Liu *et al. Bioresour. Bioprocess.* (2017) 4:55
https://doi.org/10.1186/s40643-017-0185-1

Bioresources and Bioprocessing

RESEARCH

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Influence of initial glucose concentration on seed culture of sodium gluconate production by *Aspergillus niger*

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Abstract

Background: In general, high-quality seed is the prerequisite of an efficient bioprocess. However, in terms of sodium gluconate production by *Aspergillus niger*, reports have seldom focused on seed culture with rational optimization by process analysis technology, especially for carbon source effects. In this study, based on the online physiological parameter of oxygen uptake rate (OUR), and intracellular metabolite profiling, as well as cell morphology analysis, the effects of different initial glucose concentrations on seed culture by *A. niger* were investigated.

Results: The optimum initial glucose concentration was 300 g/L, corresponding to 1900 mOsm/kg, with OUR level about 70% higher than other conditions. Besides, the cells from optimized seed culture accumulated more osmoprotectants of alanine and glutamate. Interestingly, high glucose concentration could induce glucose oxidase (GOD) activity possibly by affecting the synthesis of histidine, one key component of active site of GOD. Prominently, the fermentation yield using the optimized seed culture was up to 1.198 g/g, 99% of the theoretical value, which was the best in literature.

Conclusion: The initial glucose concentration appropriately 300 g/L in seed cultivation was determined to be the most optimal. Further, this study would be helpful for guiding sodium gluconate production on industrial scale.

Keywords: *Aspergillus niger*, Sodium gluconate, Initial glucose concentration, Osmotic pressure, Seed culture, Metabolite profiling

Background

Sodium gluconate, which is commercially produced by *Aspergillus niger* in submerged fermentation, has been used in a broad range of industrial applications in chemical, food, and pharmaceutical fields (Ramachandran et al. 2006). From the point of view of biochemical reaction, it is a simple dehydrogenation process to synthesize gluconic acid without complex cell metabolism. As shown in Fig. 1, glucose is converted to glucono-δ-lactone by glucose oxidase (GOD), accompanied by the consumption of oxygen and the formation of hydrogen peroxide. Then, the glucono-δ-lactone is hydrolyzed to gluconic acid spontaneously or by lactonase, and the hydrogen peroxide is decomposed to water and oxygen (Ikeda et al. 2006). Gluconic acid is neutralized by sodium hydroxide to generate sodium gluconate. Thus, glucose, oxygen, and GOD activity are the most important elements for the sodium gluconate consumption.

During the process of sodium gluconate fermentation by *A. niger*, batch fermentation is generally adopted for commercial production. Glucose, as one of the key substrates in sodium gluconate fermentation, has a dual role of providing the required carbon source and increasing the environmental osmotic pressure. Many studies were carried out with glucose as a starting point. Kulandaivel (2014) optimized the gluconic acid production by *A. niger* by setting three different gradients of glucose concentration and other factors in continuous shaking flask. On the other hand, for high sodium gluconate titer, high

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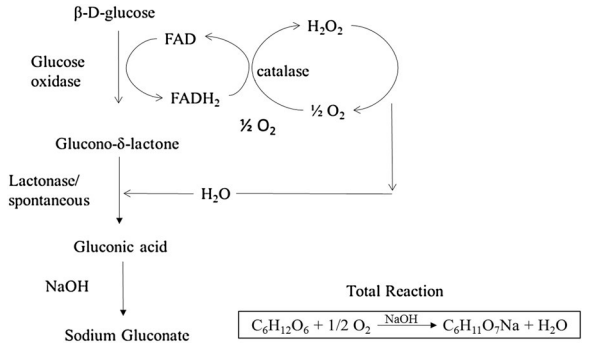

Fig. 1 Synthetic pathway of sodium gluconate by *A. niger*

initial glucose concentration and continuous addition of neutralizer (NaOH) to maintain suitable environmental pH are considered to result in strong osmotic stress. Liu et al. (2006) found that the cell growth and trehalose accumulation were decreased with the increasing initial osmotic pressure between 959 and 2108 mOsm/kg, while Wucherpfennig et al. (2011) pointed out that high environmental osmotic pressure could enhance the specific productivity of fructofuranosidase in *A. niger* SKAn 1015 notably from 0.5 to 9 U/mg/h.

Seed quality is believed to be the prerequisite for an efficient fermentation process, and an optimal process is considered to be dependent on physiological characteristics of the strain. Lee et al. (2001) enhanced the Cephalosporin C production by cultivation of *Cephalosporium acremonium* M25 using the most effective mixing ratio with a 3:7 volume ratio of early- and late-stage inocula. In terms of gluconate production, our earlier study also found that dispersed pattern of seed morphology for gluconic acid production was better than pellets, due to more than 13% improvement of volumetric oxygen transfer rate (KLa) (Lu et al. 2015a, b). Until now, although many studies on carbon source effects on fermentation process have been reported in *A. niger* (Hossain et al. 1984; Xu et al. 1989), unfortunately, few researches have been systematically focused on the effects of initial glucose concentration on metabolite profile and mycelial morphology as well as sodium gluconate production, especially for the seed culture by *A. niger*.

Based on the online physiological parameter of oxygen uptake rate (OUR), the effects of different initial glucose concentrations (100, 200, 300, 400, and 500 g/L) on seed culture by *A. niger* were investigated, and the optimal initial seed glucose concentration was determined for the first time, using the integrated analyses of intracellular metabolite profiling and mycelial morphology. Moreover, the dual physiological functions of glucose as carbon source or osmotic regulator during seed culture were further explored. The information developed in this study would be beneficial for the industrial-scale production of sodium gluconate.

Methods

Fermentation equipments

Bioreactors for seed cultivation and fermentation equipped with pH electrode (Mettler Toledo, Switzerland) and dissolved oxygen electrode (Mettler Toledo, Switzerland) were provided by Guoqiang Bioengineering Equipment Co., Ltd (Shanghai, China). Exhaust gas was measured by process mass spectrometer (Extrel, USA). All the online parameters were calculated and collected by means of real-time viewing software of Biostar (National Center of Bio-Engineering and Technology, Shanghai, China).

Microorganism and media

Aspergillus niger CICC 40350 for sodium gluconate production was kindly gifted by Shandong Fuyang Biological Technology Co., Ltd (Shandong, China). Mainly three media were involved in this study. Medium for seed culture in shake flask contained 0.64 g/L KH_2PO_4, 0.20 g/L urea, 0.02 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L corn steep liquor, and 15 g/L $CaCO_3$. Medium for seed culture in 5-L bioreactor comprised of 0.5 g/L KH_2PO_4, 0.19 g/L $(NH_4)_2HPO_4$, 0.19 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L corn steep liquor, 0.20 mL/L polyether defoamer. Medium for fermentation in 5 L bioreactor was composed of 0.17 g/L KH_2PO_4, 0.25 g/L $(NH_4)_2HPO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L corn steep liquor, 0.20 mL/L polyether defoamer. The concentration of initial glucose was regulated according to the detailed experiment. The initial pH of all above three media was adjusted to 6.5–7.0 by 250 g/L NaOH solution. The former media in shake flask was sterilized at 115 °C for 20 min, media in 5 L bioreactor was sterilized at 121 °C for 30 min and glucose solutions were separately sterilized at 115 °C for 20 min.

Culture conditions

Aspergillus niger stored in the slants was first inoculated to plate medium and cultivated at 35 °C for 60 h, then fungal colonies on the plates were selected randomly, inoculated to 250 mL eggplant bottle slant with 50 mL medium and cultured at 35 °C for 48 h until the slant covered with dense spores.

For seed culture in shake flask, the spores were harvested by washing the slant with 15 mL sterilized water, transferred to shake flask (volume, 500 mL) containing 60 mL medium with 2% inoculum size and cultivated at 37 °C and 220 rpm for 36 h.

