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Comparison of Glucose and Glycerol as Carbon Sources for ε-Poly-L-Lysine Production by *Streptomyces* sp. M-Z18

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Abstract Glucose is widely used in the production of ε-poly-L-lysine (ε-PL); however, glycerol is an emerging carbon source for ε-PL production in recent years. Glycerol is superior to glucose for ε-PL production according to batch and fed-batch fermentations by *Streptomyces* sp. M-Z18 in this study. To elaborate this difference, physiological metabolism of *Streptomyces* sp. M-Z18 on glycerol has compared with glucose during batch fermentation. The activities of key enzymes showed that aspartate kinase (ASPK) and ε-PL synthetase (Pls) in glycerol medium was higher than those in glucose, and especially the activity of Pls could enhance by 2.3- and 3.6-fold at 24 and 36 h, respectively. Moreover, metabolism flux analysis demonstrated that a 25 % higher fluxes derived from glycerol are directed into ε-PL synthesis than glucose. As a result, the mechanism of glycerol better than glucose is determined: the activities of ASPK and Pls in glycerol higher than those in glucose, and result in carbon fluxes directed into ε-PL synthesis increased. This study offers a reference to substitute glycerol for conventional glucose as carbon source for ε-PL production.

Keywords ε-Poly-L-lysine · Glucose · Glycerol · Batch and fed-batch fermentation · Metabolism flux analysis · *Streptomyces*

Introduction

ε-Poly-L-lysine (ε-PL), which typically consists of 25–35 L-lysine residues with linkages between α-carboxyl groups and ε-amino groups, is an extracellular secondary metabolite catalyzed by a highly unusual nonribosomal peptide synthetase from *Streptomyces*. ε-PL is one of only two known natural amino-acid homo-polymers, the other is γ-poly-glutamic acid. This polymer has many attractive properties such as biodegradable, water soluble, antimicrobial activity, antiphage action, endotoxin-selective removal action, and antiobesity action [1]. Currently, ε-PL is mainly used as food preservation for preventing food from the

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contaminations of Gram-positive and Gram-negative bacteria, fungi, and some viruses, etc. [2]. Meanwhile, ε-PL used as natural food preservation have been approved by the US Food and Drug Administration (GRN000135) since 2004, and used in certain countries, including Japan, Korea, and the United States for many years [3].

To meet the great demand for ε-PL in such application, numerous biotechnological methods have been developed and exploited to increase its yield and productivity. The production of ε-PL mostly originates from glucose via pH control strategy, immobilized cells, or in situ product removal technology [4–6]. Although glycerol and glucose have been proved the best carbon sources for ε-PL production, glycerol was not considered as an alternative carbon source due to its high cost of raw material in the past few decades [7]. However, with the increasing of biodiesel production from triacylglycerol oils, there has been a massive increase in the availability of glycerol, a byproduct of biodiesel manufacture (10 %, *w*/*w*) [8]. Furthermore, glycerol as carbon source has no dilution effect in fed-batch fermentation, resulting in the cost of subsequent extraction decrease in industry. Therefore, glycerol is likely to become an inexpensive and readily available carbon source for many industrial fermentation processes [9, 10].

Recently, glycerol is used as an alternative carbon source for ε-PL production. Bankar and Singhal have investigated glycerol as carbon source for ε-PL production by *Streptomyces noursei* NRRL 5126 through medium optimization [11]. Furthermore, ε-PL yield and productivity enhanced significantly through adding metabolic precursors and controlling dissolved oxygen during fermentation [12, 13]. Coincidentally, we also performed systematic media optimizations and finally determined glycerol as carbon source for ε-PL production by *Streptomyces* sp. M-Z18 [14]. Moreover, we have further increased ε-PL production through fermentation process regulation [15]. As a result, it is feasible that glycerol is used as an alternative carbon source for ε-PL production.

In this study, we find significant difference of ε-PL production from glucose and glycerol during batch and fed-batch fermentation by *Streptomyces* sp. M-Z18. Thus, the activity of key enzymes (phosphoenolpyruvate carboxylase, aspartate kinase, and ε-PL synthetase) and metabolite flux were studied, aiming at revealing the physiological metabolism difference of *Streptomyces* sp. M-Z18 in the two carbon sources, to understand the physiological changes of *Streptomyces* with different carbon sources.

Materials and Methods

Strain and Culture Conditions

Streptomyces sp. M-Z18, used throughout this study, was isolated from soil as described by Nishikawa and Ogawa [16] and has been subjected to ultraviolet and nitrosoguanidine mutagenesis as described by Hiraki et al. [17].

