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# Open fermentative production of L-lactic acid by *Bacillus* sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material

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## HIGHLIGHTS

- A strategy for production of L-lactic acid with high efficiency is proposed.
- The process was performed by a thermophilic strain from cheap biomass, corn stover.
- The process was under open conditions and using NaOH to maintain a constant pH.
- A relatively high concentration of L-lactic acid was obtained.

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## ABSTRACT

Highly efficient L-lactate production by a thermophilic strain *Bacillus* sp. NL01 was demonstrated in this study. Lignocellulosic hydrolyzates containing a high content of glucose, which was prepared from corn stover, was used as substrate for L-lactic acid production. The fermentation was carried out under open condition without sterilization and used NaOH as alkaline neutralizing reagent. In batch fermentation, 56.37 g $l^{-1}$ L-lactic acid was obtained from lignocellulosic hydrolyzates which contained the solid residues produced in enzymatic saccharification. In fed-batch fermentation, 75.03 g $l^{-1}$ L-lactic acid was obtained from lignocellulosic hydrolyzates supernatant. The yield was 74.5% and the average productivity was 1.04 g $l^{-1}$ $h^{-1}$.



## 1. Introduction

Lactic acid is a natural organic acid with a long history of applications in food, pharmaceutical, textile, and chemical industries (Gao et al., 2011; John et al., 2007; Datta and Henry, 2006). In recent years, the demand for L-lactic acid has been increasing considerably owing to its use as a monomer in the preparation of polylactic acid (PLA), a type of environment-friendly alternative to plastics (Datta and Henry, 2006; Wee et al., 2006). Lactic acid can be produced by either chemical synthesis or microbial fermentation. Compared with chemical synthesis which provides the racemic lactic acid, fermentation that can produce optically pure L- or D-lactic acid by using different organisms is desirable.

At present, there are two bottlenecks in the lactic acid production via microbial fermentation. The first one is the high cost of fermentable sugars added as substrates, and the other one is the operating cost such as sterilization and downstream separation and purification (Gao et al., 2011). To enhance the economics of lactic acid production, researchers have done extensive studies and developed many methods to solve the above two bottlenecks. To solve the first problem, many cheap and renewable raw materials, such as starch and lignocellulose from agricultural residues, and industrial and municipal wastes, had been used as substrates for lactic acid production to reduce the cost of the raw material (Li et al., 2012; Wang et al., 2010a,b; Li et al., 2010; Patel et al., 2004; Wang et al., 2009; Park et al., 2004; Wang et al., 2001). To solve the second problem, thermophilic *Bacillus* species, including *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Bacillus licheniformis*, were isolated for non-sterilization fermentation to reduce equipment requirement and energy consumption, and lower labor cost (Qin et al., 2009; Patel et al., 2006; Payot et al., 1999; Wang et al., 2011). In addition, because of the produced lactic acid, alkaline neutralizing reagent must be added to maintain a stable pH. Calcium carbonate is usually used, but it would bring out calcium lactate which required further processing to be converted to lactic acid by $H_2SO_4$. This complicates down-stream processing and generates by-product $CaSO_4$. Instead, NaOH is a simpler and cleaner alternative to $CaCO_3$ (Persson et al., 2001; Zhao et al., 2010; Qin et al., 2010).

The objective of this study is to produce L-lactic acid at low cost. According to the above guidance, we made several attempts,

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including cheap raw material, open non-sterilized fermentation, and an appropriate neutralizing reagent. Corn stover is a low-cost, renewable, and easily available feedstock in China, but large quantities of them are unutilized and burnt out every year (Chen et al., 2009). This would cause various environmental problems such as air and water pollution. As corn stover is rich in cellulose, hemicellulose, and lignin, the derived sugars are economically attractive carbohydrate feedstocks for fermentation of bulk chemicals (Ouyang et al., 2011; Pordesimo et al., 2005). Therefore, the effective utilization of corn stover not only reduces environmental pollution, but also provides abundant cheap bioenergy. In this study, lignocellulosic hydrolyzates, which is prepared from corn stover, was used as cheap feedstock. And a newly isolated thermophilic strain, *Bacillus* sp. NL01, was used as biocatalyst. The fermentation process was open and non-sterilized, and using NaOH to maintain a constant pH. After optimization of fermentation conditions, an efficient L-lactic acid fermentation process was developed.

## 2. Methods

### 2.1. Materials

Steam explosion pretreatment of corn stover (SEPOCS) was provided by Jiangsu Kangwei Biotechnology Co. (Dongtai, China). It was prepared as follows: corn stover was soaked with 3.0% (w/w) sulfuric acid for 2 h and then processed at 170 °C for 5 min. SEPOCS was washed by 10-fold distilled water (w/w), filtered, and stored in sealed plastic bags at 4 °C. The sample was used throughout the experiments.

### 2.2. Enzymatic saccharification for the preparation of lignocellulosic hydrolyzates

The solid residues obtained after steam explosion were subject to enzymatic saccharification. The substrate consistency is 10% (w/v). Two commercial enzymes, cellulase (Celluclast 1.5 l, Cat C2730) and β-glucosidase (Novozyme 188, Cat C6105), were used. The hydrolysis experiments were conducted in 50 mM citric acid buffer (pH 4.8) at 50 °C and 170 rpm for 48 h. The dosage of cellulase and β-glucosidase were 15 filter paper units (FPU) and 30 cellobiase unit (CBU) per gram residues, respectively. The resultant hydrolyzates were then isolated by centrifugation at 7000 rpm, and the supernatant was utilized as a medium component.

