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ORIGINAL PAPER

Effect of aeration strategy on the metabolic flux of *Klebsiella pneumoniae* producing 1,3-propanediol in continuous cultures at different glycerol concentrations

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Abstract The microbial production of 1,3-propaneidol (1,3-PD) by *Klebsiella pneumoniae* in continuous fermentation was investigated under low, medium and high glycerol concentrations in the absence and presence of oxygen. The production of 1,3-PD increased with increasing glycerol concentrations, reaching a maximum (266 mmol l^{-1}) under high glycerol concentration (760 mmol l^{-1}) with air sparging at 0.04 vvm. The yield of 1,3-PD, however, decreased gradually with increasing glycerol concentrations, with the highest yield (0.52 mol mol^{-1}) obtained for low glycerol concentration (270 mmol l^{-1}) under anaerobic condition. Enzyme activity assays showed that the specific activity of glycerol dehydratase was highest (0.04 U mg^{-1}) for culture sparged with 0.04 vvm air under high glycerol concentration. The specific activities of glycerol dehydrogenase and 1,3-propanediol oxidoreductase were also improved for all glycerol concentrations and in the presence of oxygen, implying that the *dha* operon was not repressed under microaerobic conditions. Analysis of metabolic fluxes showed that more carbon flux was shifted to the oxidative pathway with increasing glycerol concentrations, resulting in a reduced flux to 1,3-PD formation. However, the increases in carbon fluxes were not evenly distributed among the oxidative branches of the pathway. Furthermore, ethanol and acetic acid levels were slightly increased whereas 2,3-butanediol and lactic levels were greatly enhanced.

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Abbreviations

C_{S0}	Glycerol concentration in the feed medium mmol l^{-1}
Cs	Residual concentration of glycerol mmol l^{-1}
C_p	Product concentration mmol l^{-1}
D	Dilution rate h^{-1}
q_p	Formation rate of products mmol l^{-1}
q_s	Substrate uptake rate mmol l^{-1}
v_{BD}	Flux distribution of 2,3-butanediol mmol $mmol^{-1}$
v_{CO_2}	Flux distribution of CO_2 mmol $mmol^{-1}$
v_{EtOH}	Flux distribution of ethanol mmol $mmol^{-1}$
v_{For}	Flux distribution of formic acid mmol $mmol^{-1}$
v_{HAc}	Flux distribution of acetic acid mmol $mmol^{-1}$
v_{Lac}	Flux distribution of lactic acid mmol $mmol^{-1}$
v_{Suc}	Flux distribution of succinic acid mmol $mmol^{-1}$
v_{Pyr}	Flux distribution of pyruvic acid mmol $mmol^{-1}$
v_{Cit}	Flux distribution of citric acid mmol $mmol^{-1}$
v_{Mal}	Flux distribution of malic acid mmol $mmol^{-1}$
v_{Fum}	Flux distribution of fumaric acid mmol $mmol^{-1}$
v_{PD}	Flux distribution of 1,3-propanediol mmol $mmol^{-1}$
v_s	Consumption rate of substrate mmol $mmol^{-1}$
R_C	Carbon recovery
X	Biomass concentration g l^{-1}
$Y_{PD/s}$	Molar yield of 1,3-propanediol mol mol^{-1}
DCW	Dry cell weight
1,3-PD	1,3-Propanediol
2,3-BD	2,3-Butanediol
3-HPA	3-Hydroxypropanaldehyde

DHA	Dihydroxyacetone
TCA	Tricarboxylic acid
GDHt	Glycerol dehydratase
GDH	Glycerol dehydrogenase
PDOR	1,3-Propanediol oxidoreductase
DHAK	Dihydroxyacetone kinase
ORP	Oxidoreduction potential
DO	Dissolve oxygen
LG	Low glycerol concentration
MG	Medium glycerol concentration
HG	High glycerol concentration

Introduction

1,3-Propanediol (1,3-PD) is a valuable chemical that is used as a monomer for the production of polyesters, polyether and polyurethanes. In recent years, much attention has focused on its microbial production [4, 28]. In nature, many microorganisms, such as *Klebsiella pneumoniae*, *Citrobacters* and *Clostridia*, are able to convert glycerol to 1,3-PD, and *K. pneumoniae* has been widely investigated due to its high 1,3-PD productivity. *K. pneumoniae* is a facultative bacterium, and can dissimilate glycerol into 1,3-PD under anaerobic or aerobic conditions. The bioconversion of glycerol is mainly attributed to the *dha* system or *glp* system under anaerobic or aerobic conditions, respectively (see Fig. 1). The *dha* system is composed of four enzymes: glycerol dehydrogenase (GDH), dihydroxyacetone kinase (DHAK), glycerol dehydratase (GDHt), and 1,3-propanediol oxidoreductase (PDOR). The *glp* system is composed of two enzymes: glycerol kinase and glycerol-3-phosphate dehydrogenase [14]. Glycerol metabolism involves two pathways: the reductive and the oxidative pathways. Glycerol is first converted to 3-hydroxypropanaldehyde (3-HPA) by GDHt, which is then reduced to 1,3-PD by $NADH_2$-dependent PDOR. Through the oxidative pathway glycerol is dehydrogenated by NAD^+-dependent GDH to dihydroxyacetone (DHA), which is then phosphorylated by ATP-dependent DHAK before entering glycolysis. This pathway produces by-products such as ethanol, acetic acid, lactic acid, 2,3-butanediol (2,3-BD), also provides energy and reducing equivalents ($NADH_2$) for biomass and 1,3-PD syntheses [14].

