

Evaluating LDH and BUDB gene knockouts in *Klebsiella pneumoniae* for improved 1,3-propanediol production

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ABSTRACT

Metabolic engineering has become a powerful tool to enhance 1,3-propanediol (1,3-PDO) production. In this study, we knocked out genes involved in by-product formation in *Klebsiella pneumoniae* TWO to improve 1,3-PDO yield. We constructed Δ LDH and Δ BUDB mutants by knocking out *ldh* and *budB*, respectively. Flask experiments using 20 g/l glycerol under microaerobic conditions showed that the Δ BUDB mutant produced 8.71 g/l of 1,3-PDO with a yield of 0.51 mol/mol glycerol, outperforming the Δ LDH mutant, which produced 6.93 g/l 1,3-PDO with a yield of 0.41 mol/mol glycerol. Notably, the mutants exhibited different by-product profiles, particularly in formic acid and ethanol levels. An optimized medium supplemented with 0.0025 g/l cyanocobalamin further improved 1,3-PDO yield in the Δ budB mutant to 0.60 mol/mol glycerol. Further supplementation with a vitamin solution increased 1,3-PDO level to 10.08 g/l, yielding 0.62 mol/mol glycerol and reducing residual glycerol to 0.08 g/l. This approach resulted in a 29% increase in 1,3-PDO production compared to the wild-type strain. Our findings highlight the single knockout of the *budB* gene, combined with cyanocobalamin and a vitamin solution, significantly enhances the yield and level of 1,3-PDO with a low by-product profile, leaving no residual glycerol, offering a promising strategy for industrial bioprocessing.

1. INTRODUCTION

The increased use of fossil fuels has led to significant environmental issues and the depletion of crude oil reserves, driving the search for alternative renewable energy sources. Biodiesel has emerged as a promising alternative, produced from biomass via biochemical or thermochemical processes, which generate glycerol as a by-product. The surge in biodiesel production has consequently resulted in a substantial increase in industrial glycerol waste. Converting this glycerol into valuable chemical compounds, such as 1,3-propanediol (1,3-PDO), is a compelling solution. 1,3-PDO has diverse applications, ranging from polymers and textiles to food, medicine [1], detergents, and cosmetics [2].

The biotechnological production of 1,3-PDO offers significant advantages over conventional chemical methods, including lower costs and environmentally friendly processes [3]. Various bacterial species, such as *Clostridium butyricum* [4], *Enterobacter agglomerans* [5], *Citrobacter freundii*, *Klebsiella oxytoca* [6], and *Klebsiella pneumoniae* [7], have been studied for this purpose. Among these,

K. pneumoniae is the most extensively researched due to its high substrate tolerance, flexible carbon regulation [8], and excellent 1,3-PDO yield and productivity [9].

Klebsiella pneumoniae metabolizes glycerol via reductive and oxidative pathways. In the reductive pathway, glycerol is converted to 3-hydroxypropionaldehyde by glycerol dehydratase (GDHt) with the help of vitamin B12, and then reduced to 1,3-PDO by NADH and 1,3-propanediol oxidoreductase (*dhaT*) [9]. In the oxidative pathways, *K. pneumoniae* produces by-products such as acetic acid, lactic acid, formic acid, 2,3-BDO, and ethanol, which compete for NADH and hinder glycerol bioconversion in the reductive pathway, thus reducing 1,3-PDO levels [10].

Genetic modification, such as overexpressing key enzymes [11] or knocking out genes responsible for unwanted by-products, significantly enhances 1,3-PDO yields [12,13], demonstrating how targeted genetic interventions are likely to improve microbial performances. Excessive by-products during microbial fermentation are a critical challenge for limiting the overall yield of 1,3-PDO. The introduction of heterologous genes to reduce acetate and further reprogramming of carbon metabolism by knocking out lactate dehydrogenase, alcohol dehydrogenase, and succinate dehydrogenase is used to increase 1,3-PDO yield and purity [10]. This not only improves the process efficiency but also reduces the downstream purification costs. To enhance 1,3-PDO production, metabolic

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engineering has been employed to reduce by-product formation. In *K. pneumoniae*, eliminating lactic acid production has shown a more significant increase in 1,3-PDO levels compared to eliminating ethanol [14]. Additionally, 2,3-BDO is a major competing by-product [15], and the *budB* gene (part of the *bud* operon) is a critical factor in reducing its production [16]. However, there is limited information on the effects of single gene deletions of *ldh* and *budB* in a single isolate and their comparative metabolic profiles, particularly in 1,3-PDO production. Therefore, this study aims to identify target genes whose disruption minimally affects microbial growth while reducing by-product formation and enhancing 1,3-PDO production.

2. MATERIALS AND METHODS

2.1. Materials

Klebsiella pneumoniae strain TWO, isolated from tempeh in Indonesia and obtained from the Wilmar Benih Indonesia Collection, was used as the parent strain for glycerol fermentation experiments. *Escherichia coli* strains DH5 α and S17- λ pir were used for plasmid propagation and conjugation, respectively, with bacterial strains, plasmids, and primers listed in Table 1. *E. coli* strains were cultivated in Luria-Bertani (LB) medium containing 5 g/l yeast extract, 10 g/l tryptone, and 1 g/l NaCl. For the selection of *K. pneumoniae* mutants, M9 minimal medium was used, which consisted of 6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l (NH₄)₂HPO₄, 0.5 g/l NaCl, 15 g/l agar, 2 g/l glucose, 0.25 g/l MgSO₄·7H₂O, 0.001 g/l thiamine-HCl, and 0.147 g/l CaCl₂·2H₂O. The seed culture and fermentation media contained 20 g/l glycerol (85% v/v), 0.2 g/l yeast extract, 3 g/l (NH₄)₂SO₄, 0.2 g/l MgSO₄·7H₂O, 0.012 g/l CoCl₂·6H₂O, 14 g/l K₂HPO₄, 6 g/l KH₂PO₄, 0.2 g/l L-cysteine, and were adjusted to a final pH of 7.0 \pm 0.2, with spectinomycin and streptomycin added to the culture medium at final concentrations of 50 μ g/ml each for mutant selection. For optimization studies, malic acid, succinic acid, and cyanocobalamin (vitamin B12) were added to the fermentation medium at concentrations of 20 mM, 20 mM, and 0.0025 g/l, respectively. The trace element solution contained 0.7 g/l ZnCl₂, 1 g/l MnCl₂·4H₂O, 0.6 g/l H₃BO₃, 2 g/l CoCl₂·6H₂O, 0.2 g/l CuCl₂·H₂O, and 0.35 g/l Na₂MoO₄·2H₂O, with a separate iron solution prepared by dissolving 5 g/l FeSO₄·7H₂O in water, and a vitamin solution containing 10 g/l nicotinic acid (C₆H₅NO₂), 5 g/l thiamine-HCl, and 0.1 g/l biotin. DNA fragments were amplified using Q5 High-Fidelity DNA polymerase (New England Biolabs, UK) to ensure accurate and efficient amplification.

