Biotechnol Lett (2010) 32:1245–1250
DOI 10.1007/s10529-010-0284-y

ORIGINAL RESEARCH PAPER

Dielectric barrier discharge plasma as a novel approach for improving 1,3-propanediol production in *Klebsiella pneumoniae*

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Received: 4 February 2010 / Accepted: 16 April 2010 / Published online: 30 April 2010

Abstract Dielectric barrier discharge plasma was used to generate a stable strain of *Klebsiella pneumoniae* (designated to as Kp-M2) with improved 1,3-propanediol production. The specific activities of glycerol dehydrogenase, glycerol dehydatase and 1,3-propanediol oxidoreductase in the crude cell extract increased from 0.11, 9.2 and 0.15 U mg^{-1}, respectively, for wild type to 0.67, 14.4 and 1.6 U mg^{-1} for Kp-M2. The glycerol flux of Kp-M2 was redistributed with the flux to the reductive pathway being increased by 20% in batch fermentation. The final 1,3-propanediol concentrations achieved by Kp-M2 in batch and fed-batch fermentations were 19.9 and 76.7 g l^{-1}, respectively, which were higher than those of wild type (16.2 and 49.2 g l^{-1}). The results suggested that dielectric barrier discharge plasma could be used as an effective approach to improve 1,3-propanediol production in *K. pneumoniae*.

Keywords Dielectric barrier discharge · Enzyme activity · Fermentation · *Klebsiella pneumoniae* · Metabolic flux · 1,3-Propanediol

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Introduction

Microbial production of 1,3-propanediol (1,3-PD) has recently received extensive attention because of its large potential in commercial applications, particularly as a monomer of polyesters, polyethers or polyurethanes (Saxena et al. 2009; Xiu and Zeng 2008). *Klebsiella pneumoniae* is widely used in the production of 1,3-PD with glycerol as a substrate. However, there are still some disadvantages with using the wild-type strain to produce 1,3-PD, such as its low 1,3-PD productivity and low tolerance to high glycerol concentration (Cheng et al. 2005). Therefore, strain improvement plays an important role in enhancing tolerance to glycerol and increasing 1,3-PD production.

Various methods have been used to improve the 1,3-PD production ability of *K. pneumoniae*, such as traditional mutagenesis and breeding, as well as genetic engineering. However, new technology to improve *K. pneumoniae* with enhanced 1,3-PD

production is still being sought. Plasma discharges can generate UV radiation, electric field, charged particles and reactive species (e.g., atomic oxygen, metastable oxygen molecules, ozone, and OH) (Gaunt et al. 2006). These physical and chemical factors can temporarily alter the permeability of cell membrane (Yonson et al. 2006) and penetrate cellular envelopes of plasma-treated microbial cells to interact with intracellular biomacromolecules, such as proteins and nucleic acids, which can lead to cell death (Hou et al. 2008). Plasmas, especially non-thermal plasmas, have been extensively used in the inactivation of various microorganisms because of their low operating temperature, simple operation and innocuity. Denaturing gradient gel electrophoresis (DGGE) of the 16s rDNA amplified fragments from *Escherichia coli* cells showed that the fragments were in different positions before and after plasma discharges, due to variation in DNA sequences that resulted in different melting temperatures (Chen et al. 2008). This suggests that exposure of DNA to plasma could lead to changes in the nucleotide sequence, which have the potential to become mutations. Moreover, mutations in *Streptomyces avermitilis* have been achieved by plasma jet (Wang et al. 2009).

In our previous work, a suspension of *K. pneumoniae* treated by dielectric barrier discharge (DBD) plasma exhibited faster growth than untreated cells during batch fermentation using medium containing 6% (w/v) glycerol (Dong et al. 2009). 1,3-PD productivity for the treated *K. pneumoniae* was also increased by 59%. Moreover, by directly exposing an agar plate containing *K. pneumoniae* to DBD plasma, a strain was obtained that shows 47% increase in 1,3-PD productivity over untreated cells when fed-batch fermentation was used and the glycerol concentration in the medium was maintained at about 3% (Dong et al. 2008). The current study aimed to obtain more support for the positive effect induced by DBD plasma and to generate an excellent industrial strain of *K. pneumoniae* for accumulating 1,3-PD.