The bioconversion of glycerol to 1,3-PD is usually carried out under anaerobic conditions. However, the low biomass and target product formation rate are somewhat unsatisfactory. Recently, it was found that 1,3-PD could still be obtained by *K. pneumoniae* under microaerobic conditions [9, 17]. The productivity of 1,3-PD was enhanced from 0.8 to 1.57 g l^{-1} h^{-1} by changing the aeration condition from anaerobic to microaerobic [4]. Furthermore, the 1,3-PD concentration increased to 921 mmol l^{-1} using an anaerobic/aerobic strategy [8]. More recently, Yang et al. [29] reported on a lactic acid-deficient mutant of *K. oxytolla* that accumulated about 1,092 mmol l^{-1} 1,3-PD and 667 mmol l^{-1} 2,3-BD under microaerobic condition. According to these reports, it is suggested that oxygen, as an exogenous electron acceptor, could enhance cell growth and 1,3-PD formation. Microaerobic cultivation could also reduce culture time and the concentration of toxic fermentative by-products [5]. However, it was shown that more substrates are channeled into the oxidative pathway of glycerol metabolism under increasing air flow, leading to decrease of the yield of 1,3-PD [19]. Stoichiometric analysis shows that lower oxygen consumption rate (< 0.57 mol mol^{-1}) and optimal RQ in a range of 11.34 and 2.66 are beneficial for improving 1,3-PD productivity [6, 33]. Too little or too much oxygen supply may not be conductive to the production of 1,3-PD. These studies imply that there may exist a critical level of aeration for 1,3-PD production under microaerobic condition. Thus, it is necessary to analyze and verify the effect of oxygen on the production of 1,3-PD and to find a reliable aeration strategy.

Knowledge of cell physiology is helpful for further improving the production of 1,3-PD by applying metabolic engineering approaches to the strain. Glycerol metabolism by *K. pneumoniae* under anaerobic condition has been stoichiometrically analyzed in previous studies [25, 30, 32]. The physiological mechanism of sequential products synthesis has been studied with 0.65 vvm air sparging [31] and the metabolic fluxes of glycerol in *K. pneumoniae* have been analyzed in batch and fed-batch fermentations under anaerobic and microaerobic conditions [7, 19]. However, to our knowledge, carbon flux distribution and cell physiology have not been studied at steady states under microaerobic conditions compared with anaerobic conditions.

In this article, continuous cultures of *K. pneumoniae* were conducted at different concentrations of glycerol under anaerobic and microaerobic conditions. Activity assays of three key enzymes (GDH, PDOR, GDHt) and metabolic flux analysis were carried out to better understand the progressive shift of glycerol metabolism in *K. pneumoniae* from anaerobic to microaerobic conditions.

Materials and methods

Strain and culture conditions

Klebsiella pneumoniae (CGMCC 2028) was isolated from soil and preserved in China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The cultivation medium for continuous fermentations was a

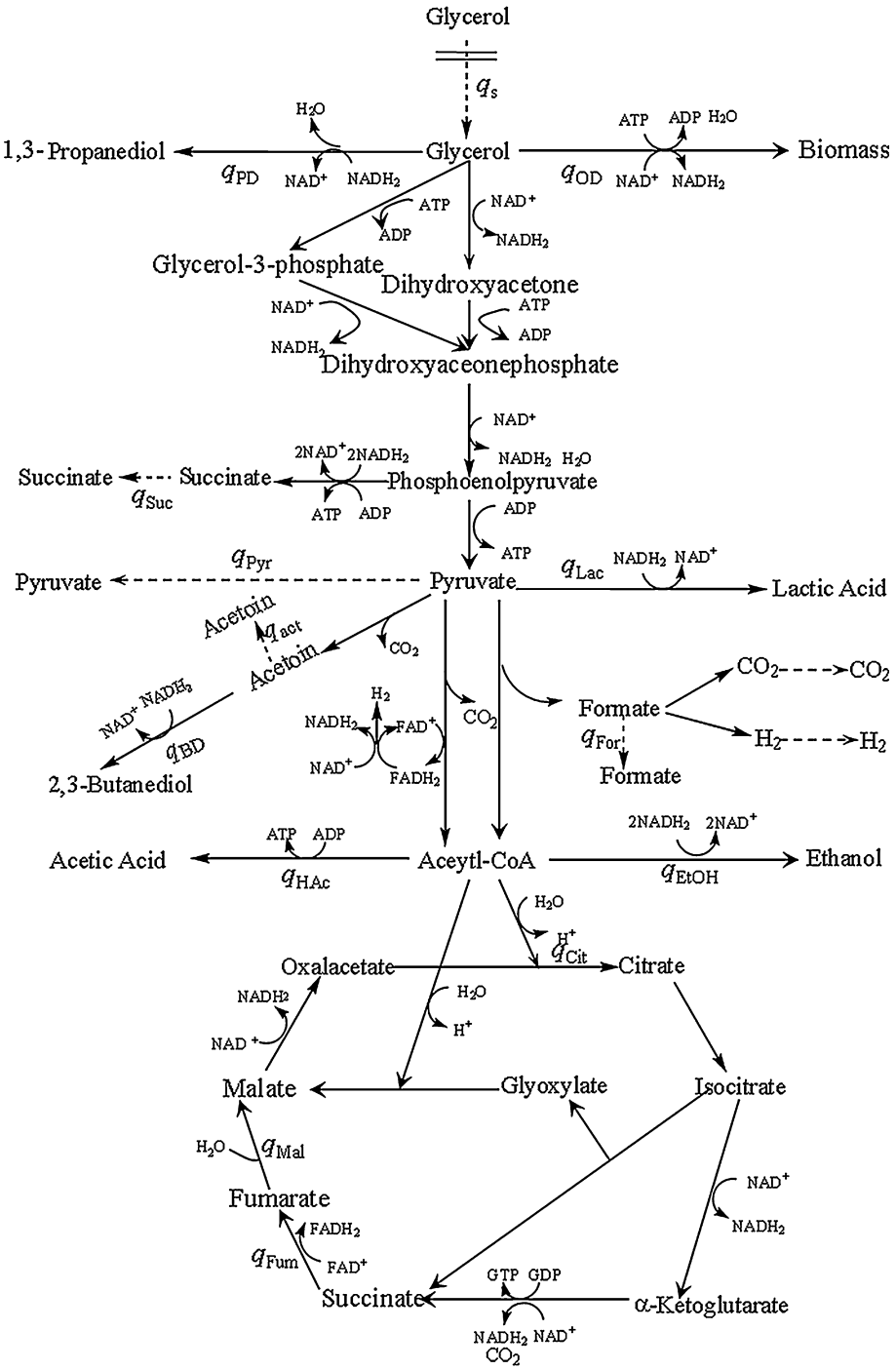

Fig. 1 Central metabolic network of 1,3-PD production in *Klebsiella pneumoniae*

chemically defined medium as described by Menzel et al. [21].

