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EFFECTS OF TEMPERATURE AND NUTRIENT FEED ON THE PRODUCTION OF OXALIC ACID BY *ASPERGILLUS NIGER*

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ABSTRACT

Oxalic acid is an important organic material used for rare earth metal extraction precipitator, metallic equipment purificant and purification of industrial minerals. Aspergillus niger *is known to be able to produce a high concentration of oxalic acid using glucose as carbon and energy sources. For further process optimization, submerged fermentation experiments were carried out to study the effect of temperature and nutrient feed on the production of oxalic acid from medium containing glucose 35g/L. An increase in temperature process from 25 to 30°C allowed the productivity to significantly increase from 75.50 to 81.06% of theoretical yield with a final oxalic acid concentration of 17.04 g/L reached after 9 days of process. When operating at more controlled fermentor with fed-batch system, both productivity and oxalic acid concentration were markedly improved (88.01% of theoretical yield and 19.52 g/L respectively). The later system gave excellent yield almost 90% of theoretical yield as a prerequisite of economical value.*

Keywords: oxalic acid, Aspergillus niger, temperature, nutrient feed, batch and fed batch fermentation

INTRODUCTION

Oxalic acid is an important basic organic material and mainly used to produce antibiotics and pharmaceutical intermediate synthesis. Another important use of oxalic acid is as precipitant extract in the the rare earth metals, tanning and cleansing agents of metal equipments. According to China Research and Intelligence Co., Ltd. (2009), the smelting of rare earth needs 1.4 tons of oxalic acid per ton. Moreover, oxalic acid is also used as a leaching agent for solubilizing heavy metals and purification of industrial minerals such as quartz, feldspar, bentonite, and gypsum (Styriakova and Stiriak, 2000; Gharieb, 2000). Due to its high reduction power, oxalic acid can also be used for the removal of iron present in kaolin as impurity (Cameselle et al., 2003). The quality of quartz sand can be improved by means of microbial leaching of iron oxide, which is especially attributed to oxalic acid excreted by the microbes (Strasser et al., 1993, Handayani, 2004). At present, the Laboratory of Biohydrometallurgy at R&D Centre for Minerals and Coal Technology is conducting a research on purification of this quartz sand using oxalic acid to produce ultrapure silicon for solar cell and electronic used. Due to the application expansion of oxalic acid, the demands for oxalic acid increased steadily over the previous years. In 2008, the demands for oxalic acid in Chinese rare earth industry were beyond 100 thousand tons and in 2009, the demands volumes of the oxalate in the world were about 450.000 tons (Wood, 2011).

The traditional industrialization production methods of oxalic acids mainly involve oxo process, glycol oxydation process, carbon monoxide coupling process, sodium formate process, propylene oxidation process (Wood, 2011). Those processes have to use sulfuric acid and lead, leading to serious threats to the environment. Therefore, it is necessary to develop another alternative and appropriate technology in the possibility of producing oxalic acid in high amount through biological process that more environmental friendly.

Oxalic acid can be produced by a variety of fungi, including saprophytic and phytopatogenic species.

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In saprophytic species such as *Aspergillus niger*, the role of oxalic acid production is related to mobilizing substrate from cell wall polysaccharides. Through acidification and chelating properties, oxalic acid may increase availability of metal ions such as iron and calcium (Ruijter et al., 1999). *Aspergillus niger* is known as a very efficient oxalic acid producer which can be illustrated by the findings of several researchers (Handayani and Suratman, 2009; Handayani, 2004; Rymowics and Lenart, 2004; Podgorski and Lesniak, 2003; Musial et al., 2006; Ruijter et al., 1999).

In a previous study, Handayani and Suratman (2009) have used *Aspergillus niger* in a research on the production of oxalid acid using glucose and sucrose media as carbon sources. The results demontrated that *Aspergillus niger* had a good capability in producing oxalic acid when grown in glucose-based media compared to sucrose. On the basis of those results, it is decided that glucose media would be used consistently for kinetics studies of oxalic acid production. The objective of the study is to further optimize the ability of this fungus to produce oxalic acid with respect to physical and nutritional requirements such as temperature and glucose feed. At this stage, none of published research has been concerned with those spesific characteristics. Clearly, a fundamental understanding of the effects of these variables become critically important, and this is the subject of the present study.