2.2. Construction of Gene Knockouts

Total genomic DNA of *K. pneumoniae* TWO was extracted using the Wizard[®] Genomic DNA purification Kit (Promega, USA). For gene knockout, the 945 bp *ldh* and 1680 bp *budB* genes were amplified by PCR using the genomic DNA of *K. pneumoniae* TWO as template DNA, with primers A1-A2 and D1-D2, respectively (Table 1). These primers were designed based on sequence information from *K. pneumoniae* (GenBank accession numbers CP011976 and CP011313, respectively). The PCR products were cloned into the pUC19 vector, yielding the constructs pNK1 and pNK3.

For *ldh* gene knockout, the entire plasmid pNK1 was amplified using primers B1-B2 and subsequently ligated to introduce an *Sma*I restriction site. The spectinomycin/streptomycin-resistance gene (*aadA*) was amplified from the plasmid pUTminiTn5-Sp/Sm using primers C1-C2. The resulting 1.1-kb PCR product was inserted into

the *ldh* gene in pNK1 at the *Sma*I site, generating pNK2. The structure of pNK2, extracted from *E. coli* DH5 α , was confirmed through restriction analysis using *Sac*I, *Nde*I, *Sac*I-*Nde*I, *Kpn*I, and *Hind*III. Sequencing with the M13 and universal pUC19 primers confirmed the absence of any introduced mutations.

For *budB* gene knockout, the 1.1-kb *aadA* gene was amplified using primers E1-E2 and inserted into the *budB* gene in pNK3, yielding pNK4. Plasmid pNK4 was transformed into *E. coli* DH5 α , and the insertion was verified by restriction analysis using *Eco*RV and *Hind*III, as well as sequencing with M13 primers to confirm the integrity of the construct.

2.3. Modification of Conjugative Plasmid

Plasmid pUTminiTn5-Sp/Sm was modified by digesting with *Bgl*II and *Sma*I to remove the transposase-encoding gene and the transposable element, respectively. Alkaline Phosphatase Calf Intestinal (NEB, UK) was used to dephosphorylate the 5' ends of the digestion products to improve ligation efficiency. Primers A3-A4 and D3-D4 were employed to amplify the inactive *ldh* and *budB* genes (designated as Δ *ldh* and Δ *budB*) from pNK2 and pNK4, respectively. The PCR products were phosphorylated using T5 polynucleotide kinase (NEB, UK) and subsequently ligated into the linearized, modified pUTmini vector using T4 DNA ligase (NEB, UK), resulting in plasmids pFS5 and pFS6.

The structures of the conjugative plasmids carrying the inactive target genes were transformed into *E. coli* S17-1 λ pir and verified by restriction analysis using *Bam*HI, *Sal*I, and a combination of *Bam*HI and *Sal*I. Sequencing with primers A3-A4 and D3-D4 confirmed that no mutations had been introduced into the constructs.

2.4. Biparental Mating

Biparental mating was conducted using *E. coli* S17-1 λ pir as the donor and *K. pneumoniae* TWO as the recipient to transfer each conjugative plasmid carrying the inactive target gene. Homologous recombination occurred between the plasmid and the target gene in the *K. pneumoniae* genome. For the selection of transconjugants, *K. pneumoniae* Δ LDH (inactive *ldh*) and Δ BUDB (inactive *budB*), M9 minimal medium containing spectinomycin and streptomycin (50 μ g/ml each) was used. Spectinomycin- and streptomycin-resistant colonies were verified using colony PCR to confirm correct recombination and obtain mutants.

2.5. Shake-Flask Fermentation

Shake-flask experiments were conducted under controlled conditions: agitation speed at 250 rpm, initial pH at 7.0 \pm 0.2, and temperature at 30°C. Both *K. pneumoniae* TWO and its mutants were initially cultured in 10 ml of LB medium (Inoculum-I) and incubated for 6 hours. Two percent of Inoculum-I was then transferred to 10 ml of seed culture medium and incubated for 16 hours. Subsequently, 2% of the seed culture was inoculated into 100 ml of fermentation medium in a 250 ml shake flask and cultured for 30 hours under microaerobic conditions with the flask closed by a screw cap.

To optimize the production of 1,3-PDO, the fermentation medium was supplemented with 20 mM malic acid, 20 mM succinic acid, and 0.0025 g/l vitamin B12 coenzyme. Additionally, to enhance glycerol consumption, 1 ml each of a trace element solution, Fe solution, vitamin solution, and their combination were added to the fermentation medium.

Table 1. Bacteria strains, plasmids, and primers used.

