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Production of 1,3-Propanediol by *Klebsiella pneumoniae*

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Abstract

1,3-Propanediol (1,3-PD) has numerous applications from polymers to cosmetics, foods, lubricants, and medicines. Recently, there are strong industrial interests in a new kind of polyester, polytrimethylene terephthalate, with 1,3-PD as a monomer. This new polyester shows significant promise for use in carpeting and textiles. In this article we introduce a mild aerobic fermentation process using a strain screened from *Klebsiella pneumoniae* ATCC 25955, which is insensitive to oxygen, to produce 1,3-PD. We also describe a two-step fermentation process starting with glucose that was converted into glycerol with a glycerol-producing yeast, followed by *K. pneumoniae* that converts glycerol into 1,3-PD without intermediate isolation and purification of glycerol.

Index Entries: 1,3-Propanediol; glycerol; *Klebsiella pneumoniae*; yeast; fermentation.

Introduction

1,3-Propanediol (1,3-PD), $HOCH_2CH_2CH_2OH$, is a clear, colorless, odorless, innocuous liquid that is miscible with water, alcohol, and ethers. As a bifunctional compound, 1,3-PD is subject to many of the same polymeric applications as other low molecular diols, such as ethylene glycol, propylene glycol, 1,3-butanediol, and 1,4-butanediol. Despite interesting applications, in the past total production of 1,3-PD remained relatively small because of its high cost. 1,3-PD of a high-purity grade costs about $26/lb for a 200-kg quantity (personal communication). Less expensive glycols are used instead of 1,3-PD for economics reasons, even though 1,3-PD provides superior properties.

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The market situation for 1,3-PD has changed significantly since recent commercialization of a new polyester operation based on terephthalic acid and 1,3-PD. The odd number of carbon atoms of 1,3-PD imparts unique properties to polymeric products. Poly(trimethylene terephthalate) is particularly appropriate for fiber and textile applications for excellent properties, such as good resilience, inherent stain resistance, and low static generation *(1,2)*. 1,3-PD is expected to become a new commodity chemical.

One of the industrial synthetic processes for 1,3-PD is the hydration of acrolein to 1,3-PD. This reaction is carried out typically under weakly acidic conditions in water with a low initial acrolein concentration of about 20%. The intermediate compound, 3-hydroxypropionaldehyde (3-HPA), can be hydrogenated in the aqueous phase directly. It is preferably extracted first with an organic solvent, such as 2-methylpropanol, and then hydrogenated separately in the organic solvent. Hydrogenation is done at 20–40 atm and 110–115°C using nickel-supported catalysts. 1,3-PD is separated from the extracting solvent or aqueous phase by distillation. The yield of desired product by these methods is relatively low, about 45% *(3)*. Another synthetic method, hydroformylation of ethylene oxide and subsequent hydrogenation to 1,3-PD, has been researched *(4)*. Low concentrations of ethylene oxide were contacted with synthesis gas, CO and H_2, in a solvent of diethyl ether at 80–100 atm and 125-180°C using bis(tributylphosphine) dicobalt hexacarbonyl complexes as catalysts.

In this article, we introduce a method of microbial production of 1,3-PD. The development of environmentally benign, or "green", processes for the production of chemicals is of increasing importance as nonrenewable resources are gradually depleted and the world population grows. Microbial fermentation processes are particularly attractive in this regard in that they typically use renewable feedstock such as glucose or sucrose and do not generate toxic byproducts. A variety of naturally occurring microorganisms ferment glycerol to 1,3-PD including species of *Klebsiella*, *Clostridia*, *Citrobacter*, *Enterobacter*, and *Lactobacillus*, but no natural microorganisms ferment sugars directly to 1,3-PD *(5)*.

