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Biosynthesis of keto acids by fed-batch culture of *Yarrowia lipolytica* WSH-Z06

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Abstract:

Both α-ketoglutarate (α-KG) and pyruvate (PYR) are important organic acids with promising applications in the food, pharmaceutical and chemical industries. During the production of α-KG by different microorganisms, PYR is always present as a by-product. Strategies have been applied to eliminate PYR accumulation since it can bring difficulties to the downstream separation process. However, modern separation technologies have already conquered this problem. Therefore, this study was aimed at simultaneously enhancing α-KG and PYR production by *Yarrowia lipolytica* WSH-Z06. Using a fed-batch strategy, in which the initial glycerol concentration was 50 $g \cdot L^{-1}$, the residual glycerol concentration was maintained 20–30 $g \cdot L^{-1}$ by constant feeding at a rate of 1.25 $g \cdot L^{-1} \cdot h^{-1}$. The titers of α-KG and PYR were increased by 9.6% and 176.8%, and reached 67.4 $g \cdot L^{-1}$ and 39.1 $g \cdot L^{-1}$, respectively. The final yield of keto acids was 0.71 $g \cdot g^{-1}$ glycerol, which is 42.0% higher than that of the optimal batch fermentation.

Keywords: Keto acids, organic acids, *Yarrowia lipolytica*, glycerol, process optimization.

1. Introduction

α-Ketoglutaric acid (α-KG) and pyruvate (PYR) are important carboxylic acids (Guo et al., 2015; Zhang et al., 2017). Both are widely applied in the food, pharmaceutical and chemical industries (Morgunov et al., 2013; Wang et al., 2005). α-KG can inhibit glutamine catabolism and stimulate protein synthesis in cells (Yao et al., 2012). The antioxidant activity of α-KG enables cells to resist sodium nitroprusside and hydrogen peroxide toxicity (Bayliak et al., 2015), and supplementation with α-KG can alleviate lipopolysaccharide-induced liver injury (Wang et al., 2015). PYR is significant clinically in treating cerebral metabolic depression (Moro et al., 2016), improving insulin secretion in diabetes mellitus patients (Inoue et al., 2016) and protecting cells as a potent antioxidant (Famili et al., 2013). The costs of both keto acids are between \$15 kg^{-1} and \$20 kg^{-1}, which are much higher than those of common organic acids such as citric acid (\$0.6 kg^{-1}), lactic acid (\$1.5 kg^{-1}) and itaconic acid (\$2 kg^{-1}). At present, commercial production of the two keto acids is mainly achieved by chemical synthesis. As the carboxyl and keto groups in the molecular structures result in long chemical synthesis routes and low yields, this leads to high prices (Stottmeister et al., 2005).

Production of the two keto acids by microorganisms using renewable bioresources as substrates has attracted increasing attention over the past two decades (Otto et al., 2011; Xu et al., 2008). Researchers have developed many different routes for producing them, such as the production of α-KG through the bioconversion of L-glutamic acid (Hossain et al., 2014), and the synthesis of PYR by oxidative dehydrogenation of lactic acid (Lomate et al., 2013). However, according to currently available data, the optimal biotechnological processes for the production of α-KG and PYR are based on *Yarrowia lipolityca* (Chernyavskaya et al., 2000) and *Candida*

glabrata (Liu et al., 2003), respectively. In our previous work, a thiamine-auxotrophic *Y. lipolytica* WSH-Z06 was screened and could accumulate α-KG with glycerol as the sole carbon source (Guo et al., 2016; Zhou et al., 2010). As for most other α-KG producers, PYR is the major by-product and can accumulate to more than 30 $g \cdot L^{-1}$ during the fermentation process.

The highly similar physical and chemical properties of α-KG and PYR make the downstream separation process very difficult and costly. Therefore, decreasing the accumulation of PYR during the production of α-KG by *Y. lipolytica* has been one of the biggest challenges. In order to enhance the production of α-KG and decrease the accumulation of PYR, various strategies have been attempted, including optimization of the culture medium (Zhou et al., 2010), optimization of the pH control strategy and fed-batch mode (Yu et al., 2012) and metabolic engineering of the central metabolic pathway or cofactor regeneration process (Yin et al., 2012). Although these strategies succeeded in reducing the accumulation of PYR, none of them could completely eliminate the by-product. During a common α-KG production process with *Y. lipolytica*, PYR could be regarded as the precursor of α-KG. PYR and α-KG were accumulated in the earlier log phase. The PYR could be further converted into α-KG after the substrate (glycerol) was depleted in the later culture phase. Simultaneously, sulfuric acid was required to maintain the pH at 3.0 in order to convert more PYR into α-KG. Several phenomena could not be negligible in this process, such as prolonging fermentation time for more than 60 h, reducing the total titer of keto acids and producing more other metabolites from PYR. In general, conversion of PYR into α-KG by prolonging the fermentation time could bring about significant increasing of the total costs, from the aspects of material, labor, energy consumption and downstream separation process.

