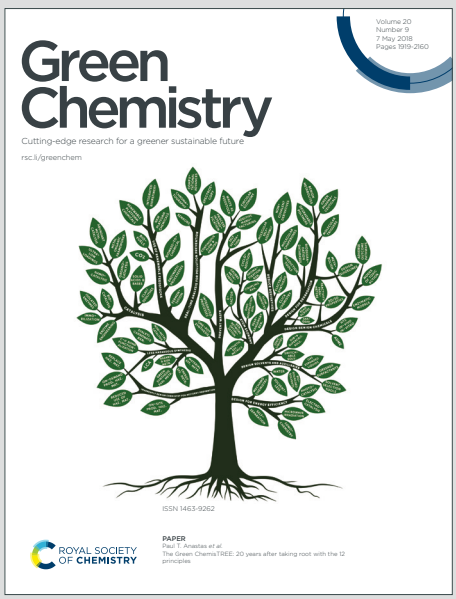
View Article Online
View Journal

Green Chemistry

Cutting-edge research for a greener sustainable future

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: Z. Guo, X. Ou, P. Xu, H. Gao, L. Zhang, M. Zong and W. Lou, *Green Chem.*, 2020, DOI: 10.1039/D0GC03044A.



View Article Online
DOI: 10.1039/D0GC03044A

Energy- and cost-effective non-sterilized fermentation of 2,3-butanediol by an engineered *Klebsiella pneumoniae* OU7 with anti-microbial contamination system

Ze-Wang Guo[a], Xiao-Yang Ou[a], Pei Xu[a], Hui-Fang Gao[b], Liao-Yuan Zhang[b], Min-Hua Zong[a], Wen-Yong Lou[a,*]

[a]*School of Food Science and Engineering, South China University of Technology, No. 381 Wushan Road, Guangzhou 510640, Guangdong, China*

[b]*College of Life Sciences, Fujian Agriculture and Forestry University, No. 15 Shangxiadian Road, Fuzhou 350002, Fujian, China*

*Correspondence: wylou@scut.edu.cn

†Electronic supplementary information (ESI) available.

View Article Online
DOI: 10.1039/D0GC03044A

Abstract

Microbial contamination is a serious challenge that needs to be overcome for the successful biosynthesis of 2,3-butanediol (2,3-BD). However, traditional strategies such as antibiotic administration or sterilization are costly, have high energy demands, and may increase the risk of antibiotic resistance. Here, we intend to develop a robust strategy to achieve non-sterilized fermentation of 2,3-BD. Briefly, the robust strain can metabolize unconventional chemicals as essential growth nutrients, and therefore, outcompete contaminant microbes that cannot use unconventional chemicals. To this end, *Klebsiella pneumoniae* OU7, a robust strain, was confirmed to rapidly exploit urea and phosphite (unconventional chemicals) as the primary sources of nitrogen (N) and phosphorus (P), and withstand deliberate contamination in the possibly contaminated systems. Secondly, metabolic engineering, pathogenicity elimination and adaptive laboratory evolution were successively performed, endowing the best strain with an excellent fermentation performance for safe 2,3-BD production. Finally, 84.53 g/L of 2,3-BD was synthesized with a productivity of 1.17 g/L/h and a yield of 0.38 g/g under the non-sterilized system. In summary, our technique reduces labor and energy costs and simplifies the fermentation process because sterilization does not need to be performed. Thus, our work will be beneficial for the sustainable synthesis of 2,3-BD.

View Article Online
DOI: 10.1039/D0GC03044A

Keywords

Biocontamination management, robust, phosphorus and nitrogen metabolism, metabolic engineering, non-sterilization fermentation

View Article Online
DOI: 10.1039/D0GC03044A

Introduction

2,3-Butanediol (2,3-BD) is a value-added chemical because of its strong demands in the manufacture of moisteners, explosives, fumigants and pharmaceuticals, perfumes, plasticizers, softening agents and printing inks.[1–3] Additionally, 2,3-BD is a potential aviation fuel with a high octane number, an excellent heating value (27.2 kJ/g), and a low freezing point.[4,5] 2,3-BD can be synthesized via chemical and biotechnological methods.[6] Recently, the sustainable synthesis of 2,3-BD via biotechnological methods has recently attracted growing attention because of the global environmental crisis and the shortage of fossil fuels.[7] However, unlike chemical processing, biosynthesis of 2,3-BD is generally conducted in a sterile system to avoid microbial contamination.

Microbial contamination affects the safety and quality of the biological product. Additionally, it results in significant economic losses to the industry. There are many reasons for microbial contamination, such as the incomplete sterilization of equipment and fermentation media, seeds carrying undesirable microbes, improperly sterilized air supply, and poor sealing of fermentation equipment. Traditionally, process sterilization and the addition of antibiotics were extensively adopted to decrease the probability of microbial contamination.[8,9] However, sterilization is a time-consuming, energy-intensive and laborious process.[10] Moreover, staff need higher-level skills to ensure successful

View Article Online
DOI: 10.1039/D0GC03044A

fermentation.[11] On the other hand, the overuse of antibiotics will promote the emergence of "superbugs" and increase the risk of antibiotic resistance,[12] which reduces the competitiveness and sustainability of the 2,3-BD biosynthesis. Therefore, there is a need to develop novel strategies to efficiently prevent biocontamination.

In previous studies, we observed a growing interest in non-sterilized fermentation because of its advantages, including i) preventing the Maillard reaction, ii) providing significant savings in the costs of energy, equipment, and labor, and iii) simplifying the fermentation process.[13] To this end, non-sterilization fermentation of lactic acid and acetoin was achieved using the thermophilic *B. coagulans* and *Bacillus* sp. H15-1, respectively.[14,15] Additionally, *B. licheniformis* was frequently reported to synthesize 2,3-BD at thermophilic conditions, which also exhibited the potential for non-sterilized fermentation.[6,16–18] Nevertheless, these reports had some restrictions related to non-sterilized fermentation of 2,3-BD. On the one hand, microbial contamination was still observed under the thermophilic fermentation since the thermophilic and spore-forming strains are ubiquitous.[9] Worse still, diacetyl and acetoin, the precursors of 2,3-BD, will volatilize easily during thermophilic fermentation. On the other hand, fermentation was conducted under mesophilic conditions (30-40°C) in the most of 2,3-BD producers, which

View Article Online
DOI: 10.1039/D0GC03044A

is infeasible for thermophilic fermentation. Without medium sterilization, it is difficult to prevent contamination completely.

Recently, essential growth nutrients (ecologically rare compounds) have been used to provide selective pressure for the dominant growth of robust strains without antibiotics. For this purpose, phosphite dehydrogenase (PtxD), an enzyme producing phosphate from phosphite (an unconventional type of phosphorus), was successfully introduced into different hosts.[19–22] Additionally, the whole melamine utilization gene cluster was assembled into *E. coli* or *Synechococcus*, endowing the modified organisms with the capability to resist harmful contamination.[19,20] Similarly, *E. coli* and *B. subtilis* were tailored by simultaneously introducing the formamide and phosphite utilization pathways, rendering them suitable for defending against microbial contamination.[9,23]

In this work, we intend to develop an effective strategy to achieve non-sterilization fermentation of 2,3-BD (**Fig. 1**). First, *K. pneumoniae* OU7, a robust strain with an endogenous anti-hybrid bacteria system, was confirmed to rapidly use urea and phosphite as the sole nitrogen (N) and phosphorus (P) sources, respectively. Second, a triple mutant of *K. pneumoniae* OU7 with inactivation of lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and phosphotransacetylase (PTA) was obtained. Moreover, the *wabG* gene was disrupted to abolish the pathogenicity of *K. pneumoniae* OU7. Lastly, the

View Article Online
DOI: 10.1039/D0GC03044A

efficiency and economic feasibility of 2,3-BD production using the engineered *K. pneumoniae* OU7 were evaluated in non-sterilized fed-batch fermentation. On the whole, the non-sterilized fermentation of 2,3-BD proposed here perfectly fulfills the principles of environmentally benign and sustainable chemistry.