The dissimilation of glycerol has been studied by many researchers and has been reviewed *(6)*. Glycerol is transported into the cell by the glycerol facilitator protein, a process that requires no energy. Equilibrium across the membrane is very fast. In the absence of external oxidants (e.g., oxygen, fumarate, or nitrate), glycerol is fermented by two parallel pathways. In one of the pathways, glycerol is transformed to dihydroxyacetone by glycerol dehydrogenase and subsequently phosphorylated by adenosine triphosphate–dependent dihydroxyacetone kinase *(7)*. Dihydroxyacetone-phosphate undergoes normal glycolysis to form pyruvate, which is further converted into various organic acids and alcohols. Glycerol is also converted through the second pathway to 3-HPA by the coenzyme B_{12}-dependent glycerol dehydratase *(8)*. 3-HPA is then converted by an $NADH_2$-linked oxidoreductase to 1,3-PD, which is then excreted from the

cell *(9)*. The physiologic purpose for the transformation of glycerol to 1,3-PD is to regenerate the oxidized form of the reducing equivalents, NAD^+, to be used in the energy-producing pathways of glycerol degradation. Fermentation of glycerol, a more reduced carbohydrate than aldoses and ketoses, requires the removal of two extra hydrogen atoms. Figure 1 illustrates these pathways and their resulting end products *(10)*. Enzymes relevant to the initial conversion of glycerol in *Klebsiella* are glycerol kinase, glycerol dehydratase, and glycerol dehydrogenase. The first is formed by the *glp* regulon and the last two by the *dha* regulon. Glycerol kinase is expressed under aerobic conditions, whereas glycerol dehydrogenase is induced only under anaerobic growth and is rapidly inactivated on shifting to an aerobic environment. Glycerol dehydrogenase is also subject to catabolite repression by glucose.

Tong and Cameron *(11)* reviewed the stoichiometry and mass balances for the production of 1,3-PD. An overall mass balance gives the maximum theoretical yields of 1,3-PD from glycerol as 0.875 mol/mol, as shown in Table 1. The theoretical yield is further constrained by the structure of the metabolic pathway since no known microorganism can ferment glycerol entirely to 1,3-PD and CO_2. Other byproducts are obligately produced, such as acetate (reducing the yield to 0.750 mol/mol), or more realistically, a mixture of byproducts, such as acetate and formate (further reducing the yield to 0.667 mol/mol).

Glycerol can be obtained by hydrolysis of natural fats and oil, and by the fermentation of sugars. A yeast culture isolated from molasses is capable of efficient glycerol production from glucose *(12)*. The price of glycerol is $0.80/lb, and 1,3-PD is $0.60/lb (personal communication). Thus, it would not be economical for bioconversion from pure glycerol to 1,3-PD. However, the price of glucose is about $0.07/lb. The yield of glycerol from glucose by yeast fermentation is 0.5 (g of glycerol/g of glucose), and the yield from glycerol to 1,3-PD by *K. pneumoniae* has been 0.5 (g of 1,3-PD/g of glycerol) or more. A two-step fermentation process employing crude glucose as the raw material can therefore still be economically attractive. A large portion of cost in the glycerol bioproduction is owing to separation and purification involving large energy expenses for evaporation and distillation under high vacuum. Another benefit of using raw glycerol is that it reduces the addition of yeast extract required in the second fermentation step by *K. pneumoniae*.

Materials and Methods

Microorganism

A strain screened from *K. pneumoniae* ATCC 25955, which is insensitive to O_2, was used in the fermentation of 1,3-PD from glycerol. For glycerol production from glucose, a *Saccharomyces* LORRE Y8 yeast culture isolated from molasses was employed.