To obtain the seed culture, a loopful of spores (approximately 2.0×10^5 spores/L) was inoculated into a 250-mL flask-shake containing 40 mL sterilized M3G medium (50 g/L glucose [Sinopharm Chemical Reagent Co., Ltd. (SCRC), Shanghai, China]; 5 g/L yeast extract [Oxoid Ltd., Hampshire, England], 10 g/L $(NH_4)_2SO_4$ [SCRC, Shanghai, China], 1.36 g/L KH_2PO_4 [SCRC, Shanghai, China], 0.8 g/L K_2HPO_4 [SCRC, Shanghai, China], 0.5 g/L $MgSO_4{\cdot}7H_2O$ [SCRC, Shanghai, China], 0.04 g/L $ZnSO_4{\cdot}7H_2O$ [SCRC, Shanghai, China], 0.03 g/L $FeSO_4{\cdot}7H_2O$ [SCRC, Shanghai, China], pH 6.8) and cultured for 24 h at 30 °C with shaking at 200 rpm and subsequently used as seed culture.

Batch fermentations were performed in a 5-L jar fermenter (Baoxing Corp. Shanghai, China) with 3.5-L working volume. The above 300 mL of pre-cultured seed was inoculated into 3.2-L sterilized fermentation medium (60 g/L glucose/industrial glycerol [Kerry Oleochemical Industrial (Shanghai) Co. Ltd., Shanghai, China], 5 g/L $(NH_4)_2SO_4$, 10 g/L beef extract [SCRC, Shanghai, China], 4 g/L KH_2PO_4, 0.8 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $FeSO_4{\cdot}7H_2O$) with an initial pH of 6.8. Agitation was provided by two Rushton turbines and varied from 200 to 900 rpm, so that it could control dissolved oxygen (DO) at 20–30 % which was monitored with O_2 sensor (InPro6800-12-220, Mettler Toledo, Zurich, Switzerland). Aeration was provided by a ring sparger with a range of 0.5 to 3.0 vvm. The pH change was detected by a pH electrode (K8S-225, Mettler Toledo, Zurich, Switzerland) during cultivation and maintained at pH 3.8 by automatically adding ammonia water (12 %, *w*/*v*) (SCRC, Shanghai, China). The fermentation temperature was maintained using a re-circulating water bath at 30 °C.

Fed-batch fermentation was performed as our previous study [15]: when the glycerol or glucose in the above ε-PL batch fermentation was below 10 g/L, a sterilized pure glycerol or 800 g/L glucose solution was automatically and pulsed added to the culture broth by peristaltic pump, to maintain the residual glycerol or glucose at about 10 g/L. In addition, when NH_4^+–N concentration was below 1 g/L, a sterilized 600 g/L $(NH_4)_2SO_4$ solution was also added to fermentation culture at the same manner as feeding glycerol or glucose to maintain the residual NH_4^+–N at about 1 g/L.

Enzyme Assays

Cell extracts for the assays of phosphoenolpyruvate carboxylase (PEPc) and aspartate kinase (ASPK) activities determination were prepared as follows [18]: cell cultivation for 24 and 36 h during fermentation were harvested and washed twice with 0.2 % (*w*/*v*) KCl and resuspended in Tris-tricarballylate buffer (200 mM), containing $MgCl_2$ (5 mM) and glycerol (20 %, *v*/*v*). The suspension was subsequently sonicated by a sonifier (SM-650D, Shunma Tech., Nanjing, China) at 0 °C for a total period of 10 min (pulse on, 1 s; pulse off, 2 s). Cell debris were removed by centrifugation at 16,000×*g* at 4 °C for 20 min, and the supernatant was then used as the cell-free crude enzyme. Protein concentrations were determined by the method of Bradford [19]. PEPc was spectrophotometrically determined by measuring the initial rate of decrease in absorbancy at 340 nm with the following composition [20]: 100 mM *N*-Tris(hydroxy methyl)methyl-2-aminoethanesulfonic acid-KOH (TES-KOH) buffer (pH 7.5), 2 mM phosphoenolpyruvate (PEP), 3.3 mM $MnSO_4$, 10 mM $NaHCO_3$, 0.15 mM NADH, 10 μg malate dehydrogenase (pig heart) and the crude enzyme in a final volume of 0.5 mL at room temperature. One unit of the enzyme is defined as the amount of enzyme causing conversion of 1 μmol NADH to NAD^+ per minute. ASPK activity was spectrophotometrically assayed by measuring the amount of aspartyl-β-hydroxamate formed in a 0.5-ml reaction mixture with the following composition [21]: 600 mM $(NH_4)_2SO_4$, 600 mM hydroxylamine-KOH (pH 7.0), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartic acid-KOH (pH 7.0), and 100 μL crude enzyme supernatant. The reaction mixtures were incubated for 10 min at 30 °C, and background activity was measured in the absence of aspartic acid. All assays were carried out under linear conditions. The amount of aspartyl-β-hydroxamate was calculated from the molar extinction coefficient of 600. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol aspartyl-β-hydroxamate per minute at 30 °C.