Experimental section

Strains, cultivation and medium

2,3-BD was produced using *K. pneumoniae* OU7, isolated in our previous work.[9] The gene-deficient mutants of *K. pneumoniae* OU7 were created in this work (**Table 1**). All strains were maintained on LB agar slants. The wild-type *K. pneumoniae* OU7 and its mutants were cultivated in auxotrophic or conventional MOPS medium. The conventional MOPS medium is comprised of Tricine (4 mM), MOPS (40 mM), $FeSO_4$ (0.01 mM), K_2SO_4 (0.276 mM), $CaCl_2$ (5×10^{-4} mM), $MgCl_2$ (0.523 mM), NaCl (50 mM), NH_4Cl (9.5 mM), and KH_2PO_4 (1.32 mM). They were also supplied with 0.5 mL of trace metal solution: 0.4 mM H_2BO_4, 0.08 mM $MnCl_2$, 0.01 mM $CuSO_4$, 0.03 mM $CoCl_2$, and 0.01 mM $ZnSO_4$. In auxotrophic MOPS medium, NH_4Cl and KH_2PO_4 were replaced by urea and KH_2PO_3, respectively. The seed or fermentation medium was obtained by adding 10 or 80 g/L of glucose to MOPS medium with 2.0 mM KH_2PO_3, 10 g/L urea, and 0.1 g/L $NiCl_2$.

Robustness analysis for *K. pneumoniae* OU7

View Article Online
DOI: 10.1039/D0GC03044A

K. pneumoniae OU7 was cultivated in auxotrophic or conventional MOPS medium. Conventional medium (with 10 g/L glucose) was prepared by adding 9.5 mM NH_4Cl and 1.32 mM KH_2PO_4 as the sources of N and P. Auxotrophic medium (with 10 g/L glucose) was prepared by adding 9.5 mM urea (with 0.1 g/L $NiCl_2$) and 1.32 mM KH_2PO_3 as the sources of N and P. The viable cell count was tested by plating culture fluid on LB-agar plates and then incubating overnight at 37 °C. Subsequently, *K. pneumoniae* OU7 and other microbes were classified by their unique colony morphology.[23]

Construction of the gene knockout strains

To construct gene knockout strains, both up- and down-stream fragments (approximately 0.95 kB) of target genes were amplified from *K. pneumoniae* OU7. Subsequently, the two fragments were combined and inserted into the suicide vector pRE112 by in-fusion cloning, and then transferred into *E. coli* S17-1 to obtain the donor strains. For the first homologous recombination, the recipient strain (300 μL) was cocultured with the donor strain (900 μL) in a non-selective LB Petri plate (37 °C, 12 h). This was then plated on selective LB agar plates (containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol) to obtain positive colonies. A single crossover strain was incubated in non-selective LB broth overnight for the second homologous recombination, then plated on 12% sucrose LB Petri plates for selection and verified by PCR method.[24] All strains and plasmids are listed in **Table 1**. All primers are listed in **Table S1**.

View Article Online
DOI: 10.1039/D0GC03044A

Enzyme activity assays

Cells of *K. pneumoniae* in different growth phases were collected by centrifugation (10000 g, 5 min, 4°C) and completely resuspended with 50 mM precooled phosphate buffer (pH 7.4). Subsequently, cells were disrupted by ultrasonication (10 min, 4°C), and the lysates were centrifuged (12000 g, 20 min, 4°C) to obtain the crude cell extracts. The LDH, ADH and PTA activity were assayed according to the method established by Guo *et al.* .[25] The PtxD and urease activity was determined as described by Ou *et al.* and Pearson *et al.* , respectively.[9,26]

Electron microscopy analysis

Surface morphology and microstructure of KP-OU7-ME3 and KP-OU7-ME4 were studied by field-emission scanning electron microscopy (FE-SEM, Hitachi, SU-8010) and transmission electron microscopy (TEM, Hitachi, H-7650), respectively. The detailed procedures for preparing bacterial specimens for FE-SEM and TEM analysis were the same as described by Jung *et al.* .[27]

Adaptive laboratory evolution (ALE)

ALE was conducted using a long-term serial transfer experiment. Briefly, every 12 h, the seed culture (1% v/v) of evolved strain was rapidly transferred into a new and fresh auxotrophic MOPS medium (containing 80 g/L glucose). The transfers were performed

View Article Online
DOI: 10.1039/D0GC03044A

until the clones showed rapid glucose consumption, fast growth, and high production of 2,3-BD.[28]

Fermentation

For seed preparation, *K. pneumoniae* OU7 from a fresh slant tube was picked into conical flasks (250-mL) containing 50-mL seed medium and inoculated overnight on a rotary shaker (37°C, 200 rpm). Subsequently, seed culture (5%, v/v) was transferred into fermentation broth.[29] Batch fermentations were performed in 250-mL shake flasks with 50-mL working volume. Fed-batch fermentation of KP-OU7-ME5 occurred in a 5-L stirred fermenter with a 3-L initial volume. Fermentation was performed at 37℃ with the pH maintained at 6.5 by automatic addition of 2 M NaOH, stirring at 300 rpm before 16 h and then switched to 200 rpm with 1.0 vvm of airflow. Additionally, 2 mM KH_2PO_3 was fed into the fermentation medium at 24, 40, and 56 h, to sustain long term growth for KP-OU7-ME5 mutant.

Analytical methods

The growth rate of cells was determined using optical cell density at a 600 nm wavelength. Measurements from a Shimadzu 00671UV-2550 spectrophotometer (Shimadzu Corporation, Japan) were converted to dry cell weight (DCW) according to the equation (DCW (g/L) = $0.6348 \times OD_{600} + 0.3257$). The intracellular NADH level or NAD^+

View Article Online
DOI: 10.1039/D0GC03044A

level was determined using the NAD(H) Assay Kit (BC0315, Solarbio, Beijing, China). HPLC analyses of 2,3-BD, glucose, lactate, acetoin, acetate, and ethanol were implemented as described in a previous study.[25] Briefly, RID-10A refractive index detector, Aminex HPX-87H column (Bio-Rad) at 65°C, a mobile phase of 5 mM H_2SO_4, and a flow rate of 0.5 mL/min was used.

Results and discussion

K. pneumoniae OU7 grew on the auxotrophic MOPS medium

The 16S rRNA sequence of the *K. pneumoniae* OU7 strain was identified. Meanwhile, further morphological and biochemical assays were also performed to identify strain (data not shown). The alignment result (**Fig. S1**) showed that the 16S rRNA sequence of *K. pneumoniae* OU7 (GenBank: MN999954.1) shared a strong identity (99%) with the that of *K. pneumoniae* ZG26 and *Klebsiella* sp. F5-1.

Previous work from our group demonstrated that the *K. pneumoniae* OU7 could exploit phosphite as the sole P source for cell growth.[9] Additionally, *Klebsiella* species have the natural capability to utilize urea as the sole N source.[30] In this study, to verify that *K. pneumoniae* OU7 could simultaneously utilize phosphite and urea as the sole P and N sources, the strain was grown in auxotrophic medium. First, we verified that *K. pneumoniae* OU7 could grow normally in the phosphite-loaded medium when the concentration of KH_2PO_3 was lower than 2 mM (**Fig. 2A and 2B**). However, there was marked inhibition

View Article Online
DOI: 10.1039/D0GC03044A

on cell growth when the concentration of KH_2PO_3 was higher than 2 mM (**Fig. 2B**). To the best of our knowledge, the reason for inhibition remains unclear, and this should be the subject of further investigation in future work.

The growth of cells was significantly affected in the urea-loaded medium when the medium did not have $NiCl_2$ (**Fig. 2A**). Luckily, cell growth returned to normal when the $NiCl_2$ was added. This phenomenon can be explained since the urease produced by *Klebsiella* species was activated by nickel.[31] Therefore, $NiCl_2$ is necessary when urea is used as the N source. Moreover, it was shown that cell growth was not affected when the concentration of urea was lower than 20.0 g/L (**Fig. 2C**). Finally, phosphite utilization gene operon (*ptxABCDE*) and urea utilization gene cluster (*ureDABCEFG*) were verified from whole genomic sequencing (**Fig. 2D and 2E**), both the PtxD and urease activity could be detected (**Fig. 2F**). Taken together, these results strongly suggest that endogenous phosphite and urea assimilating pathways exist in *K. pneumoniae* OU7.

