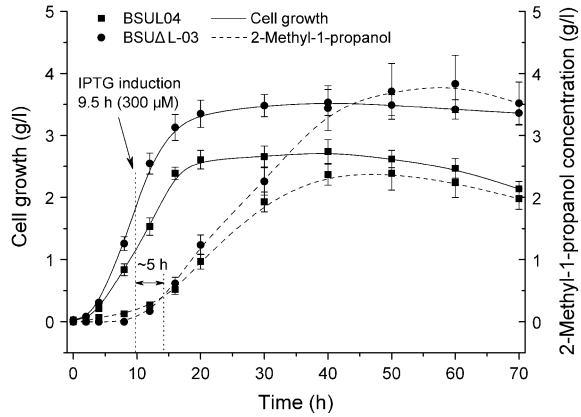

Fig. 4 Influence of inducible and constitutive biosynthetic pathways on cell growth and 2-methyl-1-propanol biosynthesis. IPTG (300 μM) was added at 9.5 h for BSUΔL-03. Strains were cultivated for 70 h. When glucose concentration fell below 1 g/l, 5 ml of 500 g glucose/l solution was added. Data shown are the means of three independent experiments and *error bars* represent standard deviations

such as CodY and TnrA (Renna et al. 1993; Tojo et al. 2005). Therefore, they were assembled in one operon to facilitate the modulation of gene expression in the present work. Promoter P_{alsSD} was chosen to control the KIV operon because it can be naturally triggered in stationary phase. This feature favors metabolic balance by decreasing the carbon flux competition between cell growth and 2-methyl-1-propanol biosynthesis. In addition, the transcriptional activity of P_{alsSD} can be regulated by the positive activator AlsR (Renna et al. 1993), acetate (Fig. 2c, d) and the oxygen level (Biswas et al. 2012). Thus, according to intra- and extra-cellular conditions, the P_{alsSD}-controlled KIV biosynthetic pathway can automatically regulate its expression to a suitable state, which serves as a pivotal signal for timely expression of Ehrlich pathway.

The transcriptional level of Ehrlich pathway was maximized by 300 μM IPTG when that of KIV operon was significantly promoted (Fig. 3). High transcriptional level of KIV operon suggests two facts: (1) The current cells are robust enough to provide adequate precursors and energy for the production of 2-methyl-1-propanol. (2) Cells are in the low-oxygen condition that favors the biosynthetic process. Thus, our induction strategy is useful to enhance the production. The transcription of the current KIV operon was slightly advanced compared to the normal profiles (Renna et al. 1993). This phenomenon may be due to the accumulation of acetate (data not shown), which activates P_{alsSD} and enables it to function

ahead of the natural schedule. Compared to the strong constitutive expression pathways, the inducible ones extended 2-methyl-1-propanol biosynthetic time for 10 h (Fig. 4). Moreover, the inducible pathways improved cell growth and production by 29 and 60 %, respectively, indicating the decreased metabolic burden and increased carbon flux through the target. These results suggest that the inducible pathways favor the biosynthetic process of 2-methyl-1-propanol by modulating genes to express in time.

Currently, we combined an auto-inducible pathway and an IPTG-inducible pathway to implement 2-methyl-1-propanol biosynthesis in engineered *B. subtilis*. Further production improvement may be obtained by a fully dynamic auto-regulated system. On one hand, the auto-inducible elements can precisely respond to cellular physiological state and dynamically modulate gene expression profile. On the other hand, inducer can be avoided during fermentation, which decreases the cell stress and lowers the production cost.

In summary, the inducible pathways improve the production of 2-methyl-1-propanol. This study demonstrates the significance of 'just-in-time' gene expression and metabolic balance in strain engineering.

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