In order to measure the activity of ε-PL synthetase (Pls), the mycelia (2 g wet weight) from a fermenter culture were harvested at the same time with the above and suspended in a total of 10 mL buffer (100 mM Tris–HCl, 20 % glycerol, 2 mM EDTA, 200 mM NaCl, and 5 mM

dithiothreitol (pH 8.0)), and then sonicated with 10 min. After centrifugation at 16,000×*g* for 20 min, the supernatant was collected as a crude extract. This supernatant was centrifuged at 160,000×*g* for 1 h, and then the precipitation (membrane fraction) was resuspended with 100 mM Tris–HCl (pH 8.0) [22]. Pls activity was measured based on Kawai et al. report [23], by monitoring the reduction of L-lysine using HPLC in a 1-mL reaction mixture with the following composition: 100 mM L-lysine, 50 mM ATP, 50 mM $MgCl_2$, 50 mM dithiothreitol, 20 μL membrane fraction solutions, 100 mM Tris–HCl (pH 8.0). The reaction mixtures were incubated for 30 min at 30 °C, and then the reaction was terminated by adding 20 % trichloroacetic acid. For the control experiment, trichloroacetic acid was added at the beginning of the experiment. After centrifugation, the residual L-lysine was determined by HPLC and calculated the activity of Pls. One unit of enzyme activity is defined as the amount of enzyme catalyzing the consumption of 1 pmol L-lysine per second at 30 °C.

All of the above chemicals, including substrates, coenzymes, and buffers, were used for enzyme activity determination were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Metabolic Reaction Model

For metabolic flux distribution analysis purpose, a simplified metabolic reaction model for ε-PL production by *Streptomyces* sp. M-Z18 under different carbon sources was considered. The primary metabolic network was reconstructed based on data about glucose and glycerol metabolism in *Streptomyces* [24]. It is reported that lysine biosynthesis may occur by two entirely distinct pathways: homocitrate pathway, mainly existed in higher fungi, synthesized lysine via α-aminoadipic acid as an intermediate; diaminopimelic acid (DAP) pathway, normally occurred in bacteria, proceeded aspartic acid to lysine via DAP [25]. The phylogenetic position of the *Streptomyces* and the reports of DAP in *Streptomycete* cell walls [26], suggesting that the DAP pathway would be operative in this genus. Therefore, the simplified models of ε-PL synthesis covered glycolytic pathway, gluconeogenesis pathway, pentose phosphate pathway, tricarboxylic acid cycle, DAP pathway, and the pathway of L-lysine conversion to ε-PL through ε-PL synthase from glucose and glycerol were constructed and showed in Fig. 3. Meanwhile, the following assumptions were made for the network models: (1) NADH converted into ATP through electronic respiratory chain oxidation for metabolites production, and NADPH used for biomass synthesis; (2) The fluxes towards cell mass of *Streptomyces* sp. M-Z18 were calculated based on precursor requirements for cell mass biosynthesis in *Escherichia coli*, this assumption has been proven acceptable by Daae and Ison [27]; (3) The composition of biomass does not changed during cell growth; (4) Reactions without metabolic bypass were merged into one reaction; (5) The flux of cell maintenance, energy, and CO_2 release and unknown metabolites synthesis is incorporated into biomass synthesis flux; (6) Pseudo-steady-state assumption was adopted for the intracellular intermediate metabolites.

Metabolic Flux Analysis

As shown in Appendix A, the models contained 34 metabolic reaction rates and 29 metabolites. In the metabolic analysis, six metabolic reactions rates, i.e., substrate consumption rate, extracellular L-lysine production rate, ε-PL production rate, glutamate production rate, threonine production rate, and specific growth rate, were calculated by measuring their concentrations variation in the period of 33–38 h, which were at the late exponential stage and showed in Table 1. As unknown metabolic reaction rates number less than metabolites, the metabolic reaction matrixes could be solely solved using software Matlab ver. R2007b (The MathWorks, Natick, MA, USA) in personal computer.