In conclusion, *K. pneumoniae* OU7, a robust strain, was confirmed to rapidly exploit phosphite and urea as P and N sources, respectively, for its growth (**Fig. 1**). One primary consideration of this work's robust strain is that phosphite is safe and inexpensive, enabling us to perform economically viable non-sterilized fermentation on an industrial scale. On the other hand, using urea as N source is favorable because: 1) urea has a low cost compared

View Article Online
DOI: 10.1039/D0GC03044A

with ammonium salts or yeast extract; and 2) unlike ammonium salts, growth in urea-containing medium suppresses the medium acidification rate.[31]

Robustness analysis of *K. pneumoniae* OU7

As shown in **Fig. 3D**, *K. pneumoniae* OU7 accounted for, on average, 94.9% of the population when cultivated in a non-sterilized auxotrophic medium. However, the average proportion of *K. pneumoniae* OU7 was only 88.5% when cultivated in a non-sterilized conventional medium (**Fig. 3A**). Additionally, the slight growth of the undesirable microorganisms observed is, as shown in **Fig. 3D**, most likely come from the starting ingredients used to prepare the medium.

Second, *K. pneumoniae* OU7 was cocultured with *E. coli* (as a typical prokaryotic microorganism) or *S. cerevisiae* (as a typical eukaryotic microorganism) to evaluate whether the robust *K. pneumoniae* OU7 was able to outcompete other microorganisms. Encouragingly, with time going on, the proportion of *K. pneumoniae* OU7 rapidly increased. Eventually, the proportion of *K. pneumoniae* OU7 approached 100% in the auxotrophic medium (**Fig. 3E and 3F**). By contrast, the proportion of *K. pneumoniae* OU7 was maintained at a relatively low level when cultivated in conventional medium (**Fig. 3B and 3C**). The above experiments demonstrated that the simultaneous utilization of phosphate and urea, which are less favorable to assimilate for common microbes, allowed the *K. pneumoniae* OU7 a capability to overcome and resist deliberate contamination (**Fig.**

View Article Online
DOI: 10.1039/D0GC03044A

1). This finding suggested that *K. pneumoniae* OU7 is indeed suitable for 2,3-BD fermentation under non-sterilized conditions.

Metabolic engineering of *K. pneumoniae* OU7

Currently, undesirable by-products (such as lactate, ethanol, and acetate) are also produced during the 2,3-BD fermentation process (**Fig. 1**), which shunts the carbon flow and dramatically decreases the efficiency of 2,3-BD synthesis.[25,28] Thus, we proposed to block pathways that competitively consume NADH to redistribute carbon flux towards 2,3-BD synthesis. Consequently, PCR analysis and enzymatic activity results confirmed that *ldhA* (encoding lactate dehydrogenase), *adhE* (encoding alcohol dehydrogenase), and *pta* (encoding phosphotransacetylase) were successfully disrupted (**Fig. S2 and Table S2**), resulting in gene-deficient mutants (**Table 1**).

To evaluate the influence of *ldhA*, *adhE* and *pta* inactivation on fermentation performances, batch fermentation was performed. Compared with the KP-OU7, the maximum DCW of KP-OU7-ME1 was elevated from 4.13 to 4.41 g/L (**Fig. 4A**). The increase in DCW may result from a decrease in lactate formation, which leads to a slower acidification rate of the medium.[32] By contrast, the maximum DCW of KP-OU7-ME2 (4.27 g/L) and KP-OU7-ME3 (4.08 g/L) was lower than that of KP-OU7-ME1 (**Fig. 4A**).

The titer of different byproducts was different in the gene-deficient mutants (**Fig. 4A)**. Firstly, the final titers of lactate in KP-OU7-ME1, KP-OU7-ME2 and KP-OU7-ME3 were

View Article Online
DOI: 10.1039/D0GC03044A

only 1.38, 1.56 and 1.41 g/L, respectively, and this corresponded to 28.11%, 31.77% and 28.72% of the KP-OU7 strain (4.91 g/L), respectively. Secondly, the deletion of ADH resulted in a nearly abolished ethanol synthesis, the ethanol concentration of KP-OU7-ME2 (0.28 g/L) decreased 88.38% compared with the parent strain (2.41 g/L). Moreover, compared with the KP-OU7 (1.81 g/L), the deletion of the *pta* gene caused a slight reduction of acetate production (1.43 g/L) in the KP-OU7-ME3 strain. Taken together, the simultaneous inactivation of LDH, ADH and PTA could effectively alleviate lactate, ethanol and acetate accumulation.

As shown in **Fig. 4A**, the final titer of acetoin in KP-OU7-ME1, KP-OU7-ME2 and KP-OU7-ME3 were only 2.88, 2.71 and 2.28 g/L, respectively, and this corresponded to 84.96%, 79.94% and 67.26% of the KP-OU7 strain (3.39 g/L), respectively. Encouragingly, the final 2,3-BD titer in KP-OU7-ME1, KP-OU7-ME2 and KP-OU7-ME3 reached 22.66, 22.87 and 23.41 g/L, respectively, which corresponded to a dramatic increase of 18.08%, 19.18% and 22.0%, respectively, compared with the parent strain (19.19 g/L) (**Fig. 4A**). The reasons for these higher titers of 2,3-BD are: i) deletion of *ldhA*, *adhE* and *pta* partially blocking the lactate, ethanol, and acetate pathway and supplying more carbon flux for the mutants to produce 2,3-BD, and ii) the slightly increase in the NADH/NAD^+ ratio that was observed in the gene-deficient mutants (**Fig. 4D**) which resulted in the decreased accumulation of acetoin.

View Article Online
DOI: 10.1039/D0GC03044A

2,3-BD biosynthesis is highly linked with the intracellular NADH/NAD^+ ratio. In **Fig. 4B and 4C** (all strains), both NADH and NAD^+ concentrations were continuously declined during the growth stage (≤ 32 h). However, in the non-growth stage, the levels of NADH and NAD^+ were maintained relatively constant. Furthermore, compared with the wild strain, the inactivation of *ldhA*, *adhE* and *pta* resulted in the NADH/NAD^+ ratios being maintained at a higher level (**Fig. 4D**). These results indicated that the disruption of NADH-dependent pathways would affect the ratio of intracellular NADH/NAD^+ and facilitate the synthesis of 2,3-BD.

Removal of the pathogenic factor by inactivating the virulence-related *wabG* gene

Currently, the practical application of *K. pneumoniae* for producing 2,3-BD is severely hindered by its pathogenicity (generally defined as biosafety class II microorganism), which exhibits potential harm to users and increases the cost of industrial-scale production.[27] Previous studies have shown that the virulence factors of *K. pneumoniae* strains include lipopolysaccharide (LPS), capsular polysaccharide, siderophores, and fimbriae.[33] Among them, LPS is a major component of the cell wall, which is considered an essential pathogenic determinant of *K. pneumoniae*. As shown in **Fig. S3C**, LPS comprises three sections, O-antigenic polysaccharide (O-PS), core oligosaccharide (OS), and lipid A. Particularly, O-antigen is responsible for the resistance of the bacteria to complement-mediated killing. The outer core LPS is also involved in the

View Article Online
DOI: 10.1039/D0GC03044A

attachment of capsules to the surface.[27,34,35] Previously, extensive success stories demonstrated that the deletion of the *wabG* gene (encoding glycosyltransferase) involved in LPS biosynthetic could eliminate the pathogenicity of *K. pneumoniae*.[27,36]

Here, the core oligosaccharide (OS) biosynthetic gene cluster was verified and annotated from whole genomic sequencing (**Fig. S3A and S3B**). Subsequently, based on KP-OU7-ME3, the *wabG* gene was disrupted, yielding the strain KP-OU7-ME4. Additionally, surface morphologies of KP-OU7-ME3 and KP-OU7-ME4 were examined by SEM. Predictably, the bacterial surface of KP-OU7-ME3 was surrounded by a thick capsular polysaccharides (CPS) layer. Moreover, the bacterial boundaries were not clear because of mucoid CPS (**Fig. 5A**). By contrast, KP-OU7-ME4 showed a nonmucoid colony morphology (**Fig. 5B**), consistent with the previous studies.[27] On the other hand, the KP-OU7-ME3 exhibited a clear and smooth outer membrane (**Fig. 5C**). However, in KP-OU7-ME4, the outer membrane's contour was irregular and unclear (**Fig. 5D**). In other words, the integrity of the outer membrane was destroyed. Taken together, the LPS of *K. pneumoniae* OU7 was successfully tailored to abolish its pathogenicity.