### 2.3. Microorganisms and culture conditions

Strain NL01 was isolated from the soil samples in hot spring environments and used in this study. It was tentatively identified as *Bacillus* species according to its biolog analysis and 16S rRNA sequence. The strain was deposited at the China Center for Type Culture Collection (CCTCC No: M 2011468).

*Bacillus* sp. NL01 was maintained on GYC agar slant containing ($g\,l^{-1}$): glucose 20, yeast extract 1, corn steep liquor powder 2.5, $NH_4Cl$ 1, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCO_3$ 10. The slant was incubated at 50 °C for 24 h and stored at 4 °C. The medium for inoculation contained ($g\,l^{-1}$): glucose 100, yeast extract 2.5, corn syrup powder 1.2, $MgSO_4{\cdot}7H_2O$ 0.4, $(NH_4)_2SO_4$ 3, $KH_2PO_4$ 0.22, $MnSO_4{\cdot}H_2O$ 0.03, $FeSO_4{\cdot}H_2O$ 0.03, $CaCO_3$ 70. The starting pH was adjusted to 7.2. The seed culture was prepared as follows: a loop of cells from the fully grown slant was inoculated into 30 ml of the above medium in 100 ml Erlenmeyer flasks and incubated for 12 h at 50 °C with 180 rpm. Then, the seed culture was inoculated into Erlenmeyer flasks or bioreactors for lactic acid production.

### 2.4. Batch and fed-batch fermentation

Batch and fed-batch fermentations of *Bacillus* sp. NL01 were carried out in a 7-l bioreactor (New Brunswick Scientific, BioFlo 110, USA) containing 4 l fresh medium. The fermentation was at 50 °C and 300 rpm without aeration. For fed-batch fermentations, pulse feeding strategy was used to add substrate for convenience. About 100 g solid glucose powder was fed only once at 42 h when the concentration of residual sugars was below 5 $g\,l^{-1}$. For both batch and fed-batch fermentations, the starting pH was adjusted to 7.2 and then controlled at a certain value by adding NaOH solution (10 M). Fermentations were conducted open and without sterilization. Samples were collected periodically to determine the concentrations of L-lactic acid and residual glucose.

### 2.5. Analytical methods

The components of SEPOCS were determined according to the National Renewable Energy Laboratory (NREL, Golden, CO) analytical methods for biomass (Kristensen et al., 2007).

One unit of FPU is defined as the amount of enzyme required to liberate 1 mmol of reducing sugar per minute at pH 4.8 and 50 °C. β-Glucosidase activity was assayed by monitoring the release of *p*-nitrophenol from 4-nitrophenyl-β-D-glucuronide (Ouyang et al., 2011).

The concentration of glucose and lactic acid were measured by high performance liquid chromatography system (HPLC, Agilent technology 1200 series, Germany) equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and a refractive index detector. Analysis was performed with a mobile phase of 5 mM $H_2SO_4$ at a flow rate of 0.6 ml $min^{-1}$ at 55 °C.

For *B. coagulans* NL01, the maximum theoretical lactic acid yield on glucose, cellobiose, and xylose was 100%. The yield of L-lactic acid was calculated according to the following equation:

$$\text{Yield of L-Lactic acid} = \frac{\text{L-Lactic acid}}{\text{Total sugars} - \text{Residual sugars}} \times 100\%$$

## 3. Results and discussion

### 3.1. Determination of SEPOCS and lignocellulosic hydrolyzates composition

The main compositions of the SEPOCS (dry weight basis) were calculated. The major component was cellulose (56.46%). The acid insoluble lignin and acid-soluble lignin were 31.24% and 6.86%, respectively. And hemicellulose accounted for only 2.26%. After enzymatic hydrolysis, the compositions in the resultant hydrolyzates were as follows: 87.42 $g\,l^{-1}$ glucose, 7.51 $g\,l^{-1}$ cellobiose, 4.74 $g\,l^{-1}$ xylose, 2.76 $g\,l^{-1}$ glycerol, 0.83 $g\,l^{-1}$ xylitol, and 0.13 $g\,l^{-1}$ acetic acid, respectively. A schematic diagram of the entire process for preparation of the corn stover hydrolyzates was showed in Fig. 1.

### 3.2. Selection of optimal fermentation temperature and initial glucose concentration

Thermophilic *Bacillus* sp. NL01 strain was isolated at a relatively high temperature. To further determine its optical fermentation temperature, *Bacillus* sp. NL01 was incubated at 40, 45, 50, and 55 °C for L-lactic acid production. The best L-lactic acid concentration, 57.2 $g\,l^{-1}$, was obtained at 50 °C. At other temperatures, the L-lactic acid concentration was no more than 50 $g\,l^{-1}$. Therefore, all the following fermentations were conducted at 50 °C.

Next, *Bacillus* sp. NL01 was cultured under different initial glucose concentrations. Many reports had indicated that sugar osmotic