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Research Article

Optimization of fermentation conditions using T7 promoter-engineered *Escherichia coli* for isobutanol production

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Abstract. Isobutanol is a promising biofuel alternative to ethanol due to its higher energy density, lower hygroscopicity, and greater compatibility with existing fuel infrastructure. However, microbial isobutanol production remains constrained by inefficient heterologous protein expression and suboptimal fermentation conditions. This study aimed to improve isobutanol production by combining promoter engineering with fermentation parameter optimization in recombinant *Escherichia coli* BL21(DE3). The *lac* promoter in the isobutanol biosynthetic plasmid, containing *alsS*, *ilvC*, *ilvD*, and *kivd*, was replaced with the T7 promoter. Protein expression was evaluated using SDS-PAGE, while isobutanol production was quantified by GC-FID. Subsequently, pH, temperature, and fermentation time were optimized using a three-factor Box-Behnken response surface methodology design comprising 15 experimental runs. The T7 promoter enhanced recombinant protein expression and increased isobutanol production compared with the *lac* promoter system, with concentrations of 0.2766 and 0.1784 g/L, respectively. The response surface model was statistically significant ($F = 7.48$, $p = 0.0053$) and explained 67.12% of the variability in isobutanol production ($R^2 = 0.6712$). Temperature was the only statistically significant individual factor, with lower temperatures generally favoring isobutanol formation. The highest experimentally observed isobutanol titer was 0.313 g/L, corresponding to a glucose-to-isobutanol yield of 0.0281 g/g. Validation under the optimized conditions was performed through time-course fermentation measurements to monitor biomass growth and isobutanol accumulation. The results demonstrate that combining T7-based expression with fermentation optimization can improve isobutanol production in recombinant *E. coli*, although the relatively low predicted R^2 indicates that additional biological and process-related factors remain to be addressed.

Keywords: Isobutanol, Box-Behnken, Biofuel, *Escherichia coli*, T7 Promoter



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1. Introduction

The increasing global reliance on motorized transportation has accelerated the depletion of fossil fuel reserves. National energy statistics report that oil consumption reached 136.07 thousand barrels per day in 2024 (Economy, 2025), highlighting the urgent need to develop renewable and sustainable energy alternatives. Biofuels derived from biological resources have emerged as a promising class of renewable energy carriers. Among them, bioethanol has been widely implemented as a gasoline substitute; however, its high hygroscopicity and corrosiveness limit compatibility with existing fuel storage and distribution systems (Olson *et al.*, 2023). These limitations underscore the need for alternative biofuels with improved physicochemical properties.

Isobutanol has attracted considerable attention as a next-generation biofuel due to its favorable fuel characteristics. As a four-carbon branched-chain alcohol and an isomer of n-butanol, isobutanol exhibits physicochemical properties more similar to gasoline than ethanol. It is less hygroscopic and corrosive,

compatible with existing fuel infrastructure, and possesses a higher energy density and lower oxygen content, making it a strong candidate for gasoline replacement (Pastore de Lima *et al.*, 2023). Isobutanol nevertheless has attractive fuel characteristics. Its lower heating value is approximately 33.4 MJ/kg and its volumetric energy density is approximately 26.8 MJ/L, which are lower than those of gasoline but higher than those of ethanol. Isobutanol also has a high research octane number of approximately 105–105.5, relatively low vapor pressure, and lower hygroscopicity than ethanol, supporting its potential use as a gasoline oxygenate and blendstock. Its oxygen content may support more complete combustion in some operating conditions, whereas its lower heating value and higher heat of vaporization may affect volumetric fuel consumption and cold-start performance at high blend fractions (Olson *et al.*, 2023).

Unlike ethanol, isobutanol is not naturally produced by microorganisms. However, the development of a synthetic biosynthetic pathway by Atsumi *et al.* (2008) enabled microbial isobutanol production through metabolic engineering. Since

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then, a range of genetically engineered hosts—including *Saccharomyces cerevisiae* (Wess et al., 2019), *Bacillus subtilis* (Jia et al., 2012), *Corynebacterium glutamicum* (K. M. Smith et al., 2010), *Ralstonia eutropha* (Lu et al., 2012), and *Pichia pastoris* (Siripong et al., 2018) —have been developed to produce isobutanol from various carbon sources. Further improvements have been achieved through pathway optimization (Liang, Chen, et al., 2018; Noda et al., 2019), replacement of key enzymes with higher-activity variants (Atsumi et al., 2010), and the utilization of alternative substrates such as lignocellulose, CO₂, and glycerol (Lu et al., 2012; Saini et al., 2017; Weber et al., 2010). Despite these advances, fermentation conditions remain a critical determinant of achievable production yields. Recent studies have further demonstrated the diversity of metabolic strategies, host organisms, and fermentation conditions employed to improve microbial isobutanol production. A comparison of recent isobutanol production studies, including the production host, metabolic engineering strategy, substrate, fermentation conditions, and reported isobutanol titer, is presented in Table 1. These studies highlight substantial variation in both metabolic strategies and process conditions, emphasizing that isobutanol production is highly dependent on the characteristics of the engineered host and the cultivation environment.

Escherichia coli has been widely used as a host for heterologous protein expression due to its well-characterized metabolic system, rapid growth rate, and the availability of extensive molecular tools for genetic engineering (Heyde &

Nørholm, 2021). Among the various *E. coli* strains, *E. coli* BL21 (DE3) is specifically designed for heterologous protein expression. This strain carries the T7 bacteriophage RNA polymerase gene integrated into its genome, which is regulated by the lacUV5 promoter and is compatible with expression systems driven by the T7 promoter (Du et al., 2021). The T7 promoter, originally derived from bacteriophage T7, is known for its strong transcriptional activity and can direct heterologous protein production accounting for up to 50% of total cellular protein (Rosano & Ceccarelli, 2014). Therefore, the use of the T7 promoter in combination with *E. coli* BL21 (DE3) is considered an effective strategy to overcome low protein expression levels and to mitigate glucose-related repression effects during fermentation.

Fermentation parameters—including pH, temperature, oxygen availability, and medium composition—strongly influence microbial growth, metabolic flux distribution, and the formation of both desired products and by-products (Akita et al., 2024; Baez et al., 2011; Xiao et al., 2012). Among these factors, pH has been shown to exert a substantial influence on isobutanol biosynthesis. Jo et al. (2024) reported that *Klebsiella pneumoniae* achieved maximal cell growth at pH 7.0, whereas the highest isobutanol titer was obtained at pH 6.0, with by-products such as acetate and ethanol accumulating predominantly at pH 7.0. Similarly, Kim and Choi (S. Y. Kim & Choi, 2022) demonstrated strain-dependent differences in isobutanol production by engineered *Escherichia coli* at pH 8.0

Table 1
Recent Studies on Isobutanol Production: Comparison of Metabolic Strategies and Fermentation Conditions

Host	Metabolic strategy	Pathway/Gene involved/plasmid	Substrate	Cultivation condition	Isobutanol	References
<i>S. cerevisiae</i>	UV-Mutagenesis and <i>Abat1</i>	Glycolysis-Ehrlich /KIV	Glucose	YNB Medium 30°C, 200 rpm, pH 4.0 – 7.0; 72 h	20.8 mg/g	(Thammapanyaphong et al., 2026)
<i>S. cerevisiae</i>	Medium Optimization by RSM	Glycolysis-Ehrlich	Glucose	30°C, 170 rpm, 48 h	172 mg/L	(Ramli N.A., Abd Rahman R., 2021)
<i>Klebsiella pneumoniae</i>	Regulation of acetohydroxyacid synthase <i>ΔIdhAΔbudA</i>	Ehrlich pUC-tac- <i>ilvIH-ilvC-ilvD-kivd-adhA</i>	Glycerol	200 rpm, pH 6.0	1.02 g/L	(Jo et al., 2024)
<i>E. coli</i>	UV-Mutagenesis	pCDFDuet-1: <i>alsS, kivD</i> pET23a(+): <i>ilvC, ilvD, yqhD</i>	Yeast Extract	Yeast Extract Medium -YEM	5.07 g/L	(S. Y. Kim & Choi, 2022)
<i>E. coli</i>	Xylose utilization pathway + competitive pathway elimination: <i>Δeda::ackA ΔcbdAΔxdh::cyo ΔxyIFG::nuo ΔIdhAΔpflB</i>	Entner-Doudorof-Ehrlich and Dahms pathway pTrc99a- <i>xdh-yagF-yagE-aldA-aceAK</i> pCL1920- <i>adhA-kivD-alaS-ilvD-ilvC</i>	Glucose-Xylose	Luria Bertani Medium, 37°C; 200 rpm, 72 h	485.35 mg/L (from xylose) 528.72 mg/L (from glucose and xylose)	(Gu et al., 2023)
<i>E. coli</i>	Optimizing glucose recovery from bamboo biomass using RSM <i>ΔfrdBC, ΔadhE, Δpta, Δfnr, ΔIdhA</i> , and <i>ΔpflB</i>	p15A ori; KanR ; PLlacO1: <i>alsS-ilvCD</i> ColE1 ori; AmpR ; PLlacO1: <i>kivd-adhA (L. lactis)</i>	Glucose from bamboo biomass	pH 6.8; 30°C	2.28 g/L	(Nawab et al., 2025)
<i>E. coli</i>	Acetate as co-substrate for redox imbalance <i>ΔIdhAΔpflBΔfrdA ΔackAΔadhEΔlacY</i>	[pBBRori- <i>aadA1 (Sp^R)-T_L-lacI-P_{lacIQ} (814bb)</i>]-PT7A1-O34-RBS _{AS7} - <i>alsS_{Bsu}</i> -RBS _{AC7} - <i>ilvC_{Eco-6E6}</i> -RBS _{AD5} - <i>ilvD_{Eco}</i> -RBS _{AK7} - <i>kivd_{Lla}</i> -RBS _{AA2} - <i>adhA_{Lla-M5}</i> - <i>gfp-T_{P22}</i>	Glucose-acetate	Luria Bertani, anaerobic, 200 rpm, 30°C;	74 mM	(Boecker et al., 2023)

and pH 5.5, indicating that optimal pH conditions depend on genetic background.

Temperature is another critical parameter influencing fermentation efficiency through its effects on microbial growth kinetics and recombinant protein solubility, both of which are central to engineer isobutanol biosynthetic pathways. Lower cultivation temperatures often favor the production of soluble enzymes involved in isobutanol synthesis, whereas higher temperatures may enhance growth rates but impair protein folding or plasmid stability (Maksum *et al.*, 2023). Baez *et al.* (2011) observed that although engineered *E. coli* exhibited the fastest growth at 37 °C, isobutanol titers were approximately twofold higher at 30 °C, accompanied by substantially reduced acetate accumulation. In contrast, Gupta *et al.* (2022) reported higher isobutanol titers at 37 °C despite faster glucose consumption at 30 °C, highlighting the strain-specific nature of temperature effects.

The duration of fermentation can significantly influence protein production, as the highest protein yields are typically achieved during the mid- to late-logarithmic growth phase (Gupta *et al.*, 2022). Prolonged fermentation extends the stationary phase, which may reduce protein availability due to time-dependent protein degradation (M. T. Smith *et al.*, 2014). Furthermore, extended fermentation periods can lead to nutrient starvation, thereby decreasing protein production as a result of disrupted molecular and metabolic regulatory mechanisms (Rusilowicz-Jones *et al.*, 2022; Snoeck *et al.*, 2024). In addition, the accumulation of by-products such as acetate during the stationary phase may create toxic intracellular conditions, further impairing cellular function and protein expression.

Although the individual effects of pH and temperature on microbial isobutanol production have been extensively studied, their combined effects remain unexplored. Moreover, the fermentation duration may give an effect on isobutanol yield since it affects the protein stability and the production of other metabolites that may cause stress for the cell. Given that both

parameters strongly influence metabolic pathways and enzymatic activities, a systematic evaluation of their interactive effects is required to determine optimal process conditions. Response surface methodology (RSM) is a widely applied statistical approach for evaluating and optimizing fermentation parameters in bioprocess engineering (Kusuma *et al.*, 2019; Oktaviani *et al.*, 2020). Therefore, this study aims to evaluate the key fermentation parameters—specifically pH, temperature and fermentation duration as well as their interaction effect toward isobutanol production—using RSM to identify conditions that maximize isobutanol production by recombinant *E. coli* BL21(DE3) to obtain optimal combination parameters that potentially increase isobutanol production.