Currently, an increasing number of modern separation technologies have been applied in industry, in particular membrane separation technology and reduced pressure distillation. We developed two dependent processes to separate the two keto acids in culture broth: (1) Extraction of keto acids from acidified concentrated culture broth after membrane isolation process by cheap organic solvents. The organic solvents could be removed by first step vacuum distillation. Since the PYR has a much lower boiling point than that of the α-KG, the PYR could be collected by the second step vacuum distillation. The residues were mostly α-KG, and could be then purified by crystallization. (2) We screened an anion resin that could discriminate the two keto acids in acidified concentrated culture broth. Combined with simulated moving bed separator, the two keto acids could be well separated with an acceptable cost. Moreover, there is no difference between the commercial values of α-KG and PYR. Hence, considering that the prices of the two keto acids are similar, it could be more economically efficient to co-produce α-KG and PYR. However, no previous studies have aimed to simultaneously optimize the production of both keto acids by *Y. lipolytica*. This work was intended to achieve optimal production of both keto acids in a single process by establishing a reasonable fed-batch strategy. This strategy could be obtained though investigating the effects of initial glycerol concentration and residual glycerol concentrations on accumulation of keto acids in the fermentation processes.

2. Material and methods

2.1 Microorganisms

The thiamine-auxotrophic *Y. lipolytica* WSH-Z06-C3, obtained by random mutagenesis in a previous study, was used throughout the screening method (Zeng et al., 2015; Zeng et al., 2016).

2.2 Medium

The medium for slant, seed cultures contained (all in $g \cdot L^{-1}$): 20 dextrose, 10 peptone, 1 KH_2PO_4 and 0.5 $MgSO_4 \cdot 7H_2O$. In the slant medium, 20 $g \cdot L^{-1}$ agar was added (Zhou et al., 2010). The fermentation medium had the following composition (all in $g \cdot L^{-1}$): 100 raw glycerol, 3 $(NH_4)_2SO_4$, 6 Sodium acetate, 3 KH_2PO_4, 0.1 K_2HPO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 0.5 NaCl, 10 $CaCO_3$; plus 0.6 $\mu g \cdot L^{-1}$ thiamine-HCl. $CaCO_3$ was dry-heat sterilized at 121 ^oC for 30 min before being added to the medium. Thiamine-HCl was filter sterilized before addition to the medium. Other components were autoclaved for 15 min at 115 ^oC.

2.3 Culture conditions

The yeast was inoculated from an agar slant and incubated in seed culture medium in flasks. The seed culture and flask culture were grown in 500 mL shake flasks containing 50 mL of culture medium on a reciprocal shaker at 200 rpm. Batch fermentation and fed-batch fermentation were performed in a 3 L fermenter (New Brunswick Scientific, Enfield, CT, USA) with a 1.5 L working volume at 600 rpm and 2.0 vvm (volume air per volume). The pH was buffered with $CaCO_3$ during the first phase and controlled automatically at 3.0 by addition of 4 M NaOH when it decreased below 3.0. The inoculation size was 10% (v/v) and all cultivations were performed at 28 ^oC (Zeng et al., 2015). All fermentations were performed in triplicate and the results are presented as mean values. The fed-batch strategies are listed in the Results and discussion (section 3).

2.4 Determining the biomass concentration

The optical density of the culture broth was measured using a Biospe-1601 spectrophotometer (Shimadzu Co., Kyoto, Japan) at 570 nm after an appropriate dilution, and converted into dry cell weight (DCW) based on a predetermined calibration curve (OD_{570}/DCW ($g \cdot L^{-1}$) = 1/0.223) (Zhou et al., 2010).