For the starting culture, *K. pneumoniae* was seeded in a 250-ml flask containing 100 ml medium and grown for 12 h at 37°C. The fermentable cultivation was carried out in a 5-l stirring bioreactor with a working volume of 1.5 l, and equipped with controls for temperature, pH, dissolved oxygen (DO) and agitation speed. Temperature, pH and agitation speed were maintained at 37°C, 7.0 and 300 rpm, respectively. The pH was controlled by addition of 5 mol l^{-1} sodium hydroxide solution. Oxidoreduction potential (ORP) measured with a redox electrode. CO_2 was determined on-line with an infrared carbon dioxide analyzer (EGAS-L; Braun, Germany).

Fermentation was carried out with a glycerol concentration of 526 mmol l^{-1} and, once the culture reached exponential growth phase, feeding was started at a dilution rate of 0.2 h^{-1}. Steady-state was obtained after a continuous flow of at least four working volumes of the medium and when the CO_2 fraction in the exit gas stream remained constant. The glycerol concentration in the feeding medium was 270 mmol l^{-1} (low glycerol level, LG), 434 mmol l^{-1} (medium glycerol level, MG), and 760 mmol l^{-1} (high glycerol level, HG). During fermentation, the bioreactor was sparged with 0.02 vvm N_2 to ensure anaerobic condition, or with 0.02, 0.04, 0.08 vvm air to provide microaerobic conditions.

Analytical methods

Cell growth was monitored at 650 nm and converted to dry cell weight (DCW) as previously described [5]. The concentrations of 1,3-PD, ethanol, 2,3-butandiol (2,3-BD), and acetoin were measured as described by Chen et al. [5]. Glycerol was assayed by a modified titration [26]. Organic acids (formic acid, acetic acid, lactic acid, citric acid, succinic acid, fumaric acid, pyruvic acid and malic acid) were measured by HPLC [27]. The concentration of 3-HPA was determined by a colorimetric method containing tryptophan adapted from Circle et al. [11].

Preparation of cell-free extracts and enzyme specific activity measurements

Cells were harvested by centrifugation at 6,225*g* for 30 min at 4°C. The cell pellets were washed twice with a cold solution of 5 mmol l^{-1} potassium phosphate buffer (pH 7.0). For disruption, the pellets were re-suspended in the same buffer with 2 mmol l^{-1} dithiothreitol (DTT), and sonicated for 3 s with 3 s pause at a power of 400 W. This cycle was repeated 140 times. Cell debris was removed by centrifugation as described above. The supernatant was used for enzyme assay. Protein concentrations of cell extracts were determined according to Lowry et al. [18]. GDH, PDOR and GDHt specific activities were determined as described by Barbirato et al. [2].

Calculation of metabolic flux and carbon recovery (R_C)

The product formation rate (q_p) and the substrate uptake rate (q_s) were calculated as follows:

$$q_p = D \cdot C_p \quad (1)$$

$$q_s = D \cdot (C_{S0} - C_S) \quad (2)$$

Where D is the dilution rate, C_p is the product concentration, C_{S0} is the glycerol concentration in the feed medium, and C_s is the residual glycerol concentration. The metabolic flux distributions of the product (v_p) was obtained by dividing the product formation rates (q_p) by the glycerol uptake rate (q_s) and multiplying by 100.

To check the consistency of the data, a carbon recovery (R_C) calculation was carried out at each steady state obtained during the continuous fermentation. In order to proceed with that calculation, the formula $C_4H_7O_2N$ was denoted as the elemental composition of biomass, which corresponded to a molecular weigh of 101 g mol^{-1} [16]. The R_c were calculated as follow:

$$R_c = (q_{For} + q_{CO_2} + 2q_{EtOH} + 2q_{HAc} + 3q_{PD} + 3q_{HPA} + 3q_{Pyr} + 4q_{BD} + 4q_{Suc} + 4q_{Mal} + 4q_{Fum} + 4q_{CDW} + 6q_{Cit})/3q_s \quad (3)$$

Results and discussion

Effects of different culture conditions on cell growth and productions of major metabolites

The effects of glycerol concentrations and aeration on cell growth and major metabolites, e.g., 1,3-PD, 2,3-BD, ethanol, and major organic acids, are shown in Figs. 2, 3 and 4.

In the absence and presence of oxygen, the biomass was highest and lowest under MG and HG, respectively. Furthermore, the biomass increased with increases in aeration rates, with a more pronounced trend observed for HG (Fig. 2). In the case of LG, the biomass only increased when the air sparging was increased to 0.04 vvm. Thus, growth of *K. pneumoniae* was affected by both glycerol limitation and excess.

