African Journal of Biotechnology Vol. 11(50), pp. 11160-11167, 21 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1116
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Producing ergosterol from corn straw hydrolysates using *Saccharomyces cerevisiae*

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Accepted 10 April, 2012

Ergosterol is an economically important metabolite produced by *Saccharomyces cerevisiae*. In this study, the production of ergosterol by the strain using corn straw as an inexpensive carbon source was investigated. The total yield of ergosterol was determined by both the biomass and ergosterol content in yeast cells which depend not only on the hydrolyze condition, but also on the culture conditions. The corn straw hydrolyzing stability test result showed that the average content of reduction sugar (dextrose equivalent, DE) was 52.3% and the conversion efficiency of hemi-cellulose was 80.0%. Cultivation in 10 L bioreactor was carried out under the optimized corn straw hydrolysate medium. According to the fermentation stabilized experiment, the mean values of biomass and ergosterol average content were up to 8.5 gL^{-1} and 2.35%, respectively. Results on the stability of ergosterol content in yeast cell extraction solution, crystallize ergosterol productivity and the mean extraction yield values are shown to be 2.35, 2.05, 87.24%, respectively. Scanning electron microscope (SEM) was employed to observe the morphology of the as-product, the infrared furrier spectrometer (IR) was used to record the infrared spectrum of the ergosterol, and the X-ray diffractometer (XRD) was carried out to characterize its component and crystallite structure. Characterization analysis indicated that the ergosterol obtained by yeast from corn straw hydrolysates has achieved high purity, and could be used as one of the widely used feedstock for Vitamin D_2.

Key words: Corn straw, ergosterol, hydrolyze, fermentation, characterization.

INTRODUCTION

Ergosterol ($C_{28}H_{43}OH$), a white palette or needled crystal which commonly exists in species throughout the plant kingdom and yeast cell, is the main sterol presenting in the membrane of epiphyte. Ergosterol is not only an important precursor for the synthesis of Vitamin D_2, but also plays an important role in physiological function. In addition, ergosterol has been suggested as important materials for other important pharmaceutical clinical trials (He et al., 2000; Butter, 1982; Dulaney, 1957; Bok et al., 1999). It can be converted to Vitamin D_2 after being irradiated by ultraviolet light (Tuohima and Lyakhovich, 2001; He et al., 2007; Shang et al., 2006). However, an asymmetric center in the molecular structure of ergosterol results in complicated steps, low yield, high costs and difficulty in commercialization and production of ergosterol by chemical method (Xue et al., 2003).

Agricultural residues such as straw from corn grain production are an obvious source of biomass. The application of these residues that would otherwise be left in the field must be done in a sustainable fashion so as not to result in unwanted carbon emissions. Biomass could ease the current energy crisis, release environment pressures and furthermore can be converted to degradable sugar by biomass hydrolysis technology. Thus, using microorganisms extract fibers which come from corn straws to produce biochemical products is an effective shortage. Nowadays, over 2.2×10^9 tons of corn straws are produced annually in China, and they are approaches to relieve environment pollution and energy shortage. Consequently, it is an important issue for us to utilize corn straws as the fermentation product (Varga et al., 2003; Zhang et al., 2005; Jeewon, 1997; Bobleter, 1997; Van, 2001).

The cell wall fraction of corn straw is rich in cellulose

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and hemicelluloses, which contain xylose, arabinose and galactose (Song et al., 2008; Li et al., 2009; Schmidt et al., 2002; House, 1995). The low cost and high carbohydrate content of corn fiber make it an attractive potential substrate to produce ergosterol. Currently, there is no way to break down the crystal structure because of the interaction between cellulose and hemicelluloses. Therefore, the corn straw should be pretreated by a special method for the process of acid hydrolysis and fermentation (Nilvebran et al., 2001; Basaglia et al., 1992; Lourdes and David, 2002; Ehara and Shiro, 2002). In this research, producing ergosterol from corn straw hydrolysates was optimized and scaled up to a 10-L bioreactor, which is simple and economical. To our knowledge, this is the first attempt to obtain ergosterol by this method.