Materials and methods

Strains and growth conditions

Klebsiella pneumoniae (CGMCC 2028) was isolated from soil and preserved in China General Microbiological Culture Collection Center (CGMCC, Beijing, China). As *K. pneumoniae* is a facultatively anaerobic organism, it was cultured under micro-aerobic conditions to allow the cells to adapt to the presence of air since the cells were exposed to air during plasma treatment and subsequent screening.

The compositions of seed culture medium and fermentation medium were as described previously (Wang et al. 2003). Batch and fed-batch fermentations were carried out in a 5-l bioreactor with a 2-l working volume at 37°C and pH 7.0. The pH of the culture medium was adjusted to 7.0 with 5 M NaOH. The agitation speed was set at 250 rpm, and the air sparging rate was 0.02 vvm. The inoculum concentration was 10% (v/v). Glycerol was at 40 g l^{-1}. For fed-batch fermentations, glycerol was continuously fed into the bioreactor to maintain it between 10 and 20 g l^{-1} until the end of fermentation.

DBD plasma treatment and strain selection

As shown in Fig. 1, DBD plasma in air at atmospheric pressure was generated at 20 kHz and 24 kV between a pair of circular aluminum plate electrodes covered with quartz glasses. A discharge gap of 3 mm between the upper electrode and the surface of the sample suspension was selected. After irradiation, all samples (irradiated and control samples) were diluted 10^3- to 10^6-fold with sterilized potassium phosphate buffer and then plated on LB agar medium and incubated at 37°C for 24 h. Individual colonies were selected from the plate and inoculated into separate 100 ml flasks containing 10 ml seed medium. The flasks were and incubated micro-aerobically at 37°C and 150 rpm for 12 h, and the concentration of 1,3-PD in each flask was measured.

Preparation of cell-free extracts and measurement of enzymatic activities

Cell-free extracts were prepared as previously described (Dong et al. 2009). The supernatant from the extract was subjected to glycerol dehydrogenase (GDHt), glycerol dehydatase (GDH) and 1,3-propanediol oxidoreductase (PDOR) assays as described by Ahrens et al. (1998), with the exception of incorporating 1 mM $Fe(NH_4)_2(SO_4)_2$ into the reaction mixture for PDOR and GDH assays. Protein was determined by the Bradford method with bovine

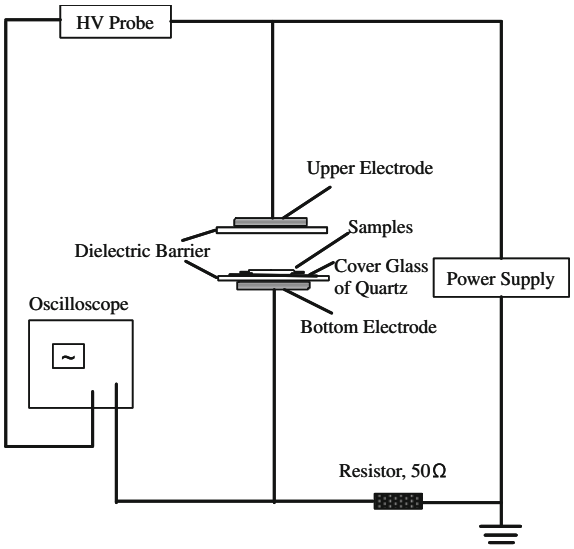

Fig. 1 Schematic diagram of the DBD plasma setup. A pair of circular aluminum plate electrodes was covered with quartz glasses. The power supply consists of a power amplifier and a transformer. Cell suspension (approximately 3×10^8 cells ml^{-1}) was spread on a piece of quartz glass. The glass plate was placed at the center of the underlying plate electrode, and then treated with low-temperature plasma

serum albumin as the standard. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol product per min.

Analytical methods

Cell dry weight (CDW) was determined as previously described (Dong et al. 2009). The molar yield of biomass was calculated according to Zeng et al. (1993). Glycerol was assayed by a modified titration method (Wang et al. 2001). 1,3-PD, 2,3-butanediol (2,3-BD), and ethanol were determined by gas chromatography. Succinic acid, lactic acid and acetic acid were determined by HPLC.