2. Materials and Methods

2.1 Strain and Plasmid

E. coli DH5α (Invitrogen) was used for plasmid construction and propagation. *E. coli* BL21 DE3 (Promega) was used for recombinant protein expression and isobutanol production. Plasmid pSB1K3-Iso was used as the template for construction of the T7 promoter-driven isobutanol expression plasmid. The characteristics of the strains and plasmids used in this study are summarized in Table 2.

2.2 Culture Condition

2.2.1. Preculture

All preculture for competent cell preparation and fermentation were prepared using the same method, or else if indicated. Single colonies were picked and inoculated into LB broth and incubated for 16–18 h at 37 °C with agitation at 150 rpm. The medium was supplemented with 30 µg/mL kanamycin, 100 µg/mL ampicillin, or no antibiotic for strains without plasmids, as appropriate (Atsumi *et al.*, 2008)

Table 2
List of strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strain		
<i>E. coli</i> DH5α	F ⁺ , endA1, hsdR17 (rK [−] , mK ⁺), <i>supE44</i> , <i>thi-I</i> , λ [−] , <i>recA1</i> , <i>gyrA96</i> , Δ <i>lacU169</i> (Φ80 <i>lacZ</i> Δ <i>M15</i>)	Invitrogen
<i>E. coli</i> BL21(DE3)	<i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> (rK [−] , mK ⁺), <i>relA1</i> , <i>supE44</i> , Δ(<i>lac-proAB</i>), [F ⁺ <i>traD36</i> , <i>proAB</i> , <i>lacI</i> ^q Δ <i>M15</i>].	Promega
D_01	<i>E. coli</i> DH5α containing pSB1K3-BB	This Study
D_02	<i>E. coli</i> DH5α containing pSB1K3-Iso	This Study
B_01	<i>E. coli</i> BL21 (DE3) containing pSB1K3-BB	This Study
B_02	<i>E. coli</i> BL21 (DE3) containing pSB1K3-IsoT7	This Study
Plasmid		
pSB1K3-BB	<i>pSB1K3</i> backbone only	This Study
pSB1K3-Iso	<i>plac-alsS-ilvCD-kivd</i> , <i>KanR</i>	(Kennardi <i>et al.</i> , 2020)
pSB1K3-IsoT7	<i>pT7-alsS-ilvCD-kivd</i> , <i>KanR</i>	This Study
pJET 1.2 blunt	<i>placUV5-eco47IR</i> , <i>AmpR</i> ,	Thermo Scientific

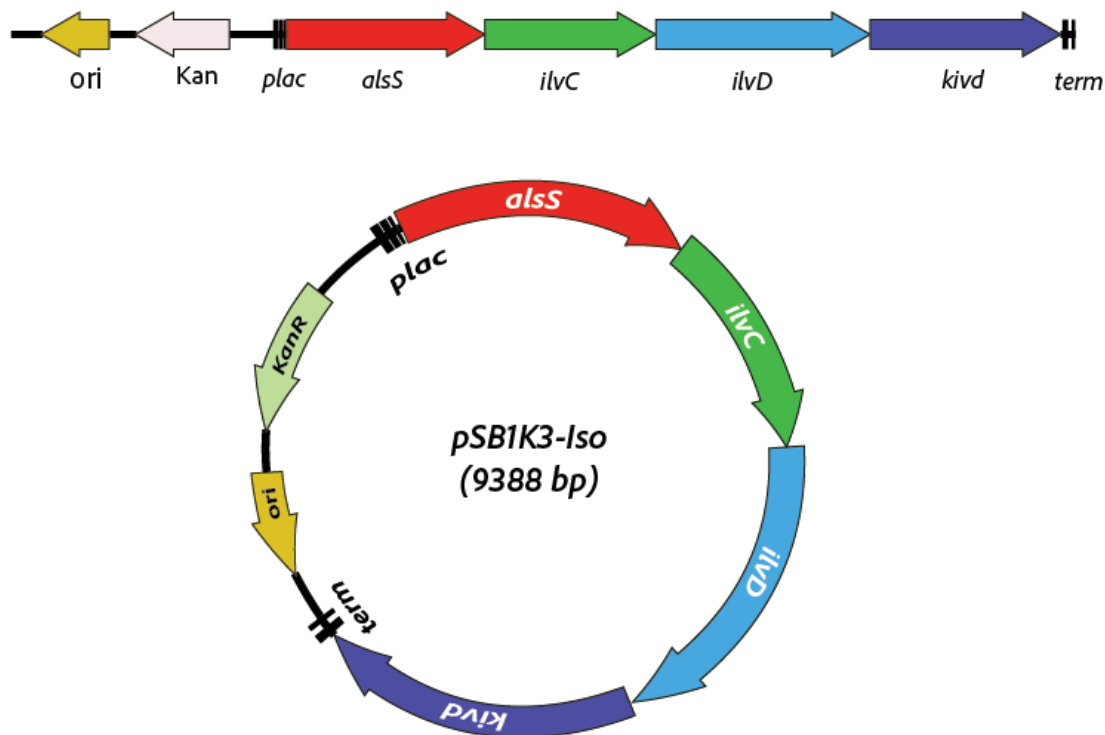


Fig. 1. Vector pSB1K3-Iso consisting of operon *lac* with *alsS*, *ilvC*, *ilvD*, and *kivd* genes controlled by *lac* promoter

2.2.2. Preparation of Competent Cells

Preculture were diluted 1:100 into fresh LB broth and incubated at 37 °C and 150 rpm until an optical density at 600 nm (OD₆₀₀) of 0.4–0.6 was reached. Cells were harvested by centrifugation at 3,000× g at 4 °C for 15 min. The supernatant was discarded, and the cell pellet was resuspended in ice-cold CCMB80 buffer by gentle pipetting and incubated on ice for 10 min. Cells were centrifuged again under the same conditions, the supernatant was discarded, and 1 mL of ice-cold CCMB buffer was added. After incubation on ice for 10 min, the cell suspension was transferred to prechilled microtubes and either used immediately or stored at –80 °C (Sambrook & Russell, 2006).

2.3.3. Transformation

Transformation was performed using the heat shock method. Up to 5% (v/v) of ligation mixture or purified plasmid DNA was added to competent cells and incubated on ice for 15–20 min. The cells were heat-shocked at 42 °C for 60 s, immediately returned to ice for 10 min, and then recovered in 500 µL fresh LB broth at 37 °C and 150 rpm for 1–2 h. Cells were centrifuged at 14,000 × g for 3 min, resuspended in 100 µL LB

broth, and spread onto LB agar plates containing appropriate antibiotics. Plates were incubated at 37 °C for 16–18 h, and transformant colonies were subsequently observed (Kennardi *et al.*, 2020).

2.3 Plasmid Construction

Plasmid pSB1K3-Iso (Fig. 1) was obtained from Kennardi, Moeis, and Andreas (Kennardi *et al.*, 2020). This plasmid contains four genes involved in isobutanol biosynthesis: *alsS*, encoding acetolactate synthase (ALS) from *Bacillus subtilis*; *ilvC*, encoding ketol-acid reductoisomerase (KARI) from *E. coli*; *ilvD*, encoding dihydroxy-acid dehydratase (DHAD) from *E. coli*; and *kivd*, encoding keto-acid decarboxylase (KDC) from *Lactococcus lactis*, all under the control of the *lac* promoter. In this study, the *lac* promoter was replaced with the T7 promoter using a PCR-based mutagenesis and assembly strategy. The primers used for plasmid construction are listed in Table 3.

Because the pSB1K3-Iso plasmid is relatively large (9,388 bp) and suitable restriction sites were limited, direct amplification using a single PCR reaction was not feasible. Therefore, a Golden Gate assembly-based cloning strategy was employed. The four genes and the plasmid backbone were amplified separately to obtain sufficient DNA yields for plasmid

Table 3
Primers used for plasmid construct

Primer name	Sequence (5'→'3')
alsS F	CCATGGAATAATTTTGTGTTAACTTTAAGGTCACACAGGAAAGTACTAGATGTTG
alsS R	ACTAGTCTAGAGAGCTTTTCGTTTTCATGAGT
ilvC F	TCTAGAGTCACACAGGAAAGTACTAGATGGC
ilvC R	CTGCAGGGATCCTTAACCCGCAACAGCAATACGT
ilvD F	GAATTCGGATCCTCACACAGGAAAGTACTAGATGCC
ilvD R	ACTAGTTTAAACCCCGAGTTTCGATT
Kivd F	GTCAGCGAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGT
Kivd R	ACTGCAGCGGCCGCTACTAGTATATAAACGCGAGAAAG
BB psbk F	CTCACCTTCGGGTGGGCCCTT
BB psbk R	CCATGGGGGAATTGTTATCCGCTACAATCCCCTATAGTGAGTCGTATTAGAATTCCAGAAATCATC
	CTTAGCGAAAGCT

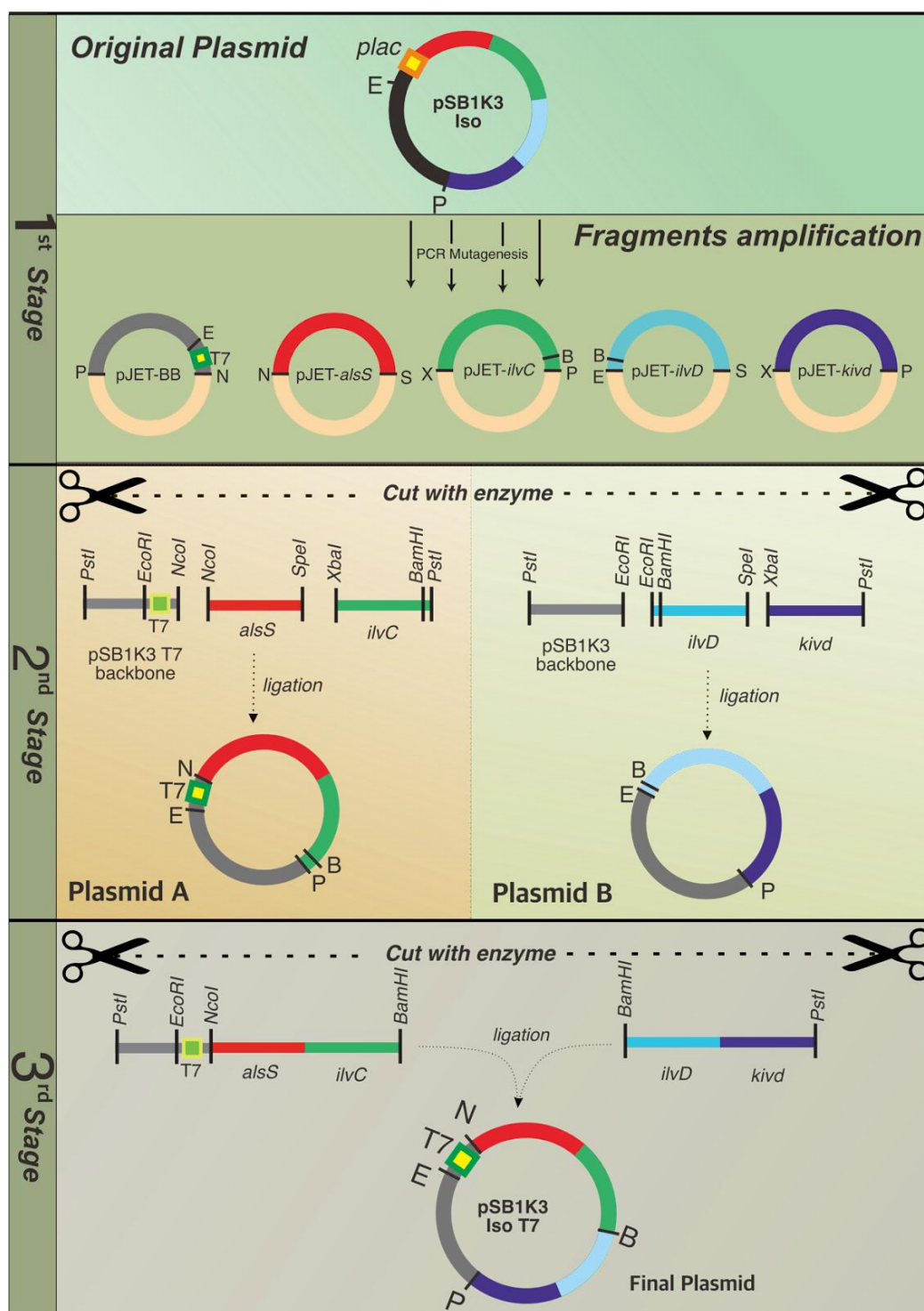


Fig. 2. Illustration of plasmid pSB1K3-Iso T7 construction. The plasmid construction consists of three stages: First stage - isobutanol genes were amplified separately and cloned into pJET-blunt; Second stage - genes in pJET were cut with appropriate enzymes and ligated to construct plasmid A and plasmid B; Third stage, plasmid A and plasmid B were cut again with appropriate enzymes and ligated to construct final plasmid pSB1K3- Iso T7.

construction. The assembly procedure consisted of three steps: (i) amplification of individual gene fragments and cloning into the pJET blunt vector; (ii) restriction digestion and assembly of plasmid A (pSB1K3 backbone containing the T7 promoter, *alsS*, and *ilvC* genes) and plasmid B (pSB1K3 backbone containing *ilvD* and *kivd* genes); and (iii) restriction digestion and ligation of plasmids A and B to generate the final expression plasmid (Fig. 2).