MATERIALS AND METHODS

Corn straws were obtained from Jilin Province, China. The yeast (*Saccharomyces cerevisiae* YELB) was cultured under aerobic and supplement conditions in our own laboratory (Liu et al., 2007). Standard ergosterol was purchased from Sigma chemical (St. Louis, MO, USA). The other chemicals purchased were of reagent purity.

Corn straw pretreatment

Corn straws were pretreated by steam at 200 to 240°C for 15 to 20 min (Song et al., 2008). Being blasted rapidly in reactor, the lignose become soft and the cellulose crystal was broken. Due to the removal of the anchor coat of lignose and hemi-cellulose, cellulose was exposed and easily hydrolyzed for the following fermentation.

Hemi-cellulose hydrolysis

Hemi-cellulose hydrolysis was prepared by using inorganic acid hydrolysis at a solid: liquid ratio of 1:10 in boiling water bathed at 90±0.5°C for 8 h. The suspension was then centrifuged to remove the unhydrolyzed residue. The residue was washed with water at 80±0.5°C to extract sugars. The supernatant and washings were then pooled together, and total reduction sugars (dextrose equivalent (DE)) were determined by spectrophotometer using a dinitrosalicylic acid (DNS) reagent (Nigam, 2002). The initial sugar composition of the corn straws hydrolytes after concentration was determined by high performance liquid chromatography (HPLC). The hemi-cellulose conversion rate computation formula is as follows (Hou et al., 2007):

$$\text{Hemi-cellulose conversion efficiency (\%)} = \frac{m_0 - m}{m_0} \times 100$$

Where, m_0 is the initial hemi-cellulose content of the corn straws (g) and m is the remaining hemi-cellulose content of the corn straws that was hydrolysis (g).

Shake flask culture

Malt wort (3.9%) was sterilized for 15 min (121°C, 0.07 MPa). Then a loopful of cells taken from a slant was inoculated in a 250 ml Erlenmeyer flask containing 30 ml of 3.9% malt wort medium, and the flask was incubated at 28°C in a rotary shaker at 200 rpm for 16 h. This culture was used as the seed culture.

Fermentation culture

First and second step were same as shake flask culture. The bioreactor (Zheng Jiang Oriental Biology Equipment Corporation, China) containing 6 L cultivation medium was sterilized at 121°C for 20 min. And then the bioreactor was inoculated with 10% (v/v) seed cultures. Cultivation was carried out at 28±1°C, at an agitation speed of 600 rpm and air flow-rate of 10 L min^{-1}. The corn straw hydrolysate was added into the bioreactor using the feedback control system and the pH was adjusted at 5.5±0.1 by automatic addition of 25% ammonia or 20% (w/w) KOH (Xue et al., 2006). Dissolved oxygen was measured with an autoclavable O_2-sensor (Mettler).

Ergosterol extraction

Five milliliters of fermentation broth (OD_{600} of 0.5, 50 times diluted) was centrifuged (4000 rpm, 10 min) to separate the yeast cells. Then the cells were transferred into a 100 ml flask followed by 8 g of KOH and 32 ml of 60% ethanol solution (v/v) added. For saponification, the mixture was treated at 80±2°C in water bath for 2 h. Finally, 25 ml of petroleum ether (boiling point range, 60 to 90°C) was added to extract the ergosterol.

Analytical methods

The total ergosterol contents were estimated by ultra-violet absorption spectral analysis at 280 nm using the following formula (Yao et al., 2007; Lees et al., 1995; Abramson and Smith, 2003; Delareauj, 2000): Egosterol (%) = CV / 1000W × 100%, where C is the ergosterol concentration in extracting samples ($mgml^{-1}$), V is the extracting solvent 100 ml and W is the dry yeast weight (g). Scanning electron microscopy images were obtained on a JS-5500LV scanning electron microscope (SEM), working at 15 and 20 KV accelerating voltages. The infrared spectrum was obtained on the infrared furrier spectrometer with KBr pellets in the 4000-400 cm^{-1} regions (Chen, 1993). The powder X-ray diffraction (XRD) patterns were recorded on a PW1700 wide Angle X-ray diffractometer (Zhou, 1997).