In batch fermentation, the maximum DCW of KP-OU7-ME3 and KP-OU7-ME4 were 4.08 and 4.15 g/L, respectively (**Fig. 5E**). These data indicated that the absence of outer core LPS did not affect the cell growth of *K. pneumoniae* OU7. Unfortunately, the 2,3-BD titer of KP-OU7-ME4 (20.75 g/L) was slightly lower than that of KP-OU7-ME3 (23.41

View Article Online
DOI: 10.1039/D0GC03044A

g/L). As described by Jung *et al.*, the main reason for the decrease of 2,3-BD titer in KP-OU7-ME4 may result from the change of outer membrane integrity, which leads to a slower utilization rate of glucose.[27] Subsequently, adaptive laboratory evolution (ALE) was performed to enhance 2,3-BD production. After 30 adaptation cycles, a maximum DCW of 4.31 g/L and 2,3-BD titer of 22.96 g/L was obtained in the endpoint strain KP-OU7-ME5 (**Fig. 5E**), which was 3.86% and 10.65% higher than that of KP-OU7-ME4 strain, respectively. Therefore, the KP-OU7-ME5 strain was used in subsequent fed-batch fermentation.

Effects of organic nitrogen on the synthesis of 2,3-BD

The yield of 2,3-BD was only 0.29 g/g in the KP-OU7-ME5 strain (**Fig. 5E**), and this was lower than the theoretical yield (0.50 g/g). This data demonstrated that the nutrients present in the MOPS medium were insufficient for 2,3-BD synthesis, even though the MOPS medium contained all nutrients for *K. pneumoniae* OU7 growth. Previous studies demonstrated a significant improvement of 2,3-BD synthesis when additional nitrogen sources were supplemented into the fermentation medium.[31]

Here, both the biomass yield and 2,3-BD titer could be enhanced when organic nitrogen was added into the MOPS medium (**Fig. S4A**). When yeast extract was added, 2,3-BD titer and DCW were 27.67 g/L and 7.60 g/L, respectively, corresponding to 121.41% and 177.57% of the control (22.79 g/L and 4.28 g/L). Unfortunately, many undesirable

View Article Online
DOI: 10.1039/D0GC03044A

microorganisms were observed when different nitrogen sources were supplemented into the MOPS medium (**Fig. S4B**), which means that non-sterilized fermentation of 2,3-BD is not suitable under these conditions. Therefore, nonsterile auxotrophic MOPS medium (without additional nitrogen sources) was used in the subsequent fed-batch experiments.

Fed-batch fermentation of KP-OU7-ME5 in the non-sterilized system

During the fermentation process, 2,3-BD synthesis is highly dependent upon the consumption of substrate.[37] Therefore, a fed-batch experiment was conducted to accumulate 2,3-BD using an periodic feeding strategy. The glucose was injected into the fermenter when its concentration was less than 10 g/L. Additionally, based on the previous studies, agitation speed was controlled using an efficient two-stage control strategy.[29] As shown in **Fig. 6A**, acetoin showed a sharp accumulation before 16 h with 300 rpm agitation speed and then continuously decreased after 16 h with 200 rpm agitation speed. The main reason is that 2,3-BD, production from acetoin, is via an NADH-linked pathway (**Fig. 1**). Before 16 h, the dissolved oxygen concentration was relatively high, leading to a down-regulation of the NADH pool (the NADH was rapidly oxidized via the electron transfer chain), resulting in acetoin accumulation. After 16 h, the level of NADH was relatively high, corresponding to the insufficient oxygen, thus rapid converting acetoin to 2,3-BD.

Acetate was formed as the main by-product with a final titer of 3.75 g/L. In contrast, the titers of ethanol and lactate were lower than 2.0 g/L throughout the fermentation process.

View Article Online
DOI: 10.1039/D0GC03044A

These phenomena suggested that other LDH, ADH and PTA manufacturing genes might exist in *K. pneumoniae* OU7. Encouragingly, 2,3-BD titer continuously increased with glucose feeding. Ultimately, 222.92 g/L of glucose was consumed, resulting in a maximum 2,3-BD titer up to 84.53 g/L with the yield of 0.38 g/g (theoretical yield of 75.84 %) and the productivity of 1.17 g/L/h. Meanwhile, under the same conditions, sterilized fermentation of KP-OU7-ME5 was also performed (**Fig. 6C**). Finally, 86.13 g/L 2,3-BD was synthesized with the yield of 0.39 g/g and the productivity of 1.20 g/L/h under the sterilized system, which displayed 1.89%, 2.56%, and 2.63% improvements in terms of titer, productivity, yield, respectively, compared with non-sterilized fermentation.

Klebsiella remained the dominant genus with a relative abundance of 95.36% at the end of non-sterilized fermentation (**Fig. 6B**). The slight growth of the contaminated microbes (relative abundance of 4.64%) most likely came from the starting ingredients used to prepare the non-sterilized medium. Moreover, the functional genes of the anti-hybrid bacteria system were located in the chromosome, which decreased the probability of horizontal gene transfer into environmental microorganisms. On the whole, these results demonstrated that non-sterilized fermentation of 2,3-BD using *K. pneumoniae* OU7 is viable and safe. At present, our system was unable to defend against phage infections, which is another vexing issues in industrial fermentations. Consequently, sterilization treatment for fermentation waste, rationally designing factories, and culture rotation are

View Article Online
DOI: 10.1039/D0GC03044A

conducive to decrease the probability of phage infections. Furthermore, in future work, the tools of abortive infection, CRISPR-Cas systems, and restriction-modification can be used to resist phage infections.[38–40]

Compared with previous reports, the 2,3-BD titer from our work was relatively low (**Table 2**). Among them, 178 g/L 2,3-BD was obtained by engineered *S. cerevisiae* with the productivity of 1.88 g/L/h and the yield of 0.34 g/g. This is the highest titer of 2,3-BD produced in a microbial fermentation process so far. However, the substrate costs increase because of this engineered strain's requirement for nutrient-rich media (which includes 20 g/L peptone and 10 g/L yeast extract) as well as antibiotics (300 mg/L hygromycin B and 0.5 mg/L aureobasidin A).[41] Additionally, it would also be necessary to sterilize the medium, which would increase the labor- and energy-costs.

Taken together, the non-sterilized fermentation of 2,3-BD presented in our work is advantageous because: (1) it is more economically because elimination the use of antibiotic addition or expensive nutrients; (2) labor- and energy-costs can be reduced as the need for sterilization has been eliminated; (3) the fermentation operation can be simplified; and (4) it is a benefit for long-lasting continuous fermentation, which often suffers from the risk of contamination resulting from constant adjustments and requires some sophistication in terms of instrument operation. In future work, the traditional bioreactor can be replaced by cement, plastic or ceramic bioreactor, as Chen's group described to reduce equipment costs

View Article Online
DOI: 10.1039/D0GC03044A

further.[11] Moreover, we believe that further improvement of 2,3-BD synthesis might be realized by optimizing the fermentation medium or overexpressing genes of the 2,3-BD biosynthesis pathway. Thus, the non-sterilized fermentation performed by a robust *K. pneumoniae* OU7 is still an excellent alternative for the sustainable synthesis of 2,3-BD.

Conclusions

The green synthesis of 2,3-BD through biotechnological methods is attractive but difficult to perform because of microbial contamination. In this work, by using the systematic engineering including the evaluation of the anti-microbial contamination system, redistribution of the metabolic flux, mutation of the pathogenicity and adaptive evolution of the fermentation performance, we successfully redesigned the *K. pneumoniae* OU7 strain for efficient 2,3-BD production. Ultimately, under the non-sterilized system, the 2,3-BD titer and yield obtained were 84.53 g/L and 0.38 g/g, respectively. From the perspective of industrial development, our work is competitive because it avoids antibiotic use and reduces the energy- and labor-costs. Additionally, our work offers an insight into the more sustainable synthesis of other chemicals using a similar strategy.