2.5 Determining the concentrations of glycerol and organic acids

The concentrations of glycerol and organic acids were determined by high-performance liquid chromatography (Agilent 1260 series, Palo Alto, CA, USA) with an Aminex HPX-87H column (Bio-Rad, Richmond, Calif). The mobile phase was 5 mM H_2SO_4 and the flow rate was 0.6 $mL \cdot min^{-1}$ at 35 ^{o}C. α-KG and PYR were detected by a UV absorbance detector at 210 nm. Glycerol was detected by a refractive index detector (Yin et al., 2012).

2.6 Calculation of kinetic parameters

Based on the experimental and fitted interpolated data, the cell growth rate, α-KG and PYR production rates, specific α-KG and PYR production rates and glycerol consumption rate were estimated as previously described (Zheng et al., 2002).

3. Results and discussion

3.1 Effects of different initial glycerol concentrations on cell growth and acid production in shaker flasks

Glycerol was the sole carbon source for cell growth and acid production, and its concentration had a substantial effect on the fermentation process. The influences of different initial glycerol concentrations (50, 100, 120, 150, 180, 200, 220 and 250

g·L^{-1}) on cell growth and keto acids production in flasks are shown in Fig. 1. The maximum biomass was obtained at an initial glycerol concentration of 100 g·L^{-1}, and decreased with the increasing of initial glycerol concentration. Acids production increased with increasing initial glycerol concentration when the initial glycerol concentration was in the range 50–180 g·L^{-1}, and decreased when the initial glycerol concentration exceeded 180 g·L^{-1}. The maximum keto acids production was obtained at 180 g·L^{-1} initial glycerol. But high initial glycerol concentrations (>150 g·L^{-1}) resulted in a biomass of less than 6 g ·L^{-1} and more residual glycerol. Moreover, the highest yield of keto acids of 0.47 g·g^{-1} glycerol was found when an initial glycerol concentration of 150 g·L^{-1} was used with α-KG and PYR concentrations of 34.2 g·L^{-1} and 17.3 g·L^{-1}, respectively.

As a result, an initial glycerol concentration of 150 g·L^{-1} was most favorable for co-production of α-KG and PYR in batch culture, whereas a high initial glycerol concentration exceeding 150 g·L^{-1} inhibited cell growth and acids production. Previous studies have shown that the optimal initial glycerol concentration for α-KG production only was 100 g·L^{-1} and the yield of α-KG was only 0.17 g·g^{-1} glycerol (Zhou et al., 2010). But the yield of keto acids increased to 0.47 g·g^{-1} glycerol when the initial glycerol concentration was 150 g·L^{-1}. It was clear that the yield of acids per gram of glycerol was significantly enhanced by a higher concentration of glycerol during co-production of the two keto acids. From an economic point of view, this strategy provides great benefits. Moreover, the fermentation period was substantially shortened under co-production, and both energy consumption and labor were reduced. Consequently, the co-production of keto acids offers great prospects for industrial applications.

3.2 Batch fermentation with 150 g·L^{-1} initial glycerol in a 3 L fermenter

The most suitable initial glycerol concentration was 150 g·L^{-1} by batch fermentation in flasks according to previous results, so the effects of batch fermentation in a 3 L fermenter when using an initial glycerol concentration of 150 g·L^{-1} were further studied (Fig. 2). The biomass accumulated slowly, and reached a stable phase at 84 h. The α-KG concentration increased until the end of fermentation, but the PYR concentration decreased and remained at a low concentration after 48 h. The amounts of α-KG and PYR produced were 61.5 g·L^{-1} and 14.2 g·L^{-1}, respectively, at the end of fermentation with a yield of 0.50 g·g^{-1} glycerol. In addition, glycerol was consumed slowly during the early stage of the fermentation, which only consumed 28.1 g·L^{-1} glycerol during the first 48 h, resulting in an extended fermentation period. These results showed that the initial glycerol concentration of 150 g·L^{-1} was not conducive to co-production of the keto acids by batch culture in a 3 L fermenter.

A relatively high substrate concentration could inhibit cell growth and result in product overproduction (Lopez-Linares et al., 2015). This effect was obvious when glucose was the sole carbon source: 80 g·L^{-1} glucose led to slow glucose consumption and lipid accumulation (Ji et al., 2014). Similarly, a relatively high initial glycerol concentration may lead to low PYR production, slow cell growth and glycerol consumption. From a previous study, the specific cell growth was found to decrease with increasing glycerol concentration when the specific cell growth rates were compared under different initial glycerol concentrations. The maximum specific cell growth rate was 0.11 h^{-1} with 50 g·L^{-1} initial glycerol, but this decreased to 0.04 h^{-1} when the initial glycerol concentration increased to 150 g·L^{-1}. Hence, a lower glycerol concentration was conducive to rapid cell growth, which is similar to

previous results (Yu et al., 2012). Therefore, lowering the glycerol concentration may be beneficial to the fermentation process.