RESULTS

Analysis of corn straw material components

The main components of corn straw are cellulose, hemi-cellulose and lignin. Table 1 show that the contents of cellulose, hemi-cellulose and lignin are 37.4, 33.1 and 15.5, respectively. In addition, it also contains ash, crude protein and water. Cellulose is wrapped between hemi-cellulose and lignin, the crystal structure of which is hard to degrade, thus hampering the action of enzyme and acid, and requiring a pretreatment of the raw material. Among many pretreatments, steam explosion is used widely, because of its low-cost, less energy consumption and pollution-free. Thus, corn straw was pretreated by the method of steam explosion.

Table 1. Component analysis of the corn straw materials.

Corn straw material	Pre-treatment (%)
Cellulose	37.4
Hemi-cellulose	33.1
Lignin	15.5
Ash	5.8
Crude protein	6.0
Water	2.2

Role of acids on the hydrolysis of corn straw

The effect of different acids on the hydrolysis of corn straw was investigated, which may participate directly or indirectly in the hydrolysis of corn straw. The tested acids were added separately to corn straw hydrolysates with 2% hydrochloric acid. The corn straw was hydrolyzed at 100°C, and the corresponding solid-liquid ratio was controlled at 7.5% after hydrolysis for 3 h. The effect of different acids on the hydrolysis of corn straw was examined and observed. The reduction sugar content was measured by the method of reduction sugar determination, and hemi-cellulose conversion efficiency was calculated by the method of fiber hydrolysis (Hou et al., 2007). The value of DE, for HCl was 44.93%, and hemi-cellulose conversion efficiency was 71.2%. However, the DE value for H_2SO_4 was 38.2%, and hemi-cellulose conversion efficiency was 66.1% (Table 2). This indicates that HCl has more positive effect on corn straw hydrolysates. The analysis indicates that the variations with the tested acids, under controlled conditions, in the content of reduction sugar, hemi-cellulose conversion efficiency are affected by hydrochloric acid. These findings are in agreements with the results of many other reports (Hui et al., 2004; Karel and Rodney, 1997), elucidating the basic role of acid in corn straw hydrolysis.

Corn straw hydrolyzed stability test

There are many factors affecting the hydrolysis of corn straw. After the usage of HCl was determined, we investigated the effects of solid-to-liquid ratio, temperature, time and HCl concentration on the hydrolysis. First, the corn straw was hydrolyzed with various HCl concentrations range of 0.5 to 2.5% in a shake flask, under the same conditions. After 3 h of hydrolysis, it was found that DE value and hemi-cellulose conversion efficiency can be prompted with the addition of HCl under the condition of keeping other factors the same. When HCl concentration was 1.5%, the maximum DE value and hemi-cellulose conversion efficiency were achieved. The ratio between solid and liquid content also has great influence on the reduction sugar content and hemi-cellulose conversion efficiency during the corn straw hydrolysis. With the purpose of developing a high hydrolysis rate, clarified corn straw was fortified with water and employed for hydrolyzing. In this experiment, when the corresponding solid-liquid ratio was 10%, a maximal hydrolysis rate was observed. Thus, 10% of the corresponding solid-liquid ratio could be used as the corn straw hydrolysate fermentation for the subsequent experiments.