Conflicts of interest

There are no conflicts to declare

Acknowledgments

View Article Online
DOI: 10.1039/D0GC03044A

This work was supported by the National Key Research and Development Program of China (number: 2018YFC1603400, 2018YFC1602100); National Natural Science Foundation of China (number: 21676104, 21878105); Science and Technology Program of Guangzhou (number: 201904010360); Fundamental Research Funds for the Central Universities (number: 2019PY15); and the Key Research and Development Program of Guangdong Province (number: 2019B020213001).

References

1. J. W. K. Oliver, I. M. P. Machado, H. Yoneda and S. Atsumi, *Proc. Natl. Acad. Sci.*, 2013, **110**, 1249–1254.
2. J. Feng, Y. Gu, P.-F. Yan, C. Song and Y. Wang, *ACS Sustain. Chem. Eng.*, 2017, **5**, 11221–11225.
3. X.-J. Ji, H. Huang and P.-K. Ouyang, *Biotechnol. Adv.*, 2011, **29**, 351–364.
4. L. Zhang, R. Singh, D. Sivakumar, Z. Guo, J. Li, F. Chen, Y. He, X. Guan, Y. C. Kang and J.-K. Lee, *Green Chem.*, 2018, **20**, 230–242.
5. J. Haider, G. R. Harvianto, M. A. Qyyum and M. Lee, *ACS Sustain. Chem. Eng.*, 2018, **6**, 14901–14910.
6. Y. Ge, K. Li, L. Li, C. Gao, L. Zhang, C. Ma and P. Xu, *Green Chem.*, 2016, **18**, 4693–4703.

View Article Online
DOI: 10.1039/D0GC03044A

7. P.-F. Yan, J. Feng, S. Dong, M. Wang, I. A. Khan and Y. Wang, *ACS Sustain. Chem. Eng.*, 2017, **5**, 11016–11023.

8. S. Gopinath, M. V Kim, T. Rakib, P. W. Wong, M. van Zandt, N. A. Barry, T. Kaisho, A. L. Goodman and A. Iwasaki, *Nat. Microbiol.*, 2018, **3**, 611–621.

9. X. Ou, X. Wu, F. Peng, Y. Zeng, H. Li, P. Xu, G. Chen, Z. Guo, J. Yang, M. Zong and W. Lou, *Biotechnol. Bioeng.*, 2019, **116**, 3333–3348.

10. W. Zeng, W. Li, L. Shu, J. Yi, G. Chen and Z. Liang, *Bioresour. Technol.*, 2013, **142**, 697–700.

11. G.-Q. Chen and X.-R. Jiang, *Curr. Opin. Biotechnol.*, 2017, **50**, 94–100.

12. H. C. Neu, *Science*, 1992, **257**, 1064–1073.

13. B. Zhao, L. Wang, C. Ma, C. Yang, P. Xu and Y. Ma, *Bioresour. Technol.*, 2010, **101**, 6494–6498.

14. Y. Zhang, X. Chen, B. Qi, J. Luo, F. Shen, Y. Su, R. Khan and Y. Wan, *Bioresour. Technol.*, 2014, **163**, 160–166.

15. Z. Xiao, R. Gu, X. Hou, J.-Y. Zhao, H. Zhu and J. Lu, *J. Chem. Technol. Biotechnol.*, 2017, **92**, 2845–2852.

16. L. Li, L. Zhang, K. Li, Y. Wang, C. Gao, B. Han, C. Ma and P. Xu, *Biotechnol. Biofuels*, 2013, **6**, 123.

View Article Online
DOI: 10.1039/D0GC03044A

17. L. Li, C. Chen, K. Li, Y. Wang, C. Gao, C. Ma and P. Xu, *Appl. Environ. Microbiol.*, 2014, **80**, 6458–6464.

18. J. O'Hair, Q. Jin, D. Yu, N. Poe, H. Li, S. Thapa, S. Zhou and H. Huang, *ACS Sustain. Chem. Eng.*, 2020, **8**, 11244–11252.

19. A. Shaw, F. Lam, M. Hamilton, A. Consiglio, K. MacEwen, E. Brevnova, E. Greenhagen, W. LaTouf, C. South, H. Dijken and G. Stephanopoulos, *Science*, 2016, **353**, 583–586.

20. T. T. Selão, A. Wlodarczyk, P. J. Nixon and B. Norling, *Metab. Eng.*, 2019, **54**, 255–263.

21. K. Motomura, K. Sano, S. Watanabe, A. Kanbara, A.-H. G. Nasser, T. Ikeda, T. Ishida, H. Funabashi, A. Kuroda and R. Hirota, *ACS Synth. Biol.*, 2018, **7**, 2189–2198.

22. S. I. González-Morales, N. B. Pacheco-Gutiérrez, C. A. Ramírez-Rodríguez, A. A. Brito-Bello, P. Estrella-Hernández, L. Herrera-Estrella and D. L. López-Arredondo, *Biotechnol. Biofuels*, 2020, **13**, 119.

23. Z.-W. Guo, X.-Y. Ou, S. Liang, H.-F. Gao, L.-Y. Zhang, M.-H. Zong and W.-Y. Lou, *ACS Synth. Biol.*, 2020, **9**, 2537–2545.

24. X. Feng, L. Jiang, X. Han, X. Liu, Z. Zhao, H. Liu, M. Xian and G. Zhao, *Microb. Cell Fact.*, 2017, **16**, 209.

View Article Online
DOI: 10.1039/D0GC03044A

25. X. Guo, C. Cao, Y. Wang, C. Li, M. Wu, Y. Chen, C. Zhang, H. Pei and D. Xiao, *Biotechnol. Biofuels*, 2014, **7**, 44.

26. M. A. Pearson, I.-S. Park, R. A. Schaller, L. O. Michel, P. A. Karplus and R. P. Hausinger, *Biochemistry*, 2000, **39**, 8575–8584.

27. S.-G. Jung, J.-H. Jang, A.-Y. Kim, M.-C. Lim, B. Kim, J. Lee and Y.-R. Kim, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 1997–2007.

28. K. Jantama, P. Polyiam, P. Khunnonkwao, S. Chan, M. Sangproo, K. Khor, S. S. Jantama and S. Kanchanatawee, *Metab. Eng.*, 2015, **30**, 16–26.

29. X.-J. Ji, H. Huang, J. Du, J.-G. Zhu, L.-J. Ren, N. Hu and S. Li, *Bioresour. Technol.*, 2009, **100**, 3410–3414.

30. S. Brisse, F. Grimont and P. A. D. Grimont, eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, Springer New York, New York, NY, 2006, pp. 159–196.

31. X.-J. Ji, H. Huang, J. Du, J.-G. Zhu, L.-J. Ren, S. Li and Z.-K. Nie, *Bioresour. Technol.*, 2009, **100**, 5214–5218.

32. T. Yang, Z. Rao, G. Hu, X. Zhang, M. Liu, Y. Dai, M. Xu, Z. Xu and S.-T. Yang, *Biotechnol. Biofuels*, 2015, **8**, 129.

33. K. Khaertynov, V. Anokhin, A. Rizvanov, Y. Davidyuk, D. Semyenova, S. Lubin and N. Skvortsova, *Front. Med.*, , DOI:10.3389/fmed.2018.00225.

View Article Online
DOI: 10.1039/D0GC03044A

34. M. Regué, L. Izquierdo, S. Fresno, N. Piqué, M. M. Corsaro, T. Naldi, C. De Castro, D. Waidelich, S. Merino and J. M. Tomás, *J. Bacteriol.*, 2005, **187**, 4198–4206.

35. E. Frirdich, E. Vinogradov and C. Whitfield, *J. Biol. Chem.*, 2004, **279**, 27928–27940.

36. L. Izquierdo, N. Coderch, N. Piqué, E. Bedini, M. M. Corsaro, S. Merino, S. Fresno, J. M. Tomás and M. Regué, *J. Bacteriol.*, 2003, **185**, 7213–7221.

37. J. Yuan, Y.-Z. He, Z.-W. Guo, H.-F. Gao, F.-B. Chen, L.-Z. Li, Y.-Y. Li and L.-Y. Zhang, *BioResources*, 2017, **12**, 4926–4942.

38. R. Sorek, C. M. Lawrence and B. Wiedenheft, *Annu. Rev. Biochem.*, 2013, **82**, 237–266.

39. H. Harvey, J. Bondy-Denomy, H. Marquis, K. M. Sztanko, A. R. Davidson and L. L. Burrows, *Nat. Microbiol.*, 2018, **3**, 47–52.