Strains, plasmids, and primers	Relevant characteristics	Melting temperature (°C)	Size (plasmid/product PCR)	Source
Strains				
<i>K. pneumoniae</i> TWO	Ampicylin ^R , Streptomycin ^R	–	–	Lab collection
ΔLDH	<i>K. pneumoniae</i> TWO with inactive <i>ldh</i> gene, Spectinomycin-Streptomycin ^R	–	–	This work
ΔBUDB	<i>K. pneumoniae</i> TWO with inactive <i>budB</i> gene, Spectinomycin-Streptomycin ^R	–	–	This work
<i>E. coli</i> DH5α	Cloning Host	–	–	Lab collection
S17-1λpir	Conjugation Host	–	–	Lab collection
Plasmids				
pUC19	Ampicylin ^R	–	2.6 kb	Lab collection
pUTminiTn5-Sp/Sm	Ampicylin ^R , Spectinomycin-Streptomycin ^R	–	7,3 kb	Lab collection
pNK1	Ampicylin ^R , pUC19 inserted <i>ldh</i> gene	–	3,5 kb	This work
pNK2	Ampicylin ^R , Spectinomycin-Streptomycin ^R , pUC19 inserted <i>Δldh</i> gene	–	4,7 kb	This work
pNK3	Ampicylin ^R , pUC19 inserted <i>budB</i> gene	–	4.3 kb	This work
pNK4	Ampicylin ^R , Spectinomycin-Streptomycin ^R , pUC19 inserted <i>ΔbudB</i> gene	–	5.2 kb	This work
pFS5	Ampicylin ^R , Spectinomycin-Streptomycin ^R , pUTmini lacking transposase encoding gene and transposable element, and inserted <i>Δdh</i> gene	–	5.8 kb	This work
pFS6	Ampicylin ^R , Spectinomycin-Streptomycin ^R , pUTmini lacking transposase encoding gene and transposable element, and inserted <i>ΔbudB</i> gene	–	6.7 kb	This work
Primers				
A1	5'-ATGGTACCATGCACACCAAAGCCCGTAAAG (KpnI)	67	0,9 kb	This work
A2	5'-TATAAGCTTTCAGGGCAGACGGGCGAT (HindIII)	67		This work
A3	5'-ATGCACACCAAAGCCCGTAAAG	62	2,0 kb	This work
A4	5'-TCAGGGCAGACGGGCGAT	63.5		This work
B1	5'- CCCGGGCAGAGCATCGACGCCTTATCC (SmaI)	72	3.5 kb	This work
B2	5'- AATCTCCAGTTCCTGCGCCAG	64		This work
C1	5'-GGACAGAAATGCCTCGACTT	57	1,1 kb	This work
C3	5'-TTATTTGCCGACTACCTTGGTG	57		This work
D1	5'-ATTATGAATTCATGGACAAACAGTATCCGGT (EcoRI)	61	1.3 kb	This work
D2	5'-ATAGGATCCTTAGAGAATCTGACTCAGATG (BamHI)	60		This work
D4	5'-ATGGACAAACAGTATCCGGT	56	2.5 kb	This work
D5	5'-TTAGAGAATCTGACTCAGATG	53		This work
E1	5'-ATACCATGGGTATTGCGGACTACCTTGGTG (NcoI)	59	1.1 kb	This work
E2	5'-ATATAGATATCGGAGAGGAAATGCCTCGACTT (EcoRV)	58		This work
F1	5'-ATGAGTATTCAACATTTCCGTG	56	3.3	This work
F2	5'-CGGCATATGACGCTACTTGCATTACAGCTT	56		This work

2.6. Analytical Methods

Cell optical density was measured at 600 nm using a spectrophotometer (Bio-Rad, USA) with appropriate dilution. Supernatants were collected by centrifuging culture samples at 13,000 rpm for 2 minutes, followed by filtration through a 0.22 μm membrane (Nylon Whatman, Uniflo). Secondary metabolites, including lactic acid, formic acid, acetic acid, 1,3-PDO, 2,3-BDO, ethanol, and glycerol, were quantified using High-Performance Liquid Chromatography (Agilent 1200). The analysis was performed with an organic acid analysis column (300 mm × 7.8 mm; Aminex HPX-87H; Bio-Rad, USA) at 60°C, and detection was done with a refractive index detector. The mobile phase used was 5 mM H₂SO₄ with a flow rate of 0.5 ml/minute.

3. RESULTS AND DISCUSSION

3.1. Confirmation of Genomic Gene Knockouts

Homologous recombination was used to knock out the 945 bp *ldh* and 1680 bp *budB* genes in the *K. pneumoniae* TWO genome. The 1138 bp *aadA* gene, encoding spectinomycin-streptomycin (Sp/Sm) resistance, was inserted into each gene, resulting in fragments of 445 bp and 499 bp for *ldh* and 969 bp and 422 bp for *budB*. The *Δldh* and *ΔbudB* genes were cloned into pUTminiTn5.Sp/Sm, modified by eliminating the transposable element and transposase gene. This vector is a suicide vector due to the R6K origin of replication. The 5.8 kb pFS5 and 6.7 kb pFS6 plasmids were transmitted from *E. coli* S17-