Table 1 The substrate consumption rate and the formation rate of metabolic product during 33–38 h when *Streptomyces* sp. M-Z18 was grown on glucose and glycerol in batch fermentation

Carbon source	Substrate consumption rate (mmol/L/g DCW/h)	Metabolic product formation rate (mmol/L/g DCW/h)				
	Glucose/glycerol ($\times10^{-1}$)	Lys ($\times10^{-4}$)	ε-PL ($\times10^{-1}$)	Glu ($\times10^{-3}$)	Thr ($\times10^{-4}$)	DCW[a] ($\times10^{-2}$)
Glucose	8.74	1.93	1.12	1.66	1.04	3.20
Glycerol	9.78	3.17	1.57	1.23	0.58	2.80

The values were the average of twice replicates. *Lys* lysine, *Glu* glutamate, *Thr* threonine

[a] DCW formation rate unit is h^{-1}

Determination of the Biomass, ε-PL, Glycerol, and Amino Acids

Samples were withdrawn from fermenter for analysis at regular intervals. The broth was centrifuged (4,500×*g*, 10 min), and the precipitate was collected, washed twice with distilled water, and dried at 105 °C to constant weight to determine the biomass of the culture (dry cell weight, DCW), and the supernatant was used to determine ε-PL concentration using the methyl orange precipitation method according to the procedure described by Itzhaki [28]. Glycerol and glucose in culture broths were measured using an HPLC system (DIONEX U-3000, Thermo Scientific, Utah, US) with a refraction index detector (Shodex RI-101, Tokyo, Japan) and an ion exchange column (Bio-Rad Aminex HPX-87H, Hercules, CA, USA). The column was eluted with 5 mM H_2SO_4 at a temperature of 60 °C and a flow rate of 0.6 mL/min. Amino acids (threonine, glutamate, and lysine) were automated precolumn derivatized by *o*-phthaldialdehyde and were analyzed with an Agilent 1100 HPLC (Agilent, Palo Alto, USA) equipped with a reverse-phase column (Zorbax Eclipse-AAA) and UV detector at 338 nm according to the procedure established by Henderson et al. [29].

Results and Discussion

Fermentation Performances in ε-PL batch and Fed-Batch Fermentations using Glucose and Glycerol as Carbon Sources

To evaluate the effects of glucose and glycerol on cell growth and ε-PL production, batch and fed-batch fermentations were conducted in 5-L fermenter by *Streptomyces* sp. M-Z18. The time courses of biomass, specific cell growth rate, ε-PL production, and specific ε-PL formation rate of batch fermentations are shown in Fig. 1, and the results of fed-batch fermentation are summarized in Table 2.

As depicted in Fig. 1a, c, the behaviors of biomass and specific cell growth rate in glucose and glycerol culture showed the same trends during batch fermentation. Meanwhile, the final biomasses obtained in the two substrates were at the same levels. It is indicated that these two substrates are available for cell growth of *Streptomyces* sp. M-Z18 and make no significant difference in biomass. However, glucose and glycerol have resulted in ε-PL production apparently different. ε-PL production from glycerol attained the highest specific ε-PL production rate at about 26 h with 0.03 h^{-1}, whereas the highest specific ε-PL production rate from glucose achieved at about 20 h with 0.02 h^{-1} (Fig. 1d). Furthermore, the specific ε-PL production rate from glycerol was higher than glucose after 15 h fermentation. As a result, the final ε-PL production from glycerol was enhanced by 44.4 % and