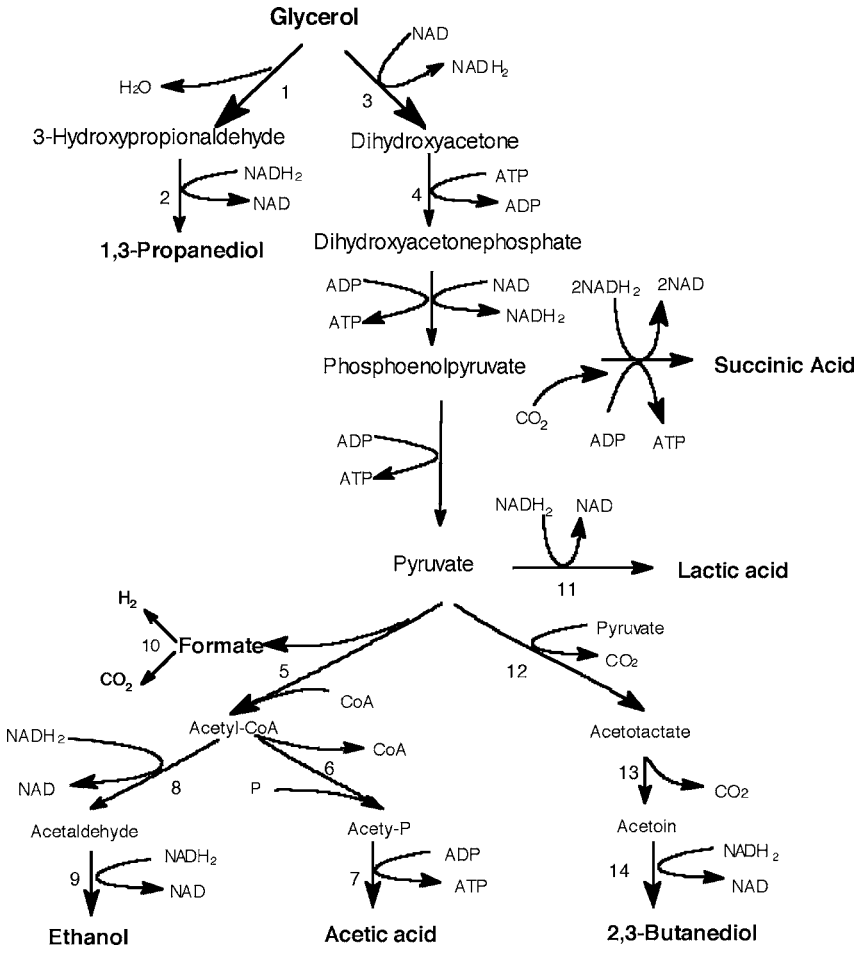

Fig.1. Pathway of anaerobic glycerol metabolism in *K. pneuomniae*.

*1. Glycerol dehydratase
2. 1,3-propanediol oxidoreductase
3. Glycerol dehydrogenase
4. Dihydroxyacetone kinase
5. Pyruvate formate-lyase
6. Phosphotransacetylase
7. Acetate kinase
8. Acetaldehyde dehydrogenase
9. Alcohol dehydrogenase
10. Formate hydrogen-lyase complex
11. D-lactate dehydrogenase
12. Acetolactate synthase
13. Acetolactate decarboxylase
14. 2,3-butanediol dehydrogenase

Culture Media

To prepare inoculum for glycerol fermentation, slant cultures were transferred into seed medium consisting of YMP medium enriched with 2 g/L of KH_2PO_4 and 20 g/L of glucose. A 10% prepared inoculant was used to start fermentation batches. The liquid medium contained 1 g/L of yeast extract, 2 g/L of KH_2PO_4, 4 g/L of urea, and 300 g/L of glucose. To prepare inoculum for 1,3-PD fermentation, slant cultures were transferred into seed

Table 1
Maximum Theoretical Yields of 1,3-PD from Glycerol

Maximum theoretical yield	Yield (mol/mol)
Overall mass balance	
8 glycerol → 7 1,3-PD + $3CO_2$ + 4 H_2O	0.875
Balance subject to metabolic constraints	
4 glycerol → 3 1,3-PD + acetate + CO_2 + $2H_2O$	0.750
3 glycerol → 2 1,3-PD + acetate + formate + H_2O	0.667

medium with 1.5 g/L of yeast extract (Difco, Detroit, MI), 1.5 g/L of malt (Sigma, St. Louis, MO), 2.5 g/L of peptone (Sigma), and 2.5 g/L of NaCl . After adjusting the sterilized medium to pH 7.10 with NaOH or HCl, the 250-mL flask with 50 mL of medium was incubated at a shaker speed of 120 rpm for 12 h at 31°C. The seed medium was transferred into 1-L fermentation medium in a 3-L reactor (New Brunswick) with 5 g/L of yeast extract (Difco), 10 g/L of $K_2HPO_4 \cdot 3H_2O$, 2 g/L of KH_2PO_4, 1 g/L of NH_4Cl, 0.5 g/L of NaCl, 0.1 g/L of $MgSO_4 \cdot 7H_2O$, 30 mg/L of $FeCl_3 \cdot 6H_2O$, 5 mg/L of $CoCl_2 \cdot 6H_2O$, 5 mg/L of vitamin B_{12}, and 30 g/L of glycerol. The fermentation temperature was kept under 31°C. When investigating anaerobic conditions, the reactor was sparged with N_2.