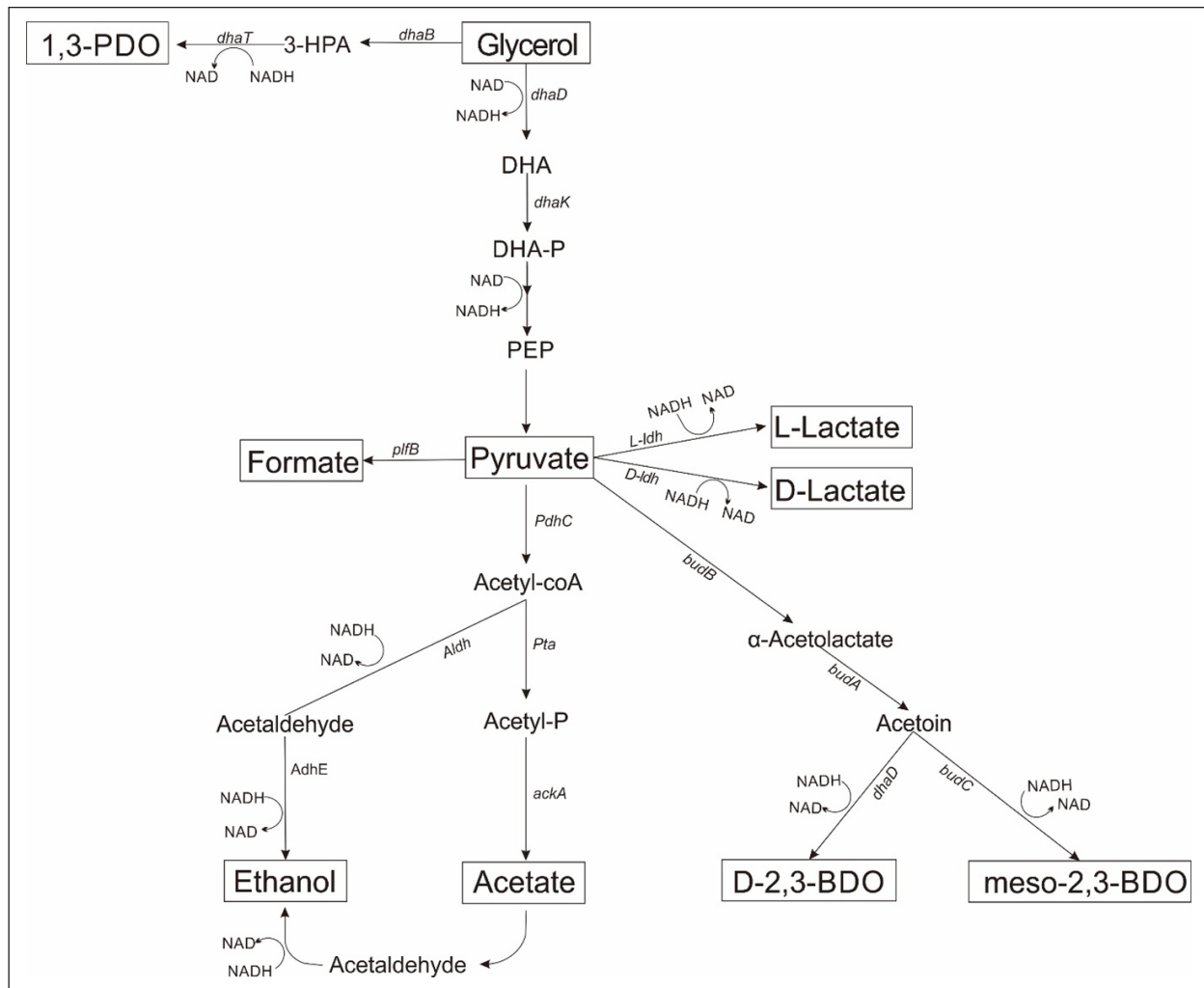


Figure 1. The metabolic pathway of glycerol in *K. pneumoniae* TWO under microaerobic condition.

1 λ pir into *K. pneumoniae* TWO, facilitating double-crossover events. Sp/Sm-resistant colonies were confirmed by colony PCR, producing 2 kb and 2.5 kb PCR products representing Δ ldh and Δ budB mutants, respectively (Fig. 2). Sequencing analysis confirmed that the inactive genes replaced the target genes (data not shown).

3.2. Impact of Argeted Gene Knockouts on Glycerol Metabolism and Cell Growth

All strains consumed 17 g/l of glycerol over varying periods. Both mutants, however, displayed slower glycerol consumption, taking 20 hours to fully deplete it due to changes in metabolic flux (Fig. 3). In the Δ LDH mutant, cell growth decreased to an OD600 of 4.5 at 12 hours, showing no significant difference from the wild type at the end of fermentation. In contrast, the Δ BUDB mutant exhibited the lowest cell growth, with OD600 values of 3.7 and 3.4 at 12 and 30 hours, respectively (Fig. 4). The decreased cell growth in the Δ BUDB mutant was associated with increased acetic acid levels [16–18]. This study confirmed that the continuous accumulation of acetic acid, a by-product of the Δ BUDB mutant's fermentation, negatively impacted cell growth. This finding was also supported by the acetic acid levels and OD600 results from the Δ LDH mutant's fermentation. These results are further discussed in detail in the by-product level section below.

3.3. Impact of Targeted Gene Knockouts on 1,3-PDO Production

The Δ BUDB mutant achieved the highest 1,3-PDO level, increasing by 11.6% with a yield of 0.51 mol/mol glycerol (Table 2). This significant increase was observed from the 16 hour of fermentation (Fig. 3). In comparison, another study reported a 1,3-PDO yield of 0.47 mol/mol glycerol in a *K. pneumoniae* mutant with triple deletions in lactate dehydrogenase (ldh), formate acetyltransferase (pflB), and acetolactate decarboxylase (buda) [19]. Similarly, a 1,3-PDO yield of 0.50 mol/mol glycerol was achieved by deleting multiple genes related to the production of 2,3-BDO, ethanol, and acetic acid (Table 3) [12]. Our study indicates that a single knockout of the budB gene is more efficient for increasing the 1,3-PDO level and yield. The increased 1,3-PDO level was achieved by blocking α -acetolactate production in the 2,3-BDO biosynthetic pathway and improving NADH availability, thereby redirecting the metabolic flux to the reductive pathway.

Consistent with another finding [16], *K. pneumoniae* TWO used in this study also has an operon with four genes: budB, budA, budC, and budR, encoding α -acetolactate decarboxylase, acetolactate synthase, acetoin reductase, and a transcriptional regulator, respectively (Fig. 1), based on our existing whole-genome database. However, the knockout of budC did not affect the 1,3-PDO level [15], while the knockout of budA decreased the 1,3-PDO level by 18% [16].

In contrast, the Δ LDH mutant resulted in an 11.2% reduction in the 1,3-PDO level, with a yield of 0.41 mol/mol glycerol. This result indicates that not all competitor genes in the by-product biosynthetic pathway can increase the level of 1,3-PDO.

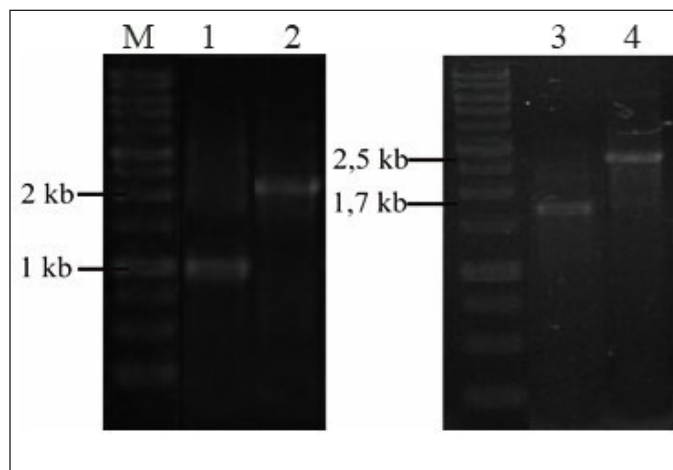


Figure 2. Verification of *K. pneumoniae* TWO wildtype and mutants by colony PCR identification. Lanes: M, 10 kb DNA Ladder Marker (SmoBio, Taiwan); 1, *ldh* (945 bp); 2, *Aldh* (2083 bp); 3, *budB* (1680 bp); 4, Δ *budB* (2529 bp).

