SHORT REPORTS



# Enhanced isobutanol production using engineered *E. coli* and *B. subtilis* host by UV-induced mutation

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#### Abstract

Recombinant *Escherichia coli* and *Bacillus subtilis* strains were engineered by simultaneous chemical and ultraviolet-induced random mutagenesis to enhance bio-alcohol production. Our study investigated the bio-alcohol production of six variants of *E. coli* (EM1–6) and *B. subtilis* mutants (BM1–6). The induced mutation in the EM variants increased isobutanol (C4 alcohol) production most effectively, whereas pH adjustment and additional L-valine feeding increased isobutanol production by the BM variants. In contrast, pH adjustment or L-valine addition negatively affected isobutanol production by the EM1 variants. The highest titer of 5.07 g/L of isobutanol from a 40 g/L yeast extract medium (YEM) was achieved by the EM1 variant, whereas 0.57 g/L of isobutanol from YEM supplemented with 5 g/L of L-valine was obtained from the BM5 variant. These results can be applied in further research on engineering production hosts and improving production titers to utilize heterogenous bioresources in the future.

### Introduction

Global warming and environmental pollution have recently become topics of increasing concern (Barecka et al. 2021; Chen et al. 2022). Therefore, the utilization of sustainable bioresources to replace fossil fuels has become a promising approach that may facilitate carbon neutrality by balancing or reducing carbon dioxide emissions (Kartik et al. 2021; Bhatia et al. 2019; Kim et al. 2020b; Martins et al. 2021). Particularly, bioconversion and fermentation technologies are at the forefront of current efforts to transform petrochemical-based industrial structures into cleaner bio-based alternatives (Park et al. 2020; Sun et al. 2022; Teekens et al. 2018; Kruger et al. 2018).

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For example, the production of bio-alcohols such as ethanol (C2), isobutanol (C4), and methyl butanol (C5) has had a long history, from basic fermentation to the development of biofuels as an energy source (Lin et al. 2014; Li et al. 2014; Choi et al. 2014; Liang et al. 2020; Muller 2014; Huo et al. 2011). However, the production of bioalcohol faces several challenges, including the sourcing of cost-effective and ecofriendly bioresources. The production of isobutanol from sugar-based biomass, for example, has been extensively studied in carbohydrate biorefineries due to the preference of microorganisms for carbon sources. Yang et al. reported isobutanol production using recombinant *Escherichia coli* strains by overexpressing NAD salvage pathway enzymes, aldehyde oxidoreductases, or glycine alleviation (Seo et al. 2016; Song et al. 2017b, c).

Moreover, research on bio-alcohol production using waste protein resources has also attracted considerable attention (Choi et al. 2014; Wernick and Liao 2013; Huo et al. 2011). Contrary to the use of carbohydrates, where fermentation pathways are utilized, the use of amino acids obtained from protein hydrolysis presents the advantage of directly converting amino acids such as valine, leucine, and isoleucine to C3–C5 bio-alcohols through deamination, which are also good carbon and nitrogen sources for microbial growth (Choi et al. 2014; Wernick and Liao 2013; Atsumi et al. 2008; Huo et al. 2011). For example, Liao et al. reported non-fermentative pathway-based isobutanol production



using recombinant *E. coli* expressing *alsS*, *ilvCD*, *kivd*, and *adhA* with deletion of several genes to achieve high yields and productivity (Atsumi et al. 2008; Baez et al. 2011). Furthermore, the waste protein included several bioresources from food-related components such as soybeans, coffee grounds, and okara, as well as industrial waste generated through fermentation (Kim et al. 2020a, b; Wernick and Liao 2013).

