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Isolation and Identification of Alkali-Resistant 1,3-Propanediol Producing Strain

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Abstract

1,3-Propanediol is a promising renewable resource produced by microbial production. It is mainly used in many synthetic reactions, particularly applied to the polymer synthesis and cosmetics industry. We described here the isolation of strain ZH-1, which has the ability of high production with 1,3-propanediol, from Fenhe River in China. It was classified as a member of *K. pneumoniae* after the study of phenotypic, physiological, biochemical and phylogenetic (16S rDNA). The initial glycerol concentration, fermentation time and pH value of strain ZH-1 were determined to be 50 $g \cdot L^{-1}$, 36 h and 8.0. Under these conditions, the practical yield of 1,3-PD was 18.53 $g \cdot L^{-1}$ and a molar yield ($mol_{1,3\text{-PD}}$ $mol_{Glycerol}^{-1}$) of 1,3-propanediol to glycerol of 0.497. In addition, we found that for the strain ZH-1, the optimum grown pH was 9.0, so we can determine that it is a new member of alkali-resistant strains.

Keywords

1,3-Propanediol, *K. pneumoniae*, Identification, Alkali-Resistant

1. Introduction

1,3-Propanediol (1,3-PD) is an important chemical raw material. As a potential intermediate product, it is widely used in fine chemicals, cosmetics, pharmaceutical and other industries. In addition, 1,3-PD can be used as a monomer in the synthesis of various polyesters, urethane polymers, etc. [1]. In recent years, the rapid development of 1,3-propanediol is due to its important and irreplaceable role as a monomer in the synthesis of polyethylene terephthalate (PTT) [2] [3]. PTT exhibits the excellent properties such as good resilience, stain resistance, low static generation, etc., and is desirable for fiber and textile applications. Finally, the biodegradability of natural plastics containing 1,3-propanediol is higher compared to that of fully synthetic polymers [4].

Currently, the method of synthesizing 1,3-PD mainly includes chemical synthesis

and microbial fermentation. Chemical synthesis of 1,3-propanediol requires high temperature (90˚C), pressure (1500PSI) as well as expensive catalysts as a precursor of ethylene oxide [5]. However, the production of 1,3-PD via microbial fermentation can be achieved under mild conditions (for example, moderate reaction condition, room temperature and atmospheric pressure) [6]. In recent years, due to the massive use of bio-diesel, crude glycerol is used as a typical industrial waste resulting from its excess [7]. Therefore, the rational use of crude glycerin can reduce the cost of production of 1,3-propanediol fermentation so as to accelerate the commercialization of microbial fermentation method. In short, microbial fermentation is environmentally-friendly and economical.

In the past, several members of the genus such as *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium butyricum* have been shown to grow anaerobically on glycerol as their sole carbon and energy source and convert it to 1,3-PD [1] [7]-[9]. *Citrobacter freundii* is strictly anaerobic and difficult to cultivate, while *Klebsiella pneumoniae* and *Clostridium butyricum* are facultative anaerobic bacteria and they have a strong glycerol tolerance and high fermentation intensity [10]. Therefore, *Klebsiella pneumoniae* and *Clostridium butyricum* are widely used in the production of 1,3-PD.

However, in all of the wild 1,3-PD producers characterized to date, there is a relatively lower fermentation pH. Silva *et al.* showed that the optimum pH range for *Klebsiella pneumoniae* GLC29 was 6.9 - 7.1 [11]. The fermentation of glycerol by *C. butyricum* was regulated under a pH of 7.0 [12]. 1,3-PD was produced by *C. pasteurianum* under a pH of 6.5 [13]. In this report, an alkali-resistant strain named *K. pneumoniae* ZH-1, which could biosynthesize 1,3-PD with a higher fermentation pH and a higher grown pH, was isolated from an anaerobic sludge in Fenhe River. Based on the initial concentration of glycerol and the fermentation time, the fermentation conditions were investigated. Initial optimization of fermentation parameters has resulted in higher yields and productivity, which can be further concentrated to make this strain viable for scale up studies and given a priority as potent wild type producer compared to other native and non-native 1,3-PD producers [14].