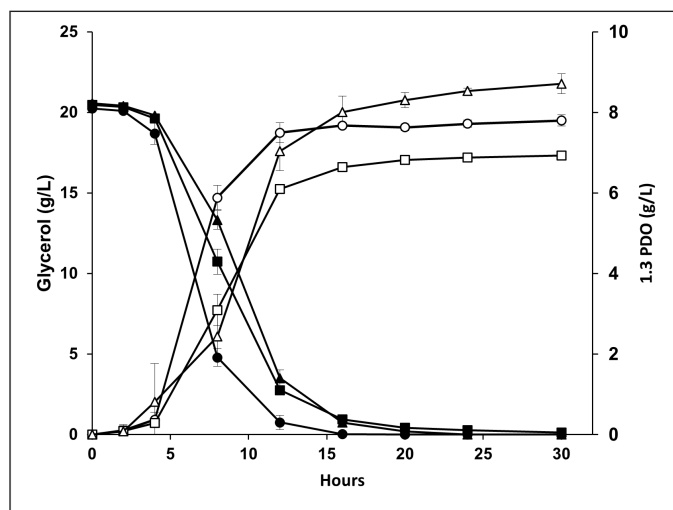


Figure 3. Glycerol consumed (black) and 1,3-PDO production (white) in a shake flask under anaerobic condition for 30 hours of *K. pneumoniae*; (○) TWO, (□) Δ LDH, and (Δ) Δ BUDB.

3.4. Impact of Targeted Gene Knockouts on by-Product Levels

To analyze the by-products of glycerol fermentation, HPLC analysis was conducted, focusing on lactic acid, formic acid, acetic acid, 2,3-BDO, and ethanol levels. Knockout of the *ldh* gene was confirmed by a 23% reduction in lactic acid levels (0.20 g/l). Acetic acid levels decreased by 53.1% (Fig. 4), while ethanol levels increased by 18.2% at the 12 hour (Fig. 5). The Δ LDH mutant ultimately exhibited the highest ethanol level (2.32 g/l) and the lowest acetic acid level (0.33 g/l). Acetic acid, an intermediate in ethanol production, is reduced to acetaldehyde by aldehyde dehydrogenase and then converted to ethanol [20]. Pyruvate accumulation led to increased ethanol production and decreased 1,3-PDO production due to NADH utilization for converting acetyl-CoA to ethanol [21]. This resulted in reduced acetic acid and 1,3-PDO levels, aligning with studies that show ethanol formation influences 1,3-PDO production [14,21]. The pyruvate accumulation also produced formic acid without NADH consumption, confirmed by the highest formic acid level (2.15 g/l) (Table 2).

The Δ BUDB mutant exhibited different by-product production behaviors from the eighth hour onward. Knockout of the *budB* gene reduced 2,3-BDO production by 87% from the fourth hour, stabilizing at 3.48 g/l by the end of fermentation (a 24% reduction) (Table 2). Consequently, the increased availability of NADH facilitated higher 1,3-PDO production, which suppressed ethanol production by 36% (1.15 g/l) and induced an 87% increase in acetic acid levels (1.20 g/l) (Table 2), leading to the low OD600 of the Δ BUDB mutant. Acetic

Table 2. Overview of cell growth, metabolites, consumed glycerol, product yield of wildtype and mutants *K. pneumoniae* TWO in shake flask under microaerobic condition at 30 hours.

	Strain		
	TWO	Δ LDH	Δ BUDB
Glycerol consumption (g/l)	20.24 ± 0,00	20.33 ± 0.20	20.56 ± 0.03
1,3-PDO (g/l)	7.8 ± 0.14	6.93 ± 0.05	8.71 ± 0.25
Lactic acid (g/l)	0.26 ± 0,00	0.2 ± 0.01	0.23 ± 0.00
Formic acid (g/l)	1.47 ± 0.15	2.15 ± 0.02	0.91 ± 0.23
Acetic acid (g/l)	0.64 ± 0,01	0.33 ± 0.03	1.20 ± 0.05
2,3-BDO (g/l)	4.57 ± 0.34	3.73 ± 0.25	3.48 ± 0.00
Ethanol (g/l)	1.8 ± 0.01	2.32 ± 0,10	1.15 ± 0.08
OD600	4.64 ± 0.03	4.47 ± 0.29	3.44 ± 0.07
1,3-PDO Yield (mol/mol glycerol) ^a	0.47	0.41	0.51

^aYield was calculated as produced metabolite (mol)/consumed glycerol (mol).

Table 3. Comparison of knockout studies in *K. pneumoniae* for improving 1,3-PDO production from glycerol.

Sources	1,3-PDO yield	Genes	Comments
This study	0.41	Δ <i>ldh</i>	Single deletion
This study	0.51	Δ <i>budB</i>	Single deletion
[12]	0.50	Δ (<i>ldhA—adhE—ack</i>)	Multiple deletion
[19]	0.47	Δ (<i>ldh—pf1B—budA</i>)	Multiple deletion
[22]	0.43	Δ (<i>budA—budB—adhE—ackA—pta—pox—frdA</i>)	Multiple deletion
[23]	0.48	Δ <i>ldh</i>	Multiple deletion
[24]	0.26	Δ (<i>dhaD—dhaK</i>)	Multiple deletion
[15]	0.48	Δ (<i>ldh—budC</i>)	Multiple deletion

acid production competed with the ethanol biosynthetic pathway for carbon flux [25]. A low formic acid level (0.91 g/l) was maintained until the end of fermentation (Fig. 5).

3.5. Redistribution of Glycerol into by-Products

The effect of deleting the *ldh* and *budB* genes on the percentage of by-products relative to glycerol consumption was investigated (g/g). All strains, including the *budB* deficient mutant, produced the highest levels of 2,3-BDO. Blocking the 2,3-BDO biosynthetic pathway significantly increased the acetic acid ratio to 5.8%, with 23.6% of the carbon flux being redirected towards other by-products (Fig. 6). There was a notable decrease in the carbon flux ratio for ethanol, formic acid,

and 2,3-BDO production. However, 2,3-BDO production persisted because acetolactate synthase, a key enzyme in this pathway, has eight isozymes. The *budB* gene, a major gene in this pathway (1680 bp) with 99% similarity to Accession Number CP011313, plays a crucial role (Fig. 7).