To eliminate the *lac* promoter sequence located upstream of *alsS* in the original pSB1K3 vector, primers for *alsS* amplification were designed to anneal directly to the coding sequence to eliminate the *lac* promoter region. The T7 promoter sequence was incorporated into the pSB1K3 backbone. Primers BB pSBK F and BB pSBK R were used to introduce *PstI*, *EcoRI*, T7 promoter, and *NcoI* restriction sites into the pSB1K3 backbone. The *alsS* fragment was amplified using primers *alsS* F and *alsS*

R, which introduced *NcoI* and *SpeI* sites at the fragment termini. Primers *ilvC* F and *ilvC* R were designed to introduce *XbaI*, *BamHI*, and *PstI* sites on the *ilvC* gene fragment. Primers *ilvD* F and *ilvD* R were designed to add *EcoRI*, *BamHI* and *SpeI* on the *ilvD* gene fragment, and primers *kivd* F and *kivd* R were designed to introduced *XbaI*, and *PstI* sites on the *kivd* gene fragment as required for assembly.

PCR amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) under the following conditions: initial denaturation at 98 °C for 3 min; 30 cycles of denaturation at 98 °C for 30 s, annealing at 53 °C for 15 s, and extension at 72 °C for 1 min 30 s; followed by a final extension at 72 °C for 5 min. The amplified fragments were cloned into the pJET blunt vector and transformed into *E. coli* DH5α using the heat shock method (Sambrook & Russell, 2006). Transformants were selected on Luria Bertani (LB) agar plates (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) supplemented with 100 µg/mL ampicillin.

To construct plasmid A, the pSB1K3 backbone containing the T7 promoter sequence cloned in pJET was digested with *NcoI* and *PstI*. The *alsS* fragment cloned in pJET was digested with *NcoI* and *SpeI*, while the *ilvC* fragment was digested with *XbaI* and *PstI*. These digested fragments were ligated to generate plasmid A, consisting of the pSB1K3 backbone with the T7 promoter and the *alsS* and *ilvC* genes. To construct plasmid B, the *ilvD* fragment cloned in pJET was digested with *EcoRI* and *SpeI*, and the *kivd* fragment was digested with *XbaI* and *PstI*. These fragments were ligated into the original pSB1K3 backbone digested with *EcoRI* and *PstI*, resulting in plasmid B containing the *ilvD* and *kivd* genes. The resulting plasmids were transformed into *E. coli* DH5α and selected on LB agar plates supplemented with 100 µg/mL kanamycin.

To construct the final expression plasmid, plasmids A and B were digested with *BamHI* and *PstI*. The fragments containing the target genes were ligated and transformed into *E. coli* DH5α, followed by selection on LB agar plates containing 100 µg/mL kanamycin. Transformant colonies were screened by colony PCR using appropriate primers. A confirmed positive colony was subcultured in LB broth supplemented with 100 µg/mL kanamycin for plasmid extraction.

2.4 Analysis of Promoter Effect on Protein Expression and Isobutanol Production

2.4.1 Protein Analysis

To analyse the effect of promoter changes on protein expression. Four strains were compared including: DH5α containing empty pSB1K3 plasmid (D_01), DH5α containing plasmid pSB1K3 with *lac* promoter (D_02), BL21(DE3) containing empty pSB1K3 plasmid (B_01) and BL21(DE3) containing pSB1K3 plasmid with T7 promoter (B_02).

Precultures were diluted 1:100 into fresh LB broth supplemented with 30 µg kanamycin and incubated at 37 °C and 150 rpm until an optical density at 600 nm (OD₆₀₀) of 0.4–0.6 was reached and then followed by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) with total concentration 1 mM. The fermentation was continued for another 3 hours. Cells then harvested by centrifugation 3,000x g, 4°C for 15 min.

Pellets were resuspended in lysis buffer (Kwon, 2019) 4 mL per gram wet weight of cells and disrupted by sonication for 15 min using a pulse program of 15 sec on and 15 sec off. Cell lysates were centrifuged at 14,000× g for 30 min, and the supernatants were collected as crude protein extracts. Pellet then washed with wash buffer (Palmer & Wingfield, 2012) two or three times, followed by centrifugation at 14,000x g for 3 min for each washing step. After washing step, solubility buffer was added 4 mL per gram wet weight of cells to extract insoluble fraction (Palmer & Wingfield, 2012). Cell was centrifuged at 14,000x g for 10 min, and the supernatant were used as sample for insoluble fraction. Protein expression was analysed by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) following the method described by Kusuma *et al.* (2019).

2.4.2 Isobutanol Analysis

To analyse the effect of promoter changes on isobutanol production, four strains were analysed with two treatments, induced or uninduced. Preculture were diluted 1:100 into 100 mL fresh LB broth and incubated at 37 °C and 150 rpm until an optical density at 600 nm (OD₆₀₀) of 0.4–0.6 was reached and then followed by IPTG induction with total concentration 0.1 mM. The fermentation was conducted for 48 hours. Broth was separated from cells with centrifugation at 3,000x g, 4°C for 15 min and supernatant was used as sample for isobutanol measurement.

Isobutanol was extracted using a salting-out method as described by Li *et al.* (2014). Fermentation broth was centrifuged at 3,000 × g to separate cells from the supernatant. Subsequently, 3 g K₂HPO₄ was added to 5 g of fermentation broth, followed by the addition of 2 g acetone after complete dissolution of the salt. The mixture was thoroughly mixed and allowed to stand until phase separation occurred, resulting in three distinct phases. The upper organic phase (approximately 3.5 mL) was collected for isobutanol analysis.

Isobutanol concentration was determined using gas chromatography with flame ionization detection (GC–FID) equipped with an SH-Rxi-5ms column (30 m × 0.25 mm). Pure isobutanol (EMSURE) was use as standard for measurement. Nitrogen (N₂) was used as the carrier gas at a flow rate of 0.75 mL min⁻¹. The injector and detector temperatures were set to 250 °C, and the oven temperature program was initiated at 60 °C (Honig *et al.*, 2015).

2.5 Determination of Optimum Culture Condition

Table 4
Experimental range of the three variables studied using box-Behnken design

Factors	Symbol	Levels		
		Low (-1)	Intermediate (1)	High (+1)
acidity (pH)	X	4.5	5.5	6.5
Fermentation temperature (°C)	Y	30	33.5	37
Duration of fermentation (h)	Z	24	48	72

Optimization of culture conditions for isobutanol production was performed using a Box–Behnken response surface methodology (RSM) design with factor levels of −1, 0, and +1, generated using Minitab Statistical Software (Minitab LLC, State College, PA, USA). Three independent variables—pH, temperature (°C), and fermentation time (h)—were evaluated according to the experimental design shown in Table 4. A total of 15 experimental runs, including three center points, were conducted in randomized order.

Fermentation experiments were performed in laboratory flasks according to the experimental design. A 16 h preculture grown in LB medium was inoculated at 1% (v/v) into 50 mL LB medium supplemented with 30 µg/mL kanamycin and 20 g/L glucose in 250 mL Erlenmeyer flasks. All fermentations were conducted in duplicate, with conditions adjusted according to each experimental run. Preculture were diluted into 1:100 into 50 mL, and growth at 37°C, 150 rpm until OD₆₀₀ reach 0.4-0.6 and then temperatures were adjusted into the experiment run settings, accordingly.

Initial and final cell concentrations were determined using the total plate count (TPC) method (Chen *et al.*, 2024). Samples (100 µL) were collected at the beginning and end of fermentation, serially diluted, and plated on LB agar. Plates were incubated at 37 °C for 24 h, and those containing 30–300 colonies were used to calculate viable cell counts (CFU/mL) using the following equation:

$$TPC = (B \times D) / V \quad (1)$$

In this equation, *B* represents the average number of colonies counted from three replicate plates at the same dilution (CFU), *D* represents the dilution factor of the selected plate, and *V* represents the sample volume plated at the first dilution step (mL). In this study, *V* was 0.1 mL.

To facilitate the presentation of large viable cell counts, results are commonly expressed as log₁₀ CFU/mL (log CFU/mL) in microbial enumeration. Conversion from CFU/mL to log CFU/mL was performed using the following equation:

$$N = 10^x \quad (2)$$

$$\log N \text{ CFU/mL} = x \log \text{CFU/mL} \quad (3)$$

In this equation, *N* represents for TPC in CFU/mL, and *x* is the value of log CFU/mL.

2.6 Metabolite Measurement

Glucose consumption by cells from the fermentation were analysed to measure the effect of parameters applied toward cells growth and isobutanol yield. Fermentation broth was centrifuged at 3,000 × g for 10 min, and the supernatant was collected for metabolite analysis. Glucose concentration was determined by high-performance liquid chromatography (HPLC) equipped with a Shimadzu SCR-101C column, using deionized water as the mobile phase. The flow rate was maintained at 1.0 mL min^{−1}, and the column temperature was set to 80 °C (Sabatina *et al.*, 2024).

2.7 Data Analysis

Response surface methodology (RSM) was applied to optimize isobutanol production by evaluating the combined effects of pH, temperature, and fermentation time. A three-factor Box–Behnken design with three centre points (15

experimental runs) was generated and analysed using Minitab Statistical Software version 22, with each factor set at three levels: pH (4.5, 5.5, 6.5), temperature (30, 33.5, 37 °C), and fermentation time (24, 48, 72 h).

A full second-order (quadratic) model was first fitted, and model reduction was then performed by backward elimination (α -to-remove = 0.10) while preserving model hierarchy: at each step the term with the largest p-value exceeding the threshold was removed until all remaining terms satisfied the criterion. The removal threshold was specified a priori and applied consistently to avoid selective retention of terms. Regression coefficients are reported in coded units to allow comparison of effect sizes, while the final regression equation is given in uncoded (natural) units for prediction.

The adequacy of the reduced model was assessed using the coefficient of determination (*R*²), adjusted *R*², predicted *R*² (based on PRESS), the lack-of-fit test, and residual diagnostics (normal probability plot, residuals versus fits and versus run order, studentized residuals, and Cook's distance). Term significance was evaluated by analysis of variance (ANOVA) at α = 0.05. Optimal conditions were determined by response optimization (maximizing isobutanol production), and the predicted optimum was verified experimentally; the single validation result was compared against the 95% prediction interval for an individual observation.

3. Results and Discussion

3.1 Expression of ALS, KARI, DHAD and KDC Protein

Protein expression profiles were compared among *E. coli* DH5α harbouring empty pSB1K3 (D_01), *E. coli* DH5α harbouring pSB1K3-Iso (D_02), *E. coli* BL21(DE3) harbouring empty plasmid (B_01), and *E. coli* BL21(DE3) harbouring pSB1K3-IsoT7 (B_02). Cell samples were collected at 6 h of fermentation (3 h after IPTG induction). Differences in protein expression levels among the strains were analysed by SDS–PAGE, and the comparative expression patterns are shown in Fig. 3.

