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Effects of Propionate on Streptolydigin Production and Carbon Flux Distribution in *Streptomyces lydicus* AS 4.2501*

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Abstract To achieve higher antibiotic streptolydigin productivity through metabolic regulation, propionate was fed during the fermentation of *Streptomyces lydicus* AS 4.2501. The effects of propionate feeding on streptolydigin production and intracellular fluxes were investigated. The highest streptolydigin production yield of 95.10mg·L^{-1} was obtained when 2mmol·L^{-1} of sodium propionate was added at 60h of cultivation into shake-flask culture. This yield is 23.06% higher when compared to that of a batch culture without propionate supplementation. It was also found that when propionate was added, much more organic acids were excreted. Metabolic flux analysis was performed and it demonstrated that the carbon fluxes of the pentose phosphate pathway and the anaplerotic reaction were significantly increased after propionate feeding. The carbon flux from pyruvate to acetyl-CoA was determined to be 24.7, which was 12.27% higher than that in the batch culture. This study indicated that the glucose-6-phosphate and pyruvate nodes were potential bottlenecks for increasing streptolydigin productivity. Potential targets and strategies that could be manipulated through genetic and process engineering to increase the production of streptolydigin were also suggested.
Keywords streptolydigin, propionate, feeding, metabolic flux, *Streptomyces lydicus*, antibiotic

1 INTRODUCTION

Streptomyces species are widely used to produce various antibiotics, such as streptolydigin. Streptolydigin is a tertramic acid antibiotic that is produced by *Streptomyces lydicus* through the polyketide pathway[1]. Studies on its pharmacological activities showed that streptolydigin selectively inhibited terminal deoxyribonucleotidyl transferase of leukemic cells[2]. Streptolydigin also inhibited RNA synthesis and acted on the catalytic function of RNA polymerase[3]. The elucidation of the mechanism and structural basis of its antibacterial effects facilitated the finding of novel streptolydigin-related antibiotics[4].

The metabolic flux analysis measured the inputs and outputs of a cell and calculated the fluxes using the data on metabolic pathways[5]. Studies on the primary metabolic network of *S. lividans* indicated that changes of oxygen utilization had the greatest impact on the flux calculation[6]. During chemostat cultures of *S. lividans*, metabolic flux analysis showed that if growth conditions, such as growth rates and carbon source, were altered, the carbon flux also changed dramatically[7]. In recent times, a metabolic network was constructed based on the primary and secondary metabolisms of *S. coelicolor*, and computational metabolic flux balancing was used to study the factors that affected cell growth and production of calcium-dependent antibiotics[8,9]. Moreover, metabolic flux analysis also showed that alteration of the nutrient condition dramatically influenced the availability of the C5 precursor in *S. clavuligerus*[10]. However, only a few reports were published pertaining to the effects of precursor feeding on the alteration of metabolic flux of *Streptomyces* during the antibiotic production.

In fact, precursors are important factors stimulating the production of secondary metabolites[11]. Short chain fatty acids such as acetic, propionic, and butyric acids were widely used as precursors of polyketide biosynthesis. The metabolic network of antibiotic production might be restored by supplying propionate precursor in the growth medium[12]. The biosynthetic incorporation of propionate into streptolydigin was studied earlier[13]. To identify the potential metabolic rate-limiting steps and enhance the production of streptolydigin produced by *S. lydicus* AS 4.2501, propionate feeding experiments and metabolic flux analysis were performed. These studies could provide important clues on why a precursor could stimulate the formation of an antibiotic under intracellular metabolic flux distribution. This research could also be used to establish a rational genetic strategy for overproduction of antibiotics in the *Streptomyces* species.

2 MATERIAL AND METHODS

2.1 Strain and medium

S. lydicus AS 4.2501 was used throughout this study. The strain was identified according to its morphology, physiology, and 16S rRNA. The organism was maintained on yeast malt agar slants at 4℃ in a refrigerator and was subcultured on a monthly basis.