40. S. J. Labrie, J. E. Samson and S. Moineau, *Nat. Rev. Microbiol.*, 2010, **8**, 317–327.

41. Y.-G. Lee and J.-H. Seo, *Biotechnol. Biofuels*, 2019, **12**, 204.

42. J. Fu, G. Huo, L. Feng, Y. Mao, Z. Wang, H. Ma, T. Chen and X. Zhao, *Biotechnol. Biofuels*, 2016, **9**, 90.

43. J.-J. Dai, J.-S. Cheng, Y.-Q. Liang, T. Jiang and Y.-J. Yuan, *Bioresour. Technol.*, 2014, **167**, 433–440.

View Article Online
DOI: 10.1039/D0GC03044A

44. L. Zhang, J. Sun, Y. Hao, J. Zhu, J. Chu, D. Wei and Y. Shen, *J. Ind. Microbiol. Biotechnol.*, 2010, **37**, 857–862.

45. M.-Y. Jung, H.-M. Jung, J. Lee and M.-K. Oh, *Biotechnol. Biofuels*, 2015, **8**, 106.

46. X.-J. Ji, L.-G. Liu, M.-Q. Shen, Z.-K. Nie, Y.-J. Tong and H. Huang, *Biotechnol. Bioeng.*, 2015, **112**, 1056–1059.

View Article Online
DOI: 10.1039/D0GC03044A

Tables

Table 1. Strains and plasmids used in this study.

Strains, plasmids	Description	Source
Strains		
KP-OU7	Wild strain of *K. pneumoniae* OU7, deposited in China Center for Type Culture Collection (CCTCC NO. M2017449)	Lab collection
KP-OU7-ME1	*K. pneumoniae* OU7 (Δ*ldhA*)	This study
KP-OU7-ME2	*K. pneumoniae* OU7 (Δ*ldhA*Δ*adhE*)	This study
KP-OU7-ME3	*K. pneumoniae* OU7 (Δ*ldhA*Δ*adhE*Δ*pta*)	This study
KP-OU7-ME4	*K. pneumoniae* OU7 (Δ*ldhA*Δ*adhE*Δ*pta*Δ*wabG*)	This study
KP-OU7-ME5	KP-OU7-ME4 at the 30th transfer during metabolic evolution	This study
E. coli S17-1 λ*pir*	Host strain for pRE112, *SmR TpR thi-1 pro recA* hsdR-M^{+}RP4: 2-Tc:Mu: Km Tn7 λ*pir*	Lab collection
Plasmids		
pRE112	Suicide vector, R6k origin, SacB Cm^{R} *oriV oriT*	Lab collection
pRE112-Δ*ldhA*	Suicide vector for *ldhA* knockout	This study
pRE112-Δ*adhE*	Suicide vector for *adhE* knockout	This study
pRE112-Δ*pta*	Suicide vector for *pta* knockout	This study
pRE112-Δ*wabG*	Suicide vector for *wabG* knockout	This study

View Article Online
DOI: 10.1039/D0GC03044A

Table 2. Fermentation performance of the different 2,3-BD producers by fed-batch style.

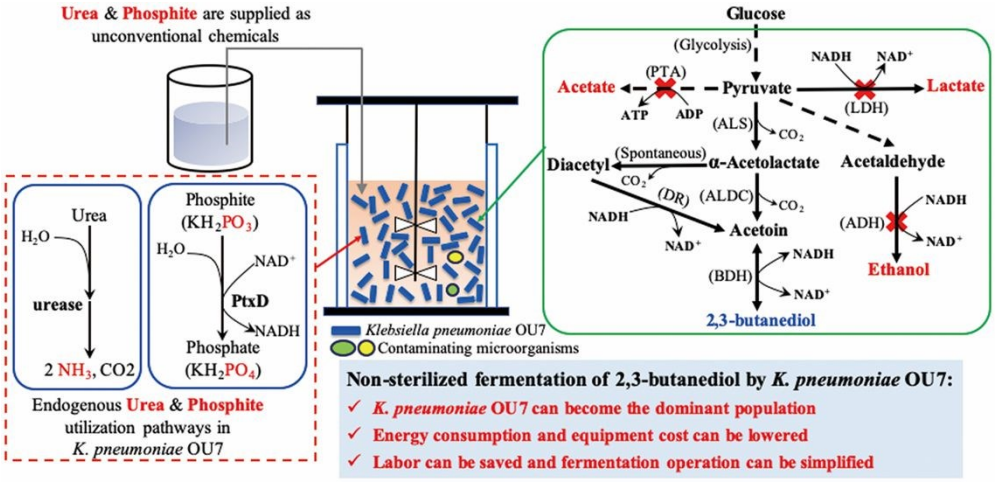
Strain	Phosphorus and nitrogen Source	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	Reference
B. subtilis	3 g/L tryptone, 20 g/L yeast extract	103.7	0.459	0.487	[42]
B. licheniformis	5.7 g/L yeast extract, 15 g/L corn steep liquor, 2 g/L $K_2HPO_4 \cdot 3H_2O$	115	2.4	0.47	[16]
P. polymyxa	10 g/L yeast extract, 4 g/L K_2HPO_4, 2 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$	71.71	1.33	0.39	[43]
S. marcescens	33.36 g/L yeast extract, 1 g/L $NH_4H_2PO_4$	152	2.67	0.463	[44]
E. aerogenes	3 g/L KH_2PO_4, 6.8 g/L Na_2HPO_4, 5.35 g/L $(NH_4)_2SO_4$, 5 g/L yeast extract, 10 g/L casamino acid	140	2.59	ns [a]	[45]
E. coli	7.56 mM $NH_4H_2PO_4$, 19.92 mM $(NH_4)_2HPO_4$	115	1.44	0.42	[46]
S. cerevisiae	10 g/L yeast extract, 20 g/L peptone	178	1.88	0.34	[41]
K. oxytoca	19.92 mM $(NH_4)_2HPO_4$, 7.56 mM $NH_4H_2PO_4$	117.4	1.20	0.49	[28]
K. pneumoniae	10 g/L yeast extract, 10 g/L KH_2PO_4, 7.2 g/L K_2HPO_4, 2 g/L $(NH_4)_2SO_4$	116	2.23	0.49	[25]
K. pneumoniae	20 g/L urea, 8 mM KH_2PO_3	84.53	1.17	0.38	This study

[a] ns: not specified.

View Article Online
DOI: 10.1039/D0GC03044A

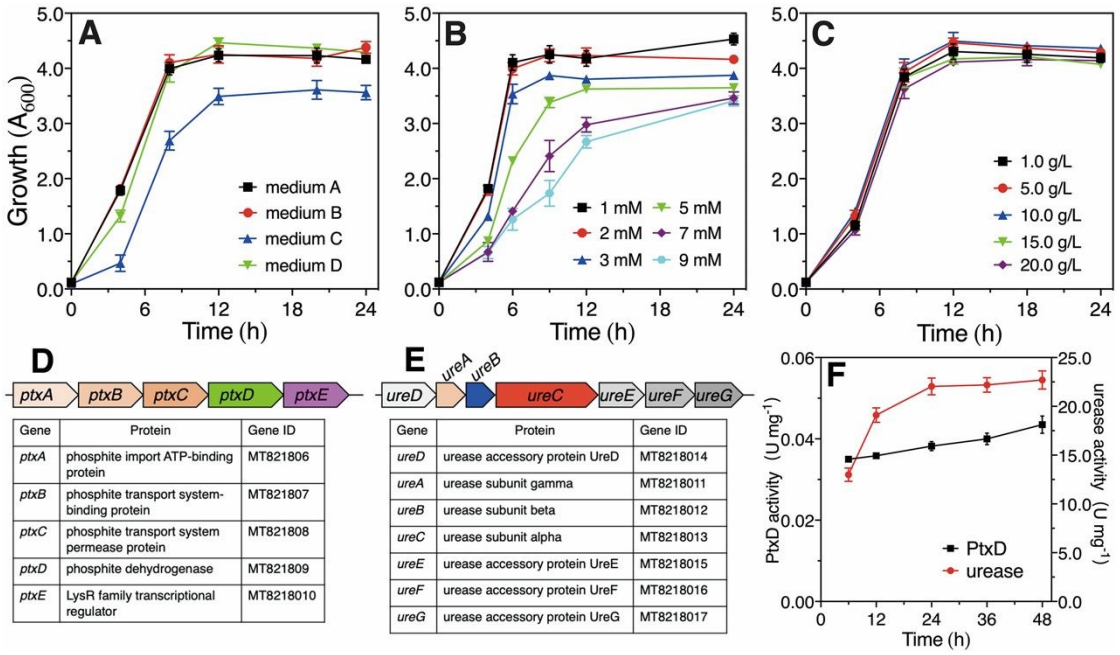
Figure captions

Fig. 1. Non-sterilized fermentation of 2,3-BD using engineered *K. pneumoniae* OU7. Phosphorous and nitrogen are the essential microbial growth nutrients and are derived from the xenobiotics (phosphite and urea). *K. pneumoniae* OU7, a robust strain with endogenous urea and phosphite assimilating pathways, was confirmed to withstand deliberate contamination without sterilization or antibiotics.