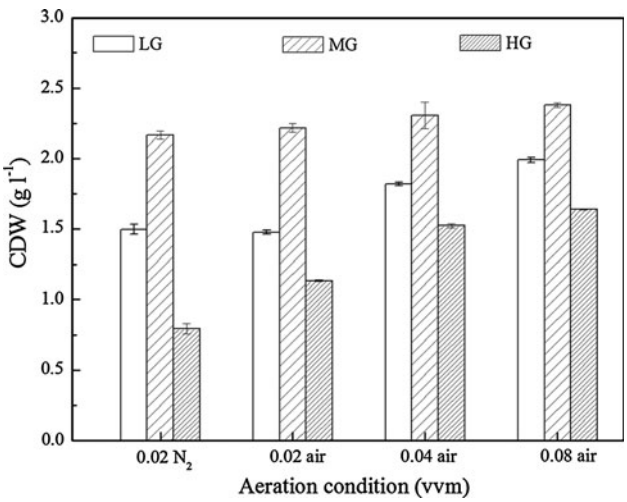

Fig. 2 Effect of aeration conditions on the DCW under different culture conditions

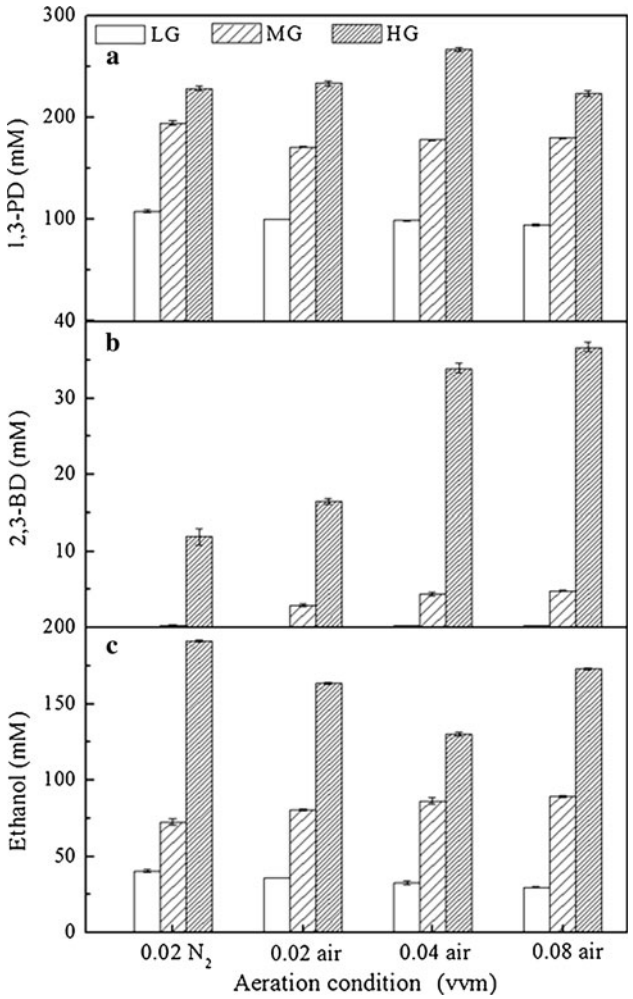

Fig. 3 Effect of aeration conditions on the concentrations of 1,3-PD, 2,3-BD and ethanol products under different culture conditions. **a** Profile of 1,3-PD concentration; **b** profile of 2,3-BD concentration; **c** profile of ethanol concentration

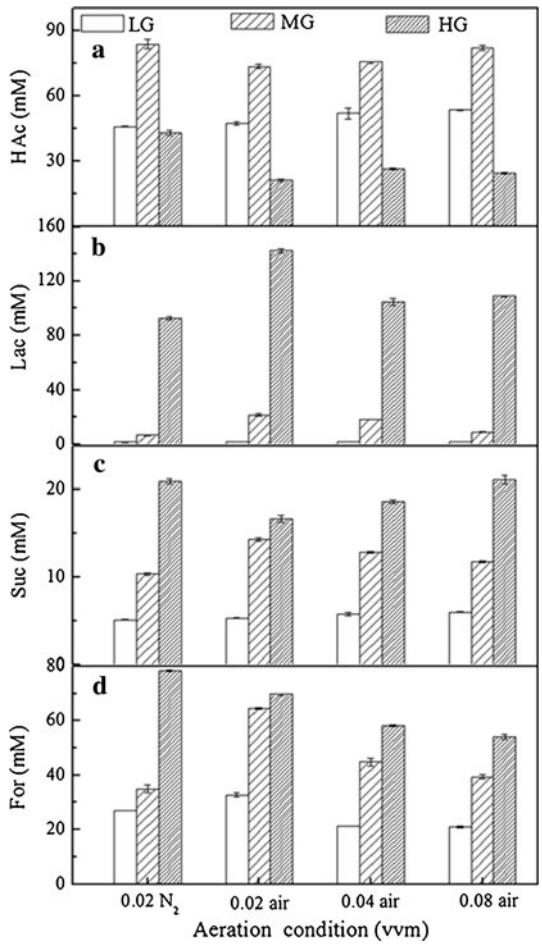

Fig. 4 Effect of aeration conditions on the concentrations of organic acids under different culture conditions. **a** Profile of acetic acid concentration; **b** profile of lactic acid concentration; **c** profile of succinic acid concentration; **d** profile of formic acid concentration

The concentration of the 1,3-PD was lowest under HG in the absence of oxygen. Among the different aeration rates, 1,3-PD reached the highest concentration (266 mmol l^{-1}) at air sparging of 0.04 vvm (Fig. 3a), representing a 17% increase over that achieved in the absence of oxygen. This concentration (266 mmol l^{-1}) was lower compared with the concentration of 1,3-PD (921 mmol l^{-1}) obtained in the anaerobic/aerobic fed-batch condition [8]. There are two reasons for the quite different results. Firstly, there exists a significant correlation with the production of 1,3-PD and cell growth [17, 23]. The DCW was about 1.5 g l^{-1} when the highest 1,3-PD was achieved (Fig. 2) in our study, yet the DCW was 3 g l^{-1} in fed-batch fermentation [8], which was almost two-fold higher than that in continuous culture. Secondly, previous studies have also shown that the concentration of glycerol could affect the productivity of *K. pneumonia* [3]. In fed-batch culture, residual glycerol is controlled in a suitable concentration (160–220 mmol l^{-1}) which helps to maintain a suitable environment for cell growth [15]. However, the residual glycerol was only 9.8 mmol l^{-1} when the concentration of 1,3-PD was highest in continuous culture (Table 1). For the above reasons, the concentration of 1,3-PD in continuous culture was lower than that in fed-batch culture. However, the productivity of 1,3-PD under this condition was 4.04 g $l^{-1}h^{-1}$ which was 2.8 times higher than that achieved in fed-batch condition [8]. Furthermore, to get the high concentration and productivity of 1,3-PD at the same time, we have designed a new multi-stage fed-continuous process to manipulate the concentration of residual glycerol and improve the activity of biomass combined the benefits of fed-batch and -continuous culture. The concentration and productivity of 1,3-PD can be increased to 601 mM and 4.6 g l^{-1} h^{-1}. This new process will be published elsewhere. Under LG and MG, 1,3-PD