2. Materials and methods

2.1. Media

For enrichment and isolation of cultures the following media was used (per litre): glycerol, 20.0 g; $(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 3.4 g; KH_2PO_4, 1.3 g; $MgSO_4$, 0.2 g; $CaCl_2$, 2×10^{-3} g; $FeSO_4$, 2×10^{-3} g; yeast extract powder 1 g; citric acid 0.42 g. Trace element solution 0.2% (v/v) was added to the enrichment media (concentration per liter of deionized water): $CoCl_2 \cdot 6H_2O$ 0.2 g; $MnCl_2 \cdot 4H_2O$ 0.1 g; $ZnCl_2$ 0.07 g; H_3BO_3 0.06 g; $Na_2MoO_4 \cdot 2H_2O$ 0.035 g; $CuCl_2 \cdot 2H_2O$ 0.02 g; $NiCl_2 \cdot 6H_2O$ 0.025 g. The initial pH at 7.0 regulated by KOH. 18.0 g agar added while the solid medium was needed [15].

2.2. Enrichment and Isolation of the Strains

The bottom silt samples were collected from Fenhe River in Shanxi Province of China.

Ten grams of silt samples were incubated anaerobically at 37˚C in 300 ml sterile plastic bottles that had been completely filled with enrichment media. After 3 days, Bacteria from each culture were streak-purified on agar plates that were then incubated in (CO_2 Incubator) anaerobic jars with a CO_2 generator (ESCO, America).

2.3. Identification of the Strains

Visual observations of both morphological and microscopic characteristics using the light microscope were conducted. Physiology and biochemistry experiments were determined according to Bergey's Manual of Determinative Bacteriology [16]. Cell size was observed by scanning electron microscope (SEM) (S570, HITACHI, Japan).

Used a commercial kit (SK8255, Sangon Biotech), genomic DNA was extracted. A pair of universal primers was prepared according to previously described methods, upstream primer (formerly named 7F), 5'-CAGAGTTTGATCCTGGCT-3'; downstream primer (named 1540R), 5'-AGGAGGTGATCCAGCCGCA-3'. DNA sequences were determined by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (China). The closest matches to the partial 16S rDNA sequence were identified based on the results of a BLAST search of the EzBioCloud data-base. The phylogenetic tree was constructed using the neighbor-joining method with the MEGA 5.0 software. Bootstrap analysis for 1000 replicates was performed to estimate the confidence of the tree topologies [17].

2.4. Determinations of Glycerol Concentration, Fermentation Time and pH

In order to determine the optimum culture environment, the initial concentration of glycerol, the fermentation time and pH value were studied. According to the result of Chao-Ling Wong *et al.*, we set the initial glycerol concentration were 20 $g{\cdot}L^{-1}$, 30 $g{\cdot}L^{-1}$, 40 $g{\cdot}L^{-1}$, 50 $g{\cdot}L^{-1}$, 60 $g{\cdot}L^{-1}$, respectively [5]. Then we take a sample every 6 hours to draw the time curve to determine the optimum fermentation time. Finally, we set the pH gradient was 5 - 10 to determine the optimum fermentation pH.

2.5. Analytical Methods

1,3-PD were analyzed by injecting 0.6 μl of reaction mixture into a gas chromatograph equipped (Aglient GC7820) with a capillary column (ON-Wax, 30 m × 0.32 mm × 0.5 μm). The flow rate of the carrier gas (nitrogen) was 25 $ml{\cdot}min^{-1}$. The column temperature was raised to 180˚C at 15˚C/min and maintained for 10 min, while injector and FID detector temperature were both 250˚C. Standard curve was drawn according to the peak area of 1,3-PD standard substance. The concentration of 1,3-PD standard substance was 2.5 $g{\cdot}L^{-1}$, 5 $g{\cdot}L^{-1}$, 10 $g{\cdot}L^{-1}$, 20 $g{\cdot}L^{-1}$, 40 $g{\cdot}L^{-1}$, respectively. Then using the standard curve ($y = 1.5E + 6x + 134372$, $R^2 = 0.9993$) to calculate the content of 1,3-PD by strain ZH-1.