For seed culture in 5-L bioreactor, the spores were harvested by washing the slant with 50 mL sterilized water and then inoculated into a 5-L stirred bioreactor

containing 3 L working volume. Aeration, temperature, and pressure were controlled at 4 vvm, 37 °C, and 0.05 MPa, respectively. To ensure the initial glucose concentration was the unique objective variable, agitation speed was set as 500 rpm for the first 10 h and then controlled at 800 rpm. The pH was maintained at 5.5 by adding 250 g/L NaOH solution.

For sodium gluconate fermentation in 5-L bioreactor, 500 mL seed broth cultured from 5-L bioreactor after 16 h was prepared to inoculate with 3-L fresh medium. The agitation speed and pH of seed culture were maintained at 500 rpm and 5.5, respectively, while the agitation speed during fermentation was set as 500 rpm at the first 6.5 h and then controlled at 800 rpm. The pH during fermentation was maintained at 5.2 by feeding 250 g/L NaOH solution. Fermentations were ended by glucose concentration less than 3 g/L, and the online parameter DO increased quickly or correspondingly, while physiological parameter OUR decreased abruptly.

Cell morphology analysis

Dispersed mycelia and mycelial pellets are two morphological forms in sodium gluconate fermentation. Due to the high agitation speed, most pellets are transformed into dispersed mycelia, which are too complicated to be quantitated. Here, mycelial pellets under different initial glucose concentrations were mainly investigated. Using photomicrography with an inverted microscope AE200 (Motic Group Co., Ltd, Shanghai, China), mycelial morphologies could be captured, imaged, and processed as described in the literature (Lu et al. 2015a, b).

The obtained images were analyzed using the professional software Image-pro Plus 6.0. Figure 2 shows a typical pellet formed during fermentation. The diameter of white circle is defined as d_{min} and the diameter of black is defined as d_{max}. Projected area (A) and filamentous ratio (Fr) around the pellets are the main parameters that we described in this study. The A is the the projected area of one pellet, and Fr was calculated according to Eq. (1), in which d_{mean} is the average of d_{min} and d_{max}.

$$\mathrm{Fr} = \left(d_{\mathrm{mean}}/d_{\mathrm{min}}\right)^2. \tag{1}$$

Calculation of OUR and carbon dioxide evolution rate (CER)

OUR and CER were calculated using Eqs. (2) and (3), respectively.

$$\mathrm{OUR} = \frac{F_{\mathrm{in}}}{V}\left[\mathrm{C}_{\mathrm{O_2in}} - \frac{\mathrm{C}_{\mathrm{inertin}}\cdot \mathrm{C}_{\mathrm{O_2out}}}{1-\left(\mathrm{C}_{\mathrm{O_2out}}+\mathrm{C}_{\mathrm{CO_2out}}\right)}\right] \cdot \frac{273}{273+t_{\mathrm{in}}}\cdot\frac{1}{1+h}\cdot P_{\mathrm{in}}\cdot 10^{-5} \tag{2}$$

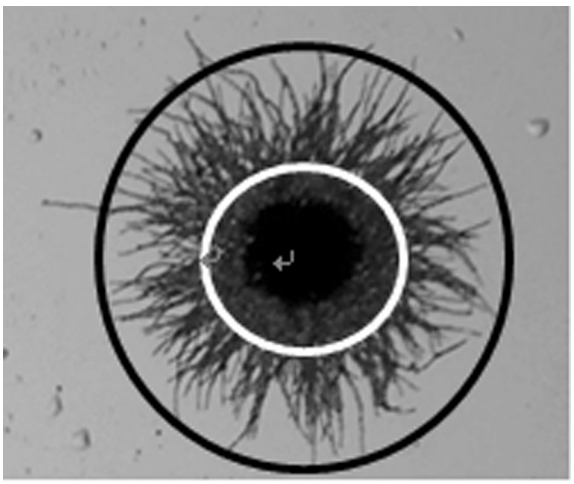

Fig. 2 Schematic diagram of a typical pellet formed in seed culture of *A. niger*

$$\mathrm{CER} = \frac{F_{\mathrm{in}}}{V}\left[\frac{\mathrm{C}_{\mathrm{inertin}}\cdot \mathrm{C}_{\mathrm{CO_2out}}}{1-\left(\mathrm{C}_{\mathrm{O_2out}}+\mathrm{C}_{\mathrm{CO_2out}}\right)} - \mathrm{C}_{\mathrm{CO_2in}}\right] \cdot \frac{273}{273+t_{\mathrm{in}}}\cdot P_{\mathrm{in}}\cdot\frac{1}{1+h}\cdot 10^{-5} \tag{3}$$

where F_{in} is the aeration rate (mmol/L); V is the broth volume (L); C_{O_2in}, C_{CO_2in}, and $C_{inertin}$ are the inlet gas concentrations of oxygen, carbon dioxide, and nitrogen in inert gas, respectively; C_{O_2out} and C_{CO_2out} are the outlet gas concentrations of oxygen and carbon dioxide, respectively; T_{in} is the temperature of inlet gas; h is the humidity of inlet gas; and P_{in} is the pressure of inlet gas.

Assays of offline parameters

The offline parameters detected in this study were dry cell weight (DCW), broth osmolality, glucose concentration, sodium gluconate concentration, and total GOD activity. To determine the DCW, 50 mL broth was filtered with qualitative filter paper and washed at least ten times with deionized water. Then, the filter paper along with the cells was dried to a constant weight at 70 °C for 24 h. The supernatant was immediately diluted appropriately for the determination of osmotic pressure, glucose, and sodium gluconate concentrations.

Osmotic pressure was determined using FM-8P Auto. F.P. Osmometer (Shanghai Medical University Instrument Factory, China) (Tian et al. 2014a, b). Glucose concentration was measured using assay kit (Shanghai Kexin Biotech Co., Ltd, China) after appropriate dilution. Sodium gluconate was assayed by high-performance liquid chromatography (HP 1100, Agilent, USA) with 0.01 M H_2SO_4 as the mobile phase at 210 nm by means

of a C18-H column (4.6 × 250 mm, No. 366-1101, Sepax Technologies, Co.) (Lu et al. 2016).

Total GOD activity was analyzed by the glucose oxidase, peroxidase-adjacent anisidine coupling reaction kinetics method. The reaction system included 2.4 mL adjacent anisidine, 0.5 mL 1% glucose solution, and 0.1 mL horse radish peroxidase, which was performed at 35 °C for 5 min. Then, 0.1 mL (broken-) filtered broth was added to the above solution before being assayed at 500 nm using a visible 721 spectrophotometer. The optical density (OD) value with an interval of 20 s for a total of 2 min was recorded, and then the maximum of slope coefficient $\Delta A/\text{min}$ between adjacent two points was determined to calculate the total GOD activity using Eq. (4)

$$\begin{aligned}\text{GOD activity} &= \frac{\frac{\triangle A}{\text{min}} * \text{total reaction volume}}{7.5 * \text{added fermentation volume} * 22.6 * 60} \\ &= 5555.2 * \frac{\triangle A}{\text{min}}\ \mu\text{LO}_2/\text{h/mL}\end{aligned} \quad (4)$$

Extraction, analysis, and quantification of intracellular metabolites

About 3 mL fresh broth was fast quenched with 40 mL precooled methanol solution (− 27.6 °C 40% w/w) and then rapidly filtered through vacuum pump onto acetic nitrocellulose membrane. To make intracellular metabolites fully inactive, 120 mL precooled quench solution was used to wash the filter cake. Then, the obtained filter cake was added into prewarmed ethanol solution (25 mL 95% v/v) and extracted for 3 min at 95 °C.