Table 1 Residual glycerol concentration, carbon recoveries (R_C) and the yield of 1,3-PD under different culture conditions

Aeration condition (vvm)	Residual glycerol (mmol)	R_c	$Y_{PD/S}$ (mol mol^{-1})
C_{S0} = 270 mmol l^{-1}			
0.02 N_2	0.98	1.07	0.52
0.02 air	0.98	1.02	0.48
0.04 air	0.98	1.04	0.48
0.08 air	0.98	1.03	0.46
C_{S0} = 434 mmol l^{-1}			
0.02 N_2	3.68	1.01	0.49
0.02 air	3.68	1.00	0.43
0.04 air	3.68	1.04	0.45
0.08 air	3.68	1.05	0.46
C_{S0} = 760 mmol l^{-1}			
0.02 N_2	7.64	0.90	0.34
0.02 air	11.41	0.98	0.37
0.04 air	9.80	0.97	0.41
0.08 air	8.48	0.97	0.33

concentration was slightly higher in the absence of oxygen, while it remained almost unchanged among the three different aeration rates. These trends were similar to those observed for the molar yield of 1,3-PD versus glycerol (Table 1). The yields decreased when the aeration shifted from anaerobic to microaerobic condition. The highest yield was 0.52 and 0.49 mol mol^{-1} in the absence of oxygen under both LG and MG. There was very low residual glycerol under both LG and MG, indicating glycerol limitation. Thus, the aeration did not influence the residual glycerol concentration of fermentations under LG or MG for the lack of substrate. While under HG, more of the residual glycerol accumulated, and the highest yield of 1,3-PD was 0.41 mol mol^{-1} with 0.04 vvm air flow sparged, which was 20.6% higher than in the absence of oxygen. In the presence of the oxygen, the residual glycerol decreased with increases in aeration rates under HG. This can be explained that more cells (Fig. 2) can consume more glycerol by the presence of excess substrate in the medium. The concentrations of 2,3-BD increased as the glycerol concentration increased, and it was highest under HG without and with oxygen. Furthermore, under MG and HG, the concentrations of 2,3-BD started to increase in the presence of oxygen, and such increase continued to rise with further increases in aeration rate (Fig. 3b). The concentrations of ethanol increased as the glycerol concentration increased and it was highest under HG in the absence and presence of oxygen. In addition, the concentration of ethanol under LG and MG remained relatively unchanged when the aeration rate was increased from 0.02 to 0.08 vvm. However, under HG, the concentration of ethanol decreased when the aeration rate was increased to 0.04 vvm, and then began to increase at 0.08 vvm (Fig. 3c).

The productions of lactic acid, succinic acid and formic acid were always favored by an increase in the concentration of substrate in the medium, with the exception of acetic acid, and this was consistent with the highest productions observed under HG (Fig. 4). Under LG, the concentrations of acetic acid, lactic aicd and succinic acid remained relatively unchanged in the absence and presence of oxygen, and among the different aeration rates, while the concentrations of formic acid increased when the aeration shifted from anaerobic to microaerobic condition, but began to decrease when the aeration rate was increased to 0.04 vvm, and then remained relative unchanged at 0.08 vvm. Under MG, the concentrations of lactic acid, succinic acid and formic acid increased when the aeration shifted from anaerobic to microaerobic condition, and then began to decrease with increasing airflow rates, but the trend of acetic acid contrasted those of the other three organic acids. Furthermore, the concentration of formic acid declined, but the concentration of succinic acid increased under HG with increasing air flow rate in the absence of oxygen.

Effects of glycerol and different aeration conditions on metabolic flux distribution

Based on the central carbon metabolic network as depicted in Fig. 1, the specific metabolic flux under different culture conditions are listed in Table 2. Under LG and MG, the sum of the main products, including 1,3-PD, ethanol, acetic acid and biomass accounted for almost 90% of the whole carbon source. While, under HG, the main products were 1,3-PD, ethanol, lactic acid and 2,3-BD, but the carbon flux to 1,3-PD formation decreased compared to that under LG and MG.

The production of lactic acid was enhanced by the presence of excess substrate in the medium. This can be explained by the tendency of the organism to get rid of excess glycerol as well as to reduce the effect of more toxic products that might increase beyond the tolerable levels to the cells [30]. For each acetic acid molecule formed from the pyruvate pathway, an extra ATP is simultaneously formed, which can be used in biomass synthesis. When glycerol becomes excessive, the inhibition by substrate would retard cell growth, and the requirement for the ATP would decrease. Therefore, the generation of acetic acid would decrease, and the carbon flow is directed to other pathways such as the syntheses of ethanol and lactic acid, which do not form ATP, resulting in the contest for $NADH_2$ between these pathways and that of 1,3-PD pathway [4]. As a result, the yield of the 1,3-PD was reduced with increasing glycerol concentrations (Table 1).