3.3 Effects of glycerol concentration on keto acids production in a 3 L fermenter

To further determine the effects of decreasing the glycerol concentration (to less than 150 $g \cdot L^{-1}$) on cell growth and acids production, different glycerol concentration ranges were required. These ranged from of glycerol concentration 10 to 50, 60 to 100 and 130 to 150 $g \cdot L^{-1}$ during the first 36 h, when initial glycerol concentrations of 50, 100 and 150 $g \cdot L^{-1}$, respectively, were employed during batch fermentation in a 3 L fermenter. Table 1 shows the biomass, α-KG production, PYR production and residual glycerol concentration for initial glycerol concentrations of 50, 100 and 150 $g \cdot L^{-1}$ during the first 36 h in a 3 L fermenter. The biomass, α-KG production and PYR production increased when the glycerol concentration range decreased during the same time frame, reaching 8.2, 10.4 and 17.3 $g \cdot L^{-1}$, respectively, for glycerol concentrations between 10 and 50 $g \cdot L^{-1}$. The yield of keto acids was highest for the higher glycerol concentration range, but the glycerol was consumed slowly.

The kinetic characteristics of cell growth, α-KG production, PYR production and glycerol consumption were further investigated. The profiles of cell growth rates with various initial glycerol concentrations were similar, and all of the maximum cell growth rates were obtained at about 13 h. Using a glycerol concentration range of 10-50 $g \cdot L^{-1}$, the cell growth rate was highest, with the maximum reaching 0.39 $g \cdot L^{-1} \cdot h^{-1}$. This result proved again that a low glycerol concentration range was beneficial to cell growth. The profiles of α-KG and PYR production rates exhibited similar trends, while the maximum values of the acid production rates were different at various glycerol concentration ranges. For a glycerol concentration range of 10–50

$g \cdot L^{-1}$, both α-KG and PYR production rates were the highest, and the peak values were 0.33 $g \cdot L^{-1} \cdot h^{-1}$ and 0.66 $g \cdot L^{-1} \cdot h^{-1}$, respectively. The above studies indicated that a low glycerol concentration range was also beneficial to the accumulation of keto acids. Moreover, the glycerol consumption rate for the high glycerol range was much lower.

Consequently, it was appropriate to use a relatively low glycerol concentration for cultivation to increase the cell growth rate and keto acids production rates, which could shorten the lag phase and enhance acids production. From the results obtained, an ideal fermentation situation for co-production of α-KG and PYR involved maintaining a low glycerol concentration during the entire fermentation process. So a method to decrease the glycerol concentration during the fermentation was required with an unchanged total amount of glycerol.

Fed-batch culture is a widely used mode of operation that overcomes substrate inhibition and increases production (Ji et al., 2014; Kim et al., 2009), and it is also applicable when glycerol is used as the carbon source (Zhao et al., 2002; Zhu et al., 2010). Based on the above results, a novel fed-batch fermentation method was applied to enhance keto acids production, in which a low glycerol concentration was maintained by reducing the initial glycerol concentration and feeding glycerol constantly later at an appropriate rate.

3.4 Determining the optimal initial and residual glycerol concentrations for the fed-batch fermentation

Based on a kinetic analysis of the effects of glycerol concentration on cell growth and acids production, a low glycerol concentration range of 10–50 $g \cdot L^{-1}$ was found to be beneficial to cell growth and acids production. The optimal initial glycerol

concentration and the residual glycerol concentration maintained during the later phase should be determined when a fed-batch fermentation strategy is applied (Kim et al., 2013). Using initial glycerol concentrations of 20, 30, 40 and 50 $g \cdot L^{-1}$, the glycerol concentration increased to 150 $g \cdot L^{-1}$ with feeding at 24 h in flasks (Table 2). It turned out that the biomass decreased with lower initial glycerol concentrations, and the fermentation period showed a similar trend. The reason may be that an initial glycerol concentration that is too low cannot meet the requirements of cell growth at the beginning, as the glycerol concentration was 0 at 24 h when the initial concentration was 20 $g \cdot L^{-1}$ (Yu et al., 2012). The maximum α-KG and PYR productions of 42.7 and 13.3 $g \cdot L^{-1}$, respectively, were obtained at an initial glycerol concentration of 50 $g \cdot L^{-1}$, with the highest yield of keto acids of 0.37 $g \cdot g^{-1}$ glycerol. As a result, an initial glycerol concentration of 50 $g \cdot L^{-1}$ was chosen as optimal in fed-batch mode.