Protein bands obtained from the B_02 strain appeared more intense than those from the previously used D_02 strain. Only three distinct protein bands were observed, likely due to the similar molecular weights of ALS and KDC, which resulted in co-migration and limited separation on the SDS–PAGE gel. Nevertheless, the results clearly demonstrated that the use of the T7 promoter enhanced protein expression. The isobutanol production from the two strains were compared and we found that B_02 strain produced more isobutanol compared to D_02 strain (0.1784 g/L vs 0.2766 g/L). Based on these findings, the BL21(DE3)-IsoT7 strain was selected for subsequent isobutanol production experiments.

The soluble and insoluble fractions were also investigated to evaluate the possibility of inclusion body formation. The soluble and insoluble fraction of D_02 and B_02 strains comparison was shown in Fig. 4. The SDS PAGE showed that promoter T7 enhances protein expression on both soluble and insoluble fraction. At soluble fraction, KDC (arrow a) and KARI (arrow d) protein productions were increase compared to D_02 strain. Meanwhile, at insoluble fraction, KDC was not found as inclusion body, while the KARI protein at insoluble fraction was significantly increased. These findings imply that isobutanol production in the T7 expression system remains suboptimal, likely because KARI is not fully active; a large fraction of the recombinant enzyme is sequestered in inclusion bodies rather than in a soluble, functional form.

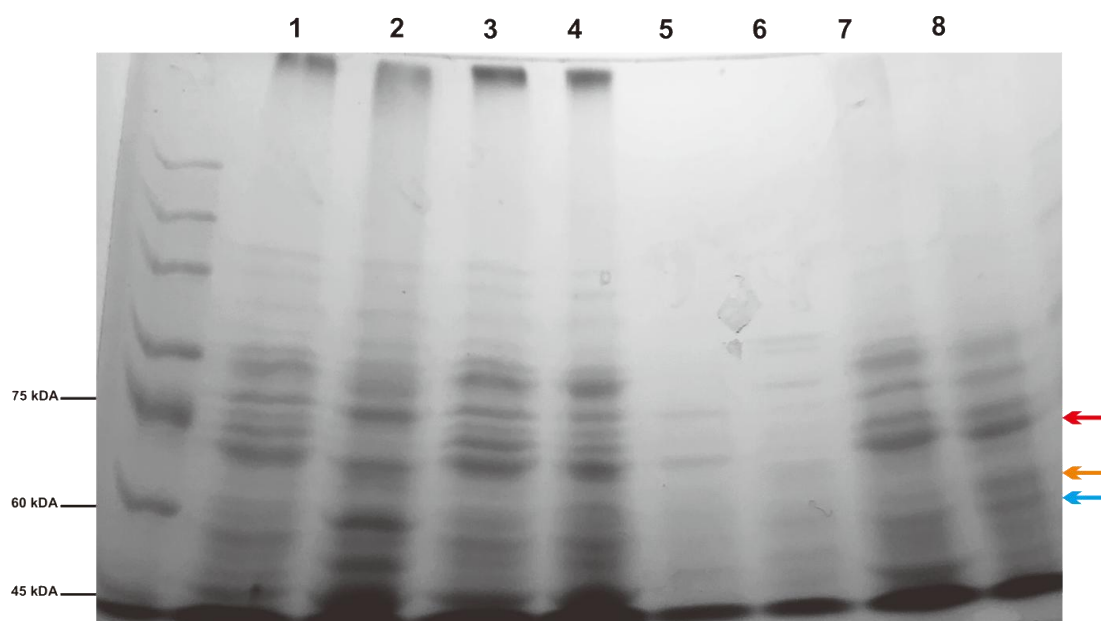


Fig. 3. Expression of ALS, KARI, DHAD, and KDC proteins in different *Escherichia coli* strains. DH5 α carrying the pSBK backbone (D_01) (1 - no induction; 2 - with induction), DH5 α harbouring the isobutanol plasmid under the control of the *lac* promoter (D_02) (3 - no induction; 4 - with induction), BL21(DE3) carrying the pSB1K3 backbone (B_01) (5 - no induction; 6 - with induction), and BL21(DE3) harboring the isobutanol plasmid under the control of the T7 promoter (B_02) (7 - no induction; 8 - with induction) were analysed in the presence or absence of IPTG induction. ALS (62 kDa) is indicated by orange arrows, KARI (54 kDa) by blue arrows, DHAD (65 kDa) by red arrows, and KDC (61 kDa)

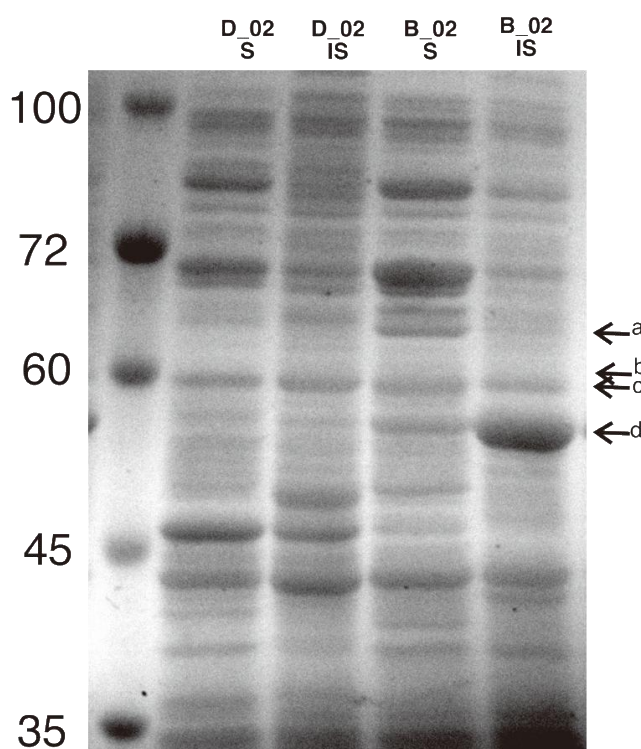


Fig. 4. Comparison of soluble (S) and insoluble (IS) fraction of protein expressed by *lac* promoter (D_02) and T7 promoter (B_02). Arrow on the right side pointing the position of KDC (a), ALS (b), DHAD (c) and KARI (d). The SDS PAGE showed that heterologous protein expressions were enhanced by promoter T7 compared to protein expression by *lac* promoter. DHAD and KARI protein bands were found thicker on the protein expressed by T7 system. A high portion of KARI protein was found on insoluble fraction, showing that not all of KARI protein expressed have been contributed to the isobutanol conversion.

To evaluate the effects of pH and temperature on protein production, we compared the soluble and insoluble fractions of proteins produced by B_02 strain subjected to different pH and temperature treatments (Fig. 5). SDS-PAGE analysis indicated that elevated temperature was associated with greater inclusion

body formation. However, the influence of pH was not clearly defined, as acidic conditions at higher temperatures appeared to suppress soluble protein accumulation, whereas at lower temperatures pH seemed to favor soluble protein formation. The work of Kim, Wu and Lee (2021) presents the possibility of

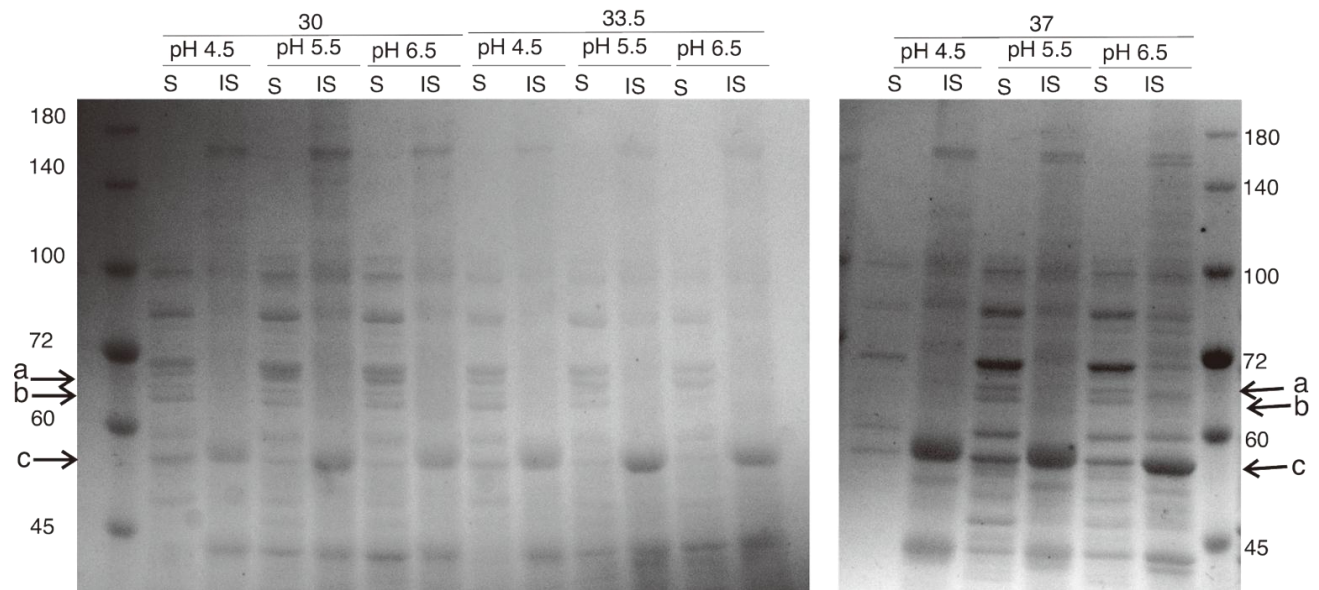


Fig. 5. The comparison of soluble (S) and insoluble (IS) fraction of heterologous protein expressed by *E.coli* BL21(DE3) harbouring isobutanol genes controlled by T7 promoter (B_02) affected by various acidity and temperature levels. SDS PAGE showed that inclusion bodies formation tends to increase at higher temperature. The acidity effect on protein expression was in conclusive, acidity seems to decrease soluble protein content at higher temperature, but at lower temperature, acidity seems more beneficial to soluble protein formation.

cell responds toward acidic pH stress through the utilization of specialized chaperone protein that help to prevent the aggregation that might occurred in this study. However, according to Palmer and Wingfield (2012), proteins also retain less residual structure when exposed to higher pH, which might lower the aggregation protein.

3.2 Optimization of Culture Condition

Isobutanol production was assessed at a laboratory scale using Erlenmeyer flasks under the conditions specified by the experimental design as described in Table 5. Isobutanol was measured by GC-FID, the retention time for isobutanol was recorded at the range 3.2-3.3 sec (Fig. 6). The resulting isobutanol concentrations were consider as main response,

while bacterial cell counts and glucose consumption were served as additional response variables for each treatment. The predicted isobutanol value from regression model also presented in Table 5. The bacterial preculture was inoculated at a ratio of 1:100 (v/v) relative to the total medium volume, following the method of Atsumi *et al.* (2008), resulting in an initial cell density of approximately 10^4 – 10^5 CFU mL⁻¹. At the end of fermentation, total bacterial counts varied among treatments, reflecting the influence of the experimental variables. The highest final cell density (10^7 – 10^8 CFU mL⁻¹) was observed at 33.5 °C, pH 5.5, and a fermentation time of 48 h. In general, total bacterial counts decreased with decreasing pH. Although this effect was not statistically significant, acidic conditions were observed to influence bacterial viability, as low

Table 5
Experimental conditions and response of Isobutanol content and number of bacteria

Independent variables				Actual Response			Predicted Responses
Run	Temperature (°C)	pH	Fermentation duration (h)	Final number of bacteria (log cfu/ml)	Glucose consumed (%)	Isobutanol content (g/L)	Isobutanol content (g/L)
1	30	5.5	24	10,22	53.25	0.230 ±0.023	0.284
2	30	4.5	48	8,43	55.68	0.313±0.007	0.224
3	30	6.5	48	9,18	78.42	0.268±0.035	0.287
4	30	5.5	72	9,49	91.36	0.211±0.014	0.227
5	33.5	4.5	24	7,05	4.13	0.128±0.01	0.180
6	33.5	6.5	24	8,88	70.51	0.293±0.034	0.243
7	33.5	5.5	48	11,62	79.53	0.195±0.018	0.183
8	33.5	5.5	48	10,81	78.01	0.152±0.011	0.183
9	33.5	5.5	48	10,61	76.42	0.185±0.013	0.183
10	33.5	6.5	72	9,89	49.01	0.180±0.025	0.186
11	33.5	4.5	72	7,79	95.48	0.157±0.025	0.123
12	37	5.5	24	8,54	39.61	0.210±0.012	0.140
13	37	4.5	48	T.F.C	0	0.021±0.015	0.080
14	37	6.5	48	10,35	57.98	0.129±0.018	0.142
15	37	5.5	72	N.D	92.77	0.084±0.006	0.082

N.D* no living bacteria found; T.F.C* too few to count. Predicted isobutanol were calculated from the linear regression model in actual factors.