Results and discussion

Effect of DBD plasma on 1,3-PD production

The survival rate of DBD plasma-treated cells decreased with increases in treatment time, but a slight recovery occurred at 90 s (Table 1). Only about 1% of the cells remained alive at 210 s. The survival rate thus showed a saddle-shaped curve that was also observed in our earlier studies (Dong et al. 2009, 2008; Hou et al. 2008).

Surviving cells that produced more 1,3-PD than wild type were considered as positive isolates. The highest percentage (31%) of positive isolates was obtained from a treatment time of 120 s (Table 1). Since the screening process used only viable cells, those cells that died as a result of severe damage induced by DBD plasma were not used to determine the proportion of isolates with improved 1,3-PD production. As a result, a much higher percentage of positive isolates was obtained than expected. One-third of the positive isolates produced 50% more 1,3-PD than wild type and, among these, one strain (designated as Kp-M2) produced the highest yield of

Table 1 Distribution fraction of strains with different 1,3-PD titer after exposure to DBD plasma

Exposure time (s)	Survival rate (%)	Distribution fraction of strains with different 1,3-PD titer (%)								
		1,3-PD concentration (g l^{-1})								
		Below 2.5	2.5–3.5	3.6–4.5	4.6–5.5	5.6–7	7.1–8	8.1–9	9.1–10	Above 10.1
30	43 ± 7	–	–	1	7	79	7	4	2	–
60	10 ± 3	1	3	4	7	64	9	7	3	2
90	22 ± 4	3	6	7	10	47	11	9	5	2
120	15 ± 2	5	4	9	13	38	13	9	6	3
150	9 ± 2	4	9	12	17	35	9	7	5	2
180	4 ± 1	3	14	17	19	32	7	5	2	1
210	1 ± 0.8	7	16	22	17	31	3	2	2	–

The survival rate is defined by (T/M) × 100%, where M is the number of colonies from control cells and T is the number of surviving colonies from plasma-treated cells. Data of survival rate are the mean ± SEs from three experiments. Distribution fraction of strains with different 1,3-PD titer is defined by (N/80) × 100%, where 80 is the number of colonies selected from each plate, and N is the number of colonies from different ranges of 1,3-PD concentrations. 1,3-PD concentration for wild type is between 5.6–7 g l^{-1}

Table 2 GDH, GDHt and PDOR specific activities in the cell extracts of wild type and Kp-M2

Specific activity (U mg^{-1})	Strains	
	Wild type	Kp-M2
GDH	0.11 ± 0.03	0.67 ± 0.07
GDHt	9.2 ± 0.5	14.4 ± 0.8
PDOR	0.15 ± 0.03	1.6 ± 0.1

Kp-M2 cells were harvested from flask culture after 8 h and the specific activities of GDH, GDHt and PDOR in the cell extract were measured. Data are the means ± SEs from three experiments

* $P < 0.01$

1,3-PD. This was used for further analysis. Kp-M2 showed stable 1,3-PD production. The specific activities of GDH, GDHt and PDOR in the cell extract of Kp-M2 were all enhanced but the extent of enhancement varied for each enzyme (Table 2). Kp-M2 was therefore selected for further studies.

Batch fermentation by wild type and Kp-M2

In batch fermentation, Kp-M2 grew faster with a more rapid consumption of glycerol than the wild type (Fig. 2). It achieved a 23% increase in the final concentration of 1,3-PD compared to wild type (Table 3). Acetic acid concentration also increased by 22%, while the concentrations of ethanol, lactic acid, 2,3-BD and succinic acid all decreased relative to wild type (Fig. 2; Table 3). The enhanced production of acetic acid is favorable for 1,3-PD production (Zeng et al. 1993).