To enrich intracellular metabolites during the detection process, the extracting solution was concentrated to 1 mL. Then, 200 µL samples and 40 µL of 200 µmol/L internal standards were transferred to a glass vial. After lyophilization, 100 µL 20 mg/mL methyl-pyridine was added, and the sample was dissolved for 1 h at 60 °C. After this, 100 µL derivatives was added, and the sample in glass vial was derived for 1 h at 60 °C. The sample was centrifuged for 2 min at 10,000 rpm, and the supernatant was transferred to GC glass vial with an insert.

Finally, all the samples derived through the above procedure were analyzed using gas chromatography with mass spectrometry (GC–MS) instrument (Agilent, Santa Clara, CA, USA). Because internal standard signals did not vary substantially with samples, the standard curves could be obtained by the peak area ratio of the sample to that of the internal standard during the same total ion flow diagram. Then, then the pool sizes of intracellular metabolites were qualified referring to the corresponding standard curves and dry cell weight. Adopting normalized data followed by logarithm ($\log_2$) and R 2.15.0 process, the heat map of intracellular metabolites was performed.

Results and discussion

Effects of initial glucose concentration on seed culture of *A. niger*

Effects of initial glucose concentration on offline parameters of seed culture

During sodium gluconate production by *A. niger*, the main offline parameters were dry cell weight, osmotic pressure, residual glucose concentration, GOD activity, and sodium gluconate concentration, while the online parameters included DO, CER, and OUR. Figure 3 shows the effects of different initial glucose concentrations of 100, 200, 300, 400, and 500 g/L on the offline parameters of seed culture by *A. niger*.

The changes of DCW and osmotic pressure during seed culture are presented in Table 1 and Fig. 3a, respectively. In the early stage, since microorganism existed in the form of spores with little energy consumed, the environment was relatively stable, and there was nearly no change of osmolality. Once entering into the growth phase, a large amount of carbon source was utilized, spores germinate, resp sodium gluconate began to produce, accompanying with the osmolality increased. Due to the restriction of phosphorus in seed media, *A. niger* stopped the growth, and the DCW reached to its maximum value (Table 1). Then, when a large amount of sodium hydroxide was added to maintain the pH, the broth was diluted, and the detected DCW would be declined to some extent. At the end of the process, the osmotic pressure tended to be constant.

As shown in Fig. 3a, c, it is notable that high initial glucose concentration would significantly prolong the lag phase. The delay period of conditions not higher than 300 g/L was as short as 8 h, while those of 400 and 500 g/L were about 12 and 20 h, respectively. This result was in accordance with the findings of Wucherpfennig et al. (2011) that osmotic pressure could affect the spore germination and the high osmolality could increase time span. The interpretation was that the cells would produce compatible solutes in the early stage of seed culture to adapt to the environment and maintain the intercellular and external osmotic balance, which was more conducive to cell growth and metabolism (Schubert et al. 2007). Notably, although the fermentation performances by 300 and 400 g/L initial glucose concentration were similar, the spore germination time with 300 g/L was obviously shorter than 400 g/L. Thus, the former was adopted as the best initial glucose concentration in seed culture to save the time cost and correspondingly to improve the fermentation efficiency.

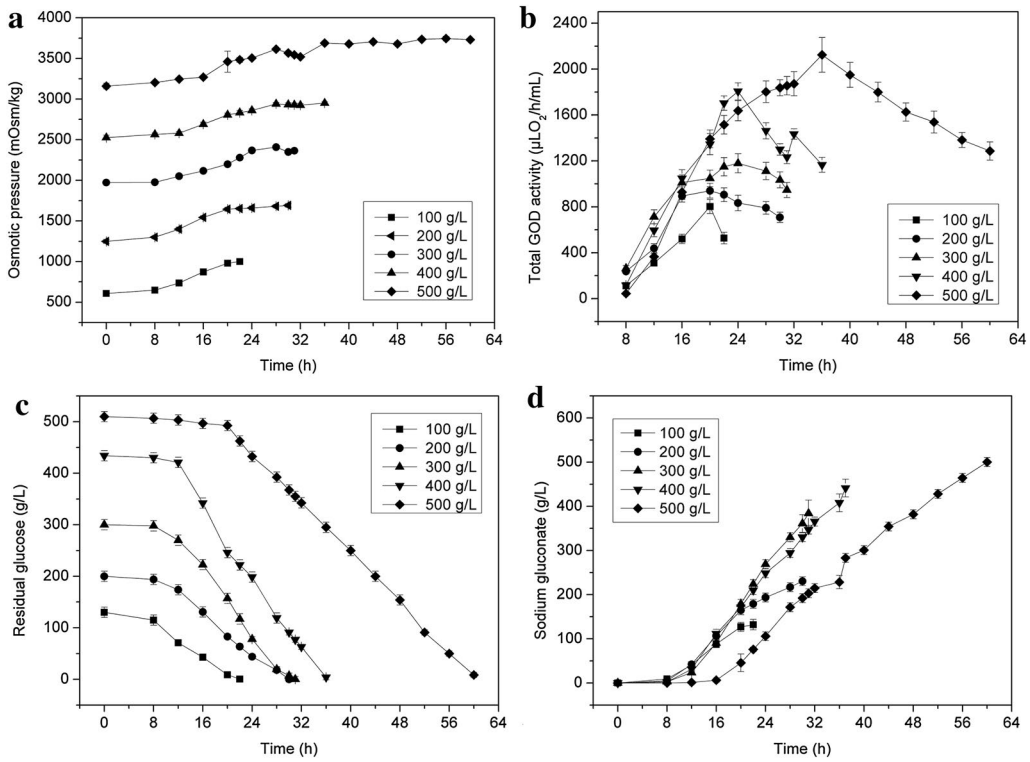

Fig. 3 Effects of different initial glucose concentrations on environmental osmotic pressure (**a**), total GOD activity (**b**), glucose consumption (**c**), and sodium gluconate production (**d**) in seed culture of *A. niger*

Table 1 Effects of different initial glucose concentrations on DCW in seed culture by *A. niger*

Time (h)	DCW[a] (g/L)				
	100 g/L	200 g/L	300 g/L	400 g/L	500 g/L
4	0.43	1.09	0.98	1.21	0.87
8	1.02	2.75	1.92	2.72	1.52
12	1.58	3.65	3.24	3.87	1.78
16	1.63	3.26	3.20	3.30	1.96
20	1.27	2.41	2.83	3.30	2.14
22	0.72	–	–	–	–
24	–	2.12	2.67	2.79	2.85
28	–	–	2.7	2.52	3.14
30	–	1.82	–	–	–
31	–	–	2.61	–	–
32	–	–	–	2.56	2.91
36	–	–	–	2.45	2.56
40	–	–	–	–	2.60
44	–	–	–	–	2.32
48	–	–	–	–	2.27
52	–	–	–	–	1.94
56	–	–	–	–	1.67
60	–	–	–	–	1.61

[a] The average value from three parallel tests

On comparing total GOD activities, as illustrated in Fig. 3b, although the overall trends were similar under different initial glucose concentrations, as they ascended rapidly to maximum values and then gradually descended until the end of fermentation, their increased degrees were markedly dependent on the initial conditions. The maximum total GOD activities under 100, 200, 300, 400, and 500 g/L glucose concentrations were 802, 940, 1179, 1806, and 2124 $\mu LO_2/h/mL$, respectively, which might be consistent with the hypotheses for enzyme repression or induction in the TCA cycle reactions as reported by Hossain et al. (1984) and Xu et al. (1989). It should be noted that, according to the Michaelis–Menten equation, the substrate concentration would affect the enzymatic reaction rate, which could not be ignored in detecting GOD activity. Therefore, in this paper, through adding a certain amount of glucose to reaction system, the enzyme activity that we measured with the same substrate concentration was much more accurate.