The glycerol is mainly used for acids production during the later phase, and a properly maintained glycerol concentration affected by the feeding rate is key to acids overproduction in fed-batch mode (Ji et al., 2014). For an initial glycerol concentration of 100 $g \cdot L^{-1}$, the residual glycerol was about 30 $g \cdot L^{-1}$ at 60 h in fed-batch fermentation mode in a 3 L fermenter. Influence of maintaining different ranges of residual glycerol concentration such as 10–20, 20–30 and 30–40 $g \cdot L^{-1}$ by feeding during the period of 72–96 h was researched. As the pH value was maintained at 3.0, which would restrain cell growth, the biomass reached a maximum value of 13.4 $g \cdot L^{-1}$ at 60 h and remained constant after 60 h at the three glycerol concentrations mentioned above. Eliminating the influence of biomass, specific changes about production of α-KG and PYR and consumption of glycerol during the 72-96 h period were shown in Table 3. During this feeding time, α-KG and PYR

production were highest when the glycerol concentration was maintained between 20 and 30 $g \cdot L^{-1}$, and the yield of acids was 0.67 $g \cdot g^{-1}$ glycerol. Although the yields at different concentrations were similar, the glycerol consumption was faster when its concentration ranged from 20 to 30 $g \cdot L^{-1}$. Consequently, keto acids production with rapid glycerol consumption was enhanced when the residual glycerol concentration was maintained between 20 and 30 $g \cdot L^{-1}$ during the feeding phase.

3.5 Development of a fed-batch fermentation with constant feeding for efficient co-production of α-KG and PYR

According to the previous results, a fed-batch fermentation approach with constant feeding for co-production of α-KG and PYR was applied as follows. With a total glycerol concentration of 150 $g \cdot L^{-1}$, the initial glycerol concentration was lowered to 50 $g \cdot L^{-1}$ to shorten the lag phase, and the glycerol concentration was maintained at 20–30 $g \cdot L^{-1}$ by feeding constantly at a rate of 1.25 $g \cdot L^{-1} \cdot h^{-1}$ during 24–104 h. Figure 3A showed the time course of the fed-batch mode. The biomass increased rapidly and reached a stable phase at 60 h, 24 h earlier than the batch fermentation. The supplied glycerol was exhausted at 132 h, which is 12 h earlier than the batch fermentation. Keto acids production continually increased until the end of fermentation. The peak values of α-KG and PYR appeared at 144 h, at 67.4 $g \cdot L^{-1}$ and 39.3 $g \cdot L^{-1}$, respectively, and the yield of keto acids and productivity attained were 0.71 $g \cdot g^{-1}$ glycerol and 0.74 $g \cdot L^{-1} \cdot h^{-1}$, respectively.

To determine the differences in the acid-producing abilities of the cells in the different modes, the specific α-KG and PYR production rates were further investigated. The specific α-KG production rates under the two modes exhibited similar trends (Fig. 3B). It is clear that the peak value of the specific α-KG production

rate was obtained at the beginning of the fermentation, and it then decreased quickly up to 24 h, after which further decreases were modest. Consequently, α-KG accumulated in the two modes throughout the fermentation process, with only minor differences in α-KG production between the two modes. In contrast, the maximum specific PYR production rate in the fed-batch mode was much higher than the maximum in batch mode. The specific PYR production rate in batch mode exhibited a similar trend to the specific α-KG production rate up to 24 h. After 48 h, the specific PYR production rate in batch mode was almost 0, but decreased from 0.04 h^{-1} to 0.01 h^{-1} in fed-batch mode (Fig. 3C). Consequently, PYR production increased until the glycerol supply was exhausted in fed-batch mode, but stopped and PYR remained at a low concentration in batch mode after 48 h.