Residual glycerol was determined by the improved Potassium Permanganate oxidation method according to Wang *et al.* [18].

Cell growth was monitored at 650 nm (OD 650) on a spectrophotometer (722S Jinghua, China).

3. Results and Discussions

3.1. Isolation and Identification of Strains

Of eleven samples collected from the sludge of Fenhe River in China, three strains were able to utilize glycerol growth anaerobically, but only one bacterial strains ZH-1 was able to form higher concentration 1,3-PD. The content of 1,3-PD in the extract can be calculated according to the GC peak area of the 1,3-PD standard substance (Figure 1). Then the Strain ZH-1 was selected for the further studies. By culturing and observed that strain ZH-1 was an aerotolerant anaerobe bacterium, the colonies white mucilaginous, colonies diameter was about 0.6 - 0.8 mm (Figure 2). The SEM studies revealed that strain ZH-1 was Chunky, no flagella bacterium, size 0.5 − 0.6 × 0.9 − 1.4 μm

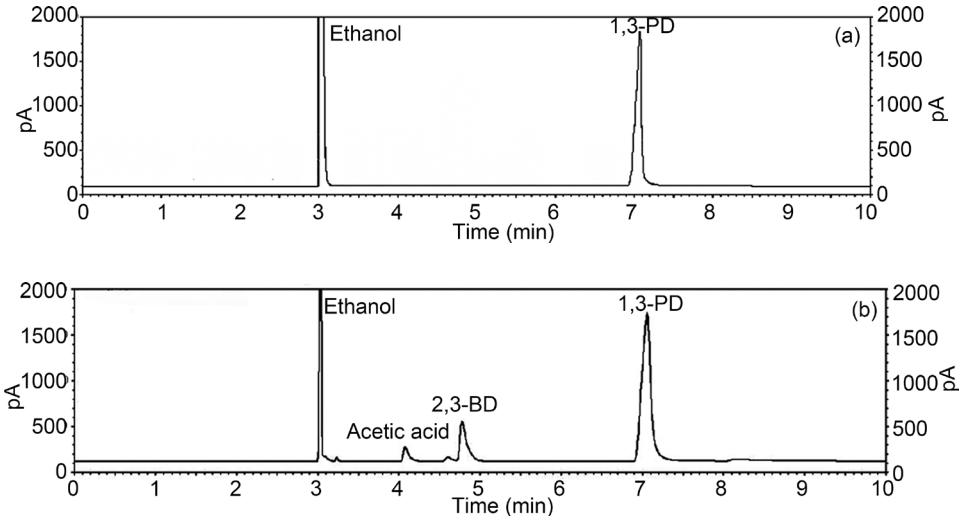

Figure 1. Schematic diagram of the 1,3-PD by GC. (a) GC of 1,3-PD standard substance; (b) GC of 1,3-PD by strain ZH-1. Ethanol is the solvent of the diluted 1,3-PD, 2,3-butanediol (2,3-BD) and acetic acid are the main impurities in the 1,3-PD by strain ZH-1.

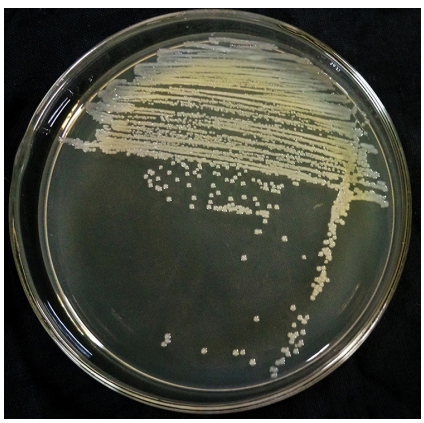

Figure 2. Colony morphology of strain ZH-1.