METHODOLOGY

Materials

The microorganism used in this research was *Aspergillus niger* that had been isolated from the quartz sample of Karimun island. The composition of media was as follows (g/L): glucose 35, $NaNO_3$ 1.5, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.025, KCl 0.025, yeast extract 1.5. The nutrient solutions were buffered with TRIS at a concentration of 1 M. The pH value was adjusted with NaOH to pH 6.0 before sterilization at 121°C for 30 minutes.

Experimental Procedures

Duplicate batch growth cultures with one control were established at 25 and 30°C at 100 rpm. Subsequently, 10 ml samples were taken periodically from the culture. The changes in the cultures including pH, optical density of the culture, dryweight of the biomass, glucose concentration and the amount of oxalic acid produced were assayed every 4 hour during the logarithmic phase and daily for 12 days during stationary phase until the glucose has fully used up.

The experiments were also conducted to compare two modes of fermentation: batch and fed-batch fermentation using a fully controlled fermentor (New Bruinswick Bio Flo Celligen 115, Figure 1). The operational parameters of fed batch run were as follows:

- working volume : 1 L
- rpm (day 1) : 100
- rpm (day 2 to 14) : 150
- air flow rate : 0.5 vvm
- temperature : 30°C
- initial glucose : 10 g/L
- addition of glucose : days 4, 5, 6, 7, 8 (5 g/L each until total of 35 g/L)
- dissolved oxygen : 20%
- pH : maintained at 6.0 using NaOH 2N
- fermentation period : 14 days

Analytical Methods

For analyses, all samples were filtrated (0.2 µm) in order to guarantee a particle free solution. The production of oxalic acid was determined by Bergermann and Elliot method, and the consumption of glucose was determined by Wang method (Wang, 2002). The cell mass of *Aspergillus niger* grown in media with different temperature were measured gravimetrically. The filtrated cell mass was dried at 80°C until the weight was constant (approximately 24 h).

The obtained data were analyzed in terms of various kinetics parameters as have been reviewed by many authors such as Podgorski and Lesniak, 2003; Kubicek et al., 1987, Ruijter et al., 1999 as follows:

1. Specific growth rate (µ)
   The growth rate was measured during the exponential growth phase of the culture. The increase in cell mass during an infinitely small time interval is proportional to the amount of cell mass present and time interval:

   $dx = \mu \times dt$

   µ is constant and can be calculated from $\mu = 0.693/dt$ where dt = doubling time.

2. Overall biomass yield (Yx/s)
   The growth yield is the increase in cell mass as a result of substrate utilization:

   $Yx/s = (x-x_o)/(s_o-s)$

   Where x_o and s_o are the initial cell mass and substrate concentration and x and s are the corresponding concentration during growth of the culture.

5. Maximum specific oxalic acid production rate (q_p)

   $Q_p = (Y_p/x)\ \mu$

   Where Y_p/x is product/cell mass formation

6. Oxalic acid yield theoretically was calculated according to the following idealized reaction:

Figure 1. Fermentor New Bruinswick Bio Flo Celligen 115

3. Overall product yield (Yp/s)
   The overall product yield can be calculated from:

   $Yp/s = (p-p_o)/(s_o-s)$

   Where p and p_o are respectively final and initial product concentration.

4. Maximum specific substrate utilization (uptake) rate (q_s)

   The utilization of substrate in a culture during an infinitely small interval time can be given by : $q_s = (1/Yx/s)\ \mu$

   1 glucose + $2CO_2$ + 2NAD -> 2 oxalic acid + 2 NADH

RESULTS AND DISCUSSION

Effect of Temperature

The effect of different temperature (25°C and 30°C) on the production of oxalic acid was studied and the changing in culture condition as a function of time can be seen in Figure 2 and 3.

In batch fermentation, the production of oxalic acid started after a lag phase of one day and reached