Although there has been considerable progress in sugar and protein refining, there is still a need to develop a host cell system suitable for the fermentation of biomass with different compositions (Choi 2021; Kim et al. 2020a; Wernick and Liao 2013; Huo et al. 2011). One possible approach to address this issue is to develop multiple suitable host strains that play independent roles in the conversion of different substrates. However, engineering a single biotransformation process that involves two different cells is difficult. This is due to differences in cell viability and responses to environmental changes such as pH or increased bio-alcohol concentration during the reaction. Any imbalance from these environmental changes during biotransformation may cause low cellular activity, resulting in low production and yields. Therefore, a major challenge in this field is an engineering and optimizing a whole-cell biotransformation system with high resistance to alcohols and the pH changes that occur during the process of bioconversion.

Therefore, this study aimed to engineer *E. coli* and *B. subtilis* hosts to increase resistance to alcohol and pH changes during the bioconversion process. First, both bacterial strains were submitted to several rounds of simultaneous chemical- and UV radiation-induced mutagenesis in the presence of isobutanol to increase their alcohol resistance and obtain high mutation rates. The six resulting variants of the *B. subtilis* (BM) and *E. coli* (EM) mutants were screened on culture plates, after which their bio-alcohol production capabilities were further investigated. Next, pH adjustment and supplementation of L-valine were conducted during bio-alcohol production using the BM and EM variants. Collectively, our findings provide important insights into the most suitable host cells and strategies for the utilization of biomass.

### **Materials and methods**

#### **Chemicals and reagents**

Yeast extract (Technical, Cat 212750, Bacto<sup>TM</sup>) was purchased from BD Bioscience (Seoul, Korea). All chemicals and reagents, including L-valine as a supplementary substrate, antibiotics for selective cell growth, and isopropyl- $\beta$ -D-thiogalactoside (IPTG) to induce the target genes



expression were obtained from Sigma-Aldrich (Suwon, Gyunggido, Korea).

#### Bacterial strains, media, and culture conditions

The *B. subtilis* 168 wild-type strain was used as a negative control and was obtained from the Korean Collection for Type Cultures (KCTC 13241). Mixed amino acid medium for cell growth and bio-alcohol substrate was prepared by mixing 40 g/L Bacto<sup>TM</sup> Yeast Extract, of which maximum theoretical yield of bio-alcohols was calculated as around 7.2 g/L by Huo et al. (Huo et al. 2011), with M9 salt in distilled water as previously described (Choi et al. 2014; Kim et al. 2020a, b). Appropriate antibiotics were added for the selective growth of *B. subtilis* [spectinomycin (100 µg/mL), chloramphenicol (5 µg/mL), and erythromycin (1 µg/mL)] and *E. coli* [kanamycin (100 µg/mL), ampicillin (50 µg/mL)].

In this study, we used recombinant *E. coli* and *B. subtilis* strains (controls), which could utilize yeast extract as a substrate and convert it into isobutanol and bio-alcohols using the isobutanol synthesis and Ehrlich pathways, respectively. *B. subtilis*  $\Delta codY\Delta bkdB$  harboring pKY11 plasmid was prepared as described in previous studies (Kim et al. 2020a, b; Choi et al. 2014). The pKY11 plasmid harboring the *lueDH*, *kivD*, and *yqhD* genes was controlled using a *P*<sub>spac</sub> promoter, and the *codY* and *bkdB* genes were deleted.

In E. coli, glucose was converted to pyruvate by glycolysis, followed by an enzymatic conversion by AlsD (AHAS) and IlvCV into acetolactate and 2-ketoisovalerate (KivD), respectively (Fig. 1a). The E. coli strain harboring pCDF-Duet-1::*alsS, kivD* and pET23a(+)::*ilvC, ilvD, yqhD* was provided by Prof. Yang's group, and detailed information has been reported previously (Song et al. 2017a, 2020). In contrast, B. subtilis converted L-valine into KivD by leucine dehydrogenase (LeuDH). The generated 2-ketovalerate was converted into isobutanol via the alcohol dehydrogenase (ADH) enzyme (Fig. 1b). Similarly, leucine and isoleucine can use this pathway for methyl butanol production. The CodY and BkdB proteins play a key role as a global regulator and 2-oxoisovalerate dehydrogenase subunit beta in branched amino acid synthesis and degradation, respectively. These proteins were inactivated in the B. subtilis  $\triangle codY \triangle bkdB$  strain (Choi et al. 2014; Kim et al. 2020a, b).