It was noteworthy that, compared to the maximum total GOD activity under the lowest initial glucose concentration of 100 g/L (600 mOsm/kg), the highest condition of GOD activity under 500 g/L (3200 mOsm/kg) increased by 165%, whereas the corresponding maximum GOD specific activity of the latter was only 13.5% higher

than that of the former with 731 $mLO_2/h/g$ (Fig. 4a). This could be possibly explained that when the initial glucose concentration was 100 g/L, the total GOD activity was low due to the limited substrate, resulting in low DCW and insufficient amount of enzyme synthesis. The extreme osmotic environment might stimulate the related cell-sensing element, so that the expression level of the gene responsible for GOD synthesis per unit cell was upregulated, thus enhancing the GOD specific activity (Hagiwara et al. 2015; Mager and Siderius 2002).

It had been reported that bulk of the key enzyme (GOD) for gluconate synthesis was localized in the cell wall, demonstrating that sodium gluconate was formed around the cell wall (Witteveen et al. 1992). If much more amounts of GOD could depart from cell wall, then the contacting area with substrate would be expanded, which would have positive effects on sodium gluconate secretion. However, free GOD activity had similar trends to those of total GOD activity (Fig. 4b), implying that the ratio of free/total GOD activity was independent on initial condition, meaning that the cells could self-stabilize GOD status under different osmotic conditions.

The effects of initial glucose concentration on substrate consumption and product synthesis during seed culture are shown in Fig. 3c, d. In the lag phase, as very little carbon source was consumed for respiratory chain, the above two curves were steady. Then, the glucose started to be quickly consumed with the product synthesis. With the increasing initial glucose concentration, both glucose consumption and sodium gluconate production rates presented to be up first and then down. Obviously, the curves of 300 and 400 g/L glucose were steeper, indicating the glucose consumption and sodium gluconate production rates were significantly higher than those of other three groups. The maximum glucose consumption rate of 13.45 g/L/h was obtained with 300 g/L, which was 1.78, 1.52, and 1.18 times those of 100, 200, and 500 g/L, respectively. Interestingly, although the lag-phase duration of seed cultured in 500 g/L initial glucose concentration was as long as 20 h, the glucose consumption rate except for delayed period was not slow, and was 57.4% higher than that of 100 g/L glucose. This further confirmed that osmotic pressure mainly affected the critical period of spore germination and cell growth. In addition, although the average glucose consumption rate of 400 g/L was the fastest, the average production rate of 400 g/L was slightly slower than that of 300 g/L with 12.39 g/L/h. Moreover, the maximum yield of 1.189 g/g was obtained at 300 g/L glucose concentration.

Effects of initial glucose concentration on the online parameters of seed culture by A. niger

For selecting better seed with more dynamic activity, this paper chose online physiological parameter OUR as a basis for judging the seed quality. On the one hand, compared with the offline parameters, OUR could real-time monitor the physiological state of the cells. On the other hand, DO declined rapidly or even lower than the critical dissolved oxygen level about 30% after the delay period, while the value of CER was relatively small during the whole process. Thus, in our previous studies, Lu et al. (2015a, b) used OUR to monitor the fermentation status in real time, and the higher OUR represented higher yield of sodium gluconate. Furthermore, Tian et al. (2014a, b) also chose OUR as a quantitative oxygen metabolism characterization and control parameters to explore the oxygen metabolism of facultative anaerobic lactic acid. Hence, OUR was chosen as an online criterion for judging seed quality ascribed to its advantages in this study.

Figure 5 shows the effects of initial glucose concentration on the online parameters of seed culture by *A. niger*. The changes of online parameters in different conditions

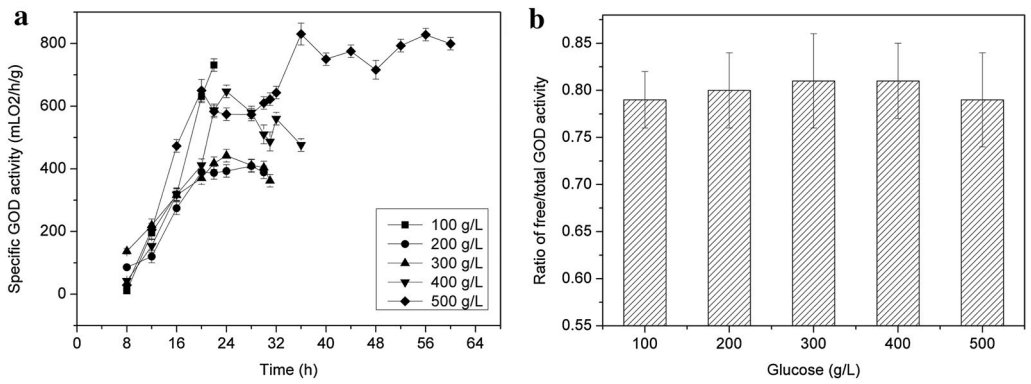

Fig. 4 Effects of different initial glucose concentrations on the specific GOD activity (**a**) and the ratio of free/total GOD activity (**b**) in seed culture of *A. niger*

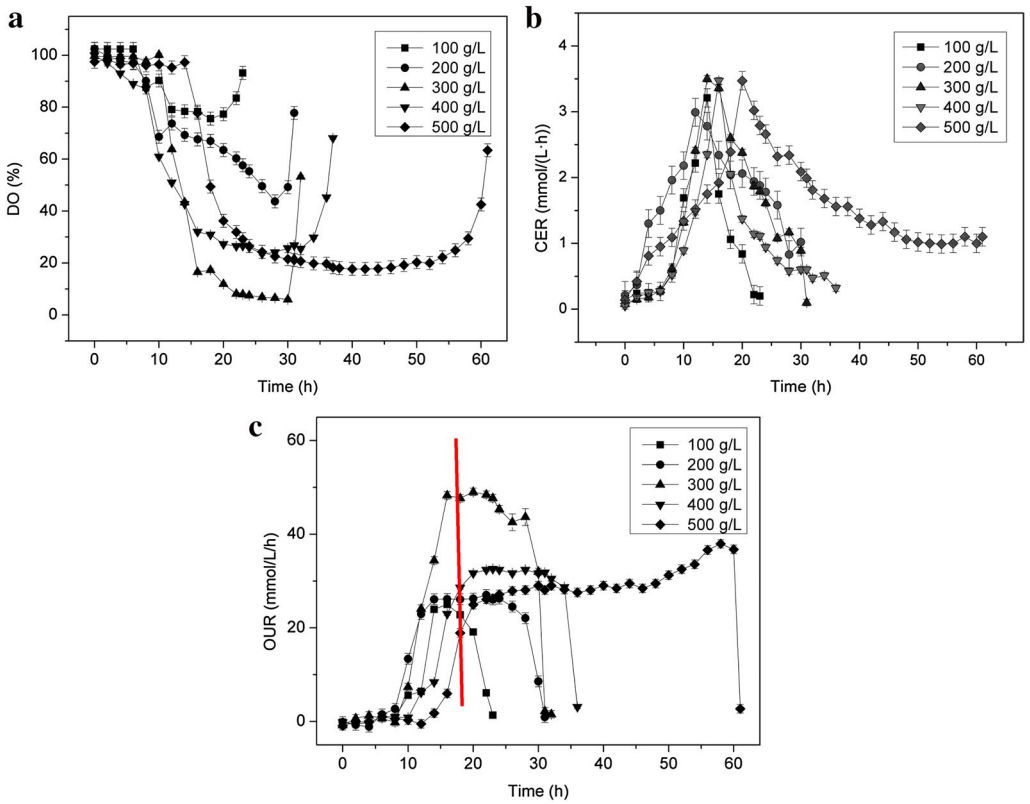

Fig. 5 Effects of different initial glucose concentrations on DO (**a**), CER (**b**), and OUR (**c**) evolutions in seed culture of *A. niger*

over time were roughly consistent with the performance of the offline parameters. As illustrated in Fig. 5a, when the initial glucose concentration was not higher than 200 g/L, the cell metabolism was weak, and the dissolved oxygen level in broth was sufficient. Whereas under initial glucose concentration greater or equal to 300 g/L, the cell metabolism became much stronger, and the DO was lower than the critical level. Since the seeds were usually transferred to fermentation after 16–18 h cultivation, this part mainly focused on the first 20-h cultivation. Clearly, the highest OUR level was obtained under 300 g/L condition, corresponding to 1900 mOsm/kg, which was appropriately 70% higher than that of other groups (red line indicated in the Fig. 3c).