View Article Online
DOI: 10.1039/D0GC03044A

Fig. 2. Growth curves of *K. pneumoniae* OU7 with 10 g/L glucose. (A) Growth curves of *K. pneumoniae* OU7 in different MOPS medium. Medium A: KH_2PO_4 (1.32 mM) and NH_4Cl (9.5 mM) as the P and N sources; Medium B: KH_2PO_3 (1.32 mM) and NH_4Cl (9.5 mM) as the P and N sources; Medium C: KH_2PO_4 (1.32 mM) and urea (9.5 mM, without $NiCl_2$) as the P and N sources; Medium D: KH_2PO_4 (1.32 mM) and 9.5 mM urea (9.5 mM, with 0.1 g/L $NiCl_2$) as the P and N sources. (B) Effect of phosphite concentration on growth of *K. pneumoniae* OU7 in auxotrophic MOPS medium (with 9.5 mM urea and 0.1 g/L $NiCl_2$). (C) Effect of urea concentration on growth of *K. pneumoniae* OU7 in auxotrophic MOPS medium (with 1.32 mM KH_2PO_3 and 0.1 g/L $NiCl_2$). (D) Phosphite utilization gene operon (*ptxABCDE*) in *K. pneumoniae* OU7. (E) Urea utilization gene cluster (*ureDABCEFG*) in *K. pneumoniae* OU7. (F) Enzyme activity assays of PtxD and urease in *K. pneumoniae* OU7.

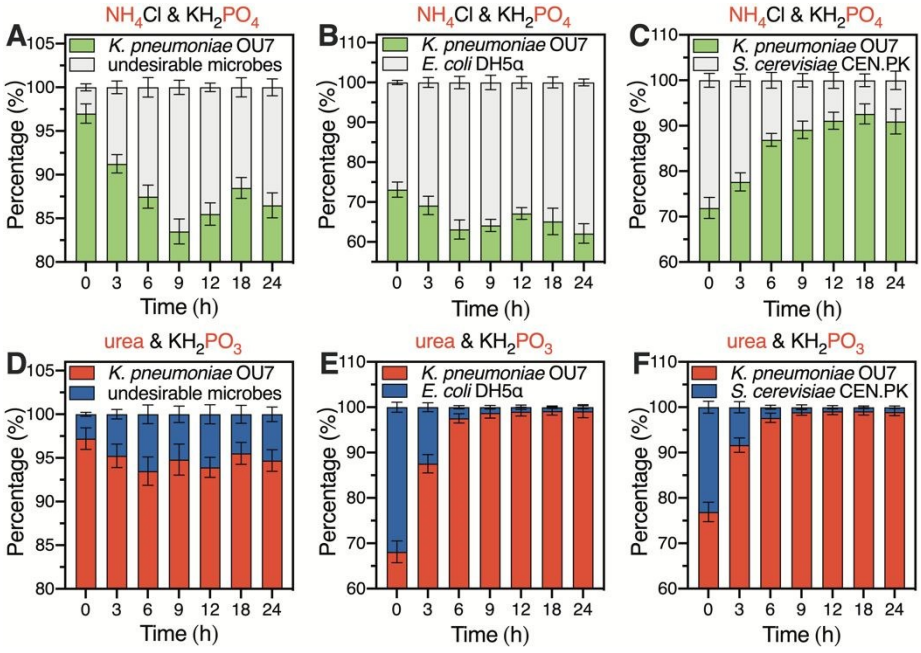


Gene	Protein	Gene ID
ptxA	phosphite import ATP-binding protein	MT821806
ptxB	phosphite transport system-binding protein	MT821807
ptxC	phosphite transport system permease protein	MT821808
ptxD	phosphite dehydrogenase	MT821809
ptxE	LysR family transcriptional regulator	MT8218010

Gene	Protein	Gene ID
ureD	urease accessory protein UreD	MT8218014
ureA	urease subunit gamma	MT8218011
ureB	urease subunit beta	MT8218012
ureC	urease subunit alpha	MT8218013
ureE	urease accessory protein UreE	MT8218015
ureF	urease accessory protein UreF	MT8218016
ureG	urease accessory protein UreG	MT8218017

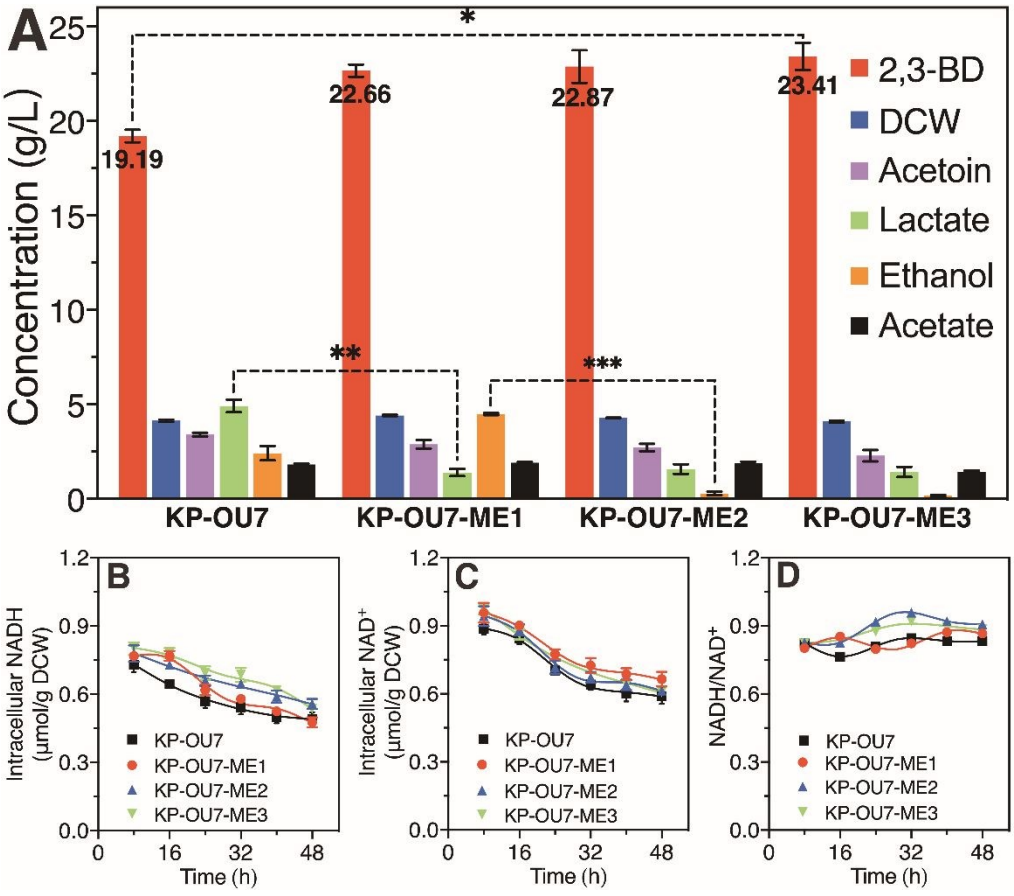
View Article Online
DOI: 10.1039/D0GC03044A

Fig. 3. Quantitative comparison of *K. pneumoniae* OU7 and undesirable microbes at non-sterilized conventional (A) or auxotrophic (D) MOPS medium. Coculture of *K. pneumoniae* and *E. coli* at sterile conventional (B) or auxotrophic (E) MOPS medium. Coculture of *K. pneumoniae* and *S. cerevisiae* at sterile conventional (C) or auxotrophic (F) MOPS medium.