The seed medium contained (g·L^{-1}) glucose 5.0, starch 30, yeast extract 2.0, peptone 4.0, K_2HPO_4 1.5, NaCl 0.5, and $MgSO_4 \cdot 7H_2O$ 0.5. The medium used for streptolydigin production, kept in a shake flask or a bioreactor, contained (g·L^{-1}) glucose 20, peptone 2.0, K_2HPO_4 1.5, NaCl 0.5, and $MgSO_4 \cdot 7H_2O$ 0.5.

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2.2 Inoculum preparation and cultivation

A loop full of cells from a slant were transferred into a 250-ml flask containing 50-ml seed medium and was allowed to grow for 48h at 28℃ at 220r·min^{-1} in a rotary shaker. Subsequently, the whole culture was centrifuged at 4800g for 10min. The cell pellet was washed twice with 50ml of sterile water, resuspended in 50ml of sterile water, and stored at 4℃ to be used as inoculums for subsequent experiments.

To produce streptolydigin, the inoculum seed (5ml) was transferred to a 250-ml flask containingwith 50ml of production medium and was cultivated at 28℃ at 220r·min^{-1} for 96h. Batch reactor cultivation was then set up in a 2-L bioreactor (Germany, B.Braum) with a work volume of 1.5L at a 5% (by volume) inoculum level (by adding 50ml of production culture to 1l medium). Cultures were aerated at 3 vvm, controlled at a temperature of 28℃, and stirred at 500r·min^{-1}. The pH was controlled at 6.70 by automatic addition of 2mol·L^{-1} KOH or 2mol·L^{-1} HCl. To supplement propionate, 10ml of aqueous solution containing 0.29g of sodium propionate was used. Generally, at 60h of cultivation, the solution was added into the reactor using a peristaltic pump.

2.3 Analytical methods

2.3.1 *Dry mass determination*

The cell culture was centrifuged at 5000*g* for 10min. The pellet was washed twice with deionized water and dried at 80℃. The mass of the pellet was determined after drying. The procedure was continued until a constant mass was obtained.

2.3.2 *Gas analysis*

The oxygen concentration in the exhaust gas from the fermentor was measured using a paramagnetic oxygen analyzer (Beijing, China, QZS-9601), and the carbon dioxide concentration was measured using an infrared gas analyzer (Beijing, China, QGS-08B).

2.3.3 *Glucose concentration*

The glucose concentration of the medium was measured using the 3, 5-dinitrosalicyclic acid method[14].

2.3.4 *Streptolydigin concentration*

After 96h of cultivation, the culture was centrifuged (5000*g*, 10min), and the liquid layer was extracted thrice with equal volumes of ethyl acetate. The extracts were then combined and concentrated under reduced pressure. The oily residue was redissolved in 5ml of methanol, filtered through a 0.45-μm syringe filter, and stored at −20℃ until it was analyzed. For HPLC analysis of streptolydigin, a reversed phase column (Waters Symmetry C18, 5μm, 4.6mm×250mm) and UV detection at 330nm were used. The mobile phase consisted of CH_3OH and 0.01mol·L^{-1} ammonium acetate mixed in the ratio 65∶35. The sample (5μl) was injected, and the mobile phase was run at a flow rate of 1ml·min^{-1}.

2.3.5 *Organic acids*

The organic acids were analyzed by HPLC with an Aminex-HPX-87H column run at room temperature using 0.1% H_3PO_4 (0.6ml·min^{-1}) as the eluant. UV detection at 210nm was used.

2.4 Metabolic network

The proposed model of the metabolic network mainly consists of the central carbon metabolic pathways. The primary metabolism is likely conserved in different *Streptomyces* species. In fact, the tricarboxylic acid (TCA) cycle, glycolysis, and pentose phosphate (PP) pathway were the same as reported in most species of *Streptomyces*[15—17]. The cell growth rate was zero during the stationary phase; therefore, the synthesis of biomass was not considered. However, at the late stationary phase, the gluconeogentic pathway was probably activated in the metabolic network because of a lower glucose concentration. The biosynthesis of streptolydigin was investigated with the help of isotope labeling experiments, and it was shown that the polyketide chain was derived from acetyl-CoA[18,19]. Propionate is an important precursor of acetyl-CoA, and the metabolism of propionate in several species of *Streptomyces* was mapped onto the KEGG database (www.kegg.com). In the proposed metabolic network, the equations for streptolydigin synthesis were grouped together into a single reaction (R20). A simplified metabolic network of *S. lydicus* AS 4.2501 is presented in Fig.1.