Moreover, we also studied the changes of the reduction sugar content and hemi-cellulose conversion efficiency during the course of corn straw hydrolyzed, and investigated the effect of temperature on the hydrolysis of corn straw. As we have known, the DE value and hemi-cellulose conversion efficiency were affected by the hydrolysis time and temperature. From the consideration of technology and fund, we chose hydrolysis time of 3 h and hydrolysis temperature of 90°C as the following up fermentation conditions. Table 3 shows the corn straw hydrolysis stability test, which was five times after determination of various factors. The hydrolyzed conditions were as follows: 1.5% of HCl concentration, 10% of solid-liquid ratio, hydrolysis time of 3 h, and at 90°C. It was concluded that the average value of DE and hemi-cellulose conversion efficiency were up to 52.3 and 80.0%, respectively. Hence, corn straw hydrolysate was successfully improved by adding HCl with the control concentration, solid-liquid ratio, time and temperature.

Fermentation stability experiment

The ergosterol biosynthesis in yeast is a complex pathway, followed by ergosterol yeast hydrolyzed corn stalk fermentation research, systematic research of the initial sugar content, corn steep water, initial pH and development cycle parameters on the fermentation and metabolism of ergosterol yeast. To our knowledge, this is the highest yield and productivity efficiency of ergosterol with corn straw hydrolysates as the sole source of carbon reported, and it has great effect on ergosterol content and cell growth of yeast in shake flasks. After 30 h, the changing trends of cell growth and ergosterol content in yeast were observed. Thus, 3.6% of corn straw hydrolysate was designated as medium. The nitrogen source and content in the medium also have great influence on cell growth and the biosynthesis of compounds in yeast (Shang et al., 2006). Biomass is widely used for its relatively low cost as carbon and nitrogen source in fermentation industries. In the test, it was observed that corn concentration steep water had a positive effect on the ergosterol biosynthesis, and can promote cell growth. For the maximal ergosterol yield, 4% of corn concentration steep water would be used as the nitrogen source for the subsequent experiments.

In addition, the yeast cell growth was affected by initial pH and cultivation time. In an effort to improve the ergosterol production, the conditions were finalized that initial pH was controlled at 7.5 with $Ca(OH)_2$, and cultivation time was around 32 h. Then the fermentation was run as described above at 28°C in a rotary shaker at

Table 2. Effects of different acids on the hydrolysis of corn straw.

No.	Different acid	DE value (%)	Cellulose conversion efficiency (%)
1	HCl	44.93	71.2
2	H_2SO_4	38.2	66.1

DE, Dextrose equivalent.

Table 3. Hydrolyzed stability test results of Corn straw.

No.	DE value (%)	Cellulose conversion efficiency (%)
1	52.2	79.5
2	51.0	79.3
3	54.3	80.4
4	50.6	80.8
5	53.3	79.8
Mean value	52.3	80.0

DE, Dextrose equivalent.

Table 4. Stability test results of fermentation.

No.	OD_{600}	Biomass (gL^{-1})	Ergosterol content (%)
1	1.26	8.54	2.39
2	1.31	8.66	2.32
3	1.20	8.51	2.37
4	1.16	8.47	2.35
5	1.21	8.42	2.33
Mean value	1.23	8.52	2.35

200 rpm for 32 h, and carried on stabilized experiment for five times as shown in Table 4. Finally, the results obtained were the arithmetic mean. The mean value of biomass was up to 8.5 gL^{-1} and ergosterol content was up to 2.35%. Hence, the ergosterol production by the engineering strain was successfully improved by feeding corn straw hydrolysates under the optimal fermentation condition.

Preparation of ergosterol crystal

We carried out ergosterol crystallization studies. The ergosterol yeast using corn straw hydrolysates as fermentation substrates was carried by saponifying, extracting, concentrating and crystallizing, and finally, white needle-like crystals were obtained. It confirmed the crystallization temperature according to the experiment of the ergosterol's solubility in diethyl ether. Figure 1 shows that -14°C is the tangential point of the curve. If the crystal temperature was higher than -14°C, the solubility of the ergosterol in diethyl ether would be high, and crystal difficult to be separated out; however, when the temperature was lower than -14°C, the solubility was low, so the solution would be easy to arrive saturation and form small crystal, which would do harm to further refrigeration, centrifugal and deposition processes. Hence, -14°C was selected to carry out the following experiments.