#### Chemical and UV-induced random mutagenesis

Two steps of chemical mutagenesis and UV mutation were carried out to induce high alcohol resistance in each strain through random mutagenesis. Ethyl methane sulfonate (EMS) was used for the first round of chemical mutagenesis (Stamato and Perez 1998; Tao et al. 2012; Tanadul et al. 2018). Initially, EMS was used for the first round of

chemical mutagenesis, where 30  $\mu$ L of EMS was added to 2 mL of seed culture medium and incubated at 37 °C for 20 min to induce random mutations in the first doubling period. This procedure was repeated five consecutive times (Tanadul et al. 2018; Tao et al. 2012; Stamato and Perez 1998). The mutated culture was diluted to 10<sup>-6</sup> and spread on a plate to select the final mutant strains.

UV-based random mutagenesis, as a second round of mutagenesis, was applied to the selected strains after the first round of mutation. In the UV mutation, 2, 5, and 10% isobutanol were added to the growing medium to screen for mutants with higher isobutanol resistance (Stamato and Perez 1998; Yi et al. 2018). The isobutanol-containing growing medium was exposed to UV radiation for 30 min at a 0.2 m distance. To stabilize the mutants, the collected cells were incubated on ice for 5 min, followed by incubation in a dark room for 30 min. Then, the mutated cells were diluted to  $10^{-6}$  and spread on a plate to screen the final mutants (Fig. 2a). The final BM 1–6 and EM 1–6 mutant strains from *B. subtilis* and *E. coli*, respectively, were selected and prepared to evaluate isobutanol production (Fig. 2b).

#### Whole-cell biotransformation of yeast extract medium for BM and EM variants

Seed cultures of the BM and EM variants (2 mL each) were incubated in YEM medium at 37 °C overnight. Then, 0.2 mL of seed culture was inoculated into 50 mL of fresh YEM medium in a 250 mL flask. The cells were incubated at 37 °C until an optical density measured at a 600 nm wavelength (OD<sub>600</sub>) occurred at approximately 0.8. Next, the target proteins of LeuDH, KivD, and ADH in the BM variants and of AlsS, IlvCD, KivD, and YqhD in the EM variants were induced with 3 and 0.1 mM of IPTG, respectively (Choi et al. 2014; Kim et al. 2020a, b; Song et al. 2017a, 2020). After induction, the bioconversion reaction continued until the highest titer was obtained. Samples were then collected for quantification.

## Quantification of bio-alcohols produced by *E. coli* and *B. subtilis* variants

To quantify the bio-alcohols produced by the BM and EM variants, each 1 mL of the collected sample was analyzed using gas chromatography. Samples were collected by centrifugation at 13,500 rpm for 15 min. Then, the aqueous supernatant was filtered to remove cell debris. The filtrate was analyzed by gas chromatography equipped with a flame ionization detector with a split injection ratio of 1:5. The samples were separated using a CP-Sil 5 CB capillary column (30 m×0.25 m inside diameter, 0.25 µm thickness; Younglin OEM GC, Seoul, Korea). Isobutanol samples with  $C \ge 2$  were analyzed, including ethanol, isobutanol,

2-methylbutanol, and 3-methylbutanol. 2-Methylbutanol and 3-methylbutanols were not clearly separated and were thus quantified together (Kim et al. 2020a, b; Choi et al. 2014).