Table 2 Metabolic flux distributions in *Klebsiella pneumoniae* under different culture conditions

Aeration condition (vvm)	v_{PD}	v_{HAc}	v_{EtOH}	v_{Lac}	v_{OD}	v_{BD}	v_{For}	v_{Suc}	v_{CO_2}	v_{HPA}	v_{Pyr}	v_{Cit}	v_{Mal}	v_{Fum}
$C_{S0} = 270$ mmol l^{-1}														
0.02 N_2	52.29	14.91	13.17	0.87	9.60	0.08	4.39	3.33	1.00	0.14	0.05	0.08	0.07	0.01
0.02 air	50.36	15.94	12.11	0.94	10.00	0.12	5.54	3.59	0.94	0.17	0.05	0.12	0.09	0.03
0.04 air	48.89	17.19	10.85	0.93	11.91	0.17	3.55	3.83	1.85	0.17	0.06	0.48	0.09	0.05
0.08 air	47.09	17.82	9.90	0.97	13.13	0.20	3.52	4.01	2.42	0.18	0.06	0.55	0.09	0.06
$C_{S0} = 434$ mmol l^{-1}														
0.02 N_2	53.15	15.28	13.25	1.89	7.82	0.12	3.19	3.78	1.12	0.13	0.03	0.19	0.03	0.01
0.02 air	45.21	12.98	14.23	5.75	7.75	1.04	5.68	5.06	1.02	0.14	0.05	0.50	0.38	0.06
0.04 air	46.33	13.13	15.06	4.78	7.94	1.54	3.90	4.47	1.73	0.14	0.05	0.44	0.34	0.04
0.08 air	47.02	14.31	15.66	2.41	8.23	1.69	3.44	4.12	2.38	0.15	0.04	0.27	0.24	0.01
$C_{S0} = 760$ mmol l^{-1}														
0.02 N_2	40.01	5.05	22.39	16.23	1.84	2.80	4.58	4.89	0.91	0.05	0.03	0.65	0.43	0.02
0.02 air	39.20	2.39	18.36	23.89	2.52	3.69	3.90	3.74	0.75	0.08	0.05	0.76	0.40	0.04
0.04 air	44.08	2.93	14.38	17.33	3.32	7.48	3.21	4.11	1.42	0.15	0.06	0.75	0.36	0.03
0.08 air	36.99	2.73	19.11	18.03	3.58	8.10	2.99	4.66	2.26	0.09	0.05	0.76	0.37	0.03

All the metabolic flux distributions of the product (v_p) are the product formation rates (q_p) divided by the glycerol uptake rate (q_s) multiplied by 100. Units of the v_p are mmol $mmol^{-1}$

Effects of glycerol and different aeration conditions on the key branch points

The alternation of the relative fluxes at three key branch points, including glycerol, pyruvate and acetyl-CoA are depicted in Tables 3, 4, and 5, respectively.

The first key point is the glycerol node, which partitioned in both oxidative and reductive pathways, as well as the biomass pathway. As shown in Table 3, the flux to the biomass enhanced with the increases in air flow but dropped off as glycerol concentration increased, due to substrate inhibition. When sparging with 0.02 vvm N_2, 0.02, 0.04, and 0.08 vvm air, the biomass fluxes decreased by 80.1, 74.8, 72.7 and 72.1%, respectively, under HG condition compared to that under LG condition. The highest decrease in biomass flux occurred with N_2 sparging, indicating that the cell growth was more liable to be suppressed by glycerol than by oxygen.

Our results confirmed that the flux to the reductive pathway decreased while the flux to the oxidative pathway increased with increasing aeration rates under LG or MG conditions. However, this trend was not obvious under HG condition, which implied that the regulation of metabolism was connected not only with the oxygen supply but also with the concentration of substrate.

The second key point is the pyruvate node, which switches into 2,3-BD, formic acid, lactic acid and acetyl-CoA. The channeling of pyruvic aicd into these different routes is obviously affected by the cultivation conditions. According to Table 4, the relative flux to acetyl-CoA and formic acid decreased while the flux to 2,3-BD and lactic acid increased with increases in glycerol concentrations. Thus, too much 2,3-BD and lactic formation will certainly reduce the flux from glycerol to 1,3-PD, since these metabolites also consume $NADH_2$. The flux of formic acid decreased with increasing air supply at the pyruvate node. This could be due to the negative effect of oxygen on the expression of the *pfl* gene encoding pyruvate formate-lyase (PFL) [20].

Under MG and HG, the relative fluxes from pyruvate to acetyl-CoA in the absence of oxygen were higher than those in the presence of oxygen (Table 4). The fluxes of acetyl-CoA under HG condition decreased by almost 50% compared to that under LG condition. However, without oxygen, the total absolute fluxes from glycerol to ethanol and acetic acid maintained at about 28% for all glycerol concentrations, but the total absolute fluxes from glycerol to 2,3-BD and lactic acid under HG improved by 2–19% over that obtained under LG (Table 2). The increases in 2,3-BD and lactic acid fluxes accounted for the sharp decrease in 1,3-PD production and biomass under HG. Therefore, under anaerobic condition and different substrate concentrations, the flux distribution was mainly adjusted to 2,3-BD and lactic acid rather than to ethanol and acetic acid.

In the presence of oxygen, the fluxes from glycerol to acetyl-CoA were similar between LG and MG. However, under HG condition, the flux to acetyl-CoA declined with increasing air sparging, while the flux to lactic acid was greatly enhanced. These results suggested that the metabolism of pyruvate is mainly regulated by the fluxes to 2,3-BD and lactic acid, and the enzyme system that

Table 3 Relative metabolic fluxes analysis of glycerol branch point

Aeration condition (vvm)	Glycerol (mmol mmol^{-1}, %)	Reductive pathway (mmol mmol^{-1}, %)	Oxidative pathway (mmol mmol^{-1}, %)	Biomass (mmol mmol^{-1}, %)
$C_{S0} = 270$ mmol l^{-1}				
0.02 N_2	100.00	52.30	38.11	9.60
0.02 air	100.00	50.53	39.47	10.00
0.04 air	100.00	49.06	39.03	11.91
0.08 air	100.00	47.27	39.59	13.13
$C_{S0} = 434$ mmol l^{-1}				
0.02 N_2	100.00	53.28	38.90	7.82
0.02 air	100.00	45.35	46.87	7.78
0.04 air	100.00	46.47	45.59	7.94
0.08 air	100.00	47.17	44.60	8.23
$C_{S0} = 760$ mmol l^{-1}				
0.02 N_2	100.00	40.06	58.10	1.84
0.02 air	100.00	39.28	58.20	2.52
0.04 air	100.00	44.23	52.45	3.32
0.08 air	100.00	37.08	59.34	3.58