In order to study the influence of temperature on the crystal quality of ergosterol, constant temperature (CT) and temperature drop (TD) were employed to crystal. SEM was used to observe the morphology of the as-product (Figure 2). Figure 2A is the SEM image of the standard sample. Figure 2B observed by SEM suggests that it could obtain tiny crystal when the method of TD is used. A speculated possible reason is that the saturation would adhere to the crystal surface by the shear force on the solution border when the saturated solution flew fast through the surface of the crystal, and the volume of the saturation increases gradually and could be separated from the surface by the shear force, then the un-separated particles become a part of the crystal lattice at the same time to grow up, hence resulting in competition between nucleating and growing of the crystal. Figure 2C suggests that it could obtain big sized and averaged crystal when the method of CT is used. So we could draw a conclusion that CT could result in perfect crystal.

In addition, considering the other trace in which sterols

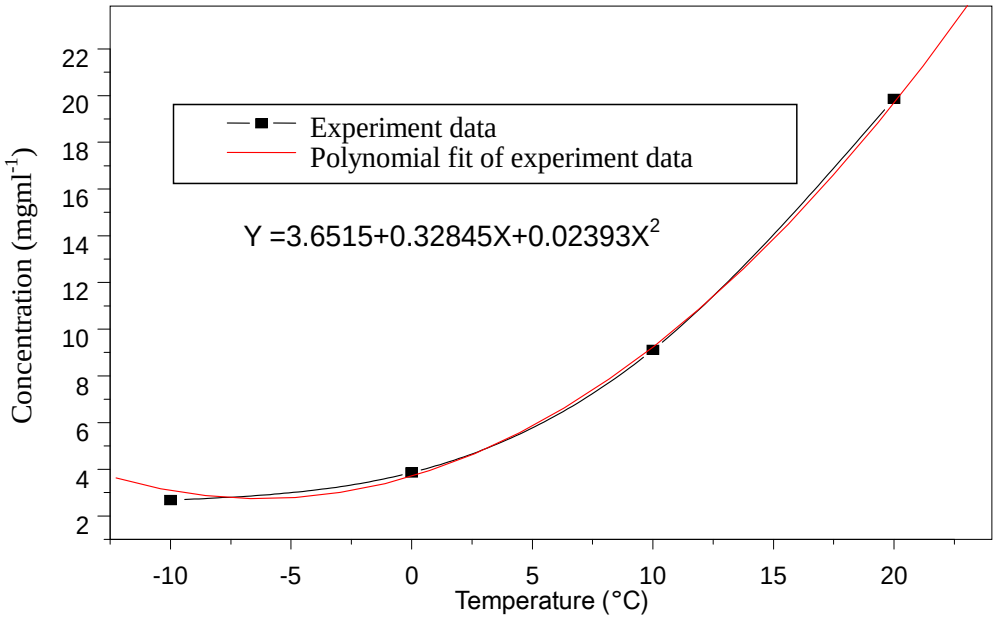

Figure 1. Solubility curve of ergosterol crystal temperature.