In summary, the performances of shorter lag phase time, higher GOD activity, faster glucose consumption rate, the highest productivity and yield, and the highest OUR level in 300 g/L condition were much better than those under other conditions.

Influences of initial glucose concentration on intracellular metabolism of *A. niger* in seed culture

In this section, the effects of initial glucose concentration on seed culture were analyzed from the view of metabolites, and the intracellular metabolite profiling was used to characterize the changes of metabolism at different time points. Figure 6 shows the heat map of intracellular metabolites under the above conditions, while Fig. 7 represents the profiles of metabolites pool size at 16 h during seed culture. In total, 23 kinds of intracellular metabolites were detected by gas chromatography–mass spectrometry with selected ion mode, including the most of amino acids, organic acids in tricarboxylic acid cycle, and some sugar phosphates.

As shown in Fig. 6, the majority of intracellular metabolites had undergone significant fluctuations under different initial glucose concentrations. Intracellular metabolite levels in group C and group D were relatively lower, while the other three groups had higher levels in seed culture. Combined with macroscopic parameter analysis, the reason was possibly as follows. The amounts of cell viability, glucose consumption rate, and the yield of sodium gluconate in group C and group D were higher, where more substrates were directly converted to products on the cell wall and less metabolic flux flowed into the cell. Similarly, the groups A, B, and E with low or high initial glucose concentrations had low glucose consumption rate and product yield, and more metabolic flux

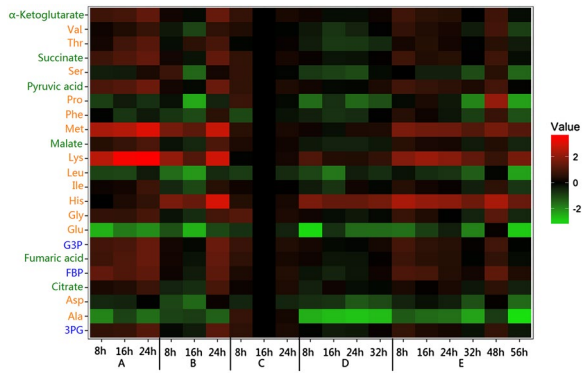

Fig. 6 Heat map of intracellular metabolites during seed culture process with different initial glucose concentrations

flowed to synthesize metabolites responding to environmental stresses for facilitating cell growth. In general, there was a relatively complete strategy for microorganism to respond to osmotic stress, containing the self-synthesis or exogenous uptake of compatible solutes and osmoprotectants, metabolic flux redistribution, and membrane fluidity variations (Schubert et al. 2007; Saum and Müller 2007; Simonin et al. 2008; Varela et al. 2003).

In addition, the quantitative pool sizes of intracellular metabolites were in coordination with each other (Fig. 7). For example, the intracellular concentration of fructose-1, 6-diphosphate (FBP) was higher on the both the group A and group E, and smaller in the middle three groups of B, C, and D. Moreover, the intracellular levels of glyceraldehyde 3-phosphate (G3P) and 3-phosphogluconic acid (3PG) also existed in similar state. It was worth noting that the pool sizes of intracellular alanine (Ala) and glutamic acid (Glu) achieved the maximum 1.5 μmol/g DCW under the optimal 300 g/L glucose. As reported in a majority of the literature, Ala and Glu played the role of osmotic protectors (Rathinasabapathi et al. 2000; Komarova et al. 2002).

Under extreme conditions of group A and group E under 100 and 500 g/L glucose concentrations in seed culture, corresponding to the initial osmotic pressures of 600 and 3200 mOsm/kg, respectively, the pool sizes of amino acids like glycine (Gly), lysine (Lys), valine (Val), methionine (Met), and threonine (Thr), and central carbon metabolism intermediates such as pyruvic acid (Pyr), 3PG, G3P, FBP, α-ketoglutarate (αKG), malate, succinate (Suc), and fumaric acid (Fum) were 2–3 times of those under 200, 300, and 400 g/L of glucose conditions, corresponding to 1200, 2500, and 2500 mOsm/kg osmotic pressures, respectively. These results suggested that extreme conditions could accelerate more energy to synthesize a series of proteins or enzymes for the better cell growth and metabolism, while the EMP pathway and TCA cycle continued to rise, which was also supported by the report of Varela et al. (2003).

Interestingly, among the detected intracellular metabolites, the concentration of histidine, the only one substance, increased with the increased initial glucose concentration, which was well consistent with the total GOD activity evolution, demonstrating that some correlations might be present between them. It was reported that the amino acid residue His was related to GOD, in which His-520 and His-563, two of the main active sites of GOD, played a catalytic role by joining to substrate and coenzyme FAD through forming hydrogen bonds with the 1-OH of glucose during the reaction (Witt et al. 2000).

Effects of initial glucose concentration on mycelium morphology in seed culture by *A. niger*

The macroscopic environment determined microscopic morphology of cells, in turn, the latter of which acted on the mixing and mass transfer through the change of the rheological properties of the broth, thus affecting the biosynthesis of products. In a 5-L fermenter, the agitation of sodium gluconate fermentation process was as high as 800 rpm, leading to *A. niger* mycelia easily be broken up. Therefore, the effects of different initial glucose concentrations on mycelium morphology in seed culture were studied in shake flask.

Due to the restriction of oxygen supply, the cell morphology in the shake flask necessarily existed in the form of pellets. Figure 8 shows the quantitative parameters of cell morphology for 24 h under different initial glucose concentrations. The projected areas (A) under first three groups, respectively, with 100, 200, and 300 g/L glucose concentrations were approximately three times of those in 400 and 500 g/L conditions. The filamentous ratio (Fr) rose up gradually with initial glucose concentration increased. With the integrated analysis of online and offline parameters, the seeds obtained from 300 to 400 g/L glucose were better. However, the condition of 300 g/L glucose in shake flask was more advantageous for culturing good-quality seed. The reasons could be attributed to, first, that the projected area of seed cultured from 400 g/L glucose was too small to readily respond to the harsh osmotic stress, due to low cell activity and weak mycelia; second, that the pellets formed under 300 g/L glucose were powerful and could withstand external stress from shear and osmolality. Besides, the Fr of seed with looser and more dispersed mycelia in 300 g/L glucose could increase the contact area with oxygen, which was conducive to improving the oxygen transfer coefficient, K_La, thereby enhancing the mass transfer and oxygen supply. The results were not consistent with those of Lu et al. and this might have been caused by the different