The reason for the differences in PYR production may be that the glycerol concentration affects enzyme activities in some metabolic pathways. Ahrens detected that the glycerol concentration could affect the extracellular enzyme activity of three key enzymes, glycerol dehydrogenase, glycerol dehydratase and 1,3-propanediol oxidordeuctase, during the production of 1,3-propanediol by *Klebsiella pneumonia* with glycerol as the carbon source (Ahrens et al., 1998), and Yang further enhanced 1,3-propanediol production by controlling the optimal glycerol concentration during different fermentation stages (Yang et al., 2006). Moreover, pyruvate kinase (PK), one of the rate-limiting enzymes in the glycolysis, catalyzes the transformation of phosphoenolpyruvate to pyruvate, and the flux from phosphoenolpyruvate to pyruvate affects PYR production. Menzel found that the activity of PK was either limited by its substrate and coenzyme ADP or inhibited by factors such as intracellular pyruvate and ATP at high glycerol concentrations (Menzel et al., 1998). As a result, a low glycerol concentration may be beneficial to PYR production as it increases the activity of PK.

The fermentation parameters using the different fermentation modes are shown in Table 4. Compared with co-production by batch fermentation, the yield of keto acids and the keto acids productivity increased by 42.0% and 39.6%, respectively, in the fed-batch fermentation. When only α-KG production was optimized in the same fed-batch mode, the fermentation period was extended by 29.4%, and pH control with sulfuric acid was needed, which led to greater labor and resources consumption. Although α-KG production increased slightly, the keto acids yield and productivity decreased by 36.6% and 94.7%, respectively. Considering both keto acids are worth \$15–\$20 kg^{-1}, it would be uneconomic to aim solely for α-KG production.

In addition, the maximum biomass of the batch mode (14 $g \cdot L^{-1}$) was higher than the fed-batch mode (12.3 $g \cdot L^{-1}$). This may be due to restrained cell growth at low pH (less than 4). When the initial glycerol concentration was decreased to 50 $g \cdot L^{-1}$, the pH value declined quickly with higher acids production rates. The pH declined to 4 at 32 h in fed-batch mode but at 50 h in batch mode. So the initial glycerol concentration could affect the biomass according to the different rates of decline of the pH. From our previous studies, a biomass of 11–12 $g \cdot L^{-1}$ could meet the needs of acids production in the later phase (Yu et al., 2012). However, more biomass means more nutrients are required for cell growth, and less are available for acids production. A relatively low biomass in fed-batch mode enhanced the yield of keto acids. Moreover, the dissolved oxygen concentration was higher when the biomass was relatively low under the same agitation speed and air inflow. The dissolved oxygen concentration reached 60%–70% in the later phase of fermentation in fed-batch mode, compared to 50% in batch mode, and high PYR production was achieved with a high dissolved oxygen concentration (Li et al., 2002). Hence, a relatively low biomass was conducive to keto acids production.

4. Conclusions

A fed-batch mode for enhancing co-production of α-KG and PYR with constant feeding was established. The production of α-KG and PYR reached 67.4 $g \cdot L^{-1}$ and 39.3 $g \cdot L^{-1}$, respectively, and the yield of keto acids and productivity attained 0.71 $g \cdot g^{-1}$ glycerol and 0.74 $g \cdot L^{-1} \cdot h^{-1}$, respectively. Compared with α-KG production alone, the fermentation period was shortened by 29.4%, and the keto acids yield and productivity increased by 36.6% and 94.7%, respectively, which led to lower costs and higher profits. The mode adopted in this study can potentially be applied to other industrial fermentation products to achieve higher yields and productivities.

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Figure legends

Fig. 1 Effects of initial glycerol concentrations on keto acids production.

The biomass was relatively lower with an initial glycerol concentration of 50 $g \cdot L^{-1}$, and reached the highest value at 100 $g \cdot L^{-1}$, after which it gradually decreased with further increases in glycerol concentration. The total titer of keto acids increased with an initial glycerol concentration of 50–180 $g \cdot L^{-1}$, while it decreased when the initial glycerol concentration exceed 180 $g \cdot L^{-1}$.White, DCW; gray, α-KG; black, PYR.

Fig. 2 Time courses of keto acids production by batch culture in a 3 L fermenter.

The batch mode with 150 $g \cdot L^{-1}$ initial glycerol concentration was cultivated in a 3 L fermenter at 28 °C, 600 rpm and 2.0 vvm for 144 h. Squares, DCW; black circles, α-KG; white circles, PYR; triangles, glycerol.