(Figure 3). The species can be grown on nitrogen-free media, and colonial morphology will vary with different medium components. The physiological and biochemical experiments were shown in Table 1.

We used a 16S rDNA gene sequence-based strategy to identify isolate ZH-1. Full-length (approximately 1.4 kb fragment) 16S rDNA gene was PCR amplified (Figure 4). The complete 16S rDNA of strain ZH-1 (1423 bp) was sequenced and is available at GenBank under accession number KT804692. A BLAST search of EzBioCloud database showed ZH-1 most resembled *K. pneumoniae*^{T} strain. The physiological and morphological characteristic shows that the strain ZH-1 was most closely allied to *K. pneumoniae*. A phylogenetic tree (Figure 5) was constructed based on an alignment of 1423 bp of 16S rDNA sequences.

3.2. Determination of Glycerol Concentration

1,3-PD production depends largely on glycerol—the only carbon and energy source. However, the glycerol tolerance of different strains is inconsistent. Therefore, we first studied the influence of the initial concentration of glycerol (from 20 - 60 $g \cdot L^{-1}$) on 1,3-PD production and bacterial biomass at pH 7.0 [5]. As showed in Figure 6, when the glycerol concentration was 20 - 50 $g \cdot L^{-1}$, 1,3-PD production and yield increased with the glycerol concentration. However, with the increase of the glycerol concentration from 50 to 60 $g \cdot L^{-1}$, the content of 1,3-PD has a slight decline. In addition, Jun *et al.* [19] reported that 1,3-PD production from *Klebsiella pneumoniae* DSM 4799 is suppressed

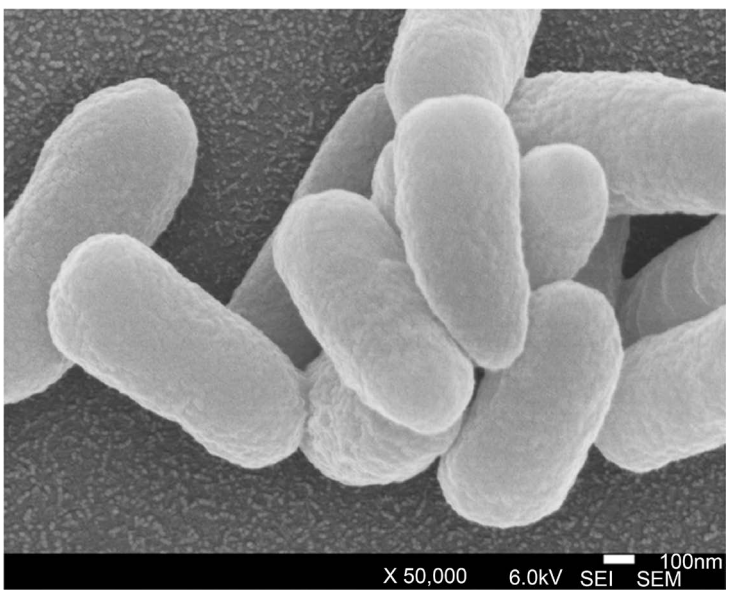

Figure 3. SEM image of strain ZH-1 (×50,000).

Table 1. Physiological and biochemical characteristics of strain ZH-1.

Gram stain	Gelatin liquefaction	Citrate	Methyl red	V.P	H_2S	Indol	Glucose
−	−	+	+	+	−	−	+

"+" indicates a positive reaction, "−" indicates a negative reaction.

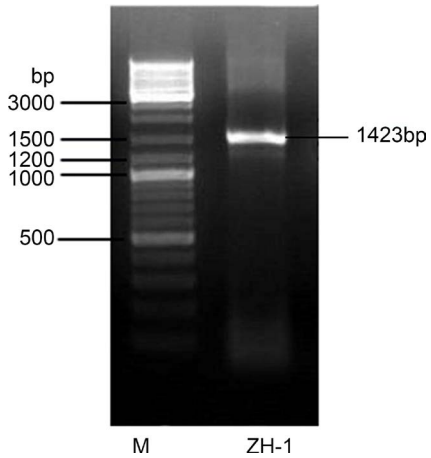

Figure 4. The electrophoretogram shows amplification of strain ZH-1 16S rDNA by a pair of universial primer 7F and 1054R.