Reaction 21 (R21) did not exist in the metabolic network in the absence of propionate feeding. Abbreviations of metabolites and their formulae and information of the bioreaction network are presented in Appendices A and B.

2.5 Metabolic flux analysis

Metabolic flux analysis was performed based on the principles of metabolite balances, biochemical constraints, and the pseudo-steady-state assumptions for intracellular intermediate metabolites. Under these conditions, no net accumulation of the intermediate metabolites occurred. Therefore, a mass balance could be obtained for each intermediate metabolite. For example, G6P was produced by reaction 1 (R1) and was consumed by reactions 2 (R2) and 6 (R6), at a rate of V1, V2, and V6, respectively. Therefore, the mass balance on G6P could be given as follows:

$$V1 - V2 - V6 = 0 \tag{1}$$

Similar balances can be written with each intermediate, and these relations can be represented in a matrix form as

$$\boldsymbol{Sv} = \boldsymbol{b} \tag{2}$$

where $\boldsymbol{S}$ is the stoichiometric matrix, $\boldsymbol{v}$ is the vector of unknown fluxes, and $\boldsymbol{b}$ is the vector corresponding to the net accumulation, input, consumption or output of the metabolites.

When propionate was supplemented, as shown in Appendix B there were 22 fluxes in the proposed metabolic network involving 16 metabolites. Namely, there are 16 mass balance equations in the proposed model. Therefore the freedom degree of the system is six. Out of the 16 equations, eight fluxes can be directly calculated from the experimental data. These linear algebraic equations of this system can be solved by using the principle of Gauss elimination[20]. Matlab was used to resolve these equations.

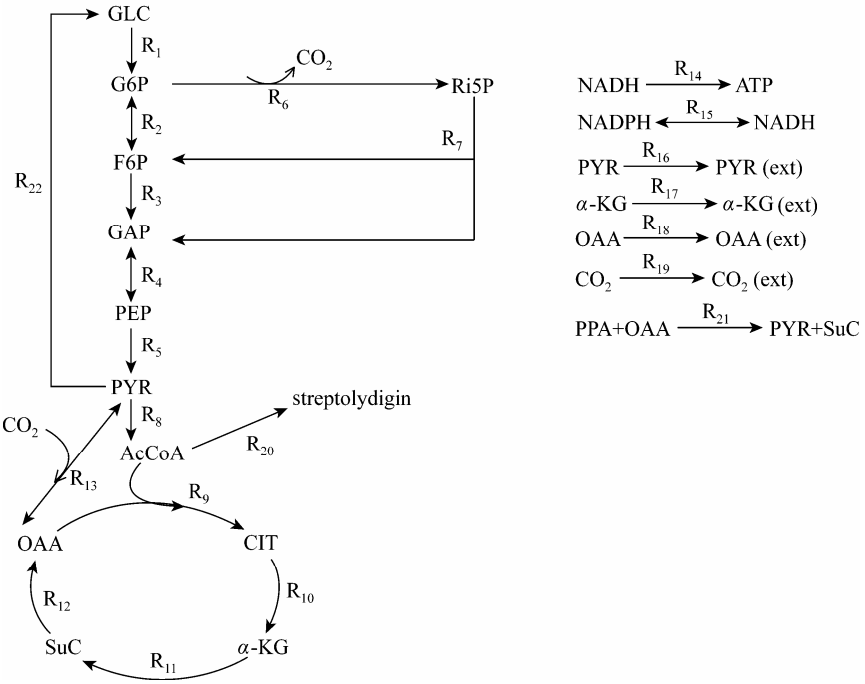

Figure 1 Schematic representation of the biochemical network described in the Appendix, employed for the metabolic flux analysis of *S. lydicus* AS 4.2501 cultures