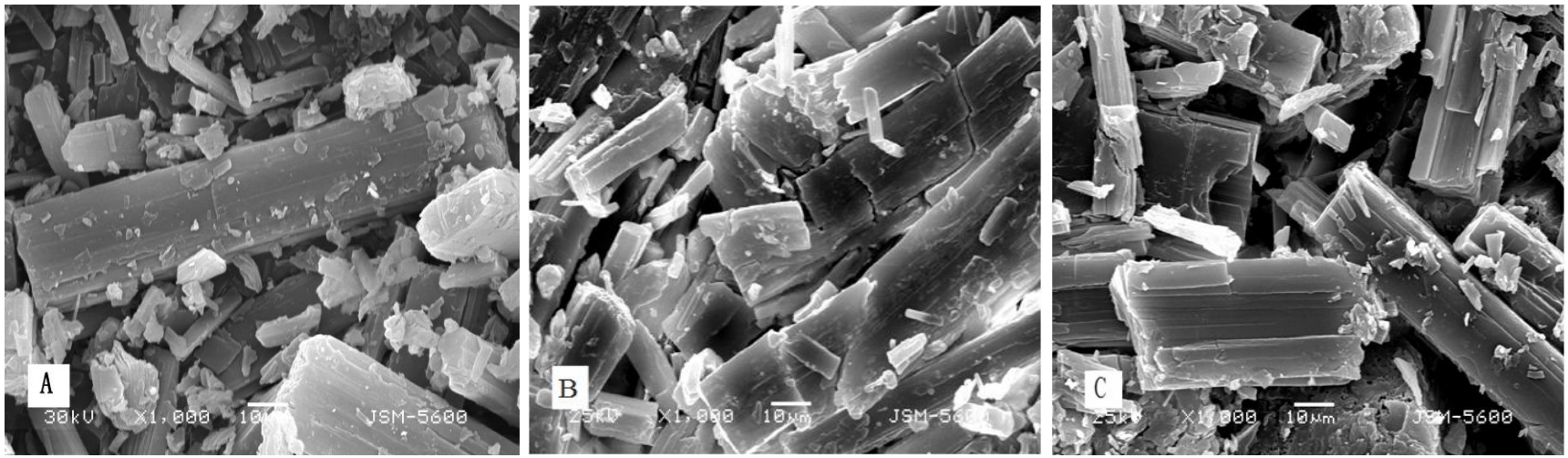

Figure 2. The SEM images of ergosterol. (A) The SEM image of ergosterol standard sample. (B) The SEM image of product with TD. (C) The SEM image of product with CT. SEM, Scanning electron microscope; CT, constant temperature; TD, temperature drop.

Table 5. The productivity and yield of ergosterol crystal.

No.	Raw material (g)	Extracts solution Content (%)	Ergosterol crystal Productivity (%)	Ergosterol yield (%)
A	2.00	2.30	2.01	87.40
B	2.00	2.41	2.07	85.90
C	2.00	2.33	2.06	88.41
Mean value	2.00	2.35	2.05	87.24