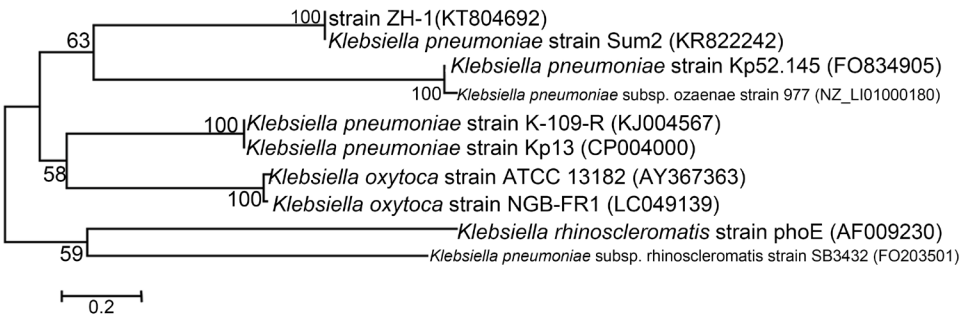

Figure 5. Phylogenetic tree shows the 16S rDNA relationships of strain ZH-1 with other bacteria that can be bioconversion of glycerol to 1,3-propanediol. The tree was constructed by MEGA 5 by the neighbor-joining method with bootstrap values calculated from 1000 trees.

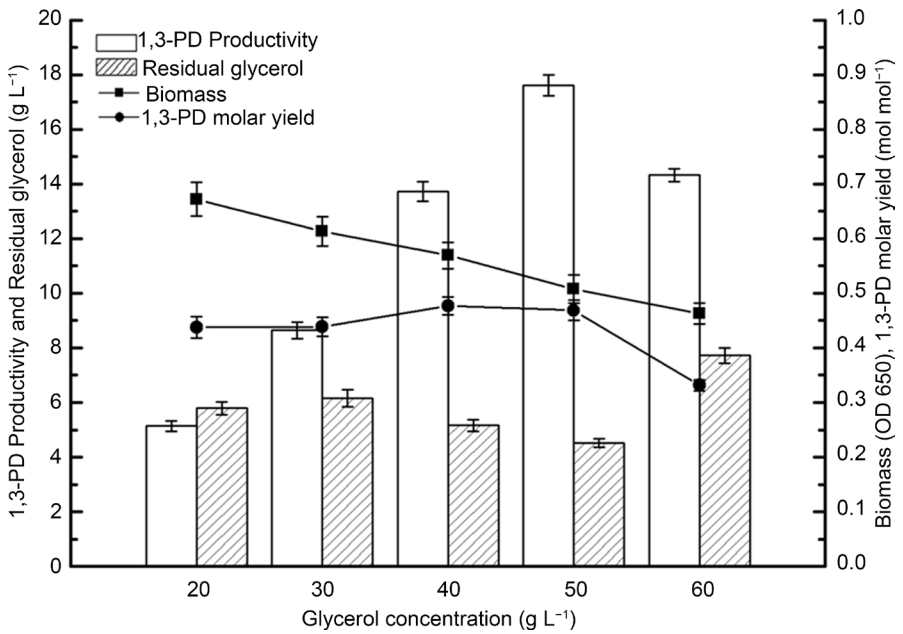

Figure 6. Effect of glycerol concentration on the yield of 1,3-PD by *K. pneumoniae* ZH-1.

when glycerol concentration was 60 g·L^{-1}. Wong *et al.* found that the ability of HE-2 strains in producing 1,3-PD was inhibited when glycerol concentration was 40 - 60 g·L^{-1} [5]. In our case, inhibition occurred when the initial glycerol concentration exceeded 50 g·L^{-1}. When the initial glycerol concentration was 50 g·L^{-1}, the 1,3-PD yield and biomass reached the maximum, and the molar conversion rate reached 0.4686 mol·mol^{-1}. Therefore, we determined the optimal initial glycerol concentration was 50 g·L^{-1}.