In contrast, the knockout of the *ldh* gene significantly increased the carbon flux towards ethanol and formic acid production, each by approximately 11%. Additionally, 1.6% and 18% of the glycerol flux were directed towards acetic acid and 2,3-BDO, respectively (Fig. 6). Blocking lactic acid production via *ldh* deletion was less effective in reducing carbon flux. The *ldh* gene (954 bp), classified under enzyme commission 1.1.1.27, catalyzes the production of L-lactate.

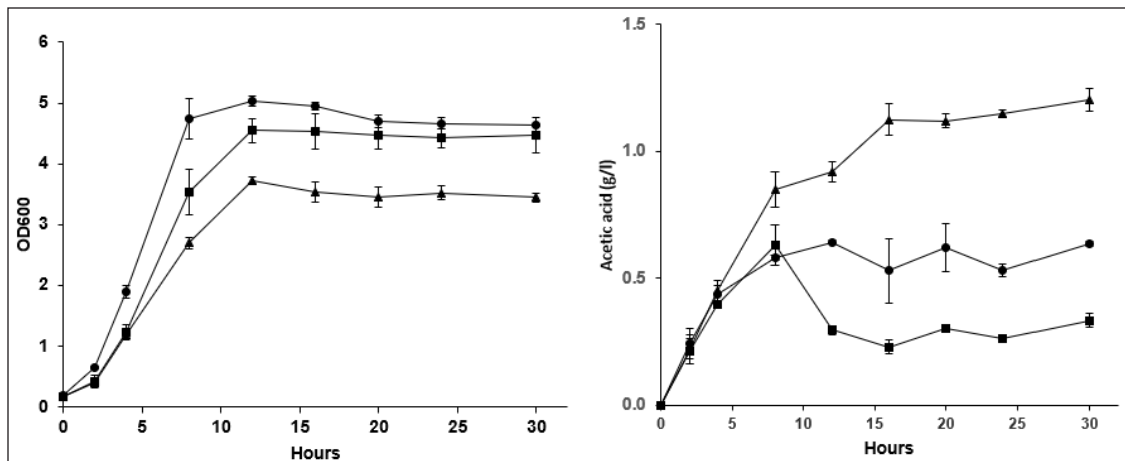


Figure 4. Cell growth and acetate production of *K. pneumoniae*; (●) TWO, (■) ALDH, dan (▲) ΔBUDB.

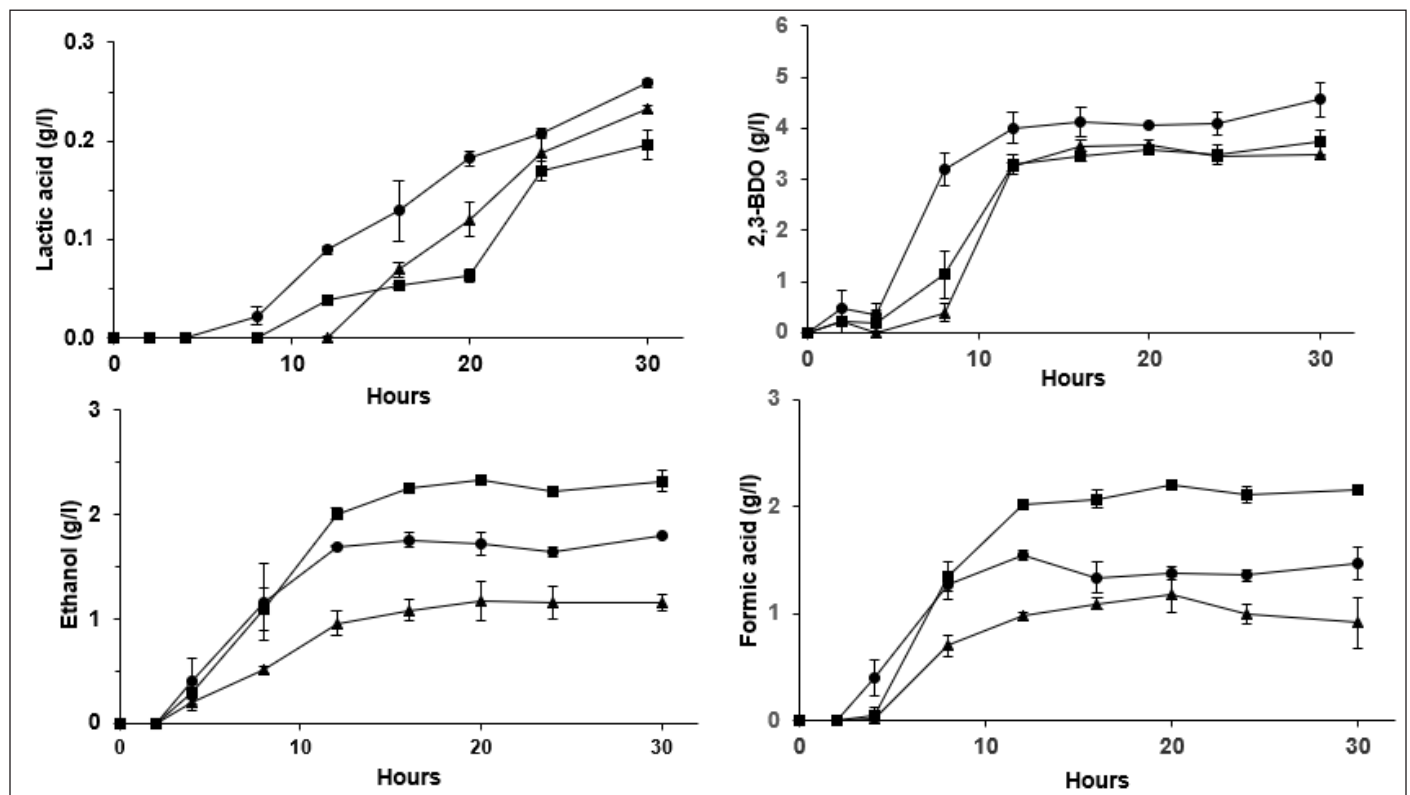


Figure 5. Lactic acid, 2,3-BDO, ethanol and formic acid, production of *K. pneumoniae*; (●) TWO, (■) ALDH, dan (▲) ΔBUDB.