### **Results and discussion**

# Host cell engineering for enhanced resistance to isobutanol by random mutagenesis

In this study, we used genetically engineered E. coli and B. subtilis control strains, which could utilize yeast extract as a substrate and convert it into isobutanol and bio-alcohols using the isobutanol synthesis and Ehrlich pathways, respectively (Kim et al. 2020a, b; Choi et al. 2014; Huo et al. 2011). The first engineering step was to increase cell resistance to isobutanol and cope with pH changes and stress, thereby increasing the viability of cells for higher alcohol production (Mori et al. 2022; Min et al. 2021; Ho et al. 2021; Ma et al. 2011). To induce high alcohol resistance for each strain through random mutagenesis, we carried out two steps of chemical mutagenesis and UV mutation, as described in the Materials and Methods (Stamato and Perez 1998; Tao et al. 2012; Tanadul et al. 2018). According to the optimal mutation dosage, the survival rate on plates was reported around 10% (Tillich et al. 2012). The final mutant strains of BM 1-6 and EM 1-6 from B. subtilis and E. coli, respectively, were selected and prepared to evaluate isobutanol production (Fig. 2b).

#### Evaluation of BM and EM variants for bio-alcohol production at pH 8.0

The evaluation of bio-alcohol production, including ethanol, isobutanol, and methylbutanol, was initially carried out for B. subtilis. The Bacillus control strain, which was used as a mutation template, produced a total of 1.13 g/L of bioalcohol. The analyzed composition was 0.77 g/L of ethanol, 0.041 g/L of isobutanol, and 0.32 g/L of methylbutanol (Fig. 3a). The *B. subtilis* mutants BM1–6 could produce an average of  $0.68 \pm 0.12$  g/L bio-alcohols at pH 8.0. The highest production (0.88 g/L) was obtained using BM2 at pH 8.0 (Fig. 3a). The alcohol resistance increased through random mutagenesis, which was driven by the 10% isobutanol concentration. However, this did not guarantee higher alcohol production by the engineered cells. The gram-positive B. subtilis has been reported to form spores in the presence of high alcohol concentrations, and this might limit alcohol production by disrupting the growth of alcohol-producing parental hosts (Bohin and Lubochinsky 1982; Choi et al. 2014; Kim et al. 2020a, b).



Interestingly, EM variants showed a dramatic increase in isobutanol production compared to the control strain. Particularly, isobutanol was a major component of bioalcohols in the E. coli system (Table 1). The average production of isobutanol by the six EM variants at pH 8.0 was  $4.74 \pm 0.41$  g/L, which was 2.2 times higher than the control strain (Fig. 3b). Particularly, the highest isobutanol

production of  $5.07 \pm 0.18$  g/L was obtained using the EM1 variant, whereas the lowest production of  $3.92 \pm 0.17$  g/L was observed in the EM4 variant at pH 8.0. These results were in stark opposition to those observed in the BM variants. Bio-alcohol production by the BM variant isolated from the 10% high isobutanol concentration decreased by 60% on average. However, the isobutanol production by the

Host strain	Genes expressed and deleted	Substrate	Bio-alcohols $(C \ge 2) (g/L)$	Host strain	Genes expressed and deleted	Substrate	Isobutanol (C4) (g/L)
Control BM1 BM2 BM3	leuDH, kivD, yqhD, ∆codY∆bkdB	40 g/L yeast extract + 5 g/L L-valine	1.13 1.05 1.46 0.83	Control EM1 EM2 EM3	alsS (AHAS), ilvCD, kivD, YqhD	40 g/L yeast extract	2.17 5.07 4.90 4.84
BM4 BM5 BM6			1.02 1.48 0.94	EM4 EM5 EM6			3.92 3.87 4.86

EM variants produced isobutanol (C4) as a major component in bio-alcohols, and therefore only isobutanol was quantified



Engineered Bacillus subtilis∆codY∆bkdB

Fig. 1 Biosynthetic pathways of  $C \ge 2$  bio-alcohols from glucose and branched chain amino acids using each engineered Escherichia coli and Bacillus subtilis strains. a The fermentative pathway of glucose by engineered Escherichia coli includes the conversion of pyruvate into acetolactate by the AHAS enzyme, followed by conversion into 2-ketoisovalerate, isobutyl aldehyde, and finally isobutanol. b Heterologous expression of leucine dehydrogenase (LeuDH), 2-ketoisovalerate decarboxylase (KivD), and alcohol dehydrogenase (ADH) in engineered Bacillus subtilis



