Preparation of levoglucosan by pyrolysis of cellulose and its citric acid fermentation

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Abstract

Levoglucosan (LG), 1,6-anhydro- β -D-glucopyranose, was produced by pyrolysis of cellulose. A response surface method was used to optimize the reaction parameters: X_1 , temperature; X_2 , time required for heating cellulose from room temperature to the designed pyrolysis temperature; and X_3 , vacuum, and a Box–Behnken design was employed for this purpose. The optimal temperature and time were found to be 388°C and 26.2 min by fixing the vacuum at 1 mm Hg. The levoglucosan prepared was fermented to citric acid by *Aspergillus niger* CBX-209, which was a mutant derived by γ -ray mutagenesis of the parent strain CBX-2. The mutant could produce increasing citric acid with increasing LG purity and had a citric acid yield of 87.5% when using purified levoglucosan as the sole carbon source in a 5 day fermentation period. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Levoglucosan; Citric acid; Response surface method; Pyrolysis; *Aspergillus niger*

1. Introduction

Studies of degradation and bioconversion of cellulose have been given much attention because of the serious problem of limitations of fossil fuel reserves. The ubiquitous cellulosic materials are huge renewable resources for the production of bulk chemicals, such as ethanol and organic acids, by fermentation (Lynd et al., 1991). However, raw materials to be used as carbon and energy sources in fermentation industries have to be available in a simple and safe way, at acceptable prices, and in a way not affecting the environment. Levoglucosan (LG), 1,6-anhydro- β -D-glucopyranose, the main component of the highly viscous syrup which condenses in an ice-water bath during pyrolysis of cellulose, is attractive today for use directly or indirectly as a potential feedstock for the fermentation industries.

Development of methodology for the pyrolysis of cellulose involving standardization of the various parameters to achieve maximum performance and optimum conditions is very necessary to get a high yield of levoglucosan. In general, the single-parameter method, a single-dimensional search which involves changing one

variable while fixing others at a certain level, is laborious and time consuming, especially when the number of variables is large. An alternative and effective approach is the response surface technique (Carvalho et al., 1999; Gomes et al., 1994; Lopez et al., 2000) which involves three major steps: performing the experiments by the approach of Box–Behnken design or central composite design, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model. In this paper, we used response surface technique to optimize reaction parameters.

Crude LG can be crystallized easily from the cellulose pyrolysis oil, and its yield can reach 15% or more, based on the dry weight of cellulose. Many strains of bacteria, yeasts and fungi (Kitamura et al., 1990; Prosen et al., 1993) can use the LG as a carbon and energy source for their growth. However, references concerning LG fermentation with high yields and concentrations of metabolites are very scarce. So far, only Nakagawa et al. (1984) has reported that *Aspergillus terreus* K26 could convert pure LG into itaconic acid in the same yield and at the same rate as from glucose. Some species of yeasts, such as *S. Castelli*, *S. diastaticus*, *C. utilis* (Prosen et al., 1993) produced ethanol from partly purified levoglucosan, but the yields were all very low (about 10% or so). In our preliminary studies we also found that a strain producing citric acid (CA), *Aspergillus niger* CBX-2,

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which fermented starch or glucose well (conversion rate $\geq 90\%$), produced very little citric acid (conversion rate $<10\%$), in a medium with either pure or partly purified LG. In the present work, we were interested in the mutagenesis and selection of *A. niger* that could produce citric acid at high yield using LG as a sole carbon and energy source as well as in the production of LG.

2. Methods

2.1. Production of LG

2.1.1. Statistical design

A Box–Behnken design with three factors (Box and Behnken, 1960) was used to study the effect of three factors (X_1 , temperature; X_2 , time required for heating cellulose from room temperature to the designed pyrolysis temperature; and X_3 , vacuum) on the production of levoglucosan.

Statistical analysis was carried out using the RSREG procedure, and then the equation was refitted by the GLM procedure of the SAS-STAT software (Cary, 1987).