Table 6.
Isobutanol Yield and Productivity for each Box–Behnken run

Run	Glucose consumed (g/L)	Yp/S (g/g)	Yield Isobutanol (% of theor.)	Productivity (g/L.h)
1	10.65	0.0216	5.3	0.0096
2	11.14	0.0281	6.9	0.0065
3	15.68	0.0171	4.2	0.0056
4	18.27	0.0115	2.8	0.0029
5	0.83	0.1550 a	37.8 a	0.0053
6	14.10	0.0208	5.1	0.0122
7	15.91	0.0123	3.0	0.0041
8	15.60	0.0097	2.4	0.0032
9	15.28	0.0121	3.0	0.0039
10	9.80	0.0184	4.5	0.0025
11	19.10	0.0082	2.0	0.0022
12	7.92	0.0265	6.5	0.0088
13	0.00	n.a	n.a	0.0004
14	11.60	0.0111	2.7	0.0027
15	18.55	0.0045	1.1	0.0012

n.a. = not applicable (no glucose consumed).

Initial glucose 20 g/L; theoretical maximum yield ≈ 0.41 g/g. Glucose consumed (g/L) = 20 g/L × (% consumed/100); Yp/s = isobutanol ÷ glucose consumed; productivity = isobutanol ÷ fermentation time. Run 5 shows a high apparent Yp/s only because very little glucose was consumed (0.83 g/L); its low titre makes this value unrepresentative of productive conditions.

pH can induce cellular stress (S. Y. Kim & Choi, 2022). *E. coli* grows optimally within a pH range of 6–8; however, the bacterium can adapt to lower pH values through acid resistance mechanisms, allowing growth at pH 4.5, albeit at reduced levels compared to optimal conditions (Li *et al.*, 2024).

The linear regression model provided a reasonably good description of the relationship between temperature, pH, fermentation time, and isobutanol production. The predicted isobutanol concentrations, calculated from the model in actual factors, followed the general trend of the observed values across the experimental domain, with most residuals (observed – predicted) falling within ±0.07 g/L. The largest positive deviation was observed in run 2 (30°C, pH 4.5, 48 h), where the actual isobutanol content (0.313 g/L) was substantially higher than the predicted value (0.224 g/L), suggesting that under these specific conditions the model underestimated production. Conversely, the model overestimated isobutanol in run 1 (30°C, pH 5.5, 24 h; observed 0.230 g/L, predicted 0.284 g/L) and run 13 (37°C, pH 4.5, 48 h; observed 0.021 g/L, predicted 0.080 g/L). The latter case coincides with a marked reduction in viable cell counts (T.F.C), indicating that the severe stress condition at high temperature and low pH may have triggered physiological responses that were not fully captured by the linear model.

Despite these deviations, the model was statistically significant ($p = 0.0053$) and exhibited non-significant lack of fit ($p = 0.1552$), supporting its adequacy for describing the main trends within the studied region. The coefficient of determination ($R^2 = 0.6712$) indicates that approximately 67% of the variability in isobutanol concentration could be explained by the three independent variables, while the remaining variation may be attributed to biological variability, measurement error, and unmodeled higher-order effects. Overall, the agreement between observed and predicted values, together with the significant model terms and adequate precision ratio (> 4), suggests that the linear model is suitable for identifying the direction and relative magnitude of factor effects on isobutanol production, although its predictive accuracy for individual runs—particularly under extreme stress conditions—should be interpreted with caution.

Under the conditions that supported measurable isobutanol production, the glucose-to-product yield ($Y_{P/S}$) was generally low, ranging from 0.0045 to 0.0281 g/g for most experimental

runs, corresponding to approximately 1.1–6.9% of the theoretical maximum yield of 0.41 g/g (Table 6). Volumetric productivity also remained relatively low, varying between 0.0012 and 0.0122 g/L.h. The highest isobutanol titer was obtained in Run 2 (30 °C, pH 4.5, 48 h), reaching 0.313 g/L with a yield of 0.0281 g/g (6.9% of the theoretical yield) and a productivity of 0.0065 g/L.h. These additional bioprocess metrics, including product yield on substrate ($Y_{P/S}$) and volumetric productivity, were calculated from the experimental endpoint data and incorporated into the results table to facilitate a more comprehensive evaluation of process performance. An exception was observed in Run 5, which exhibited the highest yield (0.1550 g/g), corresponding to 37.8% of the theoretical maximum yield. However, this condition was associated with very low glucose consumption (0.83 g/L), indicating that the apparently high conversion efficiency was achieved under limited substrate utilization rather than enhanced production capacity. In contrast, Run 11 consumed approximately 19.10 g/L glucose, representing nearly complete utilization of the initial substrate, yet produced only a small amount of isobutanol, resulting in a low $Y_{P/S}$ value of 0.0082 g/g (2.0% of the theoretical yield). This finding is not indicative of a calculation error but rather highlights inefficient carbon conversion, suggesting that a substantial proportion of the consumed glucose was directed toward biomass formation and competing metabolic pathways rather than isobutanol biosynthesis. Similarly, several other runs with glucose consumption exceeding 15 g/L also displayed low yields and productivities, further supporting this interpretation. Run 13 showed no detectable glucose consumption; therefore, the $Y_{P/S}$ value could not be calculated and was reported as not applicable (n.a.), since yield cannot be defined when substrate consumption is zero. This treatment avoids introducing misleading values through division by zero and more accurately reflects the experimental outcome.

Overall, the consistently low yields observed across the actively producing conditions suggest that carbon flux toward the isobutanol biosynthetic pathway remained limited. This observation is consistent with the use of *E. coli* BL21(DE3) as the production host, which lacks the competing-pathway deletions and extensive metabolic rewiring commonly implemented in highly engineered isobutanol-producing strains. Consequently, a significant fraction of the assimilated carbon

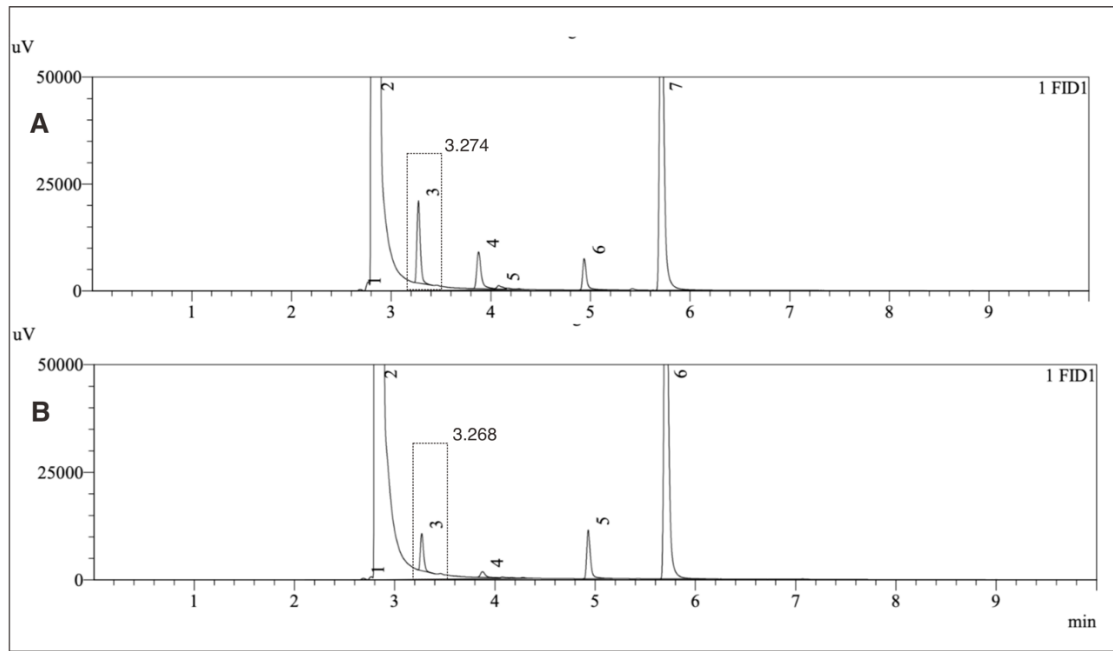


Fig. 6. Chromatogram of GC-FID showing the peak of isobutanol (marked with a dashed-line box) at retention time approx. 3.270 sec. (A) chromatogram of highest isobutanol measure; and (B) chromatogram with the fewest impurity compounds.

was likely diverted to native cellular processes, including biomass accumulation and by-product formation, thereby reducing the amount available for isobutanol synthesis. To place the obtained performance into context, the isobutanol titre, yield, and productivity values were compared with those reported in previous studies, including the pioneering works of Atsumi *et al.* (2010) and Baez *et al.* (2011), demonstrating that substantial improvements remain possible through metabolic engineering and process optimization.

It should be noted that time-course fermentation profiles, by-product quantification (e.g., acetate and ethanol), and a complete carbon balance analysis were not included in the present study. The experimental design relied on endpoint measurements, and by-product concentrations could not be reliably quantified using the available analytical approach. As a result, the distribution of carbon among biomass, isobutanol, and competing metabolites could not be fully resolved. These limitations are acknowledged in the Conclusion section and should be addressed in future studies through comprehensive fermentation monitoring, accurate by-product analysis, and carbon flux evaluation to better understand the factors limiting isobutanol production.

3.3 Regression Model for Isobutanol Production

Response surface methodology (RSM) was applied to evaluate the effects of temperature, pH, and fermentation time on isobutanol production by recombinant *E. coli* BL21(DE3). The ANOVA test for the model suggested the linear model as the most appropriate. The constants for the regression model are presented in Table 7. While the ANOVA data were presented in Table 8. The 2D-plot and 3D-surface plot were

presented in Fig. 7, the normality, cook's test and residual vs predicted test were presented in Fig. 8.

The model explained 67.12% of the variability in isobutanol production ($R^2 = 0.6712$), while the adjusted and predicted R^2 values were 0.5815 and 0.3093, respectively. Although the moderate predicted R^2 indicates that a substantial fraction of the response variability remained unexplained, the model showed an adequate signal-to-noise ratio (Adeq Precision = 8.0226). Importantly, the lack-of-fit test was not significant ($p = 0.1552$), indicating that there was insufficient evidence that the linear model was inadequate for describing the experimental response within the investigated range. The negative coefficient for temperature (-0.0206) indicates that, within the studied range ($30\text{--}37^\circ\text{C}$), increasing temperature tended to decrease isobutanol concentration when pH and fermentation time were held constant. This finding is consistent with the ANOVA result that temperature was the only statistically significant individual factor ($p = 0.0018$). The negative effect of temperature may reflect the combined influence of temperature on cellular physiology, heterologous protein expression, enzyme stability, and metabolic flux. Although higher temperatures can increase

Table 7
Coefficient for the regression model for isobutanol production

Variable	Coefficient
Constant	+0.759957
Acidity	+0.031375
Temperature	-0.020643
Fermentation Time	-0.001193

Acidity (pH), Temperature ($^\circ\text{C}$), Fermentation time (hours)

Table 8
ANOVA of regression model for isobutanol production

	SS	DF	MS	F	p-value	R2 values	
Model	0.0562	3	0.0187	7.48	0.0053	R^2	0.6712
Error	0.0275	11	0.0025			R^2 adjusted	0.5815
Total	0.0837	14				R^2 predicted	0.3093

Model p -value = 0.0053 indicates a statistically significant regression model. Lack of fit was not significant ($p = 0.1552$), suggesting adequate model fit. Adequate precision = 8.023 (>4), indicating an adequate signal-to-noise ratio.