Table 4 Relative metabolic fluxes analysis of pyruvate branch point

Aeration Condition (vvm)	Pyruvate (mmol mmol^{-1}, %)	2,3-BD (mmol mmol^{-1}, %)	Lactate acid (mmol mmol^{-1}, %)	Formic acid (mmol mmol^{-1}, %)	Acetyl-CoA (mmol mmol^{-1}, %)
$C_{S0} = 270$ mmol l^{-1}					
0.02 N_2	100.00	0.24	2.52	15.50	81.75
0.02 air	100.00	0.33	2.63	18.06	78.98
0.04 air	100.00	0.47	2.63	15.33	81.57
0.08 air	100.00	0.58	2.73	16.68	80.02
$C_{S0} = 434$ mmol l^{-1}					
0.02 N_2	100.00	0.35	5.38	12.29	81.98
0.02 air	100.00	2.82	13.76	16.03	67.39
0.04 air	100.00	4.03	11.63	13.69	70.64
0.08 air	100.00	4.27	5.94	14.39	75.41
$C_{S0} = 760$ mmol l^{-1}					
0.02 N_2	100.00	5.43	30.50	10.32	53.75
0.02 air	100.00	7.19	43.86	8.54	40.41
0.04 air	100.00	16.26	36.27	9.58	37.89
0.08 air	100.00	15.27	32.97	9.60	42.17

catalyzes pyruvate to acetyl-CoA is independent of the glycerol concentration.

The third key point is the acetyl-CoA node, which could be switched to acetic acid, ethanol and TCA. As listed in Table 5, the flux to TCA improved with increasing glycerol concentrations. When the substrate was limited (LG), the flux to TCA began to increase with increasing air flow under microaerobic condition. However, when the substrate was sufficient (MG) or excessive (HG), the opposite was observed in the presence of oxygen.

Under all aeration conditions, the ratio of ethanol to acetic acid (v_{EtOH}/v_{HAc}) did not change much when the glycerol concentration was low or medium (Fig. 5). However, under HG condition, the ethanol to acetic acid ratio dramatically increased about 4- to 7-fold. The acetic acid pathway provides the energy in the form of ATP and the ethanol pathway consumes the $NADH_2$. The variation in v_{EtOH}/v_{HAc} ratio reflected this demand for $NADH_2$ regeneration versus ATP to some extent. When glycerol is excessive, cell growth is largely inhibited. Therefore, it is

Table 5 Relative metabolic fluxes analysis of acetyl-CoA point

Aeration condition (vvm)	Ethanol (mmol mmol^{-1}, %)	Acetic acid (mmol mmol^{-1}, %)	TCA (mmol mmol^{-1}, %)
C_{S0} = 270 mmol l^{-1}			
0.02 N_2	46.31	52.465	1.23
0.02 air	42.73	56.25	1.02
0.04 air	37.79	59.86	2.34
0.08 air	34.75	62.60	2.65
C_{S0} = 434 mmol l^{-1}			
0.02 N_2	46.03	53.09	0.89
0.02 air	50.63	46.05	3.32
0.04 air	51.84	45.21	2.95
0.08 air	51.31	46.87	1.83
C_{S0} = 760 mmol l^{-1}			
0.02 N_2	78.28	17.65	4.07
0.02 air	83.41	10.87	5.72
0.04 air	78.52	16.00	5.48
0.08 air	82.87	11.82	5.31

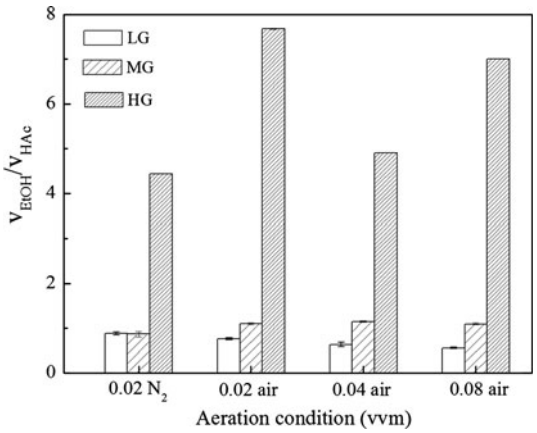

Fig. 5 Effect of aeration conditions on the ratio of v_{EtOH} to v_{HAc} under different culture conditions

unnecessary to generate much of ATP, and the cells would adjust the partitioning at the acetyl-CoA node by changing the ratio of ethanol (two $NADH_2$ consumed) to acetic acid (no $NADH_2$ to consume) to achieve the balance of ATP and reducing equivalents [24].

Effects of glycerol and different aeration conditions on oxidoreduction potential

$NADH_2$ is an important metabolic intermediate which is considered as the key co-factor for 1,3-PD production [32]. The extracellular ORP of the fermentation process could affect NAD^+ and $NADH_2$ equivalent, and this equilibrium in turn affects the production of 1,3-PD [33]. Therefore, the changes in ORP lead to increase or decrease in NAD^+/$NADH_2$ ratio [12], which can be used as an indicator of oxidoreductive status in the cell.

ORP increased with the increasing air flow (Fig. 6), which demonstrated that the environment became more oxidative under LG and MG conditions. As a result, the concentration of 1,3-PD decreased in this environment (Fig. 3a), which contradicted the trends of ORP. The ORP increased with increasing glycerol concentrations. Although cell growth is better under oxidative status [13], it still decreased due to excessive inhibition by substrate under HG as seen in Fig. 2. Unlike that of LG and MG, the trend of ORP was in accord with that of the 1,3-PD concentration under HG. This may be the reason that the maximum 1,3-PD concentration was achieved when the ORP was about −200 mV, which was very close to the reported preferable ORP (−190 mV) for the production of 1,3-PD [13].