Fig. 3 Time courses of fed-batch culture and comparison of specific acids production rates between batch mode and fed-batch mode.

A: The initial glycerol concentration was 50 $g \cdot L^{-1}$, and the residual glycerol concentration was maintained in the range 20–30 $g \cdot L^{-1}$ by feeding constantly at a rate of 1.25 $g \cdot L^{-1} \cdot h^{-1}$ during 24–104 h, Squares, DCW; black circles, α-KG; white circles, PYR; triangles, glycerol; B: The specific α-KG production rates in different modes; C: the specific PYR production rates in different modes. Black lines: the specific production rates in fed-batch mode; dashed lines: the specific production rates in batch mode.

Tables

Table 1 Effects of glycerol concentration on keto acids production by batch fermentation.

Initial glycerol concentration ($g \cdot L^{-1}$)	Biomass ($g \cdot L^{-1}$)	α-KG ($g \cdot L^{-1}$)	PYR ($g \cdot L^{-1}$)	Residual glycerol concentration ($g \cdot L^{-1}$)	yield of acids on glycerol ($g \cdot g^{-1}$)
150	5.1±0.1	5.5±0.4	10.8±0.6	129.3±2.9	0.79
100	6.3±0.2	8.2±0.6	14.1±1.1	61.6±2.4	0.58
50	8.2±0.6	10.4±0.7	17.3±1.2	12.5±0.6	0.74

Table 2 Effects of initial glycerol concentration on keto acids production by fed-batch fermentation.

Initial glycerol concentration ($g \cdot L^{-1}$)	Biomass ($g \cdot L^{-1}$)	α-KG ($g \cdot L^{-1}$)	PYR ($g \cdot L^{-1}$)	Time (h)	Yield of acids on glycerol ($g \cdot g^{-1}$)
50	8.5±0.7	42.7±1.3	13.3±0.6	156	0.37
40	7.9±0.8	40.4±0.9	13.1±0.5	168	0.36
30	7.7±0.6	38.7±0.8	13.2±0.7	168	0.35
20	6.4±0.4	36.1±0.8	12.9±0.5	192	0.33

Table 3 Effects of various residual glycerol concentrations on acids production.

Residual glycerol concentration ($g \cdot L^{-1}$)	α-KG production ($g \cdot L^{-1}$)	PYR production ($g \cdot L^{-1}$)	Glycerol Consumption ($g \cdot L^{-1}$)	Yield of acids on glycerol ($g \cdot g^{-1}$)
10-20	12.7±0.7	3.6±0.2	25.3±1.1	0.64
20-30	16.9±0.9	5.3±0.1	33.3±1.2	0.67
30-40	14.4±0.8	4.5±0.3	30.6±0.9	0.62

Table 4 Comparisons of fermentation parameters for the different fermentation modes.

Fermentation parameters	Fermentation modes			Change (%)	
	Co-production by batch (A)	Only α-KG production by fed-batch (B)	Co-production by fed-batch (C)	(C/A-1)*100	(C/B-1)*100
Fermentation period (h)	144	204	144	0	-29.4
Glycerol consumption ($g \cdot L^{-1}$)	150	150	150	0	0
α-KG production ($g \cdot L^{-1}$)	61.5±1.0	78.1±0.8	67.4±1.3	9.6	-13.7
PYR production ($g \cdot L^{-1}$)	14.2±0.4	0	39.3±0.6	176.8	-
Keto acids production ($g \cdot L^{-1}$)	75.7±1.4	78.1±0.8	106.7±1.9	42.0	36.6
Yield of keto acids on glycerol ($g \cdot g^{-1}$)	0.50	0.52	0.71	42.0	36.6
Keto acids productivity ($g \cdot L^{-1} \cdot h^{-1}$)	0.53	0.38	0.74	39.6	94.7

Highlights

- A fed-batch mode was established to enhance total titer of α-KG and PYR.
- The titer of α-KG and PYR reached 67.4 $g \cdot L^{-1}$ and 39.1 $g \cdot L^{-1}$, respectively.
- Yield of keto acids and productivity achieved to 0.71 $g \cdot g^{-1}$ and 0.74 $g \cdot L^{-1} \cdot h^{-1}$.
- Co-production of α-KG and PYR could result in lower costs and higher profits.