3.3. Determination of Fermentation Time

Fermentation time plays a critical role in the glycerol transform to 1,3-PD. When the fermentation time is too short, the desired product yield is low that cannot reach the requirements of industrial production. Fermentation time takes too long would make some of the byproducts accumulate and affect the purification of 1,3-PD. To determine optimum fermentation time of *K. pneumoniae* ZH-1, we carried out batch fermentation research. The initial glycerol concentration was 50 g/L and pH was 7.0, samples were took every 6 hours to detect 1,3-PD concentration, glycerol concentration and biomass, then the fermentation time curve was drawn. As can be seen from the growth curve of bacteria (Figure 7), 0 - 6 h showed a rapid cell growth, 6 - 18 h cells lie in the logarithmic growth phase, after 36 h, cell growth gradually slows down due to the lack of substrate glycerol. In addition, 1,3-PD formation and bacterial growth showed a positive correlation, with the cell reproduction, 1,3-PD have begun to generate, and showed a rapid growth in 0 - 18 h, 1,3-PD production stabilized after 36 h, the amount of residual glycerol was essentially the same after 36 h, so we determine the fermentation time as 36 h.

3.4. Optimum of pH Value for *K. pneumoniae* ZH-1

The changes of pH value have a significant impact on cell growth and product synthesis. Suitable pH value can increase the reaction activity, and accelerate the utilization of

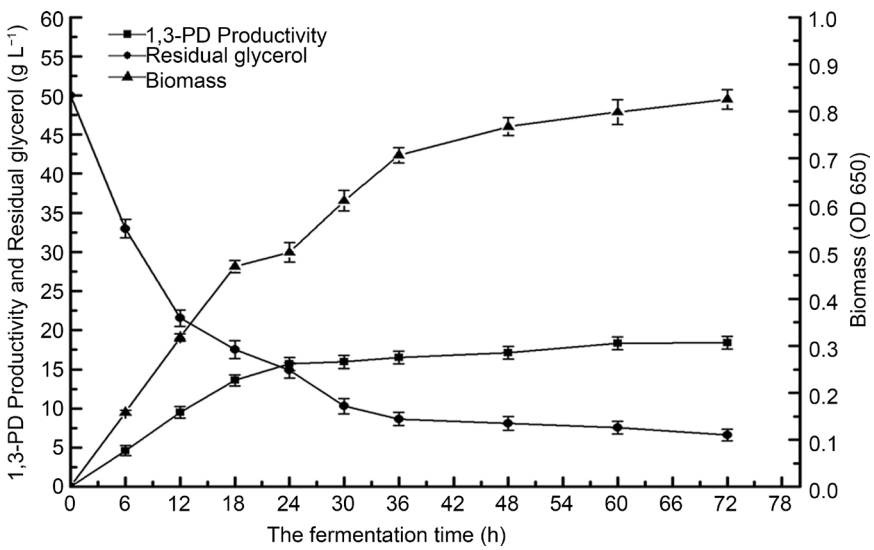

Figure 7. The fermentation time of glycerol converted to 1,3-PD for *K. pneumoniae* ZH-1.

glycerol and increased cell growth and product synthetic rate. In order the maximum production rate of 1,3-PD and the highest bacterial biomass, the pH gradient we selected to study the fermentation optimum pH was 5 to 10. The initial glycerol concentration was 50 g/L and fermentation 36 h, the 1,3-PD production and the bacterial biomass were detected. It was indicated in **Figure 8** that the maximum concentration of 1,3-PD was obtained in pH 8.0 media, and the productivity reached 18.53 $g \cdot L^{-1}$. At the same time, the molar conversion rate reached 0.497. But when pH was 9.0, the bacterial biomass reached the maximum. We can conclude that it is an alkali-resistant strain. The tolerance to higher pH could probably be related to the genetic characteristics because ZH-1 was isolated from micro-alkaline soil.