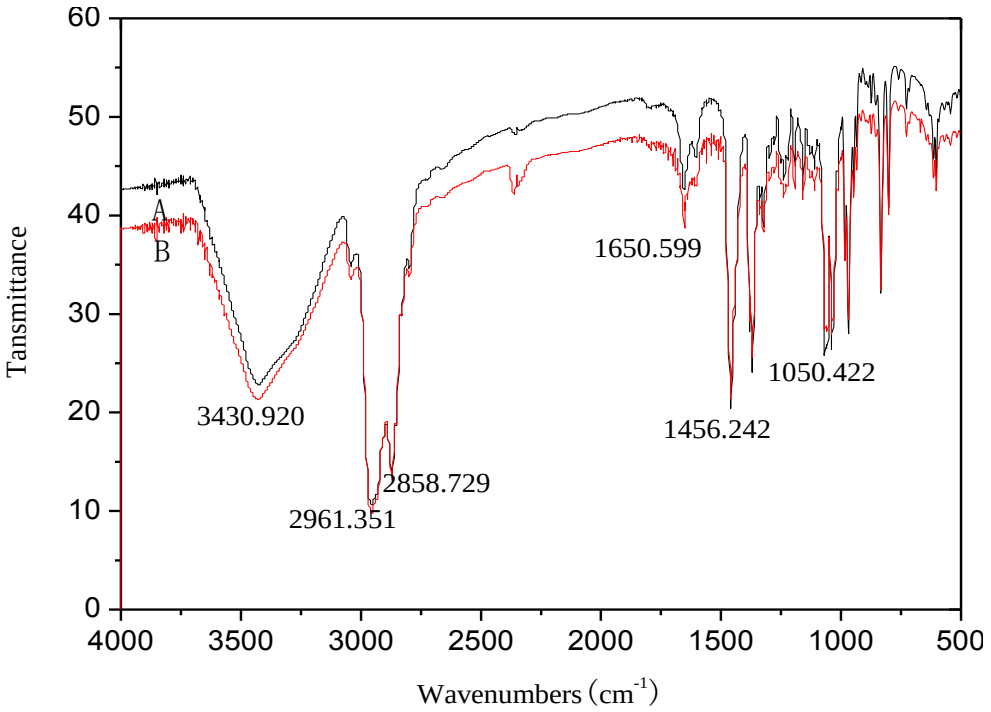

Figure 3. Infrared spectrogram of ergosterol crystal. The infrared spectrum was obtained on the infrared furrier spectrometer with KBr pellets in the region of 4000-400 cm^{-1}. FTIR, $\overline{V}$ (cm^{-1}) $^{-1}$: 3430.920, 2961.351, 2858.729 and 1650.599. A, Standard sample of ergosterol; B, the ergosterol produced by corn straw.

are often present in yeasts as free and esterified forms, alcohol was employed to carry out re-crystallization to improve its purity. Thereafter, an obvious increase in ergosterol crystal occurred. Researches on the stability of ergosterol crystallized as observed under the SEM image showed that regular and stable results were obtained (Table 5). For the ergosterol content of the yeast cell extraction solution, crystallize ergosterol yield, the mean extraction productivity reached 2.35, 2.05 and 87.24%, respectively. In this study, the best conditions of preparing ergosterol crystallize were successfully determined to increase the ergosterol crystal formation.

Ergosterol crystal characterization

Finally, in order to study the chemical bonds of the white-needled crystal, the infrared spectrum was obtained on the infrared furrier spectrometer with KBr pellets in the region of 4000 to 500 cm^{-1}. As shown in Figure 3, curve A is the spectrum of the standard, while curve B is that of the as-product. It is clear that the curve B is almost the same with the standard sample, but there is absorbance of other trace sterols in the curve too; hence we used alcohol to re-crystallize to improve its purity. Thus, it was concluded that the as-product had great a possibility to be ergosterol, although further identification was required.

The optical properties of structured ergosterol crystals were further characterized by XRD. The powder X-ray diffraction (XRD) patterns of the as-prepared product were shown in Figure 4. Curve A is the spectrum of the standard sample and curve B is that of the product. All the peaks could be indexed to the monoclinic phase ergosterol with lattice constants a=34.9, b=7.51 and