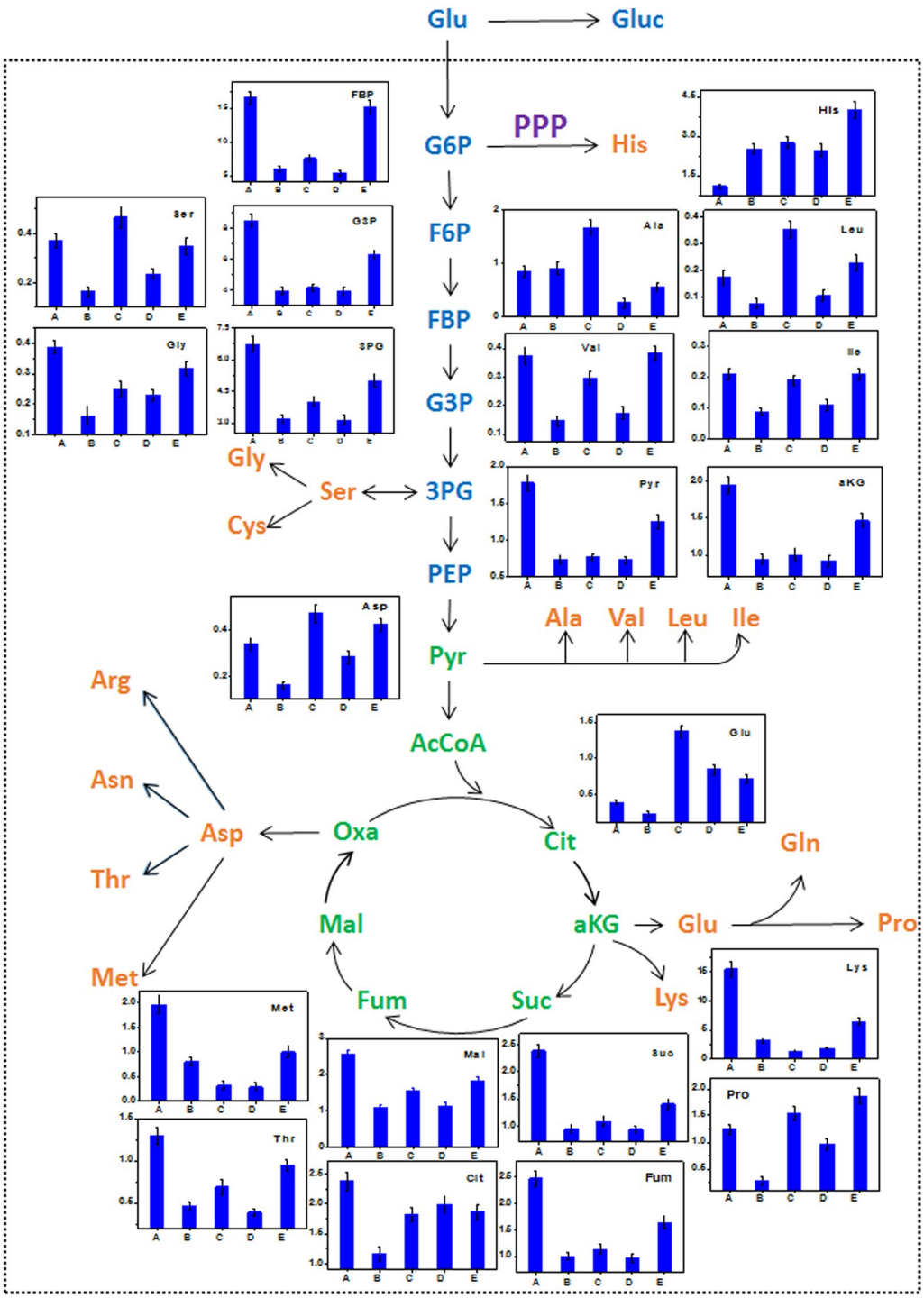

Fig. 7 Effects of different initial glucose concentrations on intracellular metabolite profiling at 16 h during seed culture of *A. niger*

reactors and regulatory strategy used in their study for the process (Lu et al. 2015a, b).

Considering the macroscopic status of seeds cultured under different initial glucose concentrations, and based on systematic analysis of its intracellular metabolite profile and cell morphology, the initial glucose concentration of 300 g/L in medium was considered to be the most suitable for culturing seed.

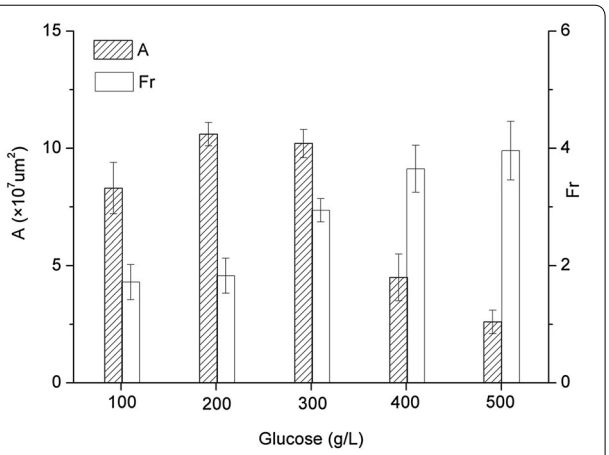

Fig. 8 Effects of different initial glucose concentrations on cell morphology at 24 h during seed culture of *A. niger* in shake flask

Verification of seed cultured from different initial glucose concentrations acting on sodium gluconate fermentation by *A. niger*

A 250 g/L glucose concentration was generally adopted for seed cultivation on industrial scale; however, the results obtained indicated that 300–400 g/L glucose concentration might be beneficial for cell morphology. tTherefore, we chose three different seeds cultured for 16 h in 250, 300, and 350 g/L glucose concentrations, corresponding, respectively, to 1500, 1900, and 2200 mOsm/kg in the following experiments for gluconate fermentation in 5-L fermentor.

Figure 9 shows the time courses of key parameters during fermentation processes with different seeds. It demonstrated that the fermentation results would were reliable and comparable. As illustrated in Fig. 9c, the performance of fermentation with seed from 300 g/L glucose in total GOD activity was superior to that of 250 g/L glucose, and the former one maintained high level during the whole process, suggesting that *A. niger* under 300 g/L glucose was much more appropriate to be applied for sodium gluconate fermentation. In combination with Table 2, it was clearly observed that the fermentation time of 300 g/L glucose in medium was the shortest with approximately 21 h, which was 2 and 6 h less than those for seeds cultured under 250 and 350 g/L glucose conditions, respectively. In comparison with the fermentations in 250 and 350 g/L, the average glucose consumption rate