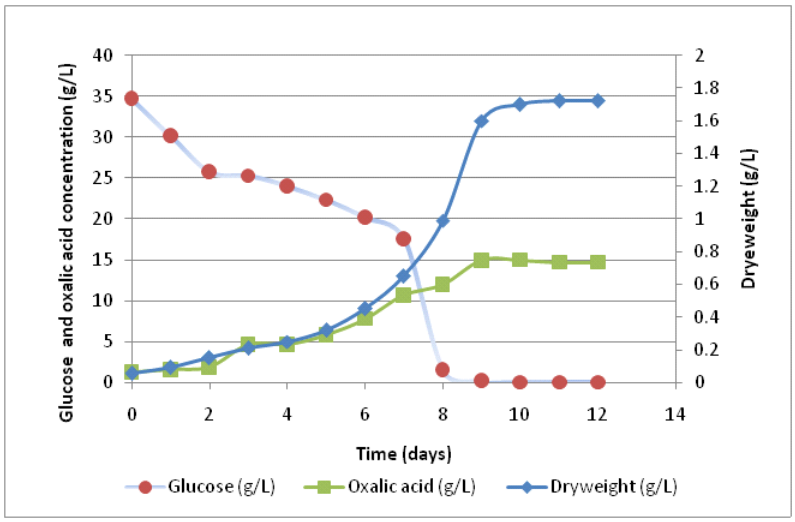

Figure 2. Kinetics profile of *Aspergillus niger* grown on glucose medium at 25°C

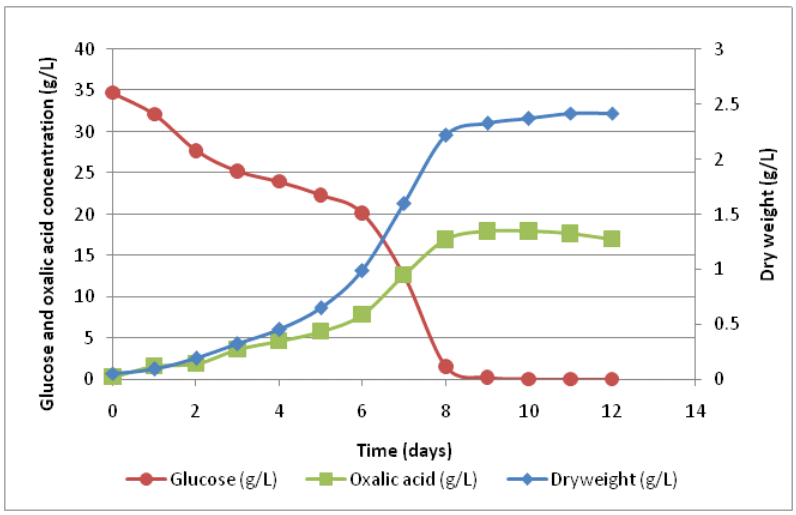

Figure 3. Kinetics profile of *Aspergillus niger* grown on glucose medium at 30°C

maximum at the onset of stationary phase or late exponential phase (Figure 2 and 3). Further increase in incubation period did not enhance oxalic acid production due to the age of fungi and depletion of glucose content in the culture broth.

There was an increase in the production of oxalic acid with the increase in temperature process. Increasing temperature process from 25 to 30°C enhanced the oxalic acid production of 12% and 17.04 g/L oxalic acid was obtained in the fermentor broth at the end of fermentation at 30°C. The maximum oxalic acid production at 30°C was achieved in a bit faster time (after 8 days of process) compared to 9 days at 25°C. All glucose was consumed and final dry cell mass was 2.4 g/L. Mycelia were found to be small round pellets (2-3 mm in diameter).

Table 1 presents a comparison of kinetics parameter data for *Aspergillus niger* grown in glucose media at different temperature (25°C and 30°C). It can be seen that all kinetics parameters were sig-

Table 1. Comparison of kinetics parameter data for *Aspergillus niger* grown in glucose media at different temperature (25°C and 30°C)

Kinetics parameters	25°C	30°C
Maximum specific growth rate (μ)	0.43	0.54
Overall biomass yield (Yx/s)	0.05	0.05
Overal product yield (Yp/s)	0.39	0.66
Maximum specific substrate uptake rate (qs)	9.04	10.81
Maximum specific oxalic acid production rate (qp)	3.48	4.33
% of theoretical yield of oxalic acid	75.5	81.06

nificantly influenced by the temperature of culture. It was observed that the biomass yield at different temperature was relatively same but other kinetics parameters (the specific growth rate and product yield, specific substrate uptake rate and specific oxalic acid production rate) increased when the temperature also increased from 25 to 30°C. This means that this strain was particularly sensitive to temperature. The temperature of 30°C gave the better results compared to that of 25°C in oxalic acid production. It reached 81.06% of theoretical yield.

Effect of Glucose Feed

One of the main problems for achieving bioreactor performance under stable conditions with filamentous fungi is limiting hyphal growth and avoiding diffusional restrictions that can put pressure on productivity. It is assumed that the fed-batch system could improve substrate conversion which in turn can increase production of oxalic acid.