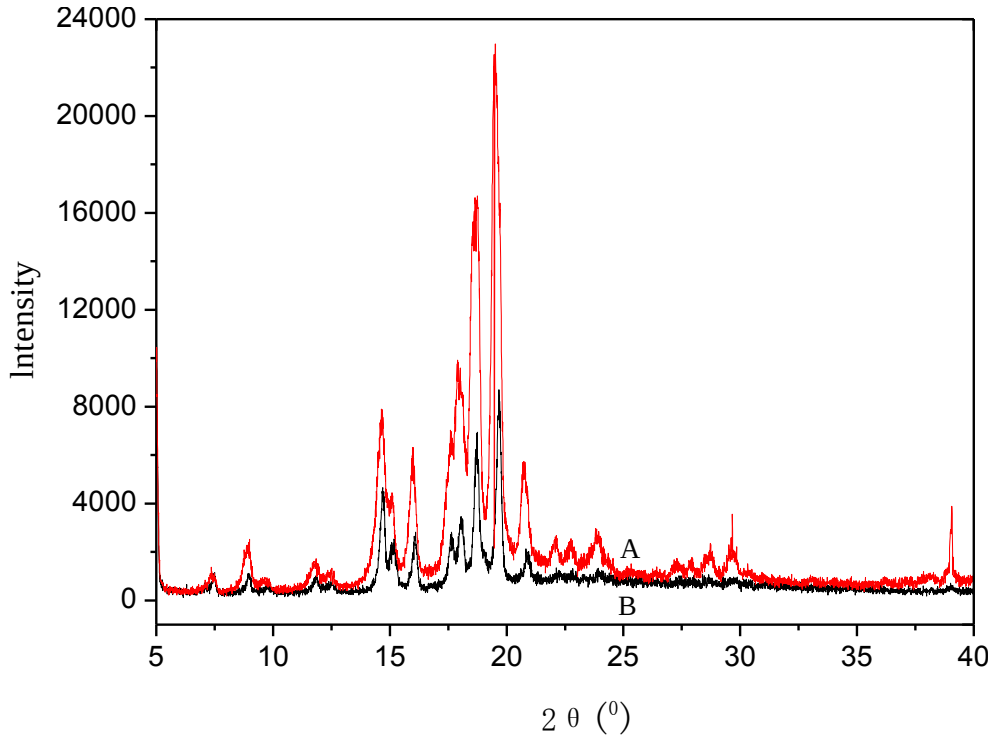

Figure 4. X-ray diffraction patterns of the ergosterol crystal. The powder X-ray diffraction (XRD) patterns were recorded on a PW1700 wide Angle X-ray diffractometer with Cu Ka radiation (λ=1.54184 Å) operated at 40 kV and 30 mA. The scanning speed was 0.02° min^{-1} in the 2θ range from 0.50 to 40.00°. A, Standard sample of ergosterol; B, the ergosterol produced by corn straw.

c=9.84, which was in good agreement with the standard card (JCPDS, 070738). There were no obvious impurities found on the surfaces of the samples, indicating that the as-prepared ergosterol crystals were relatively pure. The strong and sharp diffraction peaks suggested that the ergosterol of this process was well crystallized.

DISCUSSION

Our results suggest that corn straw could be utilized to produce ergosterol. This can be a promising sustainable development for our limited resources, and may even benefit the effort for reduction of accumulated carbon in atmosphere. From this paper, with the aim of producing ergosterol using more economical methods, optimization of hydrolysis condition and fermentation condition were determined using the corn straw as raw material that combined with the engineering of yeast strain. It was feasible to produce ergosterol from biomass using the method of deep-aerobic fermentation by yeast. Biomass strategy was used to improve the ergosterol production from yeast. The results presented herein were as follows: (i) Under the hydrolyzed condition, it obtained the reduction sugar content of 52.3%, hemi-cellulose conversion efficiency of 80.0%; (ii) By the stabilized experiment of producing ergosterol by yeast, the biomass and ergosterol content could be up to 8.5 gL^{-1} and 2.35%, respectively, thus we focused on the method of optimal hydrolysis. And fermentation conditions using the corn straw hydrolysate as the substance not only prompted cell growth, but also maintained relative high ergosterol content that led to the obvious increase of ergosterol yield.

The crystallization temperature of ergosterol was -14°C, and the perfect ergosterol crystal by the way of CT and crystallize ergosterol yield was 2.05%. The structure of ergosterol crystal was characterized. The SEM image of the as-product suggested it was well crystallized; IR spectra and XRD pattern of the ergosterol suggested it was a well crystallized monoclinic phase ergosterol. This production engineering has a low operating cost, a simple operating process, high purity of ergosterol and is in harmony with the green chemical. In summary, the use of inexpensive agricultural and industrial byproducts as carbon source will be the active direction of bioproduct production. The inexpensive corn straw is thus, an attractive carbon source to reduce fermentative production cost. This work will be significant in exploring and finding a large-scale usage for biomass, which is the products of feed and medicine, and will help to reduce the requirements of growing supplies and thus enhance more agricultural revenue. Not only is it essential to find an economically viable refining process, but also it is

environmentally friendly.

ACKNOWLEDGEMENTS

The authors are thankful to the Education Science Foundation of Jilin Province (China) under grants 2008100 and the Science and Technology Foundation of Changchun (Jilin Province, China) under grant 2009035 for providing financial assistance.

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