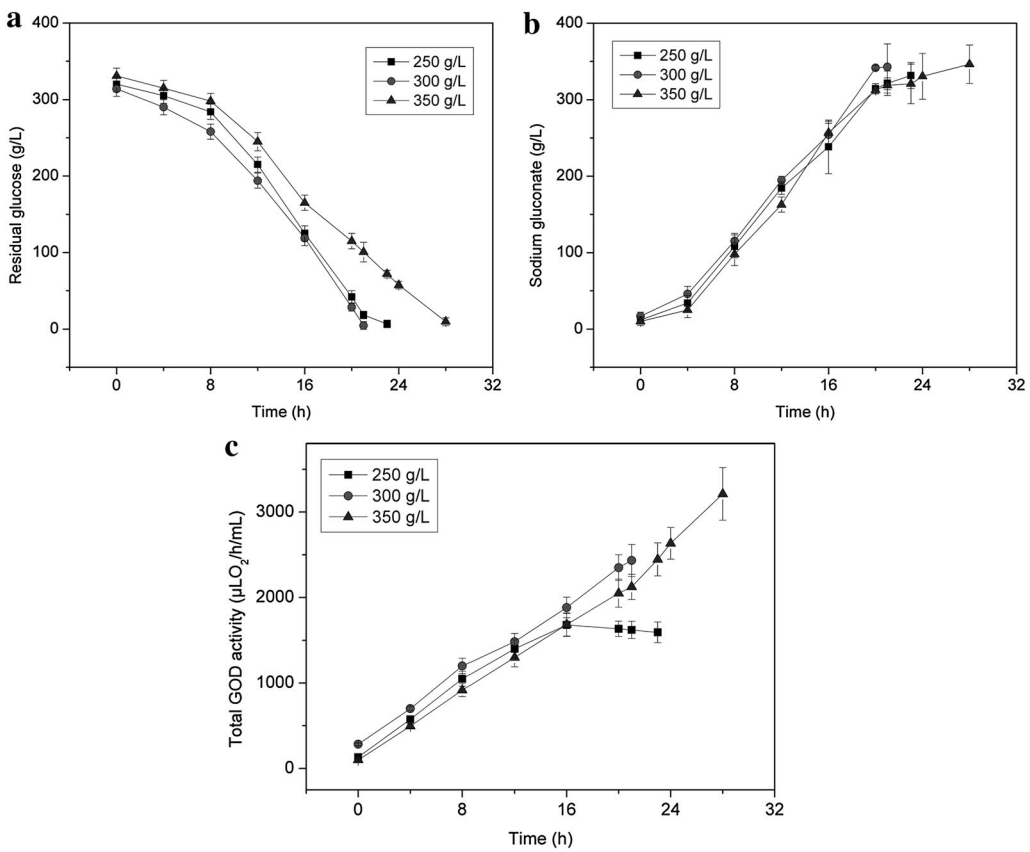

Fig. 9 Time courses of glucose consumption (**a**), sodium gluconate production (**b**), and total GOD activity (**c**) in fermentation processes with different seed cultures of *A. niger*

Table 2 Calculated parameters of sodium gluconate fermentation by *A. niger* inoculated with seed culture for three different initial glucose concentrations

Parameters	Fermentations		
	F1	F2	F3
Initial glucose in seed culture (g/L)	256 ± 5	310 ± 10	342 ± 8
Initial glucose in fermentation (g/L)	320 ± 20	314 ± 15	331 ± 25
Initial fermentation osmotic pressure (mOsm/kg)	2292 ± 30	2200 ± 20	2340 ± 45
Fermentation time (h)	23 ± 0.5	21 ± 0.5	28 ± 0.5
Maximum total GOD activity (μLO_2/h/mL)	1592 ± 121.53	2434 ± 187.31	3212 ± 308.47
Average rate of glucose consumption (g/L/h)	13.91 ± 0.54	14.95 ± 0.36	11.82 ± 0.73
Average production rate of sodium gluconate (g/L/h)	14.42 ± 0.48	16.34 ± 0.53	12.37 ± 0.51
Highest OUR value (mmol/L/h)	52.76 ± 1.21	62.34 ± 2.17	40.47 ± 2.43
Yield (g/g)	1.103 ± 0.05	1.198 ± 0.04	1.076 ± 0.09

under 300 g/L glucose was 14.95 g/L/h—7.5 and 26.5% higher, respectively. Meanwhile, the average sodium gluconate production rate under 300 g/L glucose also was the fastest, enhanced by about 13.3 and 32.1%, respectively. More importantly, the maximum overall yield of 1.198 g/g was reached by seed using 300 g/L glucose, which was 99% of the theoretical yield of 1.21 g/g, and this value was higher than any of the existing reports in the literature.

The changes of online parameters shown in the Fig. 10 were consistent with the offline results. The three fermentation processes were limited by the dissolved oxygen level with DO far below the critical level. Real-time OUR was maintained at a good level in the fermentation,

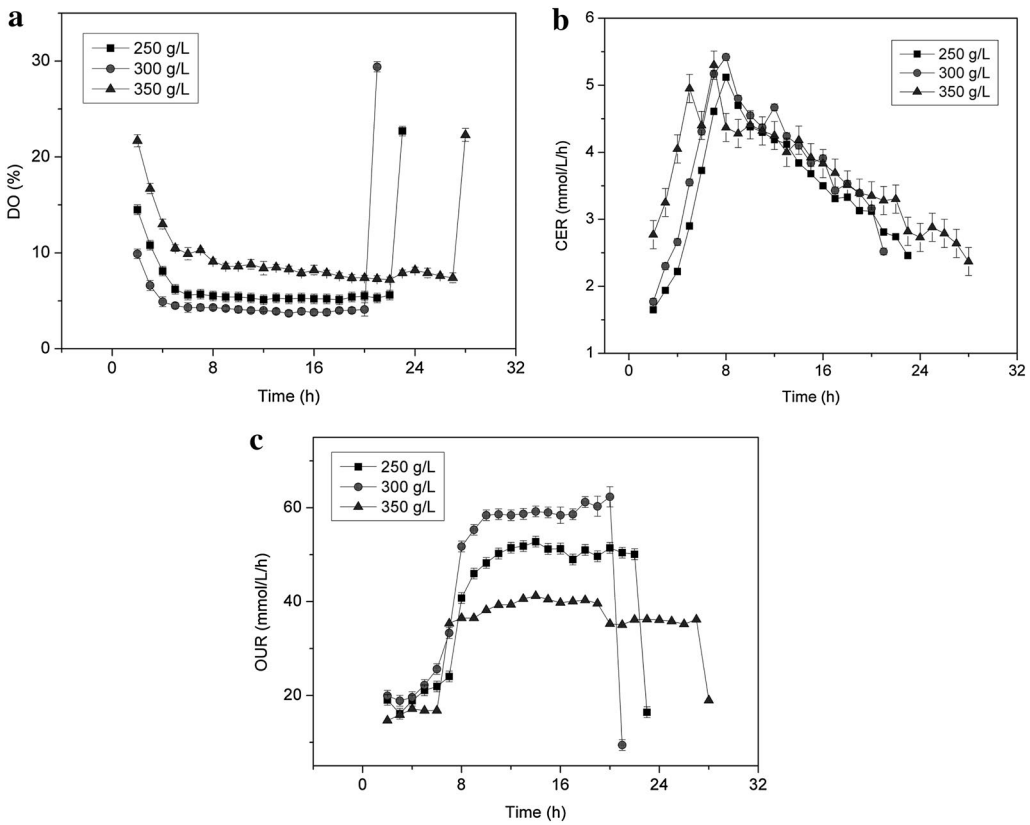

Fig. 10 Time courses of DO (**a**), CER (**b**), and OUR (**c**) in fermentation processes with different seed cultures of *A. niger*

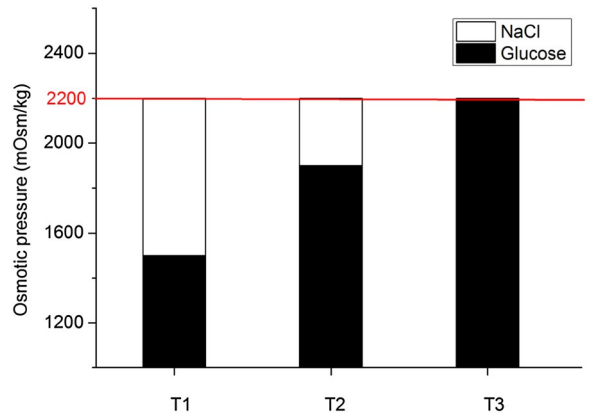

Fig. 11 Experimental design to investigate the physiological roles of glucose in seed culture of *A. niger*

and its maximum value up to 62 mmol/L/h was obtained from seed cultured in 300 g/L glucose, which was, respectively, 18 and 54% higher than those under 250 and 350 g/L glucose conditions.

On the basis of the macroscopic cultivation performances, the analyses of intracellular metabolite profile and the mycelium morphology, as well as the further verification of sodium gluconate fermentation, 300 g/L glucose was selected as the optimal initial concentration of seed culture, which was more suitable for the sodium gluconate fermentation.