After determinations of the glycerol concentration, fermentation time and pH, the glycerol molar conversion rate reached 0.497 $mol \cdot mol^{-1}$ from the original 0.34 $mol \cdot mol^{-1}$, the molar yield of 1,3-PD to glycerol was consistent with those reported in literatures and those previously reported [7] [20]-[22]. Y. M *et al.* studied fed-batch of a *K. pneumoniae* strain on combining biodiesel production by lipase with microbial production of 1,3-PD using a hollow fiber membrane. The molar yield of 1,3-PD to glycerol of 0.47 $mol \cdot mol^{-1}$ was obtained [7]. Another study on bioconversion of raw glycerol into 1,3-PD by *K. pneumoniae* showed that the molar yield of 1,3-PD to glycerol of 0.41 $mol \cdot mol^{-1}$ [23]. In this study, the strain ZH-1 had a higher molar conversion rate than some strains of the previous study, whereas compared with the study of Yang *et al.* (0.62 $mol \cdot mol^{-1}$) [24]. The 1,3-PD conversion rate of strain ZH-1 should be further improved, and we will have further research on the strain ZH-1 by adding the metabolites or some other methods.

4. Conclusion

This work showed that the alkali-resistant *K. pneumoniae* ZH-1 has the potential for

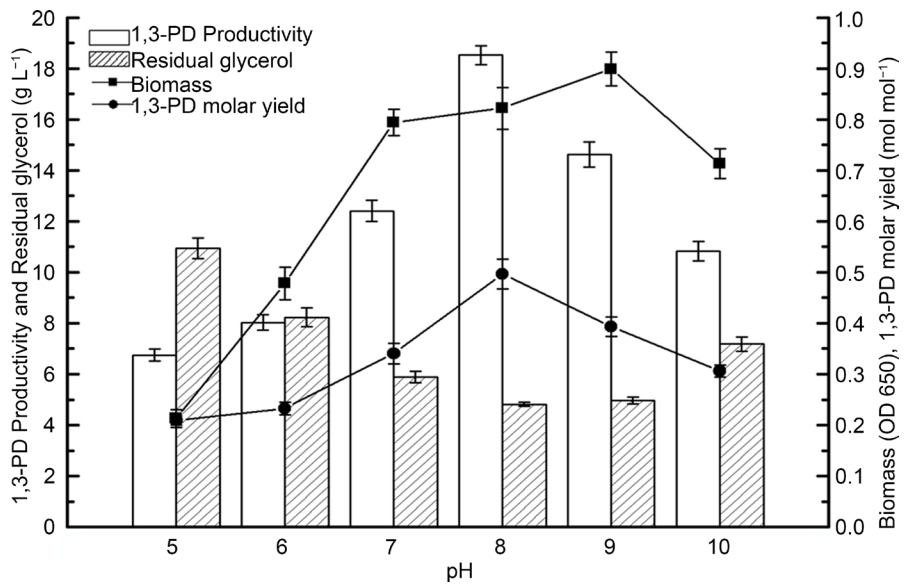

Figure 8. Effect of pH on biomass and product formation.

1,3-PD production at high efficiency under anaerobic conditions. The optimal conditions of 1,3-PD production are as follows: 50 $g \cdot L^{-1}$ glycerol, culture 36 h, pH 8.0. Under these conditions, the practical yield of 1,3-PD was 18.53 $g \cdot L^{-1}$ and the molar yield was 0.497 $mol \cdot mol^{-1}$. Compared with 1,3-PD yield from other strains, the strain *K. pneumoniae* ZH-1 has a higher pH tolerance and a higher molar conversion rate. To provide more data necessary to establish technically and economically feasible process, further investigation is needed.

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