2.1.2. Pyrolysis and LG preparation

The LG was prepared by the pyrolysis of cotton cellulose. The pyrolysis apparatus comprised a stainless steel tube furnace with a reaction volume of about 1.5 l and an insulating mantle containing glass wool, with a temperature controller. It was connected to a vacuum pump and an ice bath. Samples of 80 g cotton cellulose were pyrolyzed under various vacuum conditions and at temperatures between 320°C and 420°C. Pyrolysis oils containing LG were produced and treated by the following methods before use:

1. Crude pyrolysis oil (LGoi): The pH of crude pyrolysis oil was adjusted to 6.0 by adding lime and the supernatant was collected by centrifugation for use.
2. Activated carbon treatment (LGac): The supernatant from Step 1 was further treated by an activated carbon column to partly remove colored materials.
3. Crystal LG (LGct): The crystalline LG was prepared by the method described by Ward (1963).

2.2. Microorganism

2.2.1. Parent strain

The parent strain *A. niger* CBX-2 (Xue et al., 1994), which had a high yield of citric acid from starch or glucose, was used. The strain was maintained on levoglucosan agar slant, kept at 4°C after subculturing every month. The medium composition for conidia preparation on the agar slants was (g/l): LG, 20; wheat bran, 5; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.5; and agar, 20.

2.2.2. γ -ray treatment of spore suspension

The source of γ -rays was ^{60}Co . Samples of spore suspension (5 ml each, spore concentration: 10^7 /ml) were irradiated with a ray dose of about 10^5 R (roentgen). The suspension was then diluted 10^3 times for further use.

2.2.3. Selection of citric-acid-producing mutants

(1) Plates containing LGct 30 g/l, peptone 5 g/l and agar 20 g/l were inoculated with the spores obtained by γ -ray mutagenesis and incubated at 34°C for 5 days. (2) The colonies with typical “CA-producing” morphologies were selected and kept on LG slants. (3) The spores developed on LG slants were inoculated into the medium containing citric acid as the sole carbon source for selecting a strain that did not utilize CA. This medium contained citric acid 20 g/l, $FeSO_4$ 0.5 g/l, KCl 0.5 g/l, $MgSO_4$ 0.5 g/l, K_2HPO_4 1 g/l, $NaNO_3$ 3 g/l and had a pH of 5.5. After incubation on a rotary shaker at 400 rpm for 24 h the resulting culture was filtered and the spores that could not grow were collected for further plate screening. (4) The medium of the plates was composed of LG 30 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, KCl 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, K_2HPO_4 1 g/l, $NaNO_3$ 2 g/l, agar 20 g/l and bromocresol purple 0.4 g/l. Plates were inoculated with 0.5 ml of spore suspension prepared by Step 3 and incubated at 34°C for 5 days. The colonies showing “acid-producing circle” were harvested for further fermentation tests. Finally, the best strain producing CA was kept on LG slants on a medium containing LGct 20 g/l, wheat bran 5 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l and agar 20 g/l.

2.2.4. Fermentation

The composition of the fermentation medium per liter was: LG 80 g, peptone 5 g and pH 6.0. The medium was dispensed in 50 ml aliquots in 250 ml Erlenmeyer flasks and autoclaved for 20 min. Each flask was inoculated with 1 ml suspension having about $1-3 \times 10^5$ conidia, incubated on a rotary shaker at 400 rpm at 34°C for 5 days and the necessary analyses were made during the fermentation period.

2.3. Analysis

LG and citric acid analyses were performed on an HPLC system (Gilson 712 system, Gilson Medical Electronics, USA) equipped with a Biorad Aminex HPX-87 H column and a refractive index detector. The mobile phase was double-distilled water at 1 ml/min and 8 mM H_2SO_4 at 0.8 ml/min at 30°C. LG was also qualitatively detected by thin layer chromatography on silica gel with developing agent ethyl acetate/methanol (v/v, 3:1). The titration method was also used for routine determination of citric acid. The microbial biomass was

measured by weighing mycelia filtered and dried at 100°C to constant weight.