3 RESULTS AND DISCUSSION

3.1 Effects of propionate concentration on streptolydigin production and cell growth

Cultures grown in shake flask were used to investigate the influence of propionate concentration on cell growth and streptolydigin production by *S. lydicus* AS 4.2501. The cells were grown in the production medium that was supplemented with various concentrations (1–10mmol·L^{-1}) of sodium propionate. Culture samples were taken after 96h of cultivation to measure the streptolydigin concentration and dry cell mass. As shown in Figs.2 and 3, sodium propionate at concentrations of 3mmol·L^{-1} or higher negatively affected growth and streptolydigin synthesis, although the effects on cell growth was less dramatic than that on streptolydigin production. The maximum yield of streptolydigin at 91.6mg·L^{-1} was obtained at a propionate concentration of 2mmol·L^{-1}. At 4mmol·L^{-1}, the streptolydigin concentration decreased by 75.88%. In the meantime, a maximum biomass of 3.52g·L^{-1} was achieved at a propionate concentration of 1mmol·L^{-1}. The biomass decreased by only about 20% when the propionate was increased to 4mmol·L^{-1}. In contrast, a lower concentration of propionate is beneficial to the production of streptolydigin and to cell growth. Compared with cultures lacking propionate supplementation, streptolydigin concentration increased by about 20% when 2mmol·L^{-1} of propionate was added to the cultures, and dry cell mass increased by about 10% when 1mmol·L^{-1} of propionate was supplemented. Similarly, the beneficial effects on cell growth are less obvious than the effects on streptolydigin production.

It was observed in this study that higher propionate concentrations resulted in the increase of pH value of the fermentation broth. It is likely that cell growth and especially the biosynthesis of streptolydigin were inhibited under the conditions of higher pH.

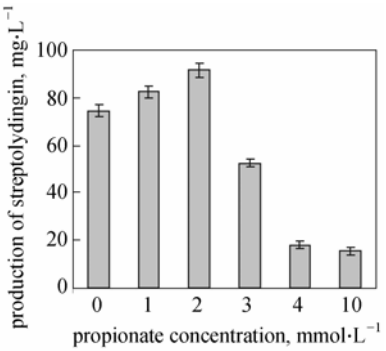

Figure 2 Effects of sodium propionate concentration on streptolydigin production in shake flask culture of *S. lydicus* AS 4.2501

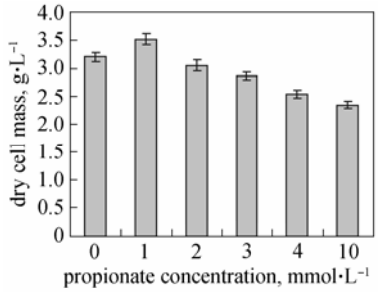

Figure 3 Effects of sodium propionate concentration on cell growth in shake flask culture of *S. lydicus* AS 4.2501

3.2 Determination of the optimal time of propionate supplementation

Generally, antibiotic production was not enhanced by the addition of precursors in the early phase of cultivation. Supplementation in the later stationary phase was more beneficial. Following the study on the effects of propionate concentration, experiments were conducted using culture fed with propionate to examine the influence of propionate supplementation time on cell growth and streptolydigin production. The sodium propionate concentration was controlled at 2mmol·L^{-1}, which was the optimal concentration for streptolydigin production. As shown in Fig.4, cell growth was inhibited slightly when propionate was supplemented at 12h compared with supplementation from 0h, but it remained at the same level or was slightly higher when added at 36h or later. Even though adding propionate at a later time generally helped cell growth, the effect was small, with less than 6% increase in dry cell mass. However, the time of addition of propionate had a dramatic effect on the production of streptolydigin. As shown in Fig.5, adding propionate before 36h increased streptolydigin production only slightly as compared with adding it at 0h, but adding it at 48h and 60h increased the production by 70% and 110%, respectively. The production of streptolydigin dropped considerably when propionate was added at 72h and 84h, indicating that 60h was the optimal time for supplementing propionate.