**(a)** 







**Fig. 2** Engineering of bio-alcohol producing host strains by random mutagenesis. **a** Screening of *Escherichia coli* and *Bacillus subtilis* variants in the presence of 2, 5, and 10% of isobutanol. After each cell was incubated with the mutational driving force of increasing isobutanol concentrations and was selected at 10% isobutanol. **b** Final mutant strains of *Escherichia coli* variants (EM1–6) and *Bacillus subtilis* variants (BM1–6) after isobutanol and UV-driven random mutations

EM variant increased by 220%. This may be due to the characteristic differences between the gram-positive *B. subtilis* and gram-negative *E. coli* strains. It may also be attributed to different alcohol resistance mechanisms and variations in activity due to random mutations (Ma et al. 2011; He et al. 2019).

# pH adjustment to resist pH changes during yeast extract biotransformation by BM variants

The pH changes were monitored as induced by the generated ammonium ions during the amino acid biotransformation process. The initial pH was adjusted to 7.0 and continued to increase to 8.0 during bio-alcohol production; this was also observed in previous studies (Kim et al. 2020a, b). Based on the isobutanol synthetic pathway in *B. subtilis*, the valine was first deaminated by the LeuDH enzyme and converted to KivD (Fig. 1). The NH<sub>3</sub> produced in this step was released into the intracellular environment and incorporated into

other transamination reactions or secreted into the extracellular environment to increase the pH of the incubation medium. This suggests that isobutanol production increases with  $NH_3$  production. To create a cellular environment capable of producing more  $NH_3$ , the pH of the culture medium was adjusted to 5.5 on the third day of the reaction, when the pH began to increase. In the isobutanol production profile of BM variants, the pH began to increase from 7.0 to 8.0 on the third day of the reaction. Here, the pH was decreased to 5.0 with phosphoric acid and was maintained at 5.5 as the reaction progressed (Fig. 4a).

The bio-alcohol production by the BM variants showed a tendency to increase when the pH was adjusted to 5.5 compared to a pH of 8.0. The bio-alcohol production was similar to that of the control strain at  $1.12 \pm 0.05$  g/L. The BM3 mutant achieved the highest total bio-alcohol production of 1.19 g/L, which was slightly higher than that by control. Particularly, the production of isobutanol and methylbutanol increased by 50 and 34% on average, respectively, compared to the control strain at  $0.07 \pm 0.01$  g/L and  $0.45 \pm 0.05$  g/L when the pH was adjusted to 5.5 (Fig. 3a, pH 5.5).

# pH adjustment to resist pH changes during yeast extract biotransformation by EM variants

Similar to the BM variants, the pH was adjusted to 5.5 on the second day of the reaction and changes in isobutanol production by the EM variants were monitored. The isobutanol production by EM variants increased to  $3.79 \pm 1.50$  g/L compared to the control strain. However, this was relatively low compared to the initially selected variant (Fig. 3b, pH 5.5). Among these variants, EM1 exhibited the highest isobutanol production of 4.94 g/L, which was similar to that achieved without pH adjustment. The time profile of isobutanol production by the EM variants showed the highest titer on the third or fourth day of the reaction and a tendency to gradually decrease on the fourth or fifth day after the reaction had commenced (Fig. 4b). Isobutanol production tended to gradually decrease when pH was adjusted to 5.5 on the second day of the reaction (Fig. 4c).