Analytical Methods

Glycerol, 1,3-PD, acetic acid, 2,3-butanediol, lactic acid, alcohol, succinic acid, and glucose were analyzed using a high-performance liquid chromatography (HPLC) system (Hitachi with a Bio-Rad HPX-87H ion-exclusion column). Cell concentration was determined by a spectrophotometer at 590 nm, correlating optical density with dry cell weight. For the dry wt measurement, 100 mL of culture was centrifuged at 4000*g* for 20 min. The pellet was washed once and centrifuged again. The residue was dried for 72 h at 80°C.

Results and Discussion

Fermentation Studies on Pure Glycerol

Comparison of Anaerobic and Aerobic Conditions

According to previous reports in the literature *(5,6,7,9)*, production of 1,3-PD when using glycerol as the sole carbon source is always done under anaerobic conditions. Usually, the delivery of N_2 or CO_2 into the fermentor is required throughout the whole process. To avoid the high cost of N_2 or CO_2 on a large scale, it will be desirable to search for a 1,3-PD production process that can be done under mild aerobic conditions. Figs. 2–4 present the concentration profiles of glycerol, 1,3-PD, acetic acid, lactic acid, and 2,3-butanediol and the absorbance of the broth related to the cell concen-

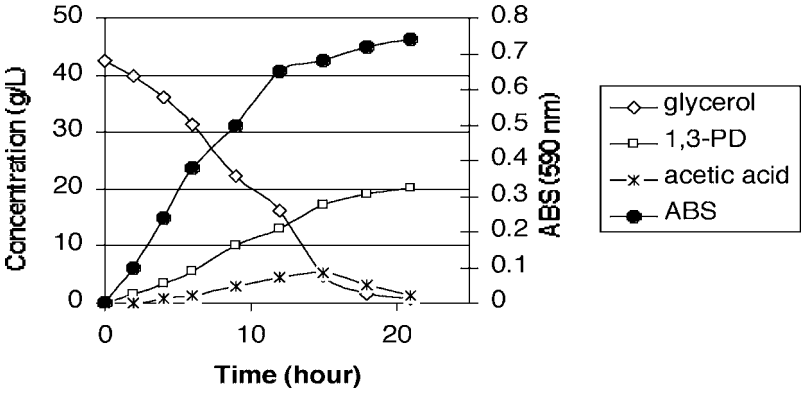

Fig. 2. Batch culture of 1,3-PD with airflow at 0.7 vvm. ABS, absorbance.

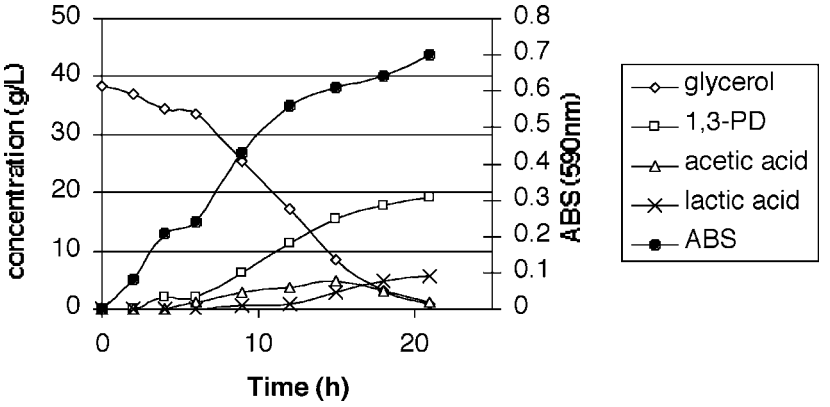

Fig. 3. Batch culture without gas flow. ABS, absorbance.

tration. These three experiments were conducted to compare the yields (mol of 1,3-PD/mol of glycerol), productivity of 1,3-PD g/(L·h) and byproduct concentrations under anaerobic and aerobic conditions (see also Table 2). In the third experiment, in which N_2 was sparged throughout the fermentation, the yield was the highest, 0.63 mol of 1,3-PD/mol of glycerol, while the productivity was the lowest, 0.8 g of glycerol/(L·h). The metabolic pathway from glycerol to 1,3-PD was not totally inhibited by the mild aerobic conditions, which was indicated in Fig. 2, while the yield was still promising with a higher productivity. In addition, the concentrations of the byproducts in Figs. 2 and 3 were fewer than in Fig. 4. In Fig. 2, there were no other byproducts, such as lactic acid and butanediol, but acetic acid was detected by HPLC. Furthermore, acetic acid was consumed at the end of fermentation.