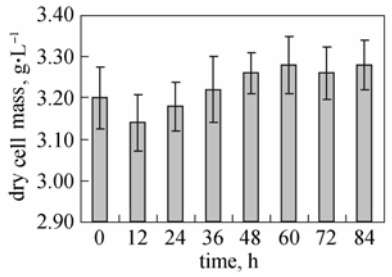

Figure 4 Effects of time of sodium propionate addition on cell growth in shake flask culture of *S. lydicus* AS 4.2501

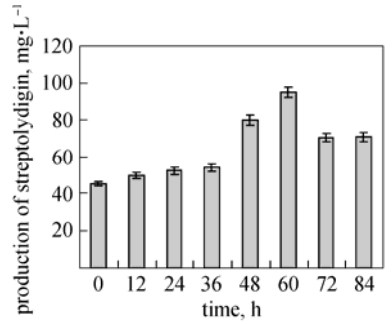

Figure 5 Effects of sodium propionate addition time on streptolydigin production in shake flask culture of *S. lydicus* AS 4.2501

The cell growth versus the time curve of propionate addition shown in Fig.4 indicated that at 48h the cells had probably reached the stationary phase. Propionate clearly showed certain inhibitory effects on cell growth when administered during the cell growth phase, which agreed with the earlier observation in the cell growth versus propionate concentration study (Fig.1). These two lines of evidence suggested that the propionate precursor was toxic to mycelia growth, even though the effect was not very severe. There might be many reasons for this low toxicity; one reason being that the excess propionyl-CoA produced by the addition of extra propionate led to cell toxicity, as suggested by Zhang *et al*.[21]. In their report, Zhang *et al*. had altered the cellular propionyl-CoA content by manipulating its metabolic pathways and had assessed its impacts on polyketide production and cell growth. Their results indicated that increased propionyl-CoA concentration inhibited cell growth and polyketide synthesis in *A. nidulans.* Therefore, addition of propionate to the medium before 36h of cultivation had a negative impact on the accumulation of biomass.

Adding propionate after the stationary phase was reached at 48h, even though the biomass had reached a plateau, the concentration of streptolydigin showed a dramatic increase, suggesting that an extra supply of propionate stimulated streptolydigin biosynthesis. With 2mmol·L^{-1} of sodium propionate addition at 60h cultivation, the yield of streptolydigin after 96h cultivation reached 95.10mg·L^{-1}. At the stationary phase, most of the propionate as the precursor of antibiotic biosynthesis is probably being used or integrated into the molecule of streptolydigin. Adding an extra precursor considerably helped the biosynthesis of streptolydigin. Although when propionate was added at 72h or 84h, the yields of streptolydigin were not substantially changed compared with those at the control time of 0h. This could either be due to the fact that the cells needed a longer time to utilize propionate to produce streptolydigin or could be due to the decline of propionyl-CoA synthetase activity.

3.3 Growth and streptolydigin production profiles in a bioreactor

To further investigate the enhancement of streptolydigin production through propionate feeding, bioreactor studies were carried out. Fig.6 showed the typical time courses of biomass and streptolydigin production in a batch culture without propionate feeding and in a culture fed with propionate at 60h in a 2-L

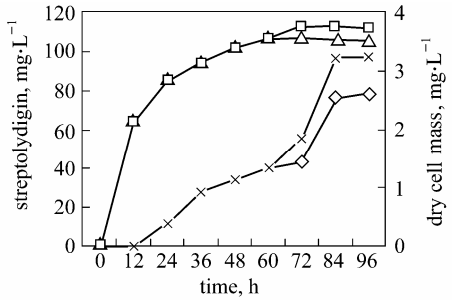

Figure 6 Time courses of cell growth and streptolydigin production in batch and propionate fed batch cultures of *S. lydicus* AS 4.2501 with glucose as carbon source