These findings contrasted with the increases in bio-alcohol production in the BM variants, including isobutanol, via pH control. This may be attributable to the difference in the bioconversion pathways between the BM and EM variants. Bio-alcohol production by BM variants is a type of biotransformation using yeast extract, which includes valine, leucine, and isoleucine as individual substrates, whereas EM variants undergo a type of fermentation process that utilizes the glycolysis pathway from glucose (Fig. 1). Particularly, the bioconversion by BM variants was initiated by the amino acid deamination reaction, which generated key intermediates of 2-keto acid for bio-alcohol production, suggesting that





Fig. 3 Conversion of 40 g/L of yeast extract: **a** by BM variants at pH 8.0, pH 5.5, and pH 5.5 with 5 g/L of L-valine addition; **b** by EM variants at pH 8.0, pH 5.5, and pH 5.5 with 5 g/L of L-valine addition

higher bio-alcohol production inevitably leads to a higher deamination cycle and  $NH_3$  release (Choi 2021).

#### Supply of additional valine during biotransformation by BM and EM variants

The manufacturing standards for BD yeast extract specify that 40 g/L of yeast extract contains approximately 3% of valine, which is approximately 1.2 g/L (Bacto<sup>TM</sup> Yeast Extract manual). This might contribute to the low isobutanol production by the BM variants. As valine was used directly as a substrate for BM biotransformation, it was deemed that increasing the valine supply may increase isobutanol production. To examine the effect of valine supply on isobutanol production, 5 g/L of L-valine was supplemented at the beginning of the reaction and on the third day of the reaction. As a result, higher isobutanol production occurred when



additional L-valine was supplied during the middle of the reaction compared to the beginning of the reaction (Table 1). Interestingly, the BM2 and BM5 variants yielded a total bioalcohol production of 1.46 and 1.48 g/L, respectively, and the isobutanol composition increased as well (Fig. 3a, valine addition). The BM2 and BM5 strains produced 0.50 and 0.57 g/L of isobutanol, respectively, accounting for 34.2a and 38.3% of the total bio-alcohol production in each case. The other BM variants yielded similar bio-alcohol amounts to those of the BM variant when the pH was adjusted to 5.5.

In contrast to the bio-alcohol production by BM variants, the addition of valine did not affect isobutanol production by the EM variants. The average isobutanol production by the EM variants was  $3.98 \pm 0.39$  g/L, which was similar to that of the EM variants with pH adjustment (Fig. 3b, pH 5.5, valine addition). This result appears reasonable, considering that the isobutanol production pathway uses the fermentation pathway



Fig. 4 Monitoring of pH changes and isobutanol production by BM and EM variants.  $\mathbf{a}$  pH changes during yeast extract biotransformation by BM variants. Isobutanol production profile  $\mathbf{b}$  without pH adjustment and  $\mathbf{c}$  with pH adjustment

rather than bioconversion. The EM variants were clearly dependent on the glucose concentration during fermentation as previously reported (Song et al. 2017a).

#### Conclusion

The BM and EM variants exhibited markedly different bioalcohol production patterns depending on the engineering strategies and biomass preferences. Previous studies have demonstrated the viability of two-cell biotransformation of plant-derived biomass into bio-alcohol by engineered *E. coli* and *B. subtilis* (Kim et al. 2020a, b). In these studies, the ratio of biomass composition between amino acids and carbohydrates varied depending on the biomass sources. As such, the optimized combination of a carbohydrate-converting *E. coli* strain and amino acid-converting *B. subtilis* is critical for efficient biomass utilization and obtaining the desired bio-alcohol production.

Biochemical production from biomass using microbial biotransformation or fermentation involves a trade-off between cell growth and target production and requires continuous energy consumption. The most efficient method is the production of high titer biochemicals from inexpensive biomass. However, most biomass sources have different carbohydrate, protein, and fat compositions, thus requiring an expensive pre-treatment process. Therefore, the utilized biomass must have a constant composition for uniform and reproducible biochemical production. We anticipate that these results will be efficiently used in future research on the engineering of production hosts and improving production titers to utilize heterogenous bioresources.

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#### Declarations

**Conflict of interest** The authors declare that there are no conflicts of interest.

**Ethical approval and consent to participate** This article does not contain any studies involving human or animal participants.

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