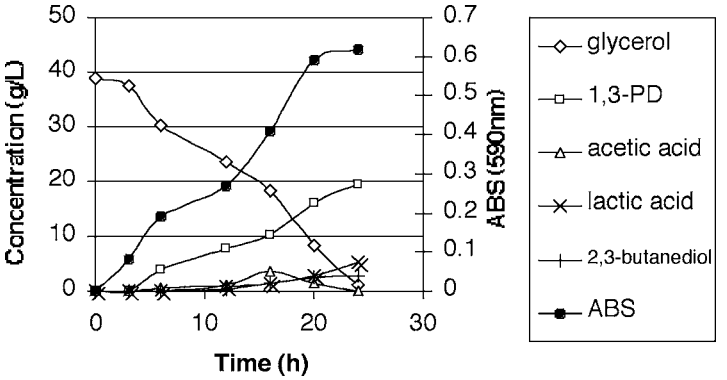

Fig. 4. Batch culture with N_2 sparged throughout the fermentation at 0.3 vvm. ABS, absorbance.

Table 2
Comparison of Yields and Productivities of Aerobic and Anaerobic Conditions

	With N_2	No gas	With air
Yield (mol 1,3-PD/mol glycerol)	0.63	0.61	0.57
Productivity (g 1,3-PD/[L·h])	0.8	1.0	1.2

Table 3
Effects of VB_{12} and $CoCl_2$ on Glycerol Fermentation

No.	Vitamin B_{12} (5 mg/L)	$CoCl_2$ (5 mg/L)	1,3-PD (g/L)
1	Added	Added	14.8
2	Added	Not added	13.7
3	Not added	Added	11.5
4	Not added	Not added	8.7

Effect of Vitamin B_{12} and $CoCl_2$

Coenzyme B_{12}–dependent enzymes are found together in glycerol and diol dehydratases. Vitamin B_{12} and Co^{2+} are important in glycerol fermentation *(9,13,14)*. Comparative experiments were done in which the temperature was set at 31°C and initial glycerol concentrations were 45.0 g/L. The results are given in Table 3. To avoid possible effects of yeast extract, no yeast extract was added, and 5 g/L of glucose was added. The broths were sampled 15 h after inoculation. The addition of vitamin B_{12} and $CoCl_2$ to glycerol fermentation had enhancing effects, even with only a small amount. In all the other fermentations, 5 mg/L of vitamin B_{12} and 5 mg/L of $CoCl_2$ were added initially.

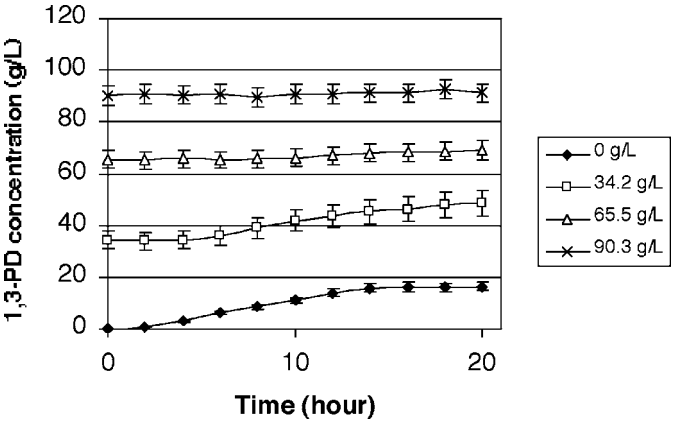

Fig. 5. Product inhibition by 1,3-PD, initial glycerol concentration of 32.5 g/L.