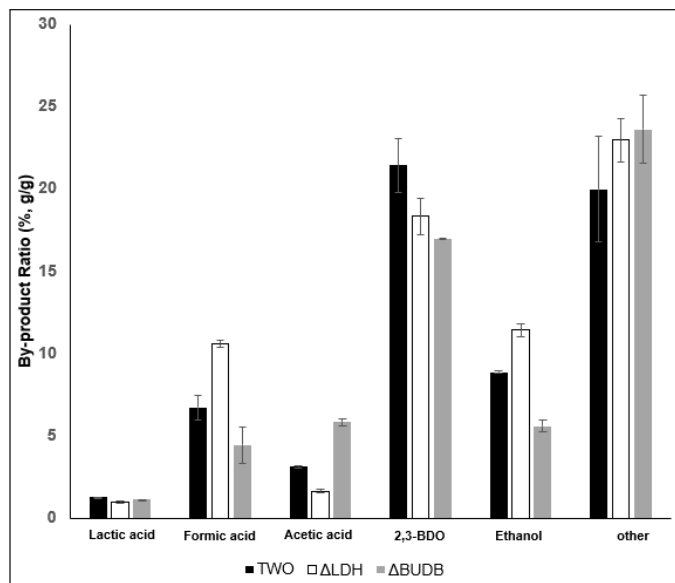


Figure 6. Conversion ratio of by-product level of consumed glycerol by *K. pneumoniae*; Black—TWO, White—ΔLDH, Gray—ΔBUDB.

Our study identified two isozymes of L-lactate dehydrogenase in the *K. pneumoniae* TWO genome. The *ldh* target gene in this study was highly similar to Accession Number CP011976, indicating that L-lactate was a minor component in the lactic acid production of *K. pneumoniae* TWO.

3.6. Impact of Malic Acid, Succinic Acid, and Vitamin B12 Supplementation

As previously explained, the NADH required for converting glycerol to 1,3-PDO is generated not only from the oxidative pathway but also from the TCA cycle. Optimizing the TCA cycle could augment the supply of reducing equivalents and promote the synthesis of 1,3-PDO [26]. This can be achieved by supplementing the TCA cycle with intermediates such as succinic acid and malic acid.

Additionally, vitamin B12 (cyanocobalamin) plays a crucial role in increasing glycerol carbon flux towards 1,3-PDO. In this study, malic acid, succinic acid, and vitamin B12 were supplemented at concentrations of 20 mM, 20 mM, and 0.0025 g/l, respectively. The results showed a decrease in both glycerol consumption and 1,3-PDO production across all treatments. However, supplementation with malic acid and vitamin B12 increased the 1,3-PDO yield to 0.55 mol/

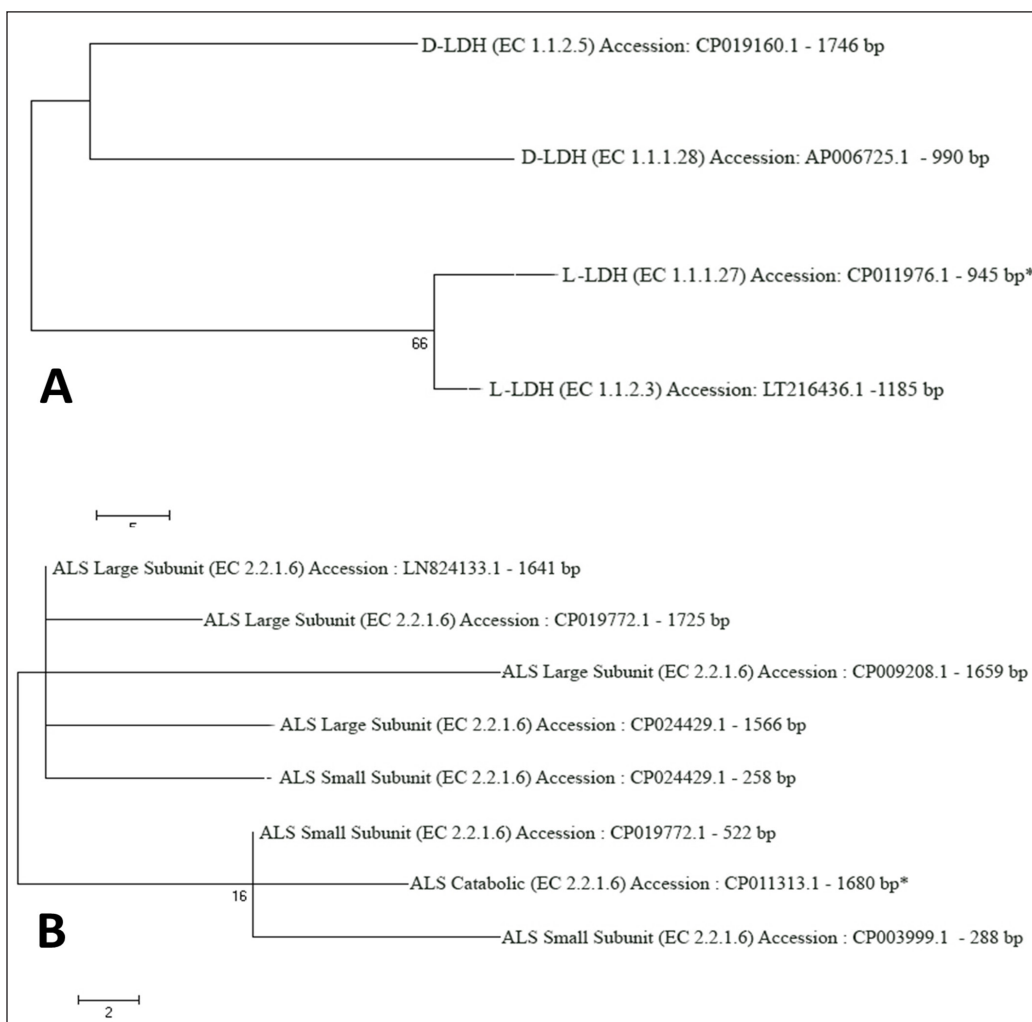


Figure 7. Phylogenetic tree of the isozymes of *K. pneumoniae* TWO: A, Lactate dehydrogenase (LDH); B, Acetolactate synthase (ALS). Asterisk: target gene that inactivated. All sequences not published. Based on similarity genes from GenBank.