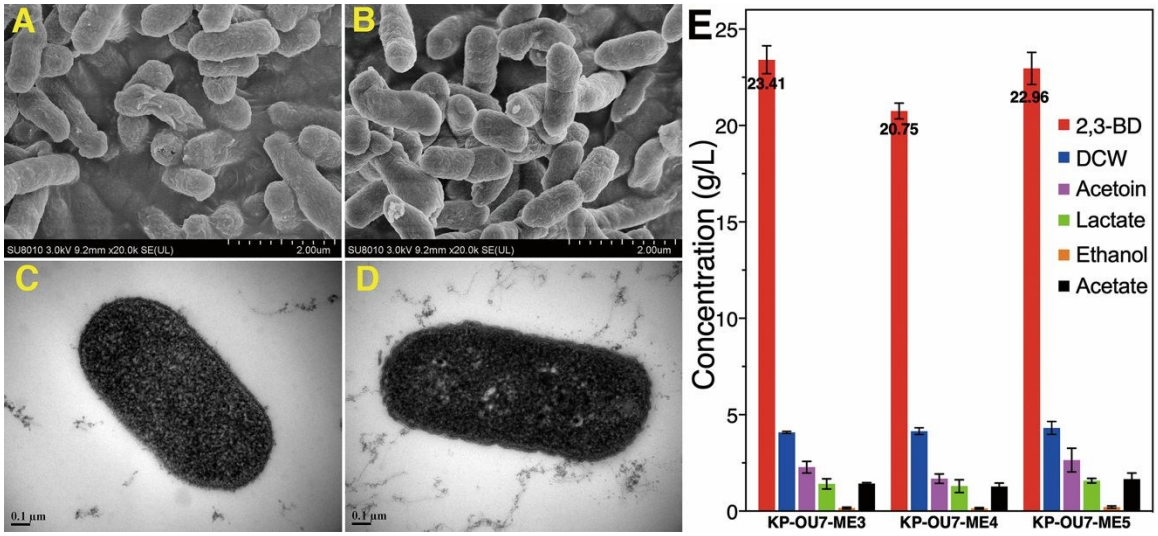
View Article Online
DOI: 10.1039/D0GC03044A

Fig. 4. (A) Metabolite profiles of *K. pneumoniae* OU7 and its mutants in auxotrophic MOPS medium (containing 80 g/L glucose) in 48-h-flask fermentation. Intracellular NADH content (B), NAD^+ content (C), and NADH/NAD^+ ratio (D) of *K. pneumoniae* OU7 and its mutants in the batch fermentation. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.



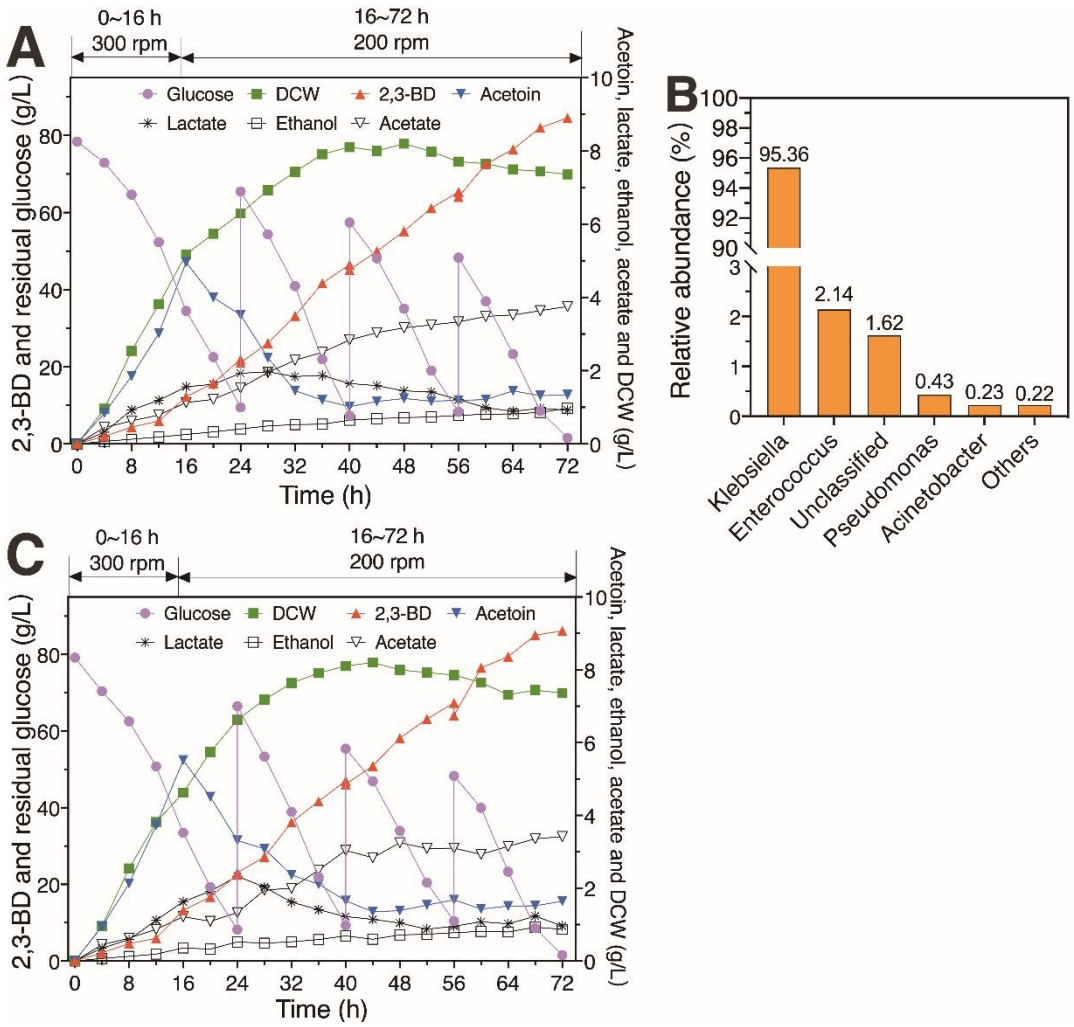
View Article Online
DOI: 10.1039/D0GC03044A

Fig. 5. FE-SEM analysis of the surface morphology of KP-OU7-ME3 (A) and KP-OU7-ME4 (B). TEM analysis of the microstructure of KP-OU7-ME3 (C) and KP-OU7-ME4 (D). (E) Metabolite profiles of KP-OU7-ME3, KP-OU7-ME4 and KP-OU7-ME5 in auxotrophic MOPS medium (containing 80 g/L glucose) in 48-h-flask fermentation.



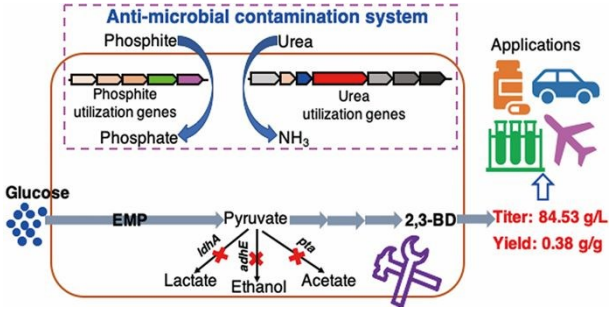
View Article Online
DOI: 10.1039/D0GC03044A

Fig. 6. (A) Non-sterilized fed-batch fermentation of 2,3-BD by KP-OU7-ME5. (B) Relative abundance (16S rRNA) of KP-OU7-ME5 and undesirable microbes in non-sterilized fermentation at 72 h. (C) Sterilized fed-batch fermentation of 2,3-BD by KP-OU7-ME5.



View Article Online
DOI: 10.1039/D0GC03044A

Table of contents entry



Non-sterilized fermentation of 2,3-butanediol by a robust *K. pneumoniae* in an energy- and cost-effective manner.