Inhibition by 1,3-PD

The extent of inhibition by 1,3-PD in glycerol fermentation was investigated. Batch fermentations were carried out with different initial 1,3-PD concentrations. All the other fermentation conditions and chemical concentrations were kept the same. Thus, decreases in fermentation rates and the increase in the lag phase can be attributed to product inhibition. The initial glycerol concentration was 32.5 g/L. The profiles in Fig. 5 indicated that there was apparent product inhibition on the glycerol fermentation, in which the initial 1,3-PD concentrations would be 0, 34.2, 65.5, and 90.3 g/L. The productivity and yield declined as the 1,3-PD concentration increased. When the concentration of 1,3-PD exceeded 60 g/L, the productivity dropped to no more than 0.2 g/(L·h) 1,3-PD, and the yield dropped to <0.15 mol/mol. The growth of microorganism was greatly suppressed at 90 g/L of 1,3-PD, and the metabolic pathway from glycerol to 1,3-PD was totally inhibited.

Fed-batch Fermentation Studies

Figure 6 presents the results of the fed-batch fermentation with two discrete additions of pure glycerol. The pH, temperature, and agitation speed were controlled at 7.1, 31°C, and 60 rpm, respectively. Thirty percent ammonia ($NH_3 \cdot H_2O$) was used for pH control. In this experiment, 2 g of yeast extract was added at 10 and 20 h. The final concentration of 1,3-PD was 46.1 g/L at 30 h. The yield of 1,3-PD was 0.51 mol/mol, and the productivity was 1.5 g/(L·h). The production of 1,3-PD is a result of the primary energy metabolism. At 20 h, the growth of the microorganism became slow, as shown by the cell dry wt. Thus, the productivity of 1,3-PD was also decreased. At the same time, the concentrations of lactic acid and ethanol were increased dramatically, as an alternate energy metabolic pathway to 1,3-PD. The final concentrations of lactic acid and ethanol were as high as 22.4 and 13.4 g/L, respectively.

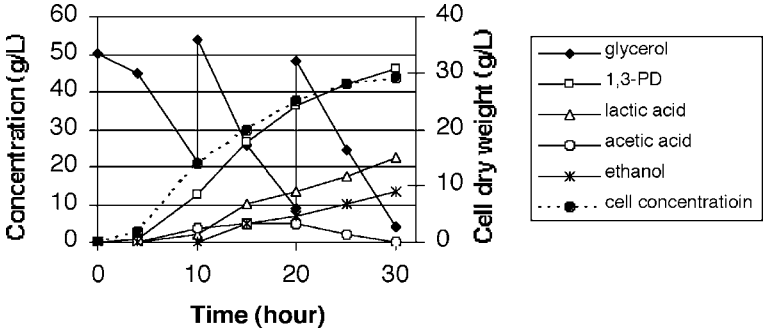

Fig. 6. Fed-batch fermentation with two discrete additions of pure glycerol.

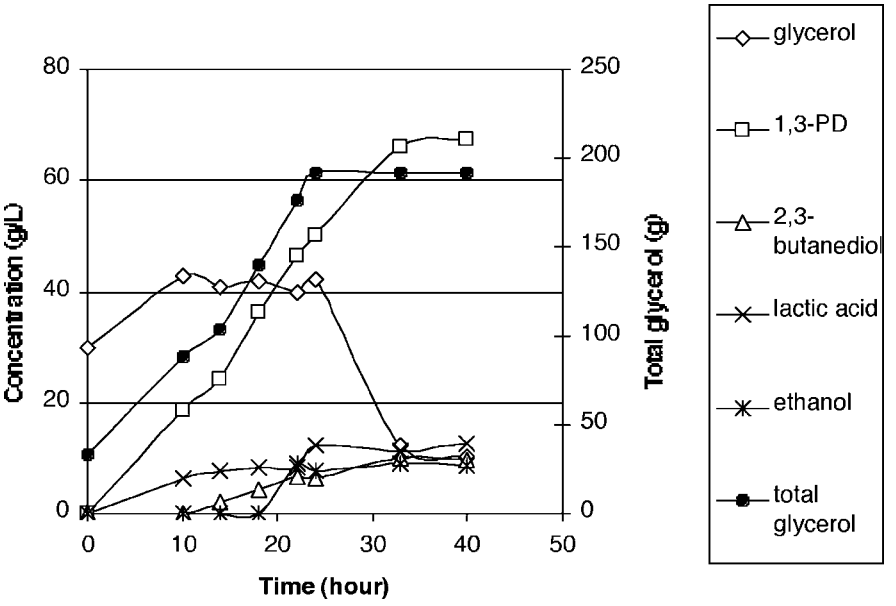

Fig. 7. Fed-batch fermentation with glycerol and ammonia mixed together.