3. Results and discussion

3.1. LG preparation by cellulose pyrolysis

A response surface method was used to optimize the pyrolysis of cotton cellulose and parameters were found to be: X_1 , 388°C and X_2 , 26.2 min by fixing X_3 at 1 mm Hg. Under these conditions, LG had an average yield of 30% (based on the dry weight of cellulose) in actual experiments. Further experiments were carried out to pyrolyze wastepaper. LG yields were found to be 10–13% under the same conditions.

3.2. Selection of strains for CA production

Our preliminary tests showed that the morphological feature of the strain CBX-2 grown in the LG medium was undesirable filamentous hyphal growth. The initial morphological feature of the outgrowth of the spore was the direct formation of germ, but the germ tubes were thinner than in glucose medium and had only very few branches which came into contact with each other loosely. The strain producing citric acid with high yield in LGct medium started with considerable spherical swelling of the spores prior to the formation of tubes.

Table 1
Selection of mutants producing citric acid by hyphal morphology^a

Mutants	CA (g/l)	Yield (% CA/ LG, w/w)	Growth morphology	
			Filamentous	Spherical
2-01	24.3	81	–	+
2-02	18.3	61	–+	–+
2-03	16.5	55	+	–
2-04	13.5	45	+	–
2-05	14.4	48	+	–
2-06	21	70	–+	–+
2-07	22.5	75	–	++
2-08	1.2	40	+	–
2-09	27.3	91	–	++
2-10	25.5	85	–	++

^a 30 g LGct/L, 5 day culture.

Table 2
Effect of LG purity on *A. niger* CBX-2 and CBX-209 biomass and citric acid yields^a

	<i>A. niger</i> CBX-2				<i>A. niger</i> CBX-209			
	LGoi	LGac	LGct	Glucose	LGoi	LGac	LGct	Glucose
Biomass (g/l)	1.57	7.02	8.18	8.51	1.48	7.42	8.07	8.60
Citric acid (g/l)	0.7	3.5	4.5	88.9	1.9	15.4	70.0	81.4
Yield ^b (%)	0.88	4.38	5.63	101	2.38	19.25	87.5	92.5

^a 80 g LG/l, 5 day shake cultivation and 88 g glucose/L, 3 day shake cultivation.

^b CA/LG or glucose, w/w.

Fig. 1. Course of the fermentation in LGct medium. (■) LG; (+) pH; (○) CA; (●) biomass.

The developed germ tubes bound tightly to form pellets. This typical morphological difference between the growth of “filamentous” and “spherical” forms was applied to select the strains with high yield of citric acid. Table 1 gives the results of the selection, which showed that the mutant CBX-209 was the best one. The value of CA/LG reached 91% and it was used in the following fermentation tests.

3.3. Effect of purity of LG on biomass and CA production

Table 2 shows the effect of various purities of LG on biomass and CA production. Good growth of strain CBX-209 was observed in LGac medium with comparable biomass to the LGct and glucose media. The significant increase of biomass as compared with the LGoi medium indicates that simple processing of the aqueous pyrolysis oil through activated carbon was able to overcome the inhibition effect of colored substances and aromatic species on biomass. Compared with CBX-2, which had poor CA production in LGoi, LGac and LGct media, CBX-209 could produce increasing citric acid with increasing LG purity (in these fermentation tests, the concentrations of LG in LGoi, LGac, LGct media were all improved to 80 g/l). This result coincides with the findings of itaconic acid fermentation by Nakagawa et al. (1984).

In the glucose medium, the yield of the mutant CBX-209 (92.5%) was somewhat less than that of CBX-2

(101%), while the mutant had a significant improvement of citric acid yield from 5.63% to 87.5% by using LG as substrate. It was obvious that the strain CBX-209 had some changes in its metabolisms of carbon source.