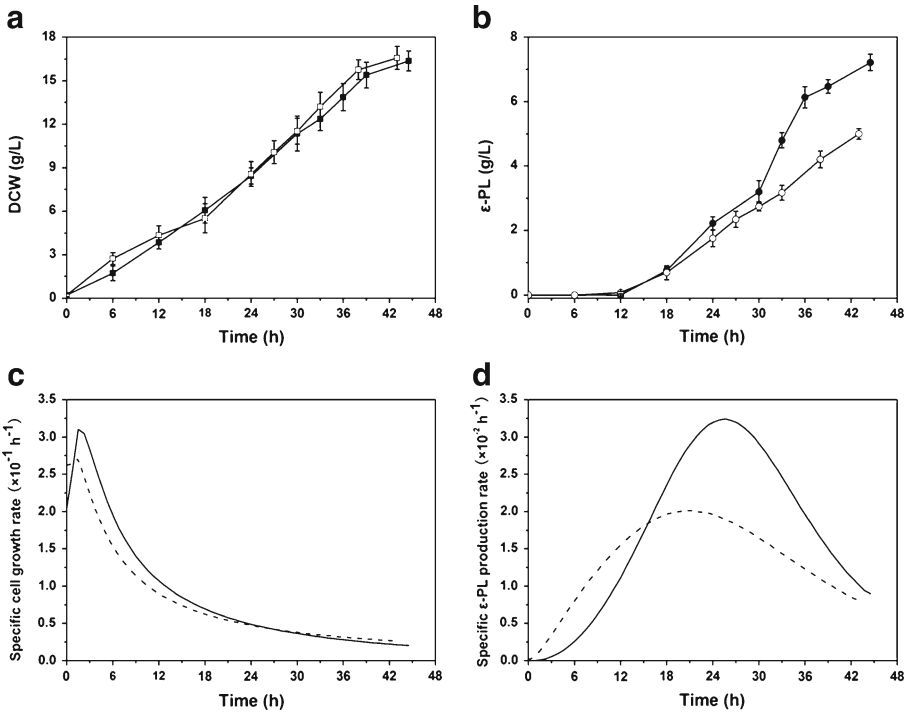

Fig. 1 Comparison of process parameters between glucose and glycerol as carbon sources for ε-PL production under batch fermentation in a 5-L fermenter by *Streptomyces* sp. M-Z18 at pH 3.8. *Filled symbols* and *solid lines* are used for the glycerol medium: cell growth (*filled squares*), ε-PL production (*filled circles*). *Empty symbols* and *dash lines* are used for the glucose medium; cell growth (*empty squares*), ε-PL production (*empty circles*). **a** Cell growth, **b** ε-PL production, **c** specific cell growth rate, **d** specific ε-PL production rate. The values were the average of three replicates. *Error bars* show the standard deviation

attained 7.22 g/L compared with glucose (Fig. 1b). Therefore, glycerol is a benefit for ε-PL production by *Streptomyces* sp. M-Z18 in batch fermentation.

Table 2 ε-PL production process parameters in glucose and glycerol under fed-batch fermentation by *Streptomyces* sp. M-Z18

Parameters	Glucose	Glycerol
Carbon source consumption (g)	885	675
Total fermentation volum (L)	4.6	3.1
Fermentation time (h)	168	173
Maximum DCW (g/L)	24.53	40.66
Maximum ε-PL concentration (g/L)	24.54	30.11
Average specific carbon source consumption rate (h^{-1})	0.139	0.069
Average specific growth rate (h^{-1})	0.012	0.012
Average specific ε-PL production rate (h^{-1})	0.012	0.009
Cell productivity (g/L/day)	3.50	5.64
ε-PL productivity (g/L/day)	3.51	4.18
Cell yield on carbon source (g/g)	0.086	0.169
ε-PL yield on carbon source (g/g)	0.086	0.132

In the case of fed-batch fermentation (Table 2), glycerol resulted in a higher ε-PL titer (30.11 g/L), a higher ε-PL productivity (4.18 g/L/day) and a higher ε-PL yield (0.132 g/g), enhanced by 22.7 %, 19.1 %, and 53.5 %, respectively, compared with glucose. Furthermore, the maximum DCW and cell productivity from glycerol were also improved by 65.8 % and 61.1 %, respectively, than from glucose. The lower biomass and cell productivity in glucose might be result from a higher dilution rate (31.4 %) (Table 2), which might induce considerable cell stress and lead to biomass and cell productivity undesirable reduction. It is noted that the biomass of ε-PL production from glucose has not exceeded to 30 g/L regardless of what fermentation approaches were performed in previous reports [5, 30, 31], including the highest ε-PL production (48.3 g/L) reported so far according to our knowledge [4]. Therefore, glycerol is also the best carbon source for ε-PL production in fed-batch fermentation due to significant increase of biomass. However, glycerol is superior to glucose for ε-PL production in batch fermentation derived from the improvement of ε-PL productivity of *Streptomyces* sp. M-Z18.

Key Enzyme Performances in ε-PL Batch Fermentation using Glucose and Glycerol as Carbon Sources

To investigate the mechanism of differences between glucose and glycerol for ε-PL production by *Streptomyces* sp. M-Z18, the activities of three key enzymes in the pathway of ε-PL synthesis, namely, phosphoenolpyruvate carboxylase (PEPC) catalyzing phosphoenolpyruvate into oxaloacetate in anaplerotic reactions, aspartate kinase (ASPK) converting aspartate into 4-phospho-L-aspartate, and ε-PL synthetase (Pls) catalyzing L-lysine into ε-PL, were analyzed at 24 and 36 h during ε-PL batch fermentation. As shown in Fig. 2, the activity of PEPC in glycerol was lower than in glucose; however, the activities of ASPK and Pls in glycerol was higher than those in glucose. These results indicated that the flux of citric acid cycle in metabolism of glycerol is low and results in anaplerotic reaction rate decrease, but the flux of ε-PL synthesis is more active. In the glucose medium, the above three enzymes showed different performances during fermentation from 24 to 36 h: the activity of PEPC declined 74.3 % (Fig. 2a), the activity of ASPK slightly decreased (Fig. 2b), and the activity of Pls increased 103.4 % (Fig. 2c). However, in the glycerol medium, the activity of PEPC declined 89.6 %, the activity of ASPK decreased 42.3 %, and the activity of Pls increased 179.6 %. With the progress of fermentation, the activities of PEPC and ASPK of *Streptomyces* sp. M-Z18 in glucose or glycerol decrease is a common phenomenon because of cell aging. However, the activity of Pls in the glycerol was enhanced by 2.3- and 3.6-fold, respectively, compared with glucose (Fig. 2b, c) at 24 and 36 h. It might be suggested that glycerol is benefit for improving the activity of Pls. In fact, multi-hydroxy compounds, such as sorbitol and trehalose, have been proved that they have some protective effect of some enzymes activities through stabilizing secondary and three-dimensional structures under extreme conditions [32, 33]. Therefore, it is reasonably speculated that glycerol could maintain Pls spatial structures through multi-hydroxy molecular structure to enhance its catalytic activities during fermentation, especially in the late phase of fermentation, encountering kinds of metabolic byproducts repression. In summary, it is demonstrated that glycerol superior than glucose for ε-PL synthesis is achieved by increasing the activity of key enzyme of Pls.