To increase 1,3-PD yield and to reduce byproduct formation, a series of experiments was conducted in which different ratios of ammonia and glycerol mixtures were used for pH control as well as for supplementation of glycerol. Glycerol and ammonia were mixed with different proportions. The initial glycerol concentration was 30.0 g/L. The pH was adjusted at 7.1 by mixing glycerol and ammonia proportionally. From 0 to 10 h, 140 g of glycerol was mixed with 50 mL of ammonia (30%). From 10 to 24 h, 150 g of glycerol was mixed with 20 mL of ammonia and 20 mL of yeast extract (0.1 g/mL). After 24 h, only ammonia was added to adjust the pH. Twenty milliliters of yeast extract was added at 24 h. No gas was sparged from 0 to 24 h, but after 24 h, air was sparged at 0.3 vvm; results are shown in Fig. 7. The final concentration of 1,3-PD was 67.3 g/L, and the yield was 0.61 mol/mol. The productivity of 1,3-PD was calculated to be 1.7 g/(L·h). Compared

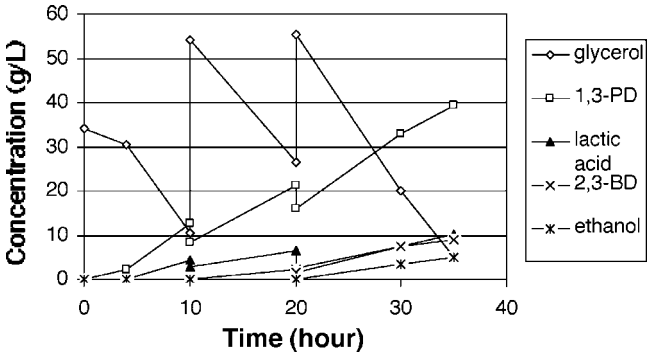

Fig. 8. Fed-batch fermentation with two discrete additions of raw glycerol.

with the former experiment from Fig. 7, the byproduct concentrations were controlled so that the yield of 1,3-PD was improved.

Production of 1,3-PD from Raw Glycerol

For the preparation of raw glycerol, the yeast strain *Saccharomyces* LORRE Y8 was used to ferment glucose under both batch and fed-batch fermentation conditions. The final glycerol concentration of a typical batch culture was 120–150 g/L, and for a fed-batch fermentation, it was 200–250 g/L. In the batch fermentation, glycerol was the only product. In the fed-batch fermentation, the byproducts and substrate, such as glucose, ethanol, butanediol, and lactic acid, were <5 g/L at the end of the fermentation. Further details have been reported elsewhere *(12)*.

The glycerol broth was autoclaved along with yeast cells. After settling for 24 h, the upper liquid was used directly as the feed for 1,3-PD fermentation without further treatment. 5 g/L of $K_2HPO_4{\cdot}3H_2O$, 1 g/L of KH_2PO_4, 1 g/L of NH_4Cl, 0.5 g/L of NaCl, 0.1 g/L of $MgSO_4{\cdot}7H_2O$, 30 mg/L of $FeCl_3{\cdot}6H_2O$, 5 mg/L of $CoCl_2{\cdot}6H_2O$, 5 mg/L of Vitamin B_{12}, were added to the crude medium: Figure 8 presents the results of a fed-batch fermentation with two discrete additions of raw glycerol. The glycerol concentration of yeast broth was 142 g/L. The final concentration of 1,3-PD was 39.4 g/L, and the yield of 1,3-PD was 0.54 mol/mol with a productivity of, 1.1 g/(L·h). Yeast extract 2 g/L (Difco) was added at the beginning of the fermentation but not in the discrete feed added later.