Table 3 Comparison of batch fermentations between wild type and Kp-M2

Parameters	Batch fermentation	
	Wild type	Kp-M2
Fermentation time (h)	9	7
Glycerol consumption (g l^{-1})	37.9	38.7
Cell dry weight (g l^{-1})	2. 6	3.5
1,3-PD concentration (g l^{-1})	16.2	19.9
Ethanol (g l^{-1})	1.5	0.25
Acetic acid (g l^{-1})	4.6	5.6
Lactic acid (g l^{-1})	0.62	0.15
2,3-Butanediol (g l^{-1})	0.59	0.11
Succinic acid (g l^{-1})	0.92	0.59
1,3-PD yield (mol mol^{-1})	0.51	0.62
1,3-PD productivity (g l^{-1} h^{-1})	1.8	2.8
Specific formation rate of 1,3-PD (g g^{-1} h^{-1})	0.70	0.82
Specific consumption rate of glycerol (g g^{-1} h^{-1})	1.6	1.6

The cultures were grown micro-aerobically with the fermentation medium containing 4% (w/v) glycerol at 37°C and 150 rpm for 7 h (Kp-M2) or 9 h (wild type)

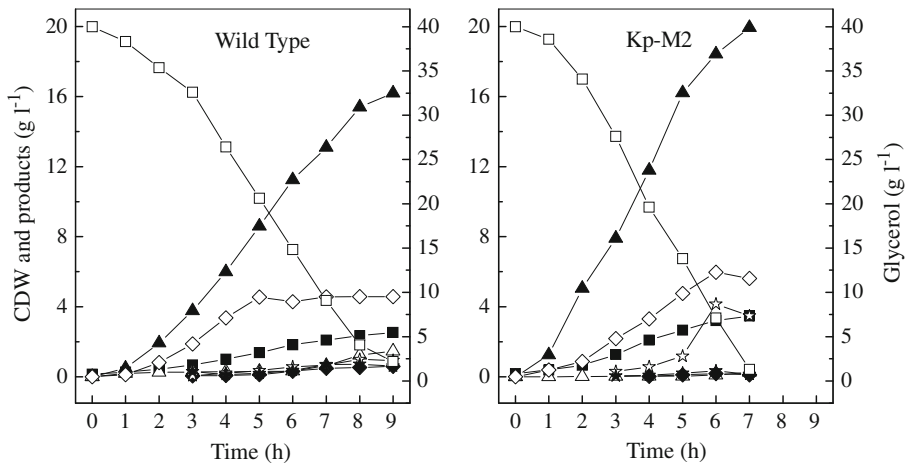

Fig. 2 Batch fermentation by wild type and Kp-M2 under micro-aerobic condition. *Closed square*: CDW; *open square*: glycerol; *closed triangle*: 1,3-PD; *open triangle*: ethanol; *open diamond*: acetic acid, *closed diamond*: 2,3-BD; *open star*: succinic acid; *closed star*: lactic acid

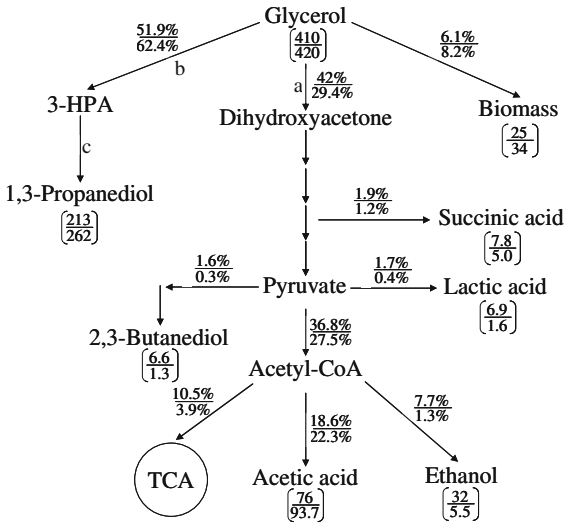

Fig. 3 Metabolic fluxes of glycerol in wild type and Kp-M2 under micro-aerobic condition. *Number in parenthesis* represents molar concentration (mmol l^{-1}); the percentage is metabolic flux of glycerol on molar base; *numerical values above the horizontal line* are from wild type and *those beneath the horizontal line* are from Kp-M2. *a* GDH; *b* GDHt; *c* PDOR

The data obtained from batch fermentations were used to analyze the glycerol fluxes in Kp-M2 and wild type. For Kp-M2, the flux to the reductive branch increased, while the flux to the oxidative branch decreased relative to wild type (Fig. 3). In addition, a redistribution of the individual fluxes to various pyruvate-derived by-products was also observed for Kp-M2, which resulted in a remarkable increase in 1,3-PD yield.