Dry cell mass: △ batch culture; □ propionate fed-batch culture
Streptolydigin concentration: ◇ batch culture;
× propionate fed-batch culture

bioreactor. It clearly showed that after propionate was added to the reactor at 60h, the rate of streptolydigin production increased substantially. However, the increase of biomass was much smaller as the cell had reached its stationary phase. After 96h of cultivation, the production of streptolydigin increased by 23.06% in the batch culture fed with propionate compared to that of the batch culture without propionate feeding. Therefore, it could be concluded that the biosynthesis of streptolydigin was strongly stimulated by propionate feeding at a late stage of cultivation. In fact, the biosynthesis of antibiotics was often influenced by the precursors supplied from the primary metabolic pathway; for example, the production of avermectin B1a increased by 12.8%—13.8% with a supplement of 0.8% propionate at 24h of cultivation[22].

3.4 Metabolic flux analysis of streptolydigin production with and without propionate supplementation

To evaluate the effects of propionate on intracellular metabolite flux, the metabolic flux distributions at the late stationary phase (72—84h) were calculated. The selection of the late stationary phase for metabolic flux analysis was attributed to the fact that the rate of streptolydigin production was the highest and that the biomass remained constant at this stage.

With propionate addition, the rates of conversion of carbon sources to products increased substantially. Moreover, excretion of organic acids was increased, and the carbon dioxide evolution rate was also faster than that in the normal batch cultivation (Table 1). A stoichiometric model was established with the experimentally determined inputs such as the rates of streptolydigin production, CO_2 production, organic acid (pyruvate, α-keoglutarate, and oxaloacetate) production, propionate consumption, and glucose consumption (Table 1). The experiments were repeated thrice, and the averaged results are shown in Fig.7. Compared with the batch culture, the cellular metabolic flux distribution in the stationary phase under propionate feeding changed considerably. At the G6P node, the carbon flux flowing toward the PP pathway was enhanced by 38.56%. The PP pathway supplied NADPH, which was one of the reducing potentials needed in the streptolydigin production process. The increased supply of NADPH was one of the reasons why propionate could stimulate the formation of streptolydigin. An enhancement in the production of antibiotics on account of an increase in the flux of the PP pathway was also reported in *Penicillin chrysogenum*. The production of penicillin also needed an increased flux in the PP pathway to meet the increased demands of cytosolic NADPH for cysteine biosynthesis[23].

It was interesting to note that the flux of the anaplerotic reaction (R13) also increased significantly. The carbon flux from the α-KG and OAA nodes flowing outside the cell in a propionate-fedbatch culture was increased 2.31 fold and 1.99-fold, respectively, compared to that in the batch culture. As the excretion of organic acids in the TCA cycle had increased considerably, and the flux from acetyl-CoA to TCA cycle had changed slightly, the anaplerotic flux had to be enhanced to maintain the balance of the whole network. Namely, propionate supplementation reduced the rigidity of the PYR node, allowing a greater flux from the PYR to the anaplerotic reaction. On the other hand, because of the increased anaplerotic flux, more acetyl-CoA would enter into the polyketide biosynthesis, thus resulting in an increased final yield of streptolydigin. This observation strongly indicated that the synthesis of streptolydigin was firmly coupled with the anaplerotic reaction. Alterations of the anaplerotic flux might be accomplished by genetically modifying the corresponding enzymes or by controlling the operating conditions. For example, it was reported that the anaplerotic function of the PEP carboxylase could be restored by the expression of pyruvate carboxylases in the *ppc* mutants[24].