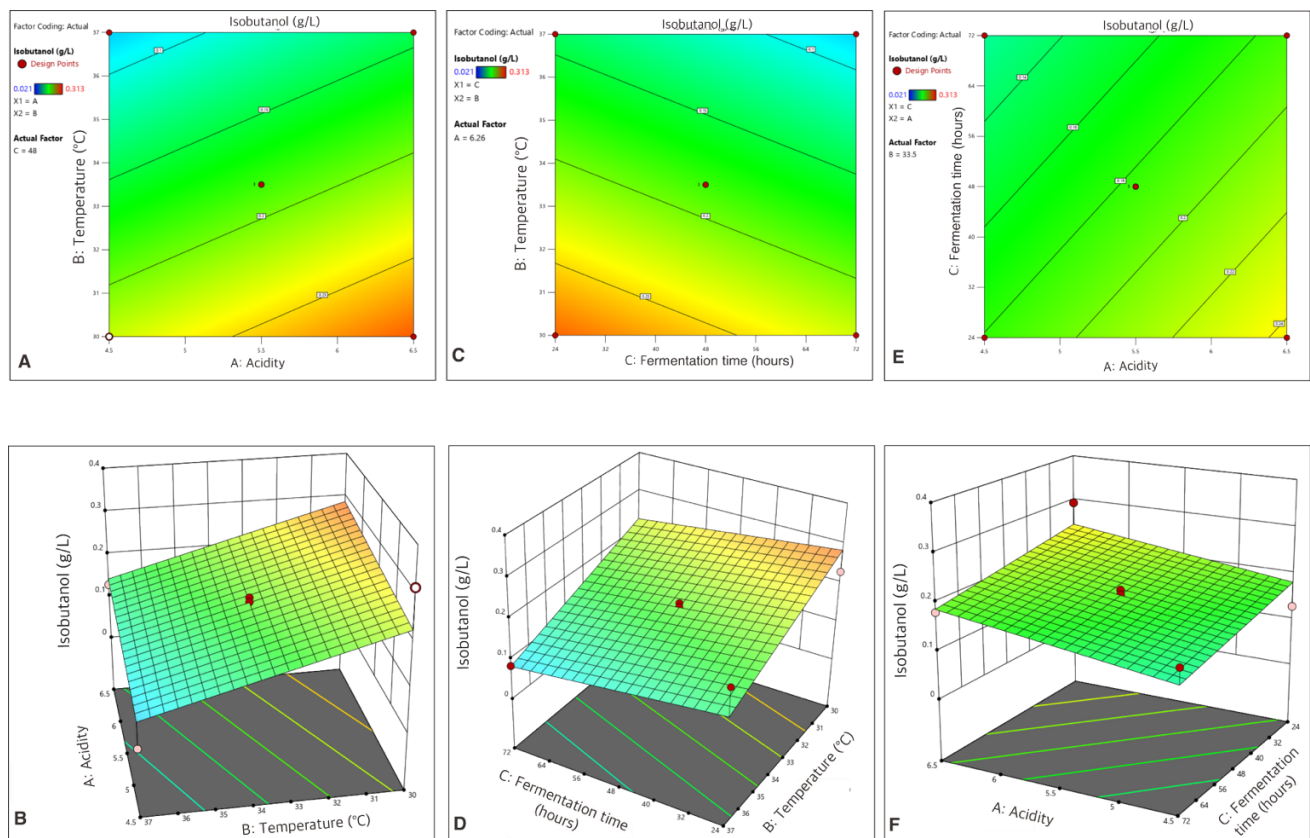


Fig. 7. 2D- Contour plot illustrating interaction of temperature and acidity (A); and its 3D-Surface plot (B); contour plot illustrating the interaction of acidity and fermentation time (C) and its 3D-Surface plot (D) and contour plot illustrating the interaction of acidity and fermentation time (C) and its 3D-Surface plot (F). The response values range from approximately 0.021 to 0.313 g/L. In the plots, the color gradient represents the predicted isobutanol concentration, with the warmer yellow region indicating a higher response and the green-to-blue region indicating a lower response. The red points represent the experimental design points used to develop and evaluate the response surface model.

metabolic activity, they may also impose greater metabolic burden and increase protein misfolding, particularly when multiple heterologous enzymes are expressed simultaneously. In an engineered isobutanol-producing system, such effects may reduce the fraction of functional pathway enzymes and consequently limit carbon flux toward the target product. Therefore, the lower-temperature region may provide a more favorable balance between cellular growth, protein folding, enzyme functionality, and product formation.

Acidity exhibited a small positive coefficient (+0.0314), suggesting a slight tendency for isobutanol production to increase with increasing pH, whereas fermentation time showed a small negative coefficient (−0.00119), indicating a marginal decrease in isobutanol with longer fermentation under these conditions. However, neither acidity nor fermentation time was statistically significant ($p = 0.1037$ and $p = 0.1339$, respectively). The inclusion of these terms in the final linear model should not be interpreted as evidence that they are biologically irrelevant; rather, their effects were insufficiently strong to explain a significant proportion of the observed response variation within the investigated experimental domain. Likewise, the R^2 value, although moderate to good, suggests that additional biological and process-related factors contribute substantially to isobutanol production. These may include metabolic burden, cofactor availability, competing metabolic pathways, acetate and other by-product accumulation, inefficient carbon partitioning, and protein misfolding or inclusion body formation. Such factors may explain why optimization of a single process parameter does not fully account for the relatively low isobutanol productivity observed in the engineered host.

The contour and three-dimensional response surface plots provide a visual representation of the relationship between the

investigated process variables and isobutanol production. Both plots show a clear gradient in the response, with the highest isobutanol production located toward the lower-temperature region. The two-dimensional contour plots and three-dimensional response surface plots (Fig. 6) illustrate the effects of acidity (A), temperature (B), and fermentation time (C) on isobutanol production. The response surfaces exhibit relatively planar characteristics, while the contour lines are generally regular and approximately parallel. These patterns suggest that the response is primarily described by linear relationships within the investigated experimental region, with no pronounced curvature or distinct interior optimum.

The overall ANOVA results indicate that the fitted model was statistically significant ($F = 7.48$, $p = 0.0053$), suggesting that the independent variables collectively explain a significant proportion of the variation in isobutanol production. The model yielded an R^2 of 0.6712, indicating that approximately 67.12% of the variability in the response was explained by the model. The adjusted R^2 was 0.5815, which accounts for the number of predictors included in the model and indicates a moderate level of explanatory capacity. However, the predicted R^2 was considerably lower (0.3093), suggesting that the model's predictive performance may be limited when applied to new observations. This difference between the adjusted and predicted R^2 values indicates that the model should be interpreted primarily as a description of the observed experimental response rather than as a highly precise predictive tool.

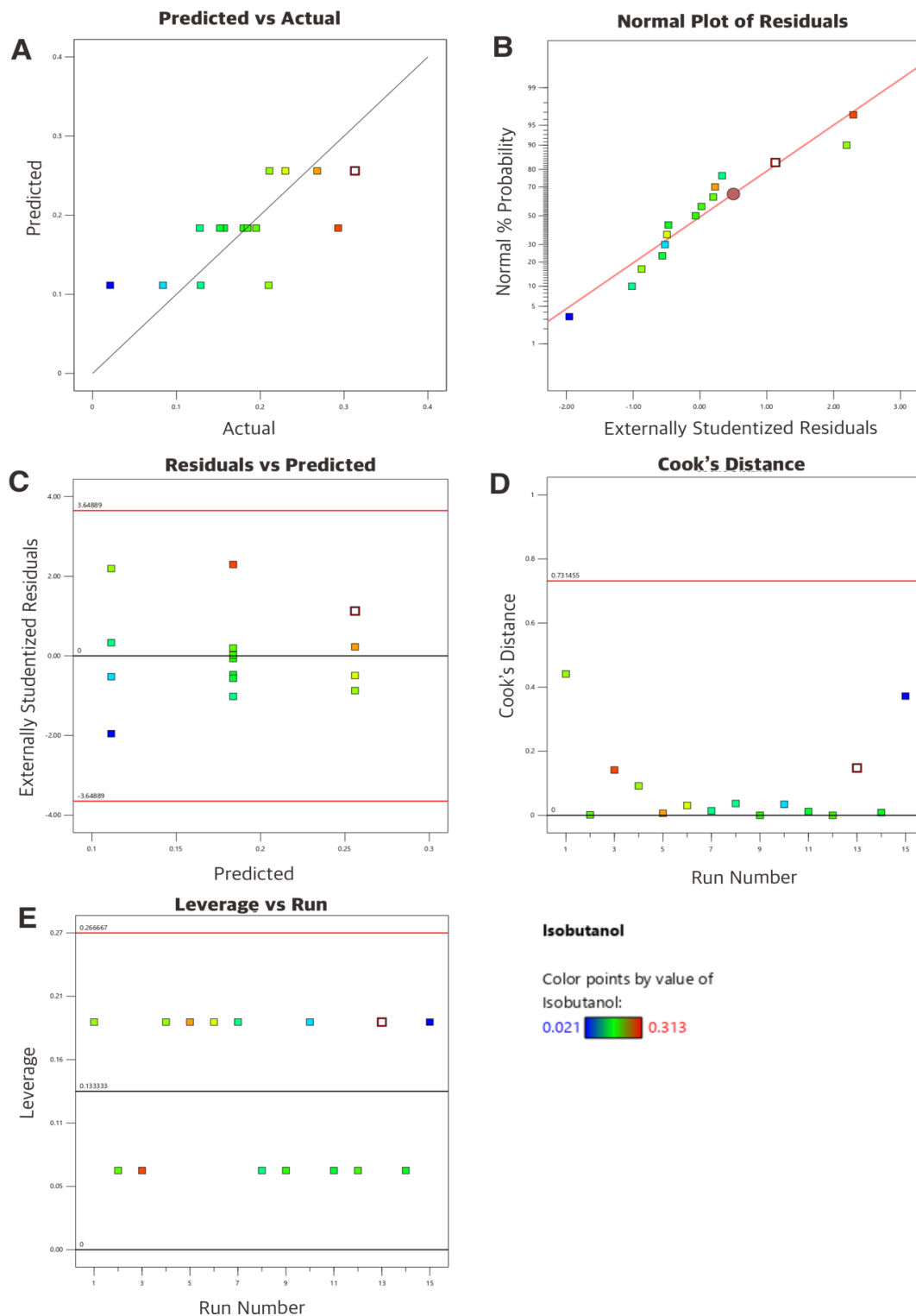


Fig 8. Comprehensive diagnostic evaluation of the reduced linear model for isobutanol production. (A) Predicted versus actual values demonstrating the agreement between model predictions and experimental observations; (B) normal probability plot assessing the normality of residuals; (C) residuals versus predicted values assessing linearity and constant variance; (D) Cook's distance assessing the influence of individual observations; an (E) leverage analysis identifying observations with potentially high leverage.

The interaction between acidity and temperature (Fig. 6A and B) shows a gradual change in predicted isobutanol concentration across the experimental domain. The contour lines indicate that the response varies more substantially along the temperature axis than along the acidity axis, suggesting that temperature has a comparatively stronger influence on isobutanol production. The corresponding three-dimensional surface displays a relatively uniform slope, supporting the

predominantly linear behavior of these factors. This graphical observation is consistent with the ANOVA results identifying temperature as the principal significant factor in the model. Nevertheless, the relatively regular contour pattern suggests that the combined effect of acidity and temperature does not produce a strong interaction within the investigated range.

The interaction between temperature and fermentation time (Fig. 6C and D) indicates a gradual variation in isobutanol

concentration across the tested conditions. The response surface suggests that lower temperatures are generally associated with higher predicted isobutanol concentrations, whereas increasing temperature is accompanied by a reduction in the predicted response. The effect of fermentation time appears less pronounced, as indicated by the relatively broad spacing and orientation of the contour lines. This suggests that temperature may exert a stronger influence on isobutanol production than fermentation time within the experimental domain. Although prolonged fermentation may affect product accumulation through changes in cellular metabolism, product loss, or physiological stress, these mechanisms cannot be confirmed solely from the response surface analysis.