Metabolic Flux Distributions in ε-PL Batch Fermentation using Glucose and Glycerol as Carbon Source

Metabolic flux analysis provides a framework for determining pathway fluxes from the stoichiometry of the pathway reactions in combination with extracellular metabolite

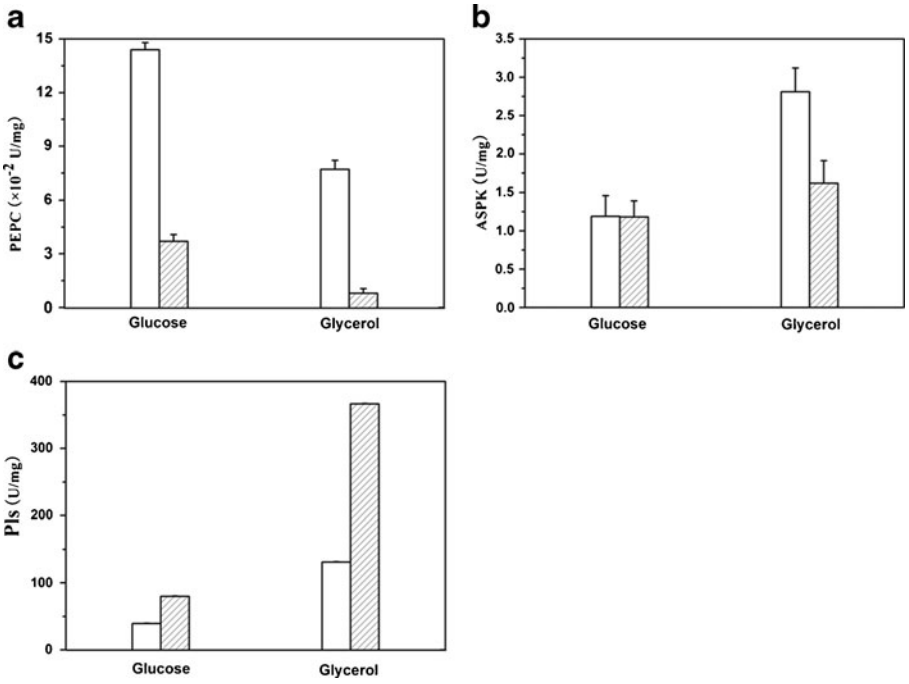

Fig. 2 Comparison of key enzyme activity of ε-PL production from glucose and glycerol by *Streptomyces* sp. M-Z18 at 24 and 36 h. *White bars* represent enzyme activity at 24 h during fermentation. *Slash bars* represent enzyme activity at 36 h during fermentation. **a** The activity of phosphoenolpyruvate carboxylase (PEPC); **b** the activity of asparate kinase (ASPK); **c** the activity of ε-PL synthetase (Pls). The values were the average of three replicates. *Error bars* show the standard deviation

measurements [35]. It emerges as a key methodology to provide information of cell physiology and metric of the cellular metabolic phenotype [36]. Therefore, we constructed a metabolic reaction model for central carbon metabolism and ε-PL synthetic pathways of *Streptomyces* sp. M-Z18 (Fig. 3), which would provide a brand new understanding of ε-PL biosynthesis in glucose and glycerol.

Based on determination of glucose and glycerol consumptions, L-lysine production (Lys), ε-PL production, glutamate production (Glu), threonine production (Thr), and specific growth rate, the corresponding rates were calculated in batch culture from 33 to 38 h, and the result was shown in Table 1. As a consequence, the relevant carbon flux distribution of *Streptomyces* sp. M-Z18 under two carbon sources were calculated in the principle of metabolic flux analysis and shown in Fig. 3. With glycerol in the medium, the carbon fluxes from oxaloacetate to aspartate and lysine to ε-PL reached 18.2 and 16.0 mmol/g DCW/h, respectively, which are increased by 16.7 % and 25 %, respectively, than glucose as carbon source. It was a delight to see that these flux analysis results were consistent with the finding of activities of ASPK and Pls enhanced by glycerol. Surprisingly, although PEPC activity in glycerol medium was lower than glucose, the carbon flux from phosphoenolpyruvate to oxaloacetate was enhanced by 16.7 %. On the other hand, carbon fluxes from glycerol directed into pentose phosphate pathway for providing cofactor NADPH used for cell and ε-PL synthesis was also improved by 16.7 %, compared to glucose as carbon source. However, less carbon fluxes from glycerol converted to byproduct of amino acids. It might be speculated that glycerol and precursor L-lysine have essentially the same redox state