The main diferences between the two modes of fermentation is that in the batch fermentation, the substrate was provided initially in high concentration, the microorganism grew in a closed system and will end up with cessation of growth due to exhaustion of an essential nutrient or an accumulation of toxic product. In batch system, the concentration of substrate, metabolites and cells vary with time. Fed batch fermentation, on the other hand, is a system for growing microorganism with some substrate at the beginning and with a continuous nutrient supply thereafter. This technique involved the controlled feeding of the culture with nutrient, and a steady state was maintained in the culture vessel as the working volume increased. The medium was fed by repeated small addition, so the amount of glucose in the medium remained at relatively low levels. In this experiment, it was operated with "variable volume" where the feed rate Fo was not the same as outflow F1 (F1=0). This mode of fermentation can avoid repressive effect of substrate or substrate inhibition of growth by adding the substrate in batch and allow periodic shifts of growth rate.

Figure 4 represents the effect of glucose feed on the production of oxalic acid in this experiment, and their kinetics paramater data are shown in Table 2.

Production of oxalic acid gradually increased somewhat when the time for addition of glucose was increased (Figure 4). From the data shown in Table 2, it can be seen that the growth rate, biomass production and product yield in fed batch system were higher than that of batch system. This result is tipical for the two systems, as ex-

Table 2. Comparison of kinetics parameter data for *Aspergillus niger* grown in glucose media in batch and fed batch fermentation at 30°C

Kinetics parameters	Batch	Fed batch
Maximum specific growth rate (μ)	0.54	0.87
Overall biomass yield (Yx/s)	0.07	0.11
Overal product yield (Yp/s)	0.66	0.71
Maximum specific substrate uptake rate (qs)	10.81	12.75
Maximum specific oxalic acid production rate (qp)	4.33	5.07
% of theoretical yield of oxalic acid	81.06	88.01

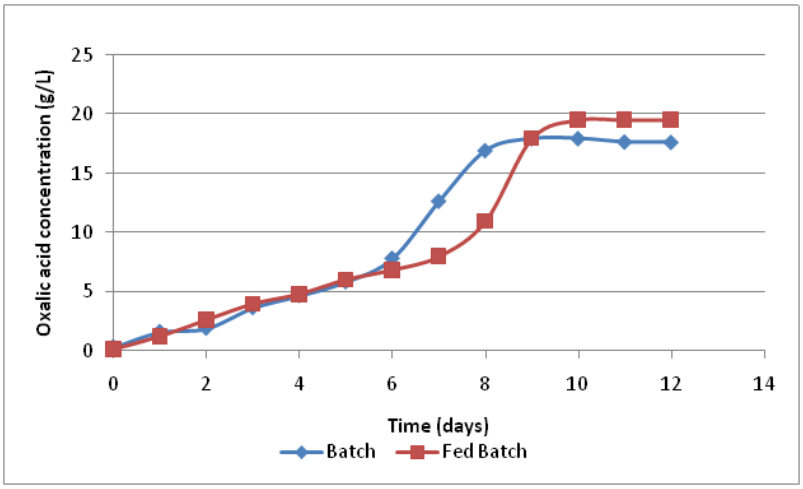

Figure 4. Profile of oxalic acid production by *Aspergillus niger* in batch and fed batch fermentaion

pected. Preprogrammed feeding of glucose can minimize the effect of catabolic repression, improve mass transfer limitation and substrate conversion and finally improve the yield as shown clearly by the results of fed batch culture in this experiment although it needed a longer time. The best results of oxalic acid production was achieved by this system with the maximum value 88% of theoretical yield and a maximum amount of 19.52 g/L of oxalic acid was obtained in the fermented broth after 10 days of fermentation.

CONCLUSION AND SUGGESTION

Using glucose based medium, the production of oxalic acid by *Aspergillus niger* reached a maximum productivity of 88% of the theoretical yield at the optimum condition studied, i.e. using fed-batch system in fully controlled fermentor at 30°C and pH 6.0. The value achieved in the present study is highly significant and can further be optimised to reach the economical value (above 90% of theoretical yield).

To improve biomass yield and productivity, the problem of substrate inhibition of growth in batch culture can be overcome by fed batch system. However, the system needs a longer time of process and growth in a long period may result in problems of contamination. As a consequent, for extended growth periods, the equipment must be reliable and of best quality to prevent mechanical breakdown, so fed batch culture requires higher initial capital costs compared to batch culture. Therefore, its aplication would be dictated by its economic value of the product.

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