The interaction between acidity and fermentation time (Fig. 6E and F) exhibits a relatively flat response surface, indicating comparatively gradual changes in isobutanol concentration across the investigated factor combinations. The contour lines suggest that acidity and fermentation time have weaker individual effects than temperature within the tested range. The absence of pronounced curvature or a localized maximum further supports the suitability of a linear model for describing the response in this region. However, the relatively low predicted R^2 indicates that factors not included in the model, experimental variability, or unexplained nonlinear behavior may contribute to the observed response.

Overall, the response surface and contour plots indicate that temperature is the dominant factor influencing isobutanol production within the investigated experimental conditions, whereas acidity and fermentation time contribute to a lesser extent. The statistically significant model confirms that the selected variables collectively affect the response, although the moderate R^2 and relatively low predicted R^2 suggest that the model has limited predictive robustness.

From a biological perspective, the preference for lower temperature may reflect the balance between cellular growth, heterologous protein expression, enzyme stability, and metabolic flux toward isobutanol. Although higher temperatures can potentially increase metabolic rates, they may simultaneously increase cellular stress and protein-folding demands (Feller, 2018). This is particularly relevant for the engineered *E. coli* system, in which several heterologous enzymes are expressed to convert central metabolic intermediates toward isobutanol. Excessively high temperature may therefore reduce the effective activity of one or more pathway enzymes through altered protein stability or folding, while also increasing cellular maintenance requirements. Consequently, the lower-temperature region may provide a more favorable physiological environment for maintaining functional pathway enzymes and directing carbon flux toward isobutanol formation (Furuya et al., 2014).

Model adequacy was further evaluated using predicted-versus-observed, residual, normal probability, Cook's distance, leverage, and lack-of-fit analyses (Fig. 8). The predicted-versus-observed plot showed that the experimental observations generally followed the predicted response trend, although several observations deviated from the reference line (Fig. 8A). This observation was consistent with the moderate explanatory capacity of the model ($R^2 = 0.4988$) and its lower predictive capability (predicted $R^2 = 0.3102$) (Table 8). The normal probability plot showed that most residuals followed the expected linear trend, with only moderate deviations at the distributional tails, indicating approximate normality of the residuals (Fig. 8B). The residual plots showed no pronounced systematic pattern, curvature, or funnel-shaped distribution, suggesting that the assumptions of linearity and approximately constant variance were reasonably satisfied (Fig. 8C).

The Cook's distance and leverage analyses indicated variation in the influence of individual observations, but no clear evidence that the model was dominated by a single highly influential observation (Fig. 8D and 8E). Therefore, there was insufficient statistical justification for excluding any experimental observation solely based on its residual, leverage, or influence. Importantly, the lack-of-fit test was not significant ($F = 7.35$, $p = 0.1257$), indicating that the discrepancy between the reduced linear model and the experimental observations was not statistically distinguishable from experimental error. Collectively, these diagnostic results support the adequacy of the reduced linear model for describing the temperature-dependent trend in isobutanol production within the investigated experimental domain. Nevertheless, the moderate R^2 and predicted R^2 values indicate that considerable response variability remains unexplained, suggesting that biological and process-related factors beyond temperature contribute to the observed variation in isobutanol production.

Although the reduced model exhibited improved predictive capability, the predicted R^2 value of 30.97% indicates that a substantial proportion of the variability in isobutanol production remains unexplained. This residual variation may arise from biological heterogeneity, experimental noise, or process variables not included in the present experimental design. Therefore, the model should be interpreted primarily as a factor-screening tool that identifies influential process parameters and general response trends rather than as a highly precise predictive model. Nevertheless, the analysis clearly demonstrates that temperature is the principal factor affecting isobutanol production under the investigated conditions, with lower cultivation temperatures promoting enhanced product formation.

3.4 Validation of Optimal Variables for Isobutanol Production

The highest isobutanol yield was obtained under the optimal parameters predicted by the statistical model, indicating that the optimized conditions were suitable for enhancing isobutanol production (Fig. 9). The optimal fermentation parameters predicted were subsequently evaluated to validate their suitability for isobutanol production. The validation experiment was conducted using the optimized conditions, and isobutanol concentration was measured at several time points throughout the fermentation process. This time-course analysis was performed to monitor the progression of isobutanol production and to determine whether the optimized conditions resulted in the expected production profile.

The agreement between the observed and predicted values indicates that the reduced model adequately captures the general response trend within the investigated experimental region. However, the model exhibited a moderate predictive capability, as reflected by a predicted R^2 value of 30.93%. This suggests that a substantial proportion of the variation in isobutanol production remains unexplained, likely due to biological variability and experimental noise. The statistical conclusions apply only within the experimental ranges investigated, namely pH 4.5–6.5 and fermentation duration of 24–72 hours. Effects that are weak, nonlinear, or expressed outside these ranges may not have been detected in the present design. In addition, the relatively limited number of experimental runs and the biological variability associated with microbial fermentation may have reduced the statistical power to identify small main effects or interactions (Arriaga, 2009; Gao et al., 2024). The reduced model explained 49.88% of the response variation ($R^2=0.4988$), while the predicted R^2 was 0.3102, indicating that a substantial proportion of the variation remained unexplained. This unexplained variation may be

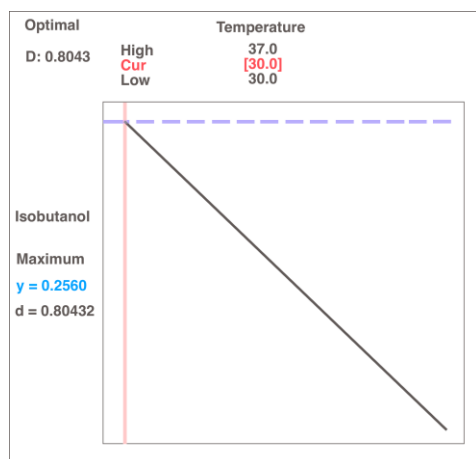


Fig. 9. The prediction of optimum value of isobutanol production. Cur: optimal value of isobutanol production variable, y: prediction of maximum isobutanol production

associated with factors not included in the model, such as inoculum condition, microbial growth phase, substrate availability, medium composition, oxygen transfer, pH changes during fermentation, and analytical variability. Consequently, the reduced model should be interpreted primarily as a factor-screening tool for identifying influential process variables and overall response trends rather than as a high-precision predictive model. Therefore, the model should be applied only within the investigated design space, and additional experiments with a broader factor range, more replicates, and inclusion of relevant biological and process variables are recommended.

Despite these limitations, the model successfully identified temperature as the only significant factor affecting isobutanol production ($P = 0.003$) and correctly predicted that lower cultivation temperatures favor isobutanol formation. Therefore, the reduced model provides a useful framework for guiding future process optimization and experimental design, while highlighting the need for additional studies to improve predictive accuracy and further elucidate the factors governing isobutanol production.

To kinetically validate the fermentation process, batch fermentation experiments were conducted with sampling at multiple time points to monitor biomass growth and isobutanol production over time. Fig. 10 shows two different kinetic behaviors during cultivation. Biomass increased rapidly during the first six hours, and the calculated specific growth rate reached a maximum of approximately 0.534 h^{-1} in the 0–3 h interval. The growth rate then decreased progressively, falling below 0.1 h^{-1} after 9 h and approaching zero after 24 h. Biomass reached approximately 1.47 g/L by 36 h and remained nearly constant until 48 h, indicating that the culture had entered a stationary or near-stationary phase.

Isobutanol production did not follow the biomass curve in a simple way. The concentration fluctuated during the early cultivation period, increased strongly between 18 and 24 h, decreased between 24 and 36 h, and increased again during the final 12 h. The most important observation is that isobutanol continued to accumulate from 36 to 48 h even though there was almost no net biomass growth. This suggests that product formation was not exclusively coupled to cell division and may have continued through non-growth-associated metabolism or delayed conversion of intracellular precursors.

The data should nevertheless be interpreted with caution because the standard deviations for isobutanol are relatively

large. To determine the kinetic mechanism more rigorously, future experiments should use more frequent sampling around the apparent transitions, quantify substrate and by-products, verify the isobutanol analytical method, and fit a Monod–Luedeking–Piret or another mechanistic model to replicate-level data. A mass balance would also help distinguish true production from product loss, re-assimilation, or measurement variation.

3.5 Biological and Metabolic Limitation of Isobutanol Production

In engineered *Escherichia coli*, isobutanol production through the 2-keto acid pathway competes directly with native mixed-acid fermentation pathways for pyruvate, acetyl-CoA, and reducing equivalents. Pyruvate is required for the acetolactate synthase-catalysed step that initiates the conversion of pyruvate to 2-ketoisovalerate, the immediate precursor of isobutanol (Atsumi *et al.*, 2010). However, under oxygen-limited conditions, pyruvate may be diverted to lactate through lactate dehydrogenase (*ldhA*) or cleaved into acetyl-CoA and formate by pyruvate-formate lyase (*pflB*). The acetyl-CoA generated by the latter reaction can subsequently be directed toward acetate production through phosphotransacetylase (*pta*) and acetate kinase (*ackA*), or toward ethanol formation through acetaldehyde/alcohol dehydrogenase (*adhE*). Acetate may also be formed through the pyruvate oxidase pathway encoded by *poxB* (Noda *et al.*, 2019; Trinh *et al.*, 2011). The accumulation of lactate, acetate, ethanol, and formate therefore reflects diversion of metabolic flux away from the desired isobutanol pathway. Lactate formation is particularly detrimental because it directly consumes both pyruvate and NADH, whereas ethanol formation consumes reducing equivalents required for cellular redox balance and potentially for the terminal alcohol-reduction step. Likewise, *pflB*-dependent formate formation competes for pyruvate and provides acetyl-CoA that may be further converted to acetate or ethanol. Accordingly, metabolic engineering strategies commonly target *ldhA*, *pflB*, *pta*, *ackA*, *poxB*, and *adhE* to decrease by-product formation and increase precursor availability for isobutanol synthesis which is not applied in our study. (Suppl.1) (Boecker *et al.*, 2023; Gu *et al.*, 2023). This native competitive pathway might contribute to low titer of isobutanol in our study. Nevertheless, these modifications should be optimized carefully because fermentative by-product pathways also contribute to ATP generation, redox homeostasis, and anaerobic growth; their complete elimination may compromise cell fitness and does not invariably improve isobutanol production (Blombach & Eikmanns, 2011).

pH may influence pyruvate production by altering intracellular pH, the energy required to maintain proton homeostasis, and the activity of glycolytic enzymes. A decrease in external pH increases cellular exposure to hydrogen ions (H^+), prompting the cell to activate the H^+ -ATPase system to expel protons from the cytoplasm. This process consumes ATP that would otherwise be available for growth, biosynthesis, and product formation. In addition, changes in pH may alter the charge and conformation of glycolytic enzymes, thereby affecting their substrate affinity and the rate of pyruvate formation (Yuan *et al.*, 2022). Under mild acid stress, enhanced glycolytic activity may help generate additional ATP to maintain intracellular pH homeostasis. However, severe or prolonged acid stress may inhibit glycolytic enzymes, reduce ATP and pyruvate production, and redirect carbon toward cellular maintenance or the formation of other metabolites. The pyruvate produced can subsequently be directed toward valine biosynthesis and isobutanol formation. Moreover, acidic

environment was more likely support the formation of lactate as by product (Liang, Gao, *et al.*, 2018; Yuan *et al.*, 2022).

In the present study, pH did not significantly affect isobutanol production within the investigated range. However, this finding indicates only that changes in pH did not produce a sufficiently strong statistical difference under the conditions tested; it does not demonstrate that pH has no biological influence on pyruvate or isobutanol production. Because intracellular pH, ATP levels, glycolytic enzyme activity, and pyruvate concentration were not measured, these proposed mechanisms require further confirmation through physiological and metabolomic analyses.