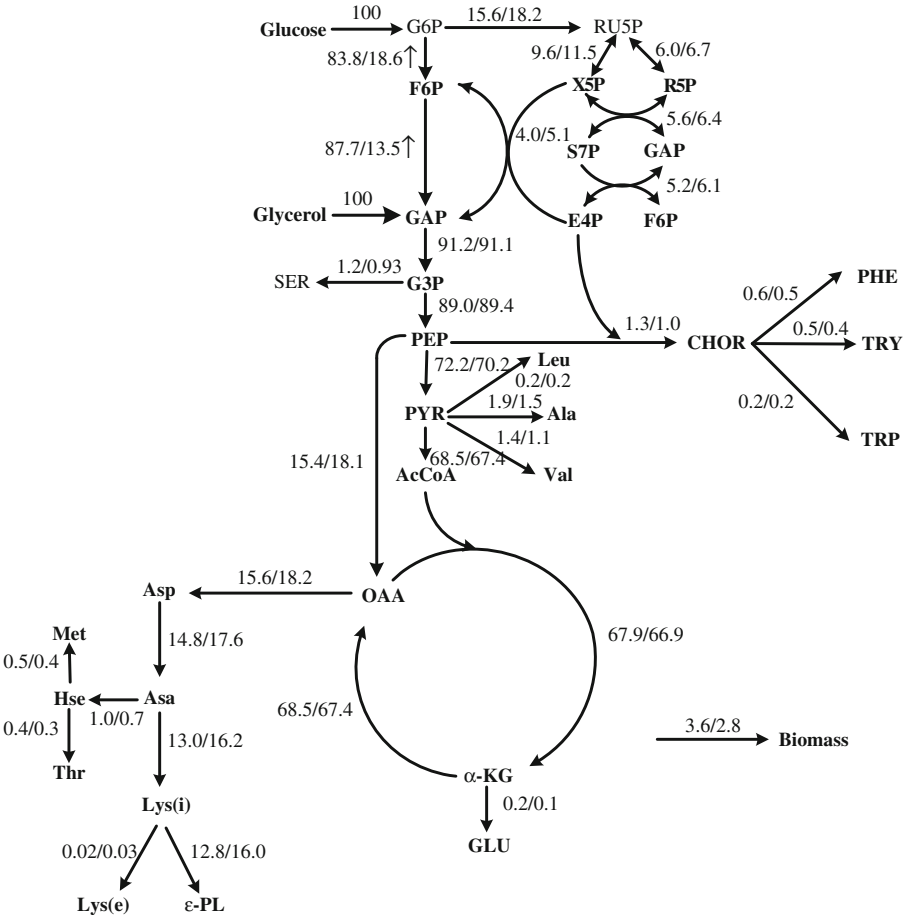

Fig. 3 Simplified metabolic network and metabolic flux distribution in ε-PL production from glucose and glycerol, respectively, between 33 and 38 h by *Streptomyces* sp. M-Z18. Abbreviations in metabolic reactions are the same as shown in Appendix A. Symbols of "15.6/18.2", etc. represent metabolic flux distributions for glucose and glycerol, respectively. Glycerol consumption rate was equivalently transformed to glucose uptake rate at carbon atom equal principle [34]

(reductance degree, 4.67), and thus can maintain a redox balance without production of another compensating metabolites during ε-PL synthesis [37]. Taken together, these results demonstrated that glycerol used as carbon source could increase carbon fluxes directed into ε-PL synthesis related pathways and reduce byproduct carbon fluxes.

Conclusion

In this study, glycerol and glucose were compared for ε-PL production in batch and fed-batch fermentations and found that glycerol was benefit for ε-PL production. However, there is a difference in glycerol better than glucose as carbon source for ε-PL production between batch and fed-batch fermentation by *Streptomyces* sp. M-Z18. To understand this difference in batch fermentation, the physiological metabolism of *Streptomyces* sp. M-Z18 on glucose and glycerol

were investigated in terms of key enzymes and metabolism flux analysis. The presented results indicated that glycerol enhances the activity of Pls and ASPK and results in carbon fluxes directed into ε-PL synthesis increased, while decreasing byproduct formation. In regard to fed-batch fermentation, glycerol was a benefit for biomass improvement because of avoiding dilution effect. As a consequence, this study offers a reference to substitute glycerol for conventional glucose as carbon source for ε-PL production. Nevertheless, it must be emphasized that further research is needed and is underway, such as the number of lysine residues of ε-PL derived from glycerol reduced and its influences on antimicrobial activity of ε-PL.