Fed-batch fermentation by wild type and Kp-M2

In fed-batch fermentations Kp-M2 also showed faster growth than wild type, and reached a plateau 16 h earlier (Fig. 4). The concentration of 1,3-PD, the yield of 1,3-PD, the productivity of 1,3-PD and the specific formation rate of 1,3-PD measured at the end of fermentation for Kp-M2 were 76.7 g l^{-1}, 0.58 mol mol^{-1}, 2.13 g l^{-1} h^{-1} and 0.32 g g^{-1} h^{-1}, respectively. Kp-M2 therefore achieved 56, 35, 55 and 10% increases in 1,3-PD concentration, yield, productivity and specific formation rate, respectively, over wild type.

1,3-PD production from glycerol is controlled mainly by GDHt and PDOR (both belong to the reductive pathway) and GDH (oxidative pathway) (Ahrens et al. 1998). Moreover, an increase in GDH activity may improve cell growth. Cell growth is positively correlated with 1,3-PD production in glycerol metabolism (Chen et al. 2003). In the case of Kp-M2, a small increase in GDHt activity (57%) coupled with a large increase in PDOR activity (11-fold) might reduce 3-HPA accumulation, causing an increase in the specific formation rate of 1,3-PD. However, the increase in GDH specific activity did not strengthen the carbon flux through the oxidative pathway. It is well known that a series of reactions and a number of enzymes are involved in the oxidative pathway. Although the specific activity of GDH was enhanced, the specific activities of other enzymes might decrease, and thereby weakening the carbon flux through the oxidative pathway.

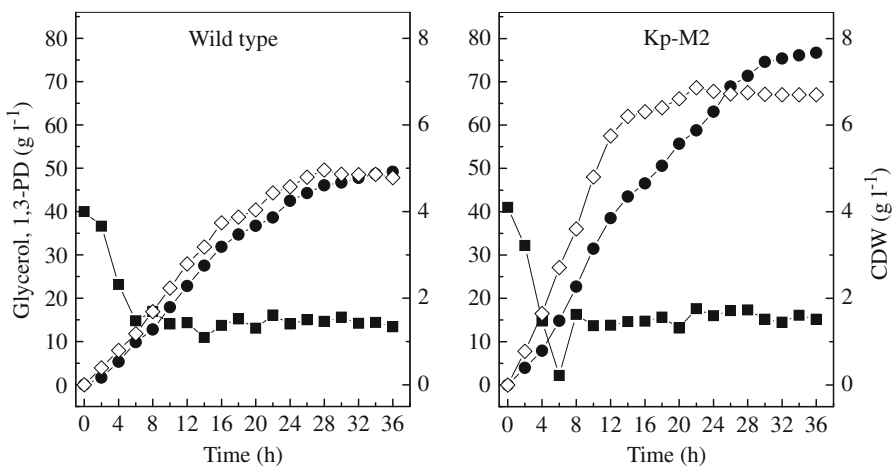

Fig. 4 Fed-batch fermentation by wild type and Kp-M2 under micro-aerobic condition. *Closed square*: glycerol; *closed circles*: 1,3-PD; *open diamond*: CDW. The initial concentration of glycerol was 40 g l^{-1}

The change that gave rise to improved 1,3-PD production observed for Kp-M2 could be considered as mutation. However, the mutation mechanism needs further study. Taken together, the results reported here indicated that it is feasible to use DBD plasma to generate similar strains with other useful traits, either for industrially related work or for more basic-oriented research.

Acknowledgements This work was supported by the National High Technology Research and Development Program of China (863 Program) (No. 2007AA02Z208) and the Major State Basic Research Development Program of China (973 Program) (No. 2007CB714306). The authors thank Mr. Tang Xiao-Hua for the screening of mutants, and Dr Alan K Chang for his help with the revision of the manuscript.

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