The GAP node located at the interface of gluconeogenesis and glycolysis appeared to be an important branch point to balance the fluxes needed to produce other intermediates for biosynthesis. However, only slight differences in the fluxes at the GAP node were observed when propionate was supplemented. Pyruvate is another central metabolite at the interface between glycolysis and TCA cycle. At the PYR node, the carbon flux flowing outside the cell was decreased by 50.94% when propionate was supplemented, while the carbon flux flowing toward the gluconeogenic pathway was decreased by only 9.09%. The flux from pyruvate to acetyl-CoA was determined to be 24.7, which was 12.27% higher compared to that in a batch culture. The carbon flux from acetyl-CoA to streptolydigin was 5.7, which was 42.5% higher compared to that in the batch culture. Acetyl-CoA was used to construct the carbon skeleton of polyketides; therefore, an increase in the intracellular levels of acetyl-CoA would have a beneficial effect on the production of streptolydigin. The regulation mechanisms of the flux from pyruvate to acetyl-CoA through the pyruvate dehydrogenase (PDH) complex are not yet clear, but efforts are made to change the activity of PDH using some strategies[25]. Modulation of the activity of PDH might increase the formation of acetyl-CoA accordingly.

Table 1 Specific rates of substrate consumption, product formation, and dry cell mass in batch and propionate fed-batch cultures of *S. lydicus* AS 4.2501

	DCW, $g \cdot L^{-1}$	qGLC	qPYR	qKG	qOAA	qCO_2	qPPA	qSTL	qO_2
normal-batch	3.55	0.390	0.118	0.0265	0.025	0.0266	0	0.019	0.220
fed-batch	3.75	0.260	0.026	0.0405	0.034	0.0175	0.019	0.030	0.112

Note: rates at late stationary phase. time=72−84h. DCW is dry cell mass. q: specific production or consumption rate (mmol $C \cdot g^{-1} \cdot h^{-1}$).

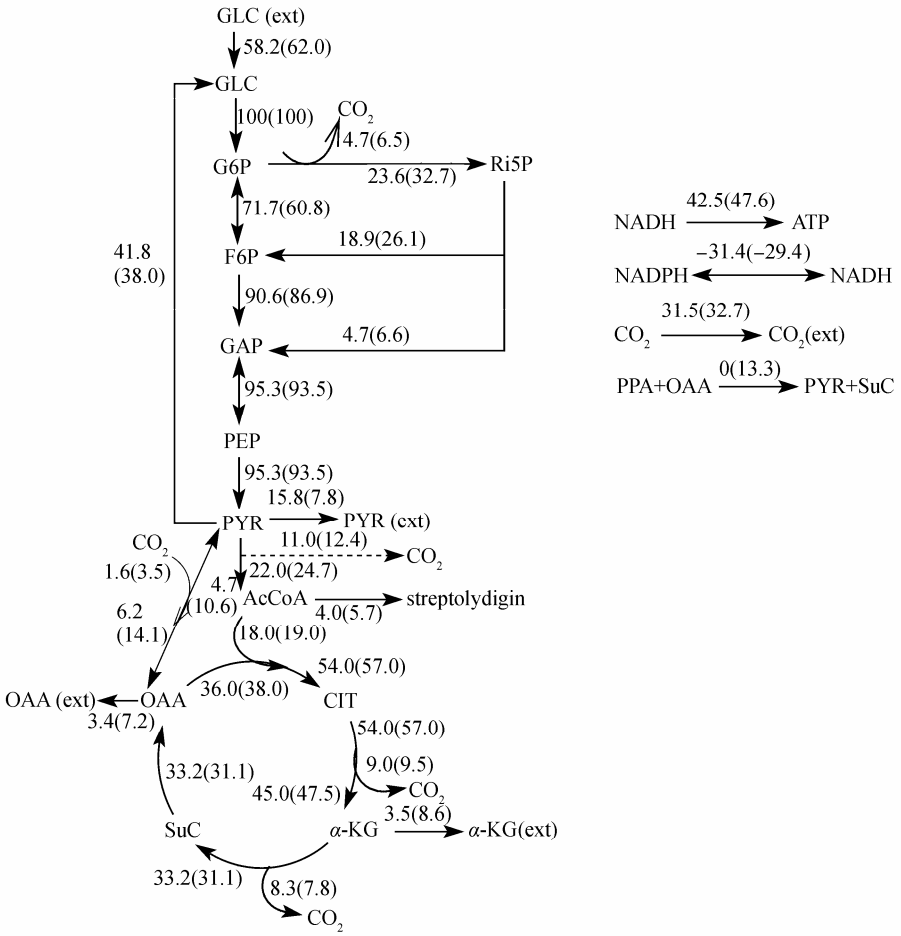

Figure 7 Metabolic flux analysis showing effects of propionate on streptolydigin production by *S. lydicus* AS 4.2501
(Flux values for cultivation with propionate supplementation are given in parenthesis)