Figure 9 shows the fed-batch fermentation of raw glycerol mixed with ammonia with the pH adjusted at 7.1. The glycerol concentration in the yeast fermentation broth was 225 g/L. From 0 to 11 h, 200 mL of raw glycerol was mixed with 30 mL of ammonia. From 11 to 34 h, the ratio of the mixture of raw glycerol and ammonia was changed, and 500 mL of raw glycerol was mixed with 20 mL of ammonia to adjust the pH. After 34 h, only ammonia was used to adjust the pH until the end of the fermentation. The final 1,3-PD concentration was 50.1 g/L. A high yield (0.61 mol of 1,3-PD/mol of glycerol) with a productivity of 1.1 g/(L·h) was achieved. The glycerol consumed was attained without the supplementation of yeast extract.

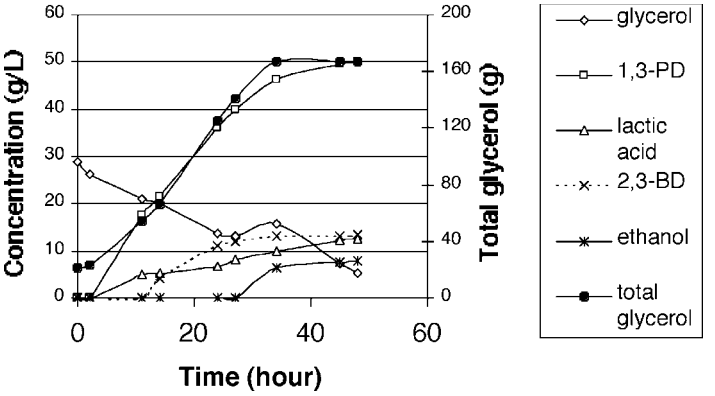

Fig. 9. Fed-batch fermentation by raw glycerol mixed with ammonia.

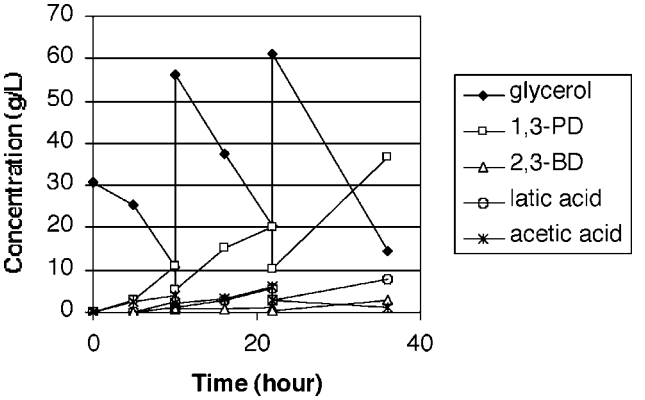

Fig. 10. Fed-batch fermentation with fixed volume.

A fed-batch fermentation of fixed volume is also shown in Fig. 10. The initial fermentation volume was 1000 mL, mixed with raw glycerol and sterilized water. At 10 h, 500 mL of broth was pumped out and replaced by 500 mL of raw glycerol (102 g/L of glycerol). At 22 h, another replacement was done. The final concentration of 1,3-PD was 39.4 g/L, which is not high. However, the yield of 1,3-PD was 0.70 mol/mol of glycerol consumed, with a productivity of 1.5 g/(L·h). Byproducts such as lactic acid, 2,3-butanediol, and acetic acid produced under this fermentation condition were less than under typical fermentations. No ethanol was detected in this case. The experimental results implied that the production of 1,3-PD was well related to bacterial growth, particularly during the log phase. The fed-batch fermentation of fixed volume would keep the microorganism growth in the log phase, demanding the 1,3-PD metabolic pathway as a major regeneration route of NAD^+. In other experiments, by examining cell concentration, it was found that the microorganism was in the stationary phase after 12 h or longer. Thus, the metabolic pathways of 2,3-butanediol, lactic acid, and

ethanol would be important to the microorganism in the regeneration NAD^+, especially in the later phase of the fermentation. Using this method, the byproduct concentration could be effectively controlled, resulting in enhancement of product yield.

Conclusion

Batch and fed-batch fermentation studies of glycerol to 1,3-PD were conducted using *K. pneumoniae* ATCC 25955 under mild aerobic conditions. In addition, a two-step fermentation process was studied in which glycerol produced from glucose by yeast was used to generate 1,3-PD. This two-step process provides a promising route to produce 1,3-PD directly from abundant agricultural products.

Acknowledgments

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