Temperature may affect isobutanol production by changing central carbon metabolism and the NADH/NAD⁺ redox balance in native *E. coli*. Under oxygen-limited conditions, pyruvate can be converted to lactate, formate, acetate, or ethanol through mixed-acid fermentation (Förster & Gescher, 2014). These pathways help maintain cellular energy and redox balance: lactate and ethanol formation consume NADH and regenerate NAD⁺, whereas acetate formation contributes to ATP production but has a different effect on reducing-equivalent balance (Yuan *et al.*, 2022). Therefore, the distribution of these by-products reflects how the cell manages carbon flux, ATP generation, and NADH reoxidation. Temperature stress may alter the activity and expression of enzymes involved in these pathways and may increase the energy required for cellular maintenance and heat-shock responses. As a result, higher temperatures may redirect pyruvate and reducing equivalents away from the valine-associated pathway and toward competing products such as acetate, formate, lactate, or ethanol, potentially reducing isobutanol formation (Shimizu, 2013). However, because these by-products and the NADH/NAD⁺ ratio were not measured in this study, this explanation remains hypothetical and should be verified through targeted metabolite and redox analyses.

The utilization of cofactor NADH and NADPH also play a significant role, which both cofactors were needed in the isobutanol conversion process. The reconstruction of *E. coli* metabolism to favor the Entner–Doudoroff (ED) pathway improved the metabolic redox balance in accordance with the cofactor requirements of the isobutanol biosynthetic pathway. Deletion of *pgi* and *gnd* suppressed carbon flux through the Embden–Meyerhof (EM) and pentose phosphate pathways (PPP), respectively, thereby redirecting glucose-derived carbon toward the ED pathway and subsequently toward isobutanol biosynthesis. Under these conditions, the CFTi51 strain produced 11.8 g/L isobutanol with a yield of 0.24 g/g glucose. Noda *et al.* (2019) attributed this improvement to the reduction of carbon loss through the PPP and the enhancement of carbon flux from glucose toward isobutanol through the ED pathway.

This metabolic configuration is particularly relevant because the ED pathway provides a more favorable redox balance for isobutanol biosynthesis. Whereas the EM pathway primarily generates NADH, the ED pathway generates both NADH and NADPH, with one mole of each reduced cofactor produced per mole of glucose. These cofactors correspond to the reducing equivalents required by the isobutanol biosynthetic pathway. Thus, restricting glucose metabolism to the ED pathway not only redirects carbon toward the desired product but also provides a metabolic environment that is more compatible with the redox requirements of isobutanol formation.

The productivity of recombinant isobutanol-producing *Escherichia coli* depends not only on the transcriptional expression of the heterologous pathway genes but also on the soluble and functional expression of the corresponding enzymes. Overexpression of isobutanol-pathway proteins may

exceed the folding capacity of the host cell, moreover, proteins in isobutanol pathway were consider large protein (54-65 kDA) which difficult to fold, resulting in protein misfolding and aggregation into inclusion bodies (Goyal & Chaudhuri, 2015). This limitation was confirmed by insoluble protein extraction which is shows the presence of thick protein KARI in insoluble fraction compared to soluble fraction. Inclusion bodies contain predominantly insoluble recombinant proteins and may substantially reduce the intracellular concentration of catalytically active enzymes, even when total protein expression appears high. Misfolding or low solubility of any enzyme in this pathway can create a metabolic bottleneck and decrease the conversion of pathway intermediates to the final product (Charoenpunthuwong *et al.*, 2023). Consequently, pyruvate may be redirected toward native mixed-acid fermentation pathways, leading to the formation of acetate, lactate, ethanol, and formate rather than isobutanol. In addition, high-level recombinant protein expression imposes a metabolic burden by competing for cellular energy, amino acids, ribosomes, and molecular chaperones, thereby potentially impairing cell growth and reducing the resources available for product synthesis (Zhang *et al.*, 2022).

Strategies to minimize inclusion-body formation include lowering the induction temperature, decreasing inducer concentration, controlling promoter strength, optimizing expression timing, and balancing the expression of individual pathway enzymes (Wang *et al.*, 2014). Co-expression of molecular chaperones, particularly the DnaK–DnaJ–GrpE and GroEL–GroES systems, may further enhance correct folding and solubility of recombinant proteins (Utami *et al.*, 2023). Therefore, optimization of isobutanol production should evaluate not only total expression but also the soluble and enzymatically active fractions of the expressed pathway proteins.

3.6 Process Engineering Considerations and Industrial Translation of Bio-Isobutanol Production

In the present study, the observed isobutanol concentration of 0.313 ± 0.007 g/L at the selected condition provides evidence of biological production; however, this titer remains a laboratory-scale outcome and should not by itself be interpreted as evidence of industrial feasibility. Future studies should quantify residual substrate and the major by-products over the fermentation course to calculate carbon recovery, product yield, and specific or volumetric productivity. Such measurements would identify whether further gains in isobutanol production arise from improved carbon partitioning toward the target pathway, increased substrate uptake, reduced by-product formation, or changes in biomass production.

Scale-up introduces operational factors that cannot be fully captured in small-scale experiments. At pilot and industrial scale, oxygen-transfer limitations, mixing heterogeneity, heat removal, pH gradients, foam formation, inoculum consistency, feed strategy, and contamination control may alter cellular physiology and redirect metabolic flux away from isobutanol. Because isobutanol is toxic to microbial cells, product accumulation can additionally impair membrane function, inhibit growth, and reduce productivity. Consequently, successful scale-up will require both robust host engineering and process engineering, including controlled fed-batch strategies, high-cell-density cultivation, adaptive improvement of solvent tolerance, and online monitoring of substrate, biomass, dissolved oxygen, pH, and product formation (Tracy, 2012).

Downstream purification remains one of the major challenges for bio-isobutanol production. Fermentation broths

Biomass Growth and Isobutanol Production Kinetics

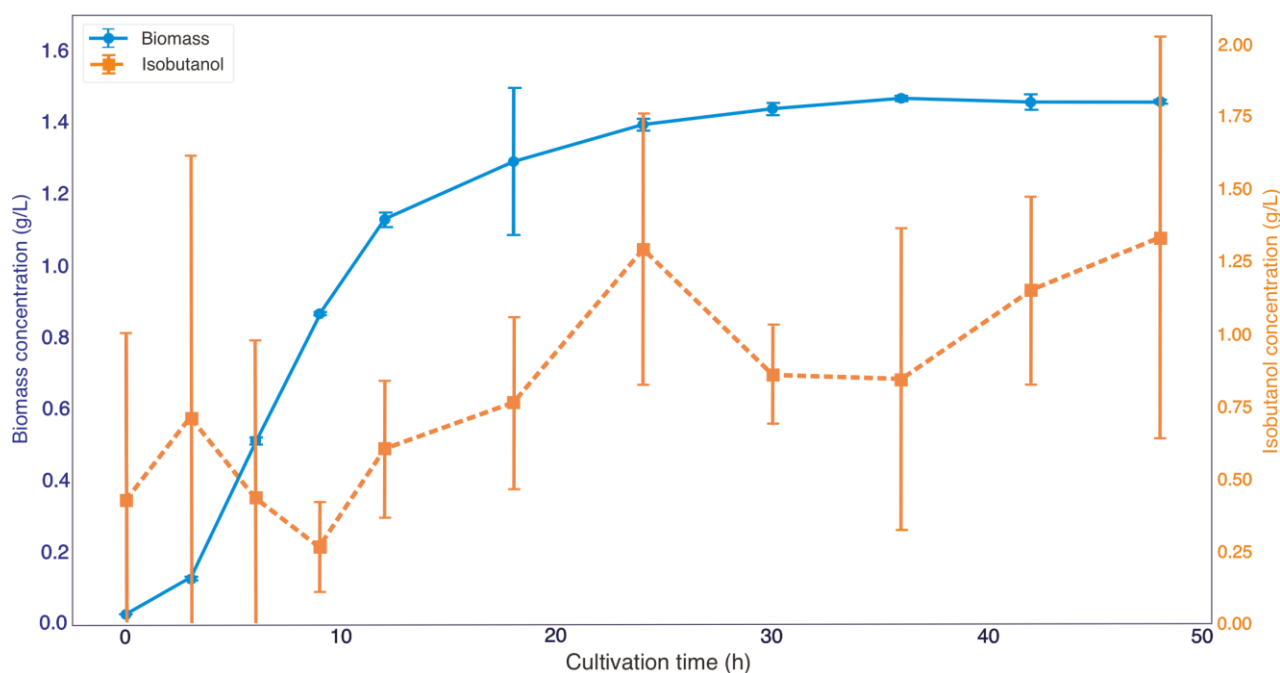


Fig. 10. Biomass growth and isobutanol concentration over cultivation time. Blue circles represent biomass and orange squares represent isobutanol.

are water-rich and commonly contain isobutanol at relatively low concentrations; conventional distillation must therefore remove large amounts of water and can require substantial energy input. Low product titers are compounded by isobutanol toxicity, which can limit the concentration achievable during fermentation. Conventional butanol separation has been reported to consume energy comparable to, or in unfavorable cases greater than, the energy embodied in the recovered product when dilute broths are processed by distillation alone (Bildea *et al.*, 2016; Fu *et al.*, 2021).

Process intensification should therefore be considered early during strain and fermentation development. Potential approaches include in situ product recovery by gas stripping, adsorption, liquid–liquid extraction, or pervaporation, followed by energy-integrated distillation or dehydration. These approaches may continuously reduce inhibitory isobutanol concentrations in the bioreactor, support higher productivity, and decrease the separation burden. Recent process simulations for industrial-scale recovery from highly dilute isobutanol broths have demonstrated that hybrid gas-stripping and vacuum-evaporation configurations coupled with azeotropic distillation can achieve product recovery above 99.9%, while heat integration and heat-pump technologies can reduce energy requirements and associated emissions. However, the performance of each recovery route depends on product concentration, separation selectivity, membrane or solvent stability, fouling, regeneration requirements, capital cost, and compatibility with continuous fermentation (Janković *et al.*, 2024).

From an industrial implementation perspective, the most realistic development pathway is likely the integration of four components: a genetically stable and solvent-tolerant production strain; a low-cost and sustainable carbohydrate feedstock; a high-titer, high-yield, and high-productivity

fermentation process; and an energy-efficient product-recovery train. Isobutanol may initially be deployed as a chemical intermediate or gasoline blendstock rather than as a neat fuel, allowing progressive adoption within existing fuel-distribution infrastructure. Nevertheless, industrial translation will require pilot-scale validation, detailed mass and energy balances, techno-economic analysis, life-cycle greenhouse-gas assessment, and evaluation of fuel-specification compliance. Accordingly, the present work should be regarded as a useful contribution to upstream fermentation optimization, while further integrated bioprocess development is needed to establish commercial viability.

4. Conclusion

This study demonstrated that the combination of genetic engineering and fermentation optimization can improve isobutanol production in recombinant *E. coli* BL21(DE3). Replacing the *lac* promoter with the T7 promoter enhanced the expression of heterologous isobutanol biosynthetic proteins and resulted in higher isobutanol production compared with the *lac*-based expression system. These findings support the suitability of the T7 expression system for the production of isobutanol-related enzymes in *E. coli*.

The optimization experiment further demonstrated that acidity, temperature, and fermentation time collectively influenced isobutanol production. The response surface model was statistically significant, although its moderate explanatory power and relatively low predicted R^2 indicate that the model does not fully describe the variability in the fermentation response. Temperature was identified as the only statistically significant individual factor, with lower temperatures generally associated with higher isobutanol production within the investigated experimental range. Validation of the optimal

parameters through time-course fermentation measurements confirmed the production profile under the predicted conditions and provided additional insight into isobutanol accumulation during fermentation.

Despite these improvements, several limitations remain. The relatively low isobutanol titer indicates that production efficiency is still limited. In addition, the low predicted R^2 suggests that other factors not included in the model may contribute substantially to the variation in isobutanol production. Potential limitations include cofactor availability, metabolic competition, and the formation of insoluble recombinant proteins, which may reduce the availability of functional biosynthetic enzymes. Furthermore, the optimization was performed within a limited range of fermentation parameters, and the validation experiment was conducted under a specific set of conditions. Future studies should therefore focus on improving enzyme solubility, balancing metabolic flux and cofactor supply, and evaluating additional fermentation and induction parameters to further enhance isobutanol production.

Overall, the findings provide a basis for developing recombinant *E. coli* as a microbial platform for isobutanol production, while highlighting the need for further optimization to achieve greater production efficiency and process robustness.

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