4 CONCLUSIONS

It was shown that adding an appropriate amount of propionate at the late stationary phase significantly increased streptolydigin production. A metabolic flux analysis was also performed to understand the mechanisms of the yield improvement of streptolydigin production through propionate feeding. It was found that the biosynthesis of streptolydigin was firmly coupled to the fluxes in the pentose phosphate and the anaplerotic pathways. Consequently, the glucose-6-phosphate and pyruvate nodes should be regarded as potential bottlenecks for increasing streptolydigin productivity. This study also suggests potential targets and strategies that could be manipulated through genetic and process engineering to increase the production of streptolydigin.

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Appendix A

Abbreviations of metabolites and their chemical formula (C-mol)

GLC (glucose): CH2.00O
G6P (glucose-6-phosphate): CH2.17P0.17O1.50
F6P (fructose-6-phosphate): CH2.17P0.17O1.50
GAP (3-phosphoglyceraldehyde): CH2.33P0.33O2.00
G3P (3-phosphoglycerol): CH2.33P0.33O2.33
PEP (phosphoenolpyruvate): CH1.67P0.33O2.00
PYR (pyruvate): CH1.33O
CIT (citric acid): CH1.33O1.17
α-KG (α-ketoglutarate): CH1.20O
SuC (succinate): CH1.50O
OAA (oxaloacetate): CHO1.25
Ri5P (ribose-5-phosphate): CH2.20P0.20O1.60
PPA (propionate): CH2.00O0.67
AcCoA (acetyl coenzyme A): [$C_2H_3O_2$-CoA] 0.5
STL (streptolydigin): CH1.38O0.28N0.06

Appendix B

Biochemical reactions involved in the model (C-mol)

Glycolysis pathway (EMP)
R1 G6P−GLC−1/6ATP=0
R2 F6P−G6P=0
R3 GAP−F6P−1/6ATP=0
R4 −GAP+1/3ATP+1/3NADH+PEP=0
R5 PYR−PEP+1/3 ATP=0
Pentose phosphate pathway (PPP)
R6 −G6P+1/3 NADPH+1/6CO2+5/6 Ri5P=0
R7 −Ri5P+4/5F6P+1/5GAP=0
TCA cycle (TCA)
R8 −PYR+2/3 AcCoA+1/3 NADH+1/3 CO_2=0
R9 −2/3OAA−1/3 AcCoA+CIT = 0
R10 −CIT+1/6 NADH+5/6 α-KG+1/6 CO_2=0
R11 −α-KG+1/5 ATP+1/5NADH+4/5 SuC+1/5 CO_2=0
R12 −SuC+5/12NADH+OAA=0
Anaplerotic reaction
R13 −3/4PYR−1/4 CO_2−1/4 ATP+OAA=0
ATP formation
R14 −NADH+2 ATP−1/2 O_2=0
R15 −NADPH+NADH=0
Transport reactions
R16 −PYR+PYR (ext)=0
R17 −α-KG+α-KG (ext)=0
R18 −OAA+OAA (ext)=0
R19 −CO_2+CO_2 (ext)=0
Biosynthesis of streptolydigin
R20 −AcCoA−7/32 NADPH−14/32 ATP+streptolydigin=0
Propionate metabolism
R21 −4/7OAA−1/7ATP−3/7PPA+4/7 Suc+3/7PYR=0
Gluconeogenic pathway
R22 −PYR−3ATP−NADPH+1/2GLC=0