3.4. Time course of CBX-209 pure LG fermentation

The time course of citric acid fermentation with purified LG as a sole carbon source is shown in Fig. 1. Accumulation of citric acid by CBX-209 could be divided into three phases. The first phase was defined as the period from inoculation to 24 h. Very little citric acid (≤ 2 g/l) was produced, while biomass increased rapidly in the culture, which maintained a relatively high pH at this period. The second phase lasted from 24 to 72 h. Excretion of citric acid increased rapidly from 2 to 43 g/l. A continuous increase of biomass was also observed even when the pH of the culture broth was very low (for example, pH was lower than 2.5 from 48 h) and reached its maximum when pH decreased to 2.0. The third phase was defined as the period from 72 to 120 h. Citric acid concentration reached its maximum (70 g/l), while biomass of this mutant was maintained at almost the same level. Since kinetics of growth and citric acid formation by this mutant in LG medium exhibit features found previously in citric acid fermentation of sucrose by Roehr et al. (1981), citric acid fermentation of LG by *A. niger* CBX-209 could be considered a “type II fermentation”.

In this research, LG was consumed at an average rate of 0.21 g/l h in the first phase, 0.90 g/l h in the second phase and 0.61 g/l h in the third phase.

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References

- Box, G.E.P., Behnken, D.W., 1960. Some new three level designs for the study of quantitative variables. *Technometrics* 2, 455–475.
- Cary, N.C., 1987. SAS/STAT™ Guide for Personal Computers. SAS Institute.
- Carvalho, C.M.L., Cabral, J.M.S., Aires-Barros, M.R., 1999. Cutinase stability in AOT reversed micelles: system optimization using the factorial design methodology. *Enzyme and Microbial Technology* 24 (8–9), 569–576.
- Gomes, D.J., Gomes, J., Steiner, W., 1994. Production of highly thermostable xylanase by a wild strain of thermophilic fungus *Thermoascus aurantiacus* and partial characterization of the enzyme. *Journal of Biotechnology* 37 (1), 11–22.
- Kitamura, Y., Abe, Y., Yasui, T., 1990. Metabolism of levoglucosan in (1,6-anhydro- β -D-glucopyranose) microorganisms. *Agricultural and Biological Chemistry* 55 (2), 515–521.
- Lopez, F., Ariza, J., Perez, I., Jimenez, L., 2000. Influence of the operating conditions on the properties of paper sheets obtained by kraft pulping of olive tree wood. *Bioresource Technology* 72 (2), 147–151.
- Lynd, L.R., Cushman, J.H., Nichols, R.J., Wyman, C.E., 1991. Fuel ethanol from cellulosic biomass. *Science* 251, 1318–1323.
- Nakagawa, M., Sakai, Y., Yasui, T., 1984. Itaconic acid fermentation of levoglucosan. *Journal of Fermentation Technology* 62 (2), 201–203.
- Prosen, E.M., Radlein, D., Piskorz, J., Scott, D.S., 1993. Microbial utilization of levoglucosan in wood pyrolysate as a carbon and energy source. *Biotechnology and Bioengineering* 42, 538–541.
- Roehr, M., Zehentgruber, O., Kubicek, C.P., 1981. Kinetics of biomass and citric acid production by *Aspergillus niger* on pilot plant scale. *Biotechnology and Bioengineering* 23, 2433–2445.
- Ward, R.B., 1963. Levoglucosan. In: Whistler, R.L., Wolfrom, M.L., BeMiller, J.M. (Eds.), *Methods in Carbohydrate Chemistry*. Academic Press, New York, pp. 394–396.
- Xue, M., Qi, H., Zhang, H., Lei, Z., Zhu, X., 1994. Citric acid production from corn starch by submerged fermentation. In: Teo, W.K., Yap, M.G.S., Oh, S.K.W. (Eds.), *Better Living Through Innovative Biochemical Engineering*. Continental Press, Singapore, pp. 104–106.