Table 4. The effect of malic acid, succinic acid, and vitamin B12 on 1,3-PDO levels and by-products of Δ BUDB mutant.

	Fermentation medium			
	Control	Succinic acid 20 mM	Malic acid 20 mM	Vitamin B12 0.0025 g/l
Glycerol consumption (g/l)	19.92 ± 0.44	16.69 ± 0.32	17.39 ± 0.40	17.45 ± 0.31
1,3-PDO (g/l)	8.71 ± 0.25	7.16 ± 0.03	7.86 ± 0.40	8.59 ± 0.67
Lactic acid (g/l)	2.03 ± 0.02	2.14 ± 0.17	4.18 ± 0.23	1.18 ± 0.02
Formic acid (g/l)	1.28 ± 0.10	0.58 ± 0.07	0.63 ± 0.03	1.17 ± 0.19
Acetic acid (g/l)	1.29 ± 0.13	0.58 ± 0.01	0.93 ± 0.19	1.82 ± 0.07
2,3-BDO (g/l)	1.61 ± 0.30	1.73 ± 0.22	1.69 ± 0.14	0.49 ± 0.04
Ethanol (g/l)	0.82 ± 0.34	0.81 ± 0.35	0.64 ± 0.22	0.00 ± 0.00
1,3-PDO yield (mol/mol glycerol) ^a	0.53	0.52	0.55	0.60
2,3-BDO yield (mol/mol glycerol) ^a	0.08	0.11	0.10	0.03

^aYield was calculated as produced metabolite (mol)/consumed glycerol (mol).

Table 5. The effects of trace element, vitamin solution, Fe solution, and its combination on the consumed glycerols and by-products of Δ BUDB mutant.

	Treatment					
	1	2	3	4	5	6
Total glycerol (g/l)	20.84 ± 1.03	19.30 ± 0.12	19.66 ± 0.09	20.76 ± 1.27	19.68 ± 0.22	19.74 ± 0.11
Glycerol residue (g/l)	1.30 ± 0.13	0.15 ± 0.12	0.23 ± 0.03	1.91 ± 0.77	0.08 ± 0.03	0.43 ± 0.04
1,3-PDO (g/l)	9.32 ± 0.34	9.67 ± 0.32	9.93 ± 0.11	9.32 ± 1.00	10.08 ± 0.33	9.73 ± 0.09
Lactic acid (g/l)	0.96 ± 0.58	1.24 ± 0.10	1.03 ± 0.15	1.91 ± 0.78	0.90 ± 0.02	1.24 ± 0.05
Formic acid (g/l)	1.37 ± 0.04	0.78 ± 0.04	1.09 ± 0.08	1.37 ± 0.16	0.96 ± 0.10	1.19 ± 0.02
Acetic acid (g/l)	1.94 ± 0.12	1.93 ± 0.07	2.14 ± 0.02	2.02 ± 0.26	2.07 ± 0.06	2.07 ± 0.02
2,3-BDO (g/l)	0.66 ± 0.07	0.89 ± 0.14	0.82 ± 0.02	0.56 ± 0.32	0.89 ± 0.20	0.69 ± 0.16
Ethanol (g/l)	0.00 ± 0.00	0.29 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1,3-PDO yield (mol/mol glycerol) ^a	0.58	0.61	0.62	0.59	0.62	0.61
2,3-BDO yield (mol/mol glycerol) ^a	0.03	0.05	0.04	0.03	0.05	0.04

^aYield was calculated as produced metabolite (mol)/consumed glycerol (mol)

1 Fermentation medium added 0.0025 g/l vitamin B12 (Control),

2 Fermentation medium added 0.0025 g/l vitamin B12 and 1 ml of trace element, vitamin solution, Fe solution,

3 Fermentation medium added 0.0025 g/l vitamin B12 and 1 ml of trace element, Fe solution,

4 Fermentation medium added 0.0025 g/l vitamin B12 and 1 ml of trace element,

5 Fermentation medium added 0.0025 g/l vitamin B12 and 1 ml of vitamin solution,

6 Fermentation medium added 0.0025 g/l vitamin B12 and 1 ml of Fe solution

mol glycerol and 0.60 mol/mol glycerol, respectively, compared to the previous yield of 0.53 mol/mol glycerol. Additionally, 2,3-BDO and lactic acid levels decreased with vitamin B12 supplementation, reducing competition for glycerol. Ethanol was not produced at all during fermentation with vitamin B12 (Table 4). These findings suggest that optimizing glycerol consumption remains a key area for further study.

3.7. Impact of Trace elements, Vitamin Solution, Iron Solution, and their Combinations

To enhance glycerol consumption, we supplemented the fermentation medium with trace elements, vitamin solution, Fe solution, and their combinations, along with vitamin B12. Only trace element supplementation alone did not reduce residual glycerol levels. The combination of 0.0025 g/l vitamin B12 with the vitamin solution significantly increased glycerol consumption, resulting in a residual glycerol level of 0.08 g/l. This treatment also produced the highest level of 1,3-PDO at 10.08 g/l, achieving

a yield of 0.62 mol/mol glycerol (Table 5). *Klebsiella pneumoniae* needs vitamin B12 as a coenzyme for the GDHt enzyme to convert glycerol into 1,3-PDO.

4. CONCLUSION

In conclusion, the single knockout of the *budB* gene in the *K. pneumoniae* TWO genome proved to be an effective strategy for enhancing 1,3-PDO production, achieving a concentration of 8.71 g/l with a yield of 0.51 mol/mol glycerol. Supplementing the fermentation medium with 0.0025 g/l vitamin B12 and a vitamin solution further increased glycerol conversion efficiency, resulting in 62% of glycerol being converted into 1,3-PDO. This combination yielded a 1,3-PDO concentration of 10.08 g/l with an improved yield of 0.62 mol/mol glycerol, accompanied by the lowest residual glycerol levels.

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6. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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8. AUTHOR CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon request.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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