Dual physiological functions of glucose

During sodium gluconate production by *A. niger*, glucose, being one of the key substrates, had dual roles of acting as not only the carbon source for cell growth and

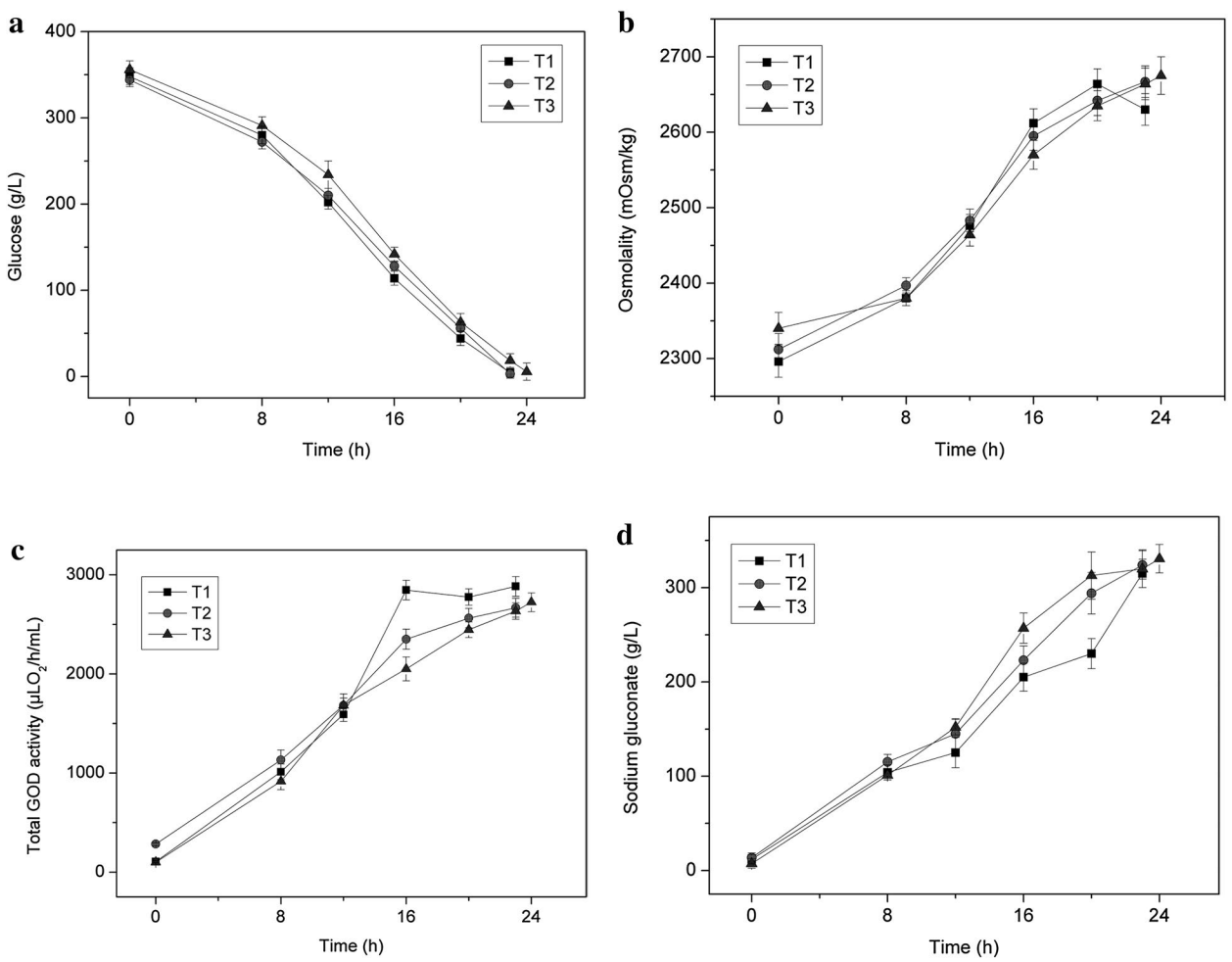

Fig. 12 Effects of different seed cultures with same initial osmolality on sodium gluconate fermentation by *A. niger*. **a** Glucose consumption, **b** environmental osmotic pressure, **c** total GOD activity, and **d** sodium gluconate production

metabolism, but also as the osmotic regulator in the medium. Previous study suggested that slightly higher osmolality was more conducive to produce sodium gluconate, whereas this paper systematically explored the effects of initial glucose concentration on seed culture by *A. niger*, and found that 300 g/L glucose, corresponding to 1900 mOsm/kg, was the optimal condition at seed stage. However, the glucose acting as carbon source or playing the increased penetration role during the process, still remained to be addressed.

Sodium chloride solutions of 0.27, 0.15, and 0 mol/L were added into different initial glucose concentrations of 250, 300, and 350 g/L one by one, and the three groups of seed culture named T1, T2, and T3 with the same initial osmotic pressure of 2200 mOsm/kg were prepared. The detailed experimental design is shown in Fig. 11. During the initial glucose concentration of 250–350 g/L, if the experimental results of three groups were similar, then this indicates that the permeability of glucose dominated; otherwise, the carbon source of glucose played a prominent role.

As illustrated in Fig. 12, the main parameters of T1, T2, and T3 were very similar, among which the culture time was within 1-h difference, and the terminal osmotic pressure and GOD total activity were about 2650 mOsm/kg and 2700 $\mu LO_2/h/mL$, respectively. The average glucose consumption rate and sodium gluconate production rate were about 15 g/L/h and 13.5 g/L/h, respectively. At the same time, the three processes showed similar yield of sodium gluconate of approximately 1.05 g/g.

The results showed that the role of glucose as an osmotic regulator was dominant under 250–350 g/L glucose concentrations, which made the research of optimized initial glucose concentration in the seed culture stage more significant.

Conclusions

Based on the physiological parameter OUR, the effects of different initial glucose concentrations on the seed culture by *A. niger* were studied for the first time. 300 g/L initial glucose concentration, which principally played a role as an osmotic regulator during the seed cultivation, was found to present the best fermentation performance. Under this condition, the maximum OUR level was about 1.7 times of the other groups. Moreover, the intracellular metabolite profiling revealed that the cells would adapt to the hyperosmotic stress with intracellular osmoprotectants' accumulation of alanine and glutamate. Consequently, 1.198 g/g of sodium gluconate yield, 99% of the theoretical value, was achieved with the optimized seed culture, and this value proved to be the highest to be reported in the literature. On the other hand, it was interesting to note that the osmolality could induce GOD activity possibly by affecting the synthesis of histidine, one of the key components of the active site of GOD.

Abbreviations

A. niger: *Aspergillus niger*; OUR: oxygen uptake rate; GOD: glucose oxidase; KLa: volumetric oxygen transfer rate; A: projected area; Fr: filamentous ratio; CER: carbon dioxide evolution rate; DCW: dry cell weight; GC–MS: gas chromatography with mass spectrometry; FBP: fructose-1,6-diphosphate; G3P: glyceraldehyde 3-phosphate; 3PG: 3-phosphogluconic acid; Ala: alanine; Glu: glutamic acid; Gly: glycine; Lys: lysine; Val: valine; Met: methionine; Thr: threonine; Pyr: pyruvic acid; αKG: α-ketoglutarate; Suc: succinate; Fum: fumaric acid.

Authors' contributions

XL conducted the experiments. XL and XT wrote the manuscript. XT, HH, WZ, YW, and CJ provided advices on the experimental design and data analysis. All authors read and approved the final manuscript.

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Acknowledgements

The authors thank the Shan Dong Fuyang Biological Technology Co., Ltd (DeZhou, China) for donating the industrial strain of *A. niger*.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included in the main manuscript.

Consent for publication

All the authors have read and approved the manuscript before submitting it to bioresources and bioprocessing.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Funding

This study was supported by the National Basic Research Program of China (973 Project) (No. 2013CB733600), and the National High Technology Research and Development Program 863 (No. 2015AA021005).

Publisher's Note

Received: 28 October 2017 Accepted: 1 December 2017
Published online: 14 December 2017

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