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Appendix A. Simplified Metabolic Model in ε-PL Fermentation by *Streptomyces* sp. M-Z18

A.1. Metabolic reactions

EMP pathways	Gluconeogenesis pathways
R1: Glucose+ATP=G6P+ADP	R1: Glycerol+ATP+NAD=GAP+ADP+NADH
R2: G6P=F6P	R2: 2GAP+ADP=F6P+ATP
R3: F6P+ATP=2GAP+ADP	R3: F6P=G6P
R4: GAP+ADP+NAD=G3P+ATP+NADH	R4: GAP+ADP+NAD=G3P+ATP+NADH
R5: G3P=PEP	R5: G3P=PEP
R6: PEP+ADP=PYR+ATP	R6: PEP+ADP=PYR+ATP
R7: PYR+NAD=NADH+CO_2+AcCoA	R7: PYR+NAD=NADH+CO_2+AcCoA

TCA pathways

R8: OAA+AcCoA=NADH+CO_2+AKG

R9: AKG +2NAD+FAD+ADP=CO_2 +2NADH+FADH+ATP+OAA

R10: CO_2+PEP+ATP=OAA+ADP

L-lysine/ε-PL biosynthesis

R11: OAA+GLU=Asp+α-KG

R12: Asp+NADPH+ATP=Asa+ADP

R13: Asa+Pyr+NADPH+SUCCOA+GLUT+ATP=SUC+α-KG+CO_2+COA+LYS(i)+NADP

R14: Lys(i)=Lys(e)

R15: Lys(i)+ATP=1ε-PL+ADP

Pentose phosphate pathways

R16: G6P+2NADP=CO_2+Ru5P+2NADPH

R17: Ru5P=X5P

R18: Ru5P=R5P

R19: R5P+X5P=GAP+S7P

R20: S7P+GAP=F6P+E4P

R21: E4P+X5P=F6P+GAP

Amino acids biosynthesis

R22: GLU+P3P+NAD=NADH+AKG+SER

R23: E4P+NADPH+2 PEP+ATP=CHOR+NADP

R24: GLU+CHOR=CO_2+AKG+PHE

R25: GLU+CHOR+NAD=CO_2+AKG+TYR+NADH

R26: SER+CHOR+GLN+PRPP=GAP+PYR+CO_2+GLU+TRP

R27: GLU+AcCoA+2 PYR+NADH=CO_2+AKG+LEU+NAD

R28: GLU+PYR=AKG+ALA

R29: GLU+2 PYR=AKG+VAL

R30: NH_3+AKG+NADH=GLU+NAD

R31: Asa+NADPH=Hse+NADP

R32: Hse+S-AcCoA+Cys=Met+HSCoA+Pry

R33: Hse+ATP+H_2O=Thr+ADP

Biomass biosynthesis

R34: 0.241THR+0.019MET+0.05LYS+0.229ASA+0.065 LEU+0.402 VAL+0.5432 ALA +0.054 TRP +0.334 SER+0.131TYR+0.176PHE+0.585GLY+0.031GLU+0.229ASP+0.11OAA +0.113S7P+ 0.113R5P+0.343 NADPH+0.165AcCoA+0.094PYR+0.039PEP+0.113G3P +0.129GAP+0.973ATP+ 0.011F6P+0.1543G6P=biomass+0.078NADH+0.2109 AKG

A.2. Abbreviations in metabolic reactions and Fig. 3

GAP: Glucose 6-phosphate; *E4P*: Erythrose 4-phosphate; *F6P*: Fructose 6-phosphate; *SER*: Serine; *G6P*: Glucose 6-phosphate; *CHOR*: Chorismate; *G3P*: Glyceraldehyde 3-phosphate; *PHE*: Phenylanine; *PEP*: Phosphoenolpyruvate; *TRY*: Tyrosine; *PYR*: Pyruvate; *TRP*: Trptophan; *AcCoA*: Acetyl coenzyme A; *LEU*: Leucine; *AKG*: α-Ketoglutarate; *ALA*: Alanine; *OAA*: Oxaloacetate; *VAL*: Valine; *Asp*: Asparate; *Hse*: Homoserine; *Asa*: Aspargine; *MET*: Methionine; *Lys*: Lysine; *THR*: Threonine; *RU5P*: Ribulose 5-phosphate ; *ATP*: Adenosine-5-triphosphate; *X5P*: Xylulose 5-phosphate; *R5P*: Ribose 5-phosphate; *S7P*: Sedoheptulose 7-phosphate; *NADPH*: Nicotinamide adenine dinucleotide phosphate, reduced; *NADH*: Nicotinamide adenine dinucleotide, reduced.

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