Effects of glycerol and different aeration conditions on key enzymes

The specific activities of GDH, GDHt and PDOR as measured in the cell extracts varied with different glycerol concentrations (Fig. 7). The specific activities of PDOR and GDH decreased whereas that of GDHt increased when the glycerol concentration increased. These results indicated that, although the genes of GDH, GDHt, and PDOR were all located on the *dha* operon, the specific activities of these enzymes were not altered coordinately. One explanation could be that the regulation of these enzymes was not only at the transcriptional level but could also be at the posttranscriptional level. Further tests are needed to draw further conclusions.

Despite a decrease in GDH activity and an increase in GDHt activity, the flux to the oxidative pathway increased according to Table 3 when the glycerol concentration increased. This result implied that the flux distribution between oxidative and reductive pathway was controlled not only by the enzymes encoded by the *dha* operon, but that there could be some other existing genes, which can stimulate glycerol catabolism via the oxidative pathway. In *K. pneumoniae*, the *glp* operon encodes another enzyme system which can utilize glycerol, and it has been shown to be induced by oxygen. Based on the above results, this gene might also be induced to express under high glycerol concentrations, resulting in increased fluxes for the oxidative pathway.

The specific activities of key enzymes were also influenced by the aeration conditions (Fig. 7). The NAD^+/$NADH_2$ ratio was enhanced because the ORP increased with increases in aeration rates (Fig. 6), and the specific activity of GDH, which depends on NAD^+ [22], was also

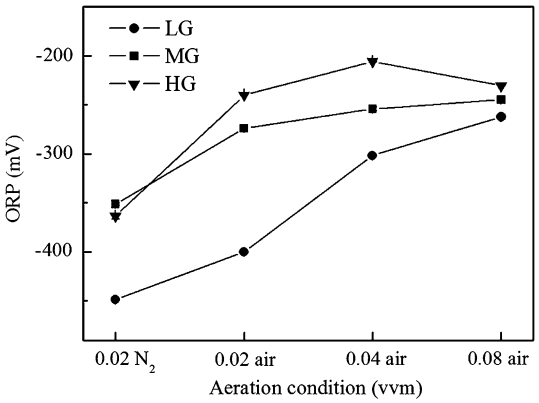

Fig. 6 Effect of aeration conditions on ORP under different culture conditions

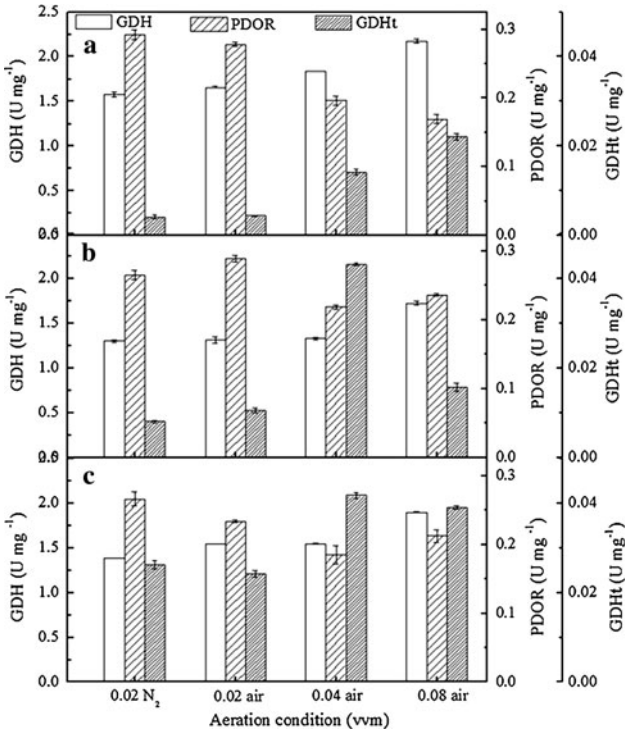

Fig. 7 Effect of aeration conditions on three key enzymes under different culture conditions. **a** LG; **b** MG; **c** HG

increased. In contrast, by enhancing air flow, the specific activity of PDOR under microaerobic conditions was less than that under anaerobic conditions. GDHt is regarded as a major rate-limiting enzyme for the consumption of glycerol and the formation of 1,3-PD in *K. pneumoniae* under HG [1]. The specific activity of GDHt was 59% higher with 0.04 vvm air sparging than that in the absence of oxygen, corresponding to the highest concentration of 1,3-PD. Given the fact that the specific activities of GDHt and GDH were enhanced, it can be concluded that the oxygen supply in our experiments was not sufficient to repress the *dha* operon, although molecular oxygen was considered to repress the expression of *dha* operon strongly [10]. In contrast, the expression of *dha* operon increased under microaerobic conditions.

Conclusion

In this work, the effects of glycerol concentration and oxygen supply on glycerol dissimilation in *K. pneumonia* were investigated in chemostat cultures at steady states. Cell growth, productions of 1,3-PD and by-products, and specific activities of three key enzymes were investigated under three glycerol concentrations and four aeration conditions at a dilution rate of 0.2 h^{-1}. From these results, the airflow rate of 0.04 vvm was preferable, corresponding to the highest 1,3-PD concentration (266 mmol l^{-1}) and a yield of 0.41 mol mol^{-1} under HG condition. However, the highest concentration and yield of 1,3-PD were achieved for LG and MG under anaerobic conditions.

Under microaerobic conditions, the availability of $NADH_2$ could theoretically be used for producing 1,3-PD. However, the experimental results showed that more carbon flux was shifted to the oxidative pathway instead of the reductive pathway, and used to produce lactic acid and 2,3-BD under microaerobic conditions. Changing the v_{EtOH}/v_{HAc} ratio is one of the ways to regulate the balance between NAD^+ and $NADH_2$. In this case, the bottleneck to enhance the concentration of 1,3-PD is not the pool of $NADH_2$, but the regulation of the distribution of carbon flow at glycerol dissimilation point.

Enzyme activity assays showed that the *dha* operon was not repressed but promoted under microaerobic conditions. Furthermore, based on the specific activities of GDH, PDOR and GDHt measured in the cell extracts, the differences in the regulation mechanism for 1,3-PD production exist not only at the level of metabolism